Effects of warming and cadmium on the feeding behaviour and growth of the aquatic invertebrate shredder *Limnephilus sp*.

Daniela Batista¹, Cláudia Pascoal¹ and Fernanda Cássio¹

¹Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.
E-mail contact: danimbatista@gmail.com

1. Introduction

Nowadays, metal contamination is still a worldwide environmental problem in both developing and developed countries [1]. Metals can compromise survival, growth and reproduction of aquatic biota at environmentally realistic concentrations [2], leading to the reduction of abundance and diversity of sensitive species [3]. In streams, invertebrate shredders play an important role in plant litter breakdown, since they actively participate in the fragmentation of plant material and decomposition of coarse particulate organic matter [4], providing a link between plant litter and higher trophic levels.

The Intergovernmental Panel on Climate Change predicts an increase in temperature [5] that may cause several changes in invertebrates, such as faster growth rates, shorter developmental time and smaller size at maturity [6], and reduce the ability of invertebrates to survive on poor nutrient diets [7].

It is probable that the combined effect of metals and increased water temperature may have strong impacts on the processes in which invertebrate shredders are involved (i.e. litter decomposition and nutrient cycling), further compromising the functioning of freshwater ecosystems [8]. In this work, we tested how leaf consumption and growth of the invertebrate shredders are affected by cadmium and whether increasing temperature modulates this relationship.

2. Materials and methods

A common species of invertebrate shredders, *Limnephilus* sp. (Tricoptera: Limnephilidae), was collected from an unpolluted site of the Cávado River (NW Portugal) and acclimated to the laboratory. Two types of feeding assays were carried out. In a feeding experiment, the animals were allowed to feed on microbially colonized alder leaves for 6 days, while exposed to increase Cd concentrations (0, 0.5, 10 mg L⁻¹) at two temperatures: 15 ºC, a temperature commonly found in streams of Northwest Portugal in spring and autumn, and 21 ºC to simulate a warming scenario. In a post-exposure feeding experiment, the animals were kept under starvation for 4 days while exposed to increasing cadmium concentrations (10 levels up to 35 mg L⁻¹) and then were released from the stressor and allowed to feed on microbially-colonized alder leaves for further 5 days. Cadmium content in leaves, cocoon and animals was analyzed by inductively coupled plasma-atomic emission spectrometry (ICP-AES) [9].

3. Results and discussion

3.1. Feeding experiment

Leaf consumption rate by the shredder decreased with the exposure to Cd, and increased with temperature. It is expected that an increase in temperature stimulates microbial biomass on decomposing leaves, which could explain the increased invertebrate feeding activity found at higher temperature in our study (Figure 1A). Temperature also stimulated the growth rate of the shredder, but Cd did not lead to any significant effect (Figure 1B).
3.2. Post-exposure feeding experiment

After metal release, leaf consumption by the shredder was severely inhibited in animals that have been previously exposed to Cd concentrations ≥ 1 mg L⁻¹. Although the exposure to Cd for 4 days had not lead to animal death, some animals lost the ability to feed and recover. Our results also suggest that the feeding rates of invertebrates could be considered as a useful criterion for evaluating toxicity.

3.3. Accumulation of cadmium on leaves and animals

After 6 days of Cd exposure, 64 % of the metal was associated with the leaves, 23 % was associated with the shredder cocoons, and 13 % with the larvae. Also, higher accumulation of Cd was found at 21 °C than at 15 °C. Cadmium accumulation in the invertebrates appeared to occur more via food than via contaminated water. Moreover, our results suggest that shredders with cocoons might have a survival advantage during short-term exposure to metals.

4. Conclusions

Our results indicate that the increase in Cd concentration and the increase in temperature (6 °C) affected the feeding behavior and the growth performance of invertebrate shredders. This may compromise at longer times the survival of sensitive shredder populations with direct impacts to plant litter decomposition and nutrient cycling in freshwater ecosystems.

5. References

The effect of temperature on the toxicity of cadmium towards Caenorhabditis elegans.

Nils J. Nørhave¹, Nina Cedergreen¹, Helen L. Hooper², David J. Spurgeon², Claus Svendsen²

¹University of Copenhagen, Faculty of Life Sciences, Thorvaldsensvej 40, 1871 Frederiksberg C, Denmark
²Centre for Ecology and Hydrology, Wallingford, Oxfordshire, OX10 8BB, England
E-mail contact: nils@dsr.life.ku.dk

1. Background

To understand the toxicity of a compound, one has to observe the toxicity in context of the environment in which it is measured. Temperature especially has been shown to be an important factor affecting toxicity, since it is the temperature that regulates the microbial degradation rates of organic compounds and the processes with which an organism take up and exclude pollutants. As a result the toxicity of some chemicals change with temperature [1-3]. However when studying metals, which are not subject to degradation, toxicokinetics (the processes determining the concentration of toxicant in the animal) and toxidynamics (the processes determining the intrinsic toxicity of the toxicant towards the animal) are the major temperature dependant processes that can cause changes in toxicity. Heugens et al. [4] and Cairns et al. [5] have reviewed the effect of changing temperature on the toxicity of a number of compounds towards aquatic organisms and found that the toxicity of metals and pesticides generally increase with temperature. The normal procedures for assessing the environmental risk of a chemical only involves testing at one temperature, but nonetheless these risk assessments are frequently applied in wide geographical areas without concern for how the different climates can affect the toxicity of the chemical.

The aim of this study was to investigate the effect of temperature on the toxicity of cadmium towards Caenorhabditis elegans, by tracking individuals from egg to death. All life cycle endpoints were combined in a population growth model. Cadmium was chosen as a stressor as it is a common pollutant resulting from anthropogenic sources and because it is representative of the group of non essential heavy metals that tends to bioaccumulate.

2. Materials and methods

C. elegans of the N2 Bristol strain was cultured on Petri dishes on a modified bacteriological agar (nematode growth medium (NGM) [6]) and fed Escherichia coli, of the uracil deficient strain OP50. Before the start of the experiment new cultures were determined at the test temperatures at least 3 months before the start of the experiment. Individuals were isolated on well plates and the daily reproduction was determined through the entire lifespan. From the reproduction data the start and end of the reproduction period was estimated for all treatments. From the lifespan, total reproduction and the reproductive period a three compartment population model [7] was made, and from this the intrinsic population growth rate was determined for all treatments.

3. Results and Conclusions

The total reproduction and lifespans (Figure 1 and 2) of C. elegans are deceptively similar for all cadmium treatments, so these endpoints on their own would suggest that there is no significant difference between the toxic effect of the concentrations used. Fertility, however, expressed as “Time to first egg”, is a most sensitive endpoint. Hence, when all endpoints are combined in a three component population growth model (Figure 4) the effect of the individual cadmium doses separate, showing that the high doses have detrimental effects at high temperatures, while population growth can still take place at the low temperatures. This demonstrates the importance of evaluating the toxic effect of chemicals on population levels rather than on single endpoints determined from individuals.

The response of C. elegans to cadmium shows how a toxic stressor can induce a change in the life cycle of exposed organisms. Exposure to a stress factor might induce production of more but smaller offspring giving the advantage of numbers, or fewer but larger offspring giving the individual offspring a better chance of fleeing or outlasting the stress factor. In the case of cadmium the latter seems to be the case. A decrease in the body size of C. elegans was observed (data not shown), making it able to stay alive but severely affecting the time, before resources to produce the first egg was achieved.
Figure 1: The average total reproduction of *C. elegans* exposed to Cd. Note that two concentrations go from having low reproduction at 11 and 15°C, to having no reproduction at 18 and 21°C. Error bars indicate standard deviation.

Figure 2: The average lifespans of *C. elegans*. There are no significant differences between the treatments with Cd. Error bars indicate standard deviation.

Figure 3: The estimated time to the production of the first egg for cadmium treated *C. elegans*. Eight and 10 mg Cd/L at 18 and 21°C could not be estimated, as the worms were infertile, and are therefore set to arbitrary “high” values. Error bars indicate standard deviation.

Figure 4: The intrinsic rate of population increase as a function of temperature for the different cadmium treatments. In cases where the reproduction start and end could not be determined or was higher than the lifespan, the lifespan was used as a substitute, resulting in a very low population growth rate.

4. References


Low temperatures enhance the chronic toxicity of Cd and Cu through different mechanisms

Nina Cedergreen¹, Kristoffer Nielsen¹, Hanna K.L. Johansson¹, Helle Marcussen¹, Peter E. Holm¹, Claus Svendsen² and David Spurgeon²

¹Department of Basic Science and Environment, University of Copenhagen, Thorvaldsensvej 40, 1871 Frederksberg, Denmark.
²Centre for Ecology and Hydrology, Maclean Building, Benson Lane, Crowmarsh Gifford, Wallingford, Oxfordshire OX10 8BB, UK
E-mail contact: ncf@life.ku.dk

1. Introduction
The toxicity of the most common metal pollutants to species from the most well investigated soil invertebrate groups (earthworms, springtail, molluscs, woodlice) has been relatively well studies under the optimal “standard” environmental conditions used in standardised toxicity tests. Field conditions are, however, far from optimal, and variation in temperature is one of the factors contributing most to the heterogeneity. The aim of this study was to assess the effect of temperature on the sensitivity of the soil dwelling worm Enchytraeus crypticus to Cd and Cu. The two metals were chosen as representatives of a non-essential heavy metal (Cd) and an essential micronutrient for which uptake and excretion mechanisms exists (Cu). Our hypothesis was that increasing temperature would result in greater toxicity, since this was the conclusion of the initial meta-analysis conducted by Heugens et al. (2001) [1]. The patterns of change in the sensitivity of the test species to the two metals were interpreted in relation to potential mechanisms through which temperature could influence the metal toxicity. Three types of processes were considered. These were temperature effects on speciation and especially the rate of “aging” of metals which results in metal becoming more strongly bound to the soil solid phase; temperature effects on toxicokinetics which could result in changes in internal concentrations if uptake and loss rates are affected differently; and toxicodynamics though temperature effects on both the production and repair of reactive oxygen species (ROS) associated damage. Additionally there may be changes in inherent sensitivity due to temperature stress on physiology.

2. Materials and methods
The toxicity of Cd and Cu to E. crypticus at different temperatures was determined using a protocol modified from the OECD 220 guideline for testing of chemical effects on Enchytraeids. Six months before the start of the experiment, worms were divided into six groups and placed in incubators at 11°C, 13°C, 15°C, 18°C, 21°C and 25°C to acclimate. The soil used for both acclimatization during rearing and for all conducted experiments was the LUFA 2.2 standard soil. For each test metal (Cd and Cu) and test temperature, a concentration series was prepared for testing, using four replicates per treatment (six for controls). Each replicate consisted of 10g dry weight of LUFA soils spiked with a stock solution of CuCl₂ or CdCl₂ in demineralised water to give the required concentration of metal in the test soils and a moisture content of 27.5%. Replicated concentrations used for Cd were 0, 16.3, 32.5, 65, 130, 260, 520, 1040 mg Cd/kg dry soil and for Cu 0, 38.13, 76.3, 52.5, 305, 610, 1220 and 2440 mg Cu/kg dry soil. To each replicate 5 adult worms were selected from the relevant temperature acclimatized culture and added to the soil surface of each replicate. Worms were then fed with autoclaved oat grains and fish food and placed at the relevant test temperature. The experiments at each temperature were terminated when it was estimated that there was between 100 – 200 juveniles in control containers based on the results of initial population growth trials. This was done in order to account for the metabolic differences at different temperatures and assess toxicity at similar population development stages. Each replicate was terminated by adding 5 ml of 98% ethanol followed by 3 mL of 1% rose Bengal solution. The soils were sieved through a coarse (1 mm) and fine (0.18mm) mesh and the stained worms counted. The number of juveniles produced per adult as a function of soil Cd or Cu was described with a log-logistic dose-response model.

In parallel, uptake kinetics of Cu and Cd was performed at 11, 18 and 25°C at soil Cd concentrations of 40 mg Cd/kg dry soil and Cu concentrations of 150 mg/kg dry soil, corresponding to somewhere between EC₁₀ and EC₅₀ depending on temperature. Five worms per treatment were taken out for determination of total tissue metal concentrations after 1, 8 and 24 h, 2, 3, 4, 7, 10, 17 and 24 days. Soil samples were collected at the end of the experiment to determine the concentration of total and dissolved metals. Dissolved metals were determined in soil water extracts, whereas total soil and worm tissue concentrations were determined.
after microwave assisted acid digestion. Cd and Cu and a range of other elements were determined using ICP-MS analysis and ETAAS

3. Results and discussion

The results showed an increase in EC$_{50}$ with increasing temperature for both Cd and Cu (Figure 1A and C). This was contrary to predicted, as most findings have found toxicity to increase with temperature [1]. Measuring tissue Cd and Cu concentrations in the worms after 24 days, showed that internal Cd concentrations increased with temperature, whereas the internal Cu concentrations decreased (Figure 1B and D).

![Figure 1](image_url)

Figure 1: The figure shows the Cd (A) and Cu (D) EC$_{50}$ for reproduction when the adults had produced approximately 50 offspring as a function of temperature. The tissue concentrations of Cd (B) and Cu (D) of control (open symbols) and treated (closed symbols) worms after 24 days are given as a proportion of the P content of the sample, as this showed to be more accurate than DW due to the varying soil content in the gut of the harvested worms. EC$_{50}$ values are given ±serr and tissue concentrations as mean±stdev.

As differences in metabolic rate between the different exposure temperatures is corrected for by varying exposure duration, this shows that the enhancement of toxicity at low temperatures (or high temperature alleviation of toxicity) for the two metals are caused by different mechanisms. The high internal concentrations of Cd at high temperature suggest that uptake, though theoretically passive, increases with temperature. The fact that the toxicity decreases with temperature, despite the increased Cd load, might be due to increased Cd immobilisation via metallothionein like proteins or increased repair systems such as oxidative stress protection, heat shock and DNA repair mechanisms with increasing temperatures. The uptake and excretion of Cu can be regulated and the results suggest that this regulation is more efficient at higher temperatures, resulting in a lower toxicity. For Cu also an increased immobilisation of Cu in the soil at higher temperatures could be a cause for the lower internal tissue concentrations. Uptake kinetic data and availability of Cd and Cu in the soils will be presented and discussed in relation to the toxicity data.

References

Combined effects of soil moisture and a fungicide on soil organisms – A study with Terrestrial Model Ecosystems

Bandow, Cornelia1,2, Coors, Anja2, Förster, Bernhard2, Ng, Ee Ling3, Römbke, Jörg2, Sousa, José Paulo 3 and Oehlmann, Jörg4

1LOEWE Biodiversity and Climate Research Centre, Senckenberganlage 25, 60325 Frankfurt/Main, Germany
2ECT Oekotoxikologie GmbH, Böttgerstraße 2-14, 65439 Flörsheim/Main, Germany
3IMAR-CMA, University of Coimbra, Department Life Science, Faculty of Science and Technology, Apartado 3046, 3001-401 Coimbra, Portugal
4Goethe University Frankfurt/Main, Institute for Ecology, Evolution and Diversity, Department Aquatic Ecotoxicology, Siesmayerstraße 70, 60054 Frankfurt/Main, Germany

E-mail contact: C.Bandow@ect.de

1. Introduction

The German Federal Environment Agency is expecting a decrease of summer precipitation of circa 16-19% in 2071-2100 in comparison to the reference period of 1961-1990 for Hesse/Germany. The precipitation in winter may increase by 10-20% within the same period [1].

The Intergovernmental Panel on Climate Change (IPCC) predicts substantial increase in the intensity of daily precipitation events in the Mediterranean region, while mean precipitation may decrease by 30-45% [2].

Changes in precipitation may lead to changes in soil moisture content. When exposed to this climate-related stressor organisms may react differently towards chemicals (e.g. pesticides). As part of the activities of the research centre BiK-F (Biodiversity and Climate Research Centre, Frankfurt, Germany) effects of combined stressors are studied in terrestrial model ecosystems (TMEs), a higher-tier semi-field study type. The research was done in cooperation with the University of Coimbra, Portugal.

In both studies the effects of two stressors (soil moisture and different concentrations of the fungicide pyrimethanil) on various structural as well as functional endpoints were investigated. The final aim of this research is to evaluate one specific aspect of Global Climate Change on soil ecosystems by comparing the combined effects of a pesticide and varying soil moisture levels on temperate and Mediterranean soil organism communities.

2. Materials and methods

Two studies were conducted. One took place in Coimbra, Portugal, the second in Flörsheim, Germany. In both cases, the TMEs consisted of intact, undisturbed soil cores with a diameter of 18 cm and a depth of 40 cm. In Germany, the soil cores were sampled in the South of Hesse at a meadow site with alluvial clay, which has not received pesticide and fertilizer input for at least 10 years. The fungicide pyrimethanil was investigated at 11 concentration levels in the TME study, each at three different moisture regimes. The concentrations ranged from 0.41 to 1270.49 mg/kg soil dry weight (10 cm depth). The second lowest concentration (0.91 mg/kg soil dry weight) reflects the maximum application rate (MAR) of this fungicide in agriculture. Two and eight weeks after application of the fungicide a sampling of 2 replicates each was conducted. In the Portuguese TME study with pyrimethanil the same endpoints were investigated, but with a Mediterranean soil and its community. The sandy loam in this study site has been kept under biological production since 2004. At the time of core extraction, the site was sowed with yellow lupin as cover crop. The design differed from the German study, with only two fungicide concentration levels (0.91 and 4.55 mg/kg soil dry weight) but four replicates per treatment. The nominal moisture levels in Germany were 30%, 50% and 70% of WHCmax. Sampling time was similar in both studies.

In this contribution, the effects of three moisture levels and different concentrations of pyrimethanil on the abundance and diversity of enchytraeids as well as the feeding activity (measured by the bait-lamina test) of the soil organisms and their vertical distribution will be presented.

The microbial endpoints are addressed in two posters, presented by Mrs. Ng, University of Coimbra, Portugal.
3. Results and discussion: Enchytraeids and bait-lamina

In total, 22 and 13 enchytraeid species were found in Flörsheim and Coimbra, respectively. Both sites can therefore be considered as “rich” in enchytraeids. In Germany, the abundance of the enchytraeids was significantly affected by the fungicide as well as by soil moisture. Yet, no interaction between these two factors on enchytraeid abundance was observed. The concentration-response curve for each moisture level in the German study is shown in Figure 1 as the mean number of enchytraeids in the upper 5 cm of the soil, eight weeks after application of pyrimethanil.

The preliminary calculated EC50 values for the endpoint enchytraeid abundance did not diverge between the nominal moisture levels and were within the range of 20-30 mg a.i./kg dw. No correlation or interaction, respectively, between soil moisture and pyrimethanil was found. In the Portuguese study, neither soil moisture nor the fungicide had an effect on enchytraeid abundance.

Feeding activity differed between the nominal moisture levels and showed an opposed effect in depth profile (Figure 2). At high moisture levels the feeding activity had its highest values in the top 4 cm, while in dry soil the activity is higher in deeper layers.

4. Conclusions

For the abundance of the enchytraeids no interaction between soil moisture and pyrimethanil could be detected, while effects of the single factors occurred. In contrast, bait-lamina, regarded as a functional endpoint, responded to the interaction of climate and chemical stressor, at least at nominal values. Further statistical evaluation of actual moisture contents, taxon-specific abundance and analytical measurements of the exposure concentrations are in progress.

5. References


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Keywords: Climate change, Enchytraeidae, Bait-lamina, Semi-field study
Antioxidant defenses and oxidative damage in amphipods under multiple stressors

Elena Gorokhova¹, Marie Löf¹, Halldór Pálmar Halldórsson², Ulla Tjärnlund¹, Magnus Lindström³, Martin Reutgard¹, Tina Elfwing¹ and Brita Sundelin¹

¹Department of Applied Environmental Science (ITM), Stockholm University, SE 10691 Stockholm, Sweden
²Súðurnes University Research Centre, University of Iceland, Garðavegur 1, 245 Sandgerði, Iceland
³Tvärminne Zoological Station, University of Helsinki, J.A. Palméns väg 260 FIN-10900 Hangö, Finland
E-mail contact: elena.gorokhova@itm.su.se

1. Introduction

Oxidative stress is a common denominator underlying many diseases and environmental insults. Both hypoxic conditions and exposure to toxic substances cause deleterious effects, directly or indirectly, via reactive oxygen species (ROS) generation [1]. These ROS-stressing agents can increase intracellular ROS to harmful levels causing structural and functional damage to lipids, proteins and DNA, up to a threshold that triggers cell death. To reduce the negative effects of the oxidative stress, an effective antioxidant defense system has evolved, including low molecular-weight antioxidants and antioxidant enzymes, in particular enzymes eliminating (i) ROS, e.g., superoxide dismutases (SOD) and catalases (CAT), (ii) lipid peroxidation products, e.g., glutathione peroxidases, and (iii) secondary radical oxidation products, e.g., glutathione S-transferases, GST. Oxidative stress results from a mismatch between the ROS production and the organism’s antioxidant capacity to mitigate the damaging effects. On the other hand, oxidative stress plays a central role as a physiological mechanism of trade-offs between growth and self-maintenance. Indeed, the metabolic costs for antioxidant defenses and stress recovery link oxidative stress to higher-level responses as any increase in investment in the antioxidant system can only come at a cost to investment elsewhere, e.g., it may decrease food intake and growth [2].

In estuarine systems, bottom hypoxia and increasing pollution load are the main stress factors causing profound alterations in benthic communities. Although hypoxic conditions often occur in polluted areas, little is known about their combined effects on estuarine organisms. The aim of this work was to test the individual and combined effects of exposure to hypoxia and to polluted sediment at environmentally realistic concentrations using the amphipod Monoporeia affinis, and to identify stress-specific biomarkers that help understanding cellular mechanisms of action and interpreting physiological and ecological responses. Further, we hypothesized that amphipods exposed to naturally occurring fluctuating hypoxia may develop adaptations to survive and recover upon reoxygenation, whereas exposure to contaminants would exacerbate oxidative stress in hypoxia-challenged animals and compromise the recovery; this hypothesis was tested using the most informative biomarkers identified in the first step.

2. Materials and methods

In Experiment 1, amphipod responses to different combinations of oxygen levels (hypoxia vs. normoxia) and contaminant (polluted vs. unpolluted sediment) were determined using a suite of biomarkers – antioxidant enzyme activities (SOD, CAT, and GST), lipid peroxidation status (TBARS), protein carbonyl content (PCC), and DNA integrity (DNA-SB). In addition to the antioxidative biomarkers, we assayed acetylcholinesterase (AChE) activity to detect the neurotoxic stress caused by organophosphates, carbamates and metals present in the polluted sediment. To assess effects at the organism level, we determined mortality and RNA:DNA ratio as a surrogate marker of individual growth.

In Experiment 2, we re-created a situation similar to an intermittent hypoxia by exposing amphipods in polluted and unpolluted sediments to low oxygen levels for 4 days with subsequent reoxygenation. During the experiment, oxidative status, AChE and RNA:DNA ratios were monitored.

3. Results and discussion

3.1. Experiment 1

There were significant increases in CAT and SOD activities and TBARS levels in response to both moderate hypoxia and contaminated sediment, while GST increased and AChE decreased in response to the contamination only. Significant positive correlations were observed among the antioxidant enzymes and...
between the enzyme activities and TBARS concentration, suggesting a complex response to the oxidative stress. No significant changes in PCC were recorded in any of the treatments. Furthermore, the negative effect of hypoxia on DNA integrity was significant; with frequency of DNA-SB increasing in animals exposed to hypoxia in contaminated sediment. Despite clear effect at the cellular and biochemical levels, no responses at the organism level were observed. Stepwise discriminant analysis (SDA) allowed 75–100% correct discrimination of amphipods according to their treatment group; this grouping aids in stress assessment in field-sampled animals as well as understanding general patterns in the data that may guide future research (Fig.1).

Of the potential biomarkers assessed in this study, CAT activity was found to be associated with hypoxia, while SOD, GST and AChE activities appear to predict best the effects of exposure to sediments containing several contaminants (e.g. heavy metals, PCBs and PAHs), and TBARS concentration is particularly indicative of combined effects of hypoxia and contamination.

3.2. Experiment 2

In Monoporeia affinis experiencing short-term moderate hypoxia and hypoxia-normoxia shifts, the exposure to heavily polluted sediment exacerbated an imbalance between antioxidant- and oxidant-generating systems. This overstressed the physiological capacity of the amphipods to generate and coordinate oxidative stress response and resulted in oxidative damage and negative effects on individual growth status. Amphipods encountering episodic moderate hypoxia in unpolluted sediments are subjected to a typical scenario of ROS production and removal, with no apparent cellular damage due to lipid peroxidation. By contrast, co-exposure to contaminants affects both the adaptive increase in the baseline activity of SOD, the key antioxidant enzyme, which is crucial for combating oxidative stress during hypoxia and hypoxia-normoxia shifts, causes persistent lipid peroxidation, and slows recovery from hypoxia.

4. Conclusions

Our results support the hypothesized potential of xenobiotics to hamper ability of benthic animals to cope with changes in oxygen regime, such as fluctuating hypoxia and migrations from hypoxic to oxygenated environments. Thus, to predict the outcome of hypoxia effects in polluted coastal areas, it is crucial to understand the interactions between antioxidant responses to contaminants and hypoxia and physiological mechanisms causing and counteracting oxidative damage.

Oxidative status assessment and multivariate analyses are instrumental for linking exposure factors to biological responses, identifying stressors and understanding response mechanisms.

5. References

Are Melting Glaciers Increasing Exposure of Alpine Wildlife to Contaminants?

John E. Elliott¹, Melanie F. Guigueno², Kyle H. Elliott², Joshua Levac², Patrick Shaw¹ Mark Wayland¹, Derek Muir¹

¹Science & Technology Branch, Environment Canada, Canada
²Department of Biology, University of Manitoba, Winnipeg, Canada

Email contact: john.elliott@ec.gc.ca

1. Introduction

Climate change is reportedly reducing the size of glaciers and icefields across the globe (Haeberli et al. 1999). As a consequence, contaminants that accumulated in glaciers through evaporation from warm regions and subsequent condensation in cold regions can be released into alpine environments (Blais et al. 1998,). Pristine environments, long distances from industrial sources, are often highly contaminated with persistent organic pollutants (POPs) and mercury due to a combination of long range transport, thermochemistry and climatic trends (Grimalt et al. 2001; Bettinetti et al. 2008) Glacial runoff can be enriched in POPs due to minimal contact with catchment soils and sediments and minimal opportunity for loss through evaporation and poor binding to organic-poor glacial sediments (Blais et al. 2001a,b; Bettinetti et al. 2008).

Ospreys (Pandion haliaetus) are apex predators in these ecosystems, feeding on fish in montane lakes (Elliott et al. 2007; Henny et al. 2008). Because they accumulate contaminants, ospreys are effective sentinels and can also be impacted by POPs and mercury (ibid.). Although ospreys are long distance migrants and winter in Lartin American countries which reportedly continued to use organochlorine pesticides after they were banned in the north, contaminant levels in eggs were not related to wintering ground exposure for ospreys satellite-tracked from the Canadian Rockies (Elliott et al. 2007). The process of contaminants exposure in alpine lakes can also be affected by changes in foodchain structure due to introduced and invasive fish species (Refn), and is explored in a companion.

We hypothesized that contamination would be greater for birds feeding at higher trophic levels and in water basins with extensive glaciation. We examined the influence of: trophic level, aquatic input, altitude, lake area, water basin size, water body type (river, reservoir or natural lake), percent of glacier in watersheds and ratio of lake size to watershed size in determining contaminant levels in eggs and blood plasma of ospreys.

2. Materials and methods

We collected 88 osprey eggs (one per nest), plasma and feather samples from 70 nestling ospreys from 59 nests nesting in 16 watersheds of the Canadian Cordillera. Organochlorine pesticides and \( \Sigma \)PCBs were analyzed at the Environment Canada National Wildlife Research Centre using gas chromatography with a mass specific detector (GC-MSD) as described by Elliott et al. (2007). For isotopic analysis, egg and plasma samples were dried, 1±0.2 mg encapsulated in tin, and sent to University of California (Davis) stable isotope facility. We used the Spatial Analyst toolbox and Hydrology toolset in ARCGIS 9.2 to measure the size of 24 watershed basins of osprey foraging lakes within the Canadian Cordillera (ESRI, www.esri.com). We calculated elevation of osprey nests, lacial areas (km\(^2\)), slope, aspect, flow direction and accumulation of water, and stream networks using flow direction and accumulation, watershed and stream network algorithms, all of which are publicly available. AIC was used to examine models that included lipid content, isotopic (\( \delta^{15}N \), \( \delta^{13}C \)) abundance and all geographic (watershed area, water body type and area, of glaciers within a watershed and elevation) as explanatory variables and DDE, PCB, HCB, chlordane, and toxaphene variables as response variables. We completed separate analyses for eggs and nestling plasma.

2. Results

Eggs. Water body type was included in all of the best models (\( \Delta AIC < 2.0 \)) for all contaminants. The ratio of watershed size to lake size was the best single-factor model for DDT (\( r^2 = 0.04 \)) and lake area was the best predictor for \( \Sigma \)PCB (\( r^2 = 0.13 \)).

Chick plasma. Individuals that fed at higher trophic levels accumulated more DDT than those that fed at lower trophic levels. Water body type was only included in the best models (\( \Delta AIC < 2.0 \)) for toxaphene and HCB. The best single-factor model for toxaphene was the size of lakes relative to the watershed size followed closely by elevation (\( r^2 = 0.56 \)), and both variables appeared along with glacier area or trophic level in the most parsimonious models. For DDT, the best single-factor model was trophic level, with elevation, aquatic input, the proportion of glaciers within a watershed, percent lipids and the size of lakes relative to overall
watershed size were all included in the most parsimonious models. For PCBs, lake and watershed area, and their interaction, played a significant role with trophic level and aquatic input playing a secondary role.

**Discussion**

Despite living in pristine environments, far from point sources of contamination, ospreys breeding in Canadian alpine lakes had relatively high levels of many contaminants. As has been shown in the past for raptors (Elliott et al. 2009), birds feeding at a higher trophic level have higher contaminant loads because of biomagnification through the food chain. After accounting for proximate factors such as trophic level and lipid content, HCB, chlordane, mercury, PCBs decreased with watershed size and toxaphene, DDT, HCB and mercury decreased with watershed size divided by lake size. Large watersheds that drain into relatively small lakes have low levels of contamination, which is opposite of what was predicted. Toxaphene, mercury, HCB, and DDT all decreased with the amount or proportion of glaciation while toxaphene and chlordane also decreased with elevation. Thus, foraging ecology (trophic level) and geography explained the large variation in osprey contaminant levels in western Canada commented on by previous authors (Elliott et al. 2007).

Nonetheless, our results do not support the hypothesis that melting glaciers are a major source of contamination for wildlife in the Canadian Rockies. Were melting glaciers an important source, we would expect lakes in watersheds with large amounts of glacial coverage and at high elevation, close to the icefields, to have the highest levels of contamination as reported for abiotic components (Blais et al. 2001a,b; Bettinetti et al. 2008). We observed the opposite patterns. We suggest that most contaminants are released during the spring snow pack melt and accumulate in lakes. Small lakes and watersheds at low elevations were most heavily impacted, possibly because contaminants were less able to disperse in small lakes and because flow rates are lowest at low altitudes, where the incline is less. Additionally, we found that lakes that were proportionally small relative to their watershed had lower levels of toxaphene, for example. That may be because water from lakes that are closer in size to their watershed area is more stagnant, and thus these lakes accumulate more contaminants than lakes that are smaller relative to their watershed, which may act as intermediates flowing into larger lakes.

3. **Conclusions**

Relatively simple models involving watershed and lake size, elevation, and the percent of glaciation explained much of the variation in contaminant levels. Despite legislation limiting the amount of legacy POPs in the environment, top predators in remote alpine ecosystems continue to accumulate substantial burdens of persistent contaminants. If current climate warmer trends continue, glaciers will continue to melt during the rest of this century. However, our data does not suggest that will lead to higher levels of contamination in alpine ecosystems. We plan to compare our data to trends in Arctic ecosystems, as well.

4. **References**


Henny, C. J.; et al. Polybrominated diphenyl ether flame retardants in eggs may reduce reproductive success of ospreys in Oregon and Washington, USA. *Ecotoxicology* 2009, 18, 802-813.

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Surface water quality and climate change issues: impact of hydrology and temperature on total organic carbon and nitrate in small scale water services

I. Delpla¹, S. Merel², E. Baurès¹, A.V. Jung³, F. Pétavy³, M. Clément¹, O. Thomas¹*

¹ Environment and Health Research Laboratory (LERES), French School of Public Health (EHESP), Avenue du Professeur Léon Bernard - CS 74312, 35043 Rennes Cedex, France
² Department of Chemical and Environmental Engineering, University of Arizona, 1133 James E. Rogers Way, Tucson, AZ 85721, USA.
³ School of Environmental Engineering (EME), Campus de Ker Lann, Avenue Robert Schumann, 35170 Bruz, France

*E-mail contact: olivier.thomas@ehesp.fr

1. Introduction

The main consequence of the actual climate change consists in a global warming of the Earth with a 0.4°C increase of mean temperature up to 2030 [1]. However, climate change is also likely to induce significant aftermaths on water through the enhancement of extreme events like floods and droughts [2-4]. Therefore, research should particularly emphasise on the impact of climate change on water quality. Indeed, declining quality of surface and groundwater resources implies declining quality of drinking water with potential risk for human health [5], particularly in small scale water services supplied from surface water that may have greater difficulties to maintain the required quality standards. The study of relationships between climatic drivers (temperature and hydrology) and water quality is consequently a useful way to assess the vulnerability of surface resources to climate change. This study aims to identify specific conditions that should increase the probability of exceedance of quality standards in drinking water resources used by small water services.

2. Materials and methods

The present study was undertaken at 3 sampling sites located on distinct small rivers used as a resource for drinking water production by small scale water services in Brittany, North-West France. Each sampling point was selected according to various criteria including the vulnerability of the resource, the water treatment plant capacity, and the amount of people supplied. The water quality parameters selected for this study (TOC and nitrates) have been chosen because of main reasons. On the one hand, they are one of the most common parameters for the assessment of water quality. On the other hand they are susceptible to be impacted the variation in hydrology and temperature induced by climate change.

3. Impact of river flow and water temperature on water quality

The impact of flow on water quality was firstly assessed. In order to avoid a huge dispersion of plots, water quality parameters were not directly presented as a function of Q but as a function of log (Q/Qm), with Qm representing the mean daily flow. As indicated in Fig. 1, flow variations have a major impact on water quality. Concentration of TOC goes down when flow increases from very low to medium rates, but it goes up when flow increases from medium to high rates. The opposite pattern was found for nitrate.

In a second time, the impact of river flow on TOC and nitrate was considered in combination with temperature. The vulnerability of surface water with respect to hydrological and thermal conditions was assessed according to the French quality standards for drinking water resource.
As indicated in Fig. 2, the first results show that the probability of exceedance of the French limit of quality for total organic carbon in surface drinking water resources is much higher at low and high flow rates associated, respectively, with high and low temperatures. In the same way, the probability of exceedance of the French limit of quality for nitrate in surface drinking water resources is much higher for medium and high flow rates associated with low temperatures. These results obtained on small rivers are consistent with previous studies conducted on larger rivers [6] [7]. The elevated levels of nitrate in these rivers could be explained by high agricultural pressures on the catchments.

4. Conclusions

The findings of this study should be useful for the administrations in charge of sanitary control but also for those in charge of drinking water production. In fact, the present conclusions should allow anticipating on the adaptation of drinking water treatment based on the thermal and hydrological conditions.

Further research should be undertaken in order to integrate other water quality parameters like microbiological contamination or concentration of micropollutants into an accurate statistical model for water quality assessment.

5. References


Acknowledgements - The authors thanks the French Health and Social Affairs Services (DDASS 35) and the ministry of Environment for the data they provided.
Multi-parametric approach to assess combined effects of pollution and climate change in West African aquatic ecosystems

Awa Ndiaye1,2, Wilfried Sanchez3, Jean D Durand1,2, Héléne Budzinski4, Olivier Palluel3, Khady Diouf5, Pape Ndiaye5, Jacques Panfili1,2

1ECOLAG, UMR 5119, place Eugène Bataillon 34095 Montpellier Cedex5, France
2IRD, UMR 5119 ECOLAG, B.P. 1386, 18524 Dakar, Sénégal
3INERIS, DRC, Unité d’écotoxicologie BP 2, 60550 Verneuil en Halatte, France
4Université Bordeaux 1, LPTC, UMR 5255, 351 Cours de la Libération, 33405 Talence, France
5IFAN, Laboratoire de biologie Marine, UCAD, B.P. 1386 18524 Dakar, Sénégal

E-mail contact: awa.ndiaye@ird.fr

1. Introduction

The complex interactions between climate change and pollutants may be particularly problematic for species living in aquatic ecosystems. In West Africa, increased saltwater or droughts have resulted in increased salinity in estuaries and freshwater ecosystems. For example, in Saloum estuary, salinity can be three-fold higher than in seawater during wet season. Salinity change can influence the species fitness and also causes a variety of physiological responses in aquatic organisms.

In addition to salinity change, environmental contaminants can affect aquatic organisms. Markers such as biochemicals markers are generally used for detecting environmental exposure and their effects on aquatic organisms and are considered such as sensitive tool for monitoring. It is well known that the use of multiple biomarkers is recommended and several factors biotic and abiotic can influence the response of these biomarkers. It is necessary to estimate impact of factor such as salinity change, seasonal variability on biomarker responses.

The first aim of this study was to assess in situ the effects of contamination of multiple sites on the West Africa aquatic ecosystem using a set of biomarker including EROD, GST, TBARS and VTG. These results were analysed in light to contaminant concentrations measured in this area. In addition we examined the relationships between biomarker responses, salinity change and the influence of seasonal changes on these responses.

2. Materials and methods

2.1. Sampling

Tilapia (Sarotherodon melanotheron) was chosen, because it is a species that found in extreme environmental conditions. Eight populations of tilapia were collected in June and October 2009. Theses sites were chosen with different level of salinity and degrees of pollutant contaminant. At each site, salinity was measured in water and PAHs, PCBs and OCPs concentrations in sediment were determined. Fish were weighted, measured, sexed and liver, brain, plasma and otoliths were conserved for analysis (Table 1).

<table>
<thead>
<tr>
<th></th>
<th>Guiers lake</th>
<th>Niayes 2</th>
<th>St Louis</th>
<th>Hann bay</th>
<th>Missira</th>
<th>Foundiouge</th>
<th>Niayes 1</th>
<th>Kaolack</th>
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<tbody>
<tr>
<td>Salinity (psu)</td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>n (M/F)</td>
<td>30 (16/14)</td>
<td>30 (21/9)</td>
<td>30 (15/5)</td>
<td>30 (14/16)</td>
<td>30 (12/18)</td>
<td>30 (12/18)</td>
<td>30 (12/18)</td>
<td>30 (12/18)</td>
</tr>
<tr>
<td>FL (mm)</td>
<td>175 ± 12</td>
<td>133 ± 14</td>
<td>170 ± 25</td>
<td>134 ± 6</td>
<td>129 ± 19</td>
<td>136 ± 17</td>
<td>147 ± 29</td>
<td>130 ± 12</td>
</tr>
<tr>
<td>W (g)</td>
<td>125 ± 26</td>
<td>48 ± 20</td>
<td>98 ± 46</td>
<td>50 ± 8</td>
<td>49 ± 23</td>
<td>47 ± 19</td>
<td>75 ± 52</td>
<td>42 ± 12</td>
</tr>
<tr>
<td>Age (months)</td>
<td>22 ± 8</td>
<td>14 ± 6</td>
<td>22 ± 7</td>
<td>19 ± 12</td>
<td>26 ± 5</td>
<td>8 ± 4</td>
<td>48 ± 14</td>
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<table>
<thead>
<tr>
<th></th>
<th>Guers lake</th>
<th>Niayes 2</th>
<th>St Louis</th>
<th>Hann bay</th>
<th>Missira</th>
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<th>Kaolack</th>
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<td>Salinity (psu)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>n (M/F)</td>
<td>30 (15/15)</td>
<td>30 (8/22)</td>
<td>30 (15/14)</td>
<td>30 (18/12)</td>
<td>30 (18/11)</td>
<td>30 (12/18)</td>
<td>30 (11/9)</td>
<td>30 (11/9)</td>
</tr>
<tr>
<td>FL (mm)</td>
<td>152 ± 18</td>
<td>142 ± 27</td>
<td>146 ± 31</td>
<td>113 ± 11</td>
<td>145 ± 19</td>
<td>129 ± 20</td>
<td>128 ± 19</td>
<td>119 ± 19</td>
</tr>
<tr>
<td>W (g)</td>
<td>80 ± 24</td>
<td>63 ± 31</td>
<td>75 ± 46</td>
<td>55 ± 9</td>
<td>65 ± 25</td>
<td>49 ± 28</td>
<td>46 ± 23</td>
<td>35 ± 15</td>
</tr>
<tr>
<td>Age (months)</td>
<td>12 ± 6</td>
<td>17 ± 12</td>
<td>14 ± 6</td>
<td>9 ± 6</td>
<td>20 ± 12</td>
<td>10 ± 9</td>
<td>10 ± 12</td>
<td>19 ± 14</td>
</tr>
</tbody>
</table>

2.2. Physiological indices and biochemical measurements

CF (Condition factor) and R1 (Grow rate) were estimated. Liver samples was used to measure EROD activity, GST and TBARS according respectively to modified methods of Flammarion et al. [1], Habig et al.
3. Results and discussion

The results obtained showed that PAHs, PCBs and OCPs levels in sediment with a maximal levels of PAHs. The higher PAHs values were found at Hann Bay (5855 ng/g dw) and Foundiougne (14378 ng/g dw). PAHs concentrations measured in others sites were ranging from 12 to 62 ng/g dw. The lowest level of DDT was found in all sites except Niayes 2 (21 ng/g dw). This result showed the recent use of this chemical in Niayes 2. For PCBs, the higher dibenzothiophen concentrations was measured at Foundiougne (51 ng/g dw) and Hann Bay (16 ng/g dw). For the contaminations profile, Hann bay and Foudiougne appear to be more impacted than other sites with the high concentration of PAH and PCB.

Physiological and biochemical parameters investigated in this study (Table 2) showed significant difference among sampled sites (p< 0.05). No gender effect was found for all parameters except for TBARS and AChE in tilapia collected during wet season. All parameters showed seasonal variation.

Table 2: Biomarker responses in tilapia collected at different sites

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Guiers lake</th>
<th>Niayes 2</th>
<th>St Louis</th>
<th>Hann bay</th>
<th>Missira</th>
<th>Foundiougne</th>
<th>Niayes 1</th>
<th>Kaloack</th>
</tr>
</thead>
<tbody>
<tr>
<td>EROD (pmol/min)</td>
<td>2.05 ± 1.16</td>
<td>11.79 ± 5.02</td>
<td>2.11 ± 1.55</td>
<td>22.78 ± 8.54</td>
<td>2.87 ± 1.95</td>
<td>1.73 ± 1.92</td>
<td>1.76 ± 1.64</td>
<td>2.73 ± 2.11</td>
</tr>
<tr>
<td>GST (U/g)</td>
<td>7007 ± 2823</td>
<td>8367 ± 3232</td>
<td>6647 ± 2010</td>
<td>8230 ± 21675</td>
<td>12214 ± 7014</td>
<td>4028 ± 2038</td>
<td>11488 ± 4665</td>
<td>7661 ± 2157</td>
</tr>
<tr>
<td>TBARS (nmol/g)</td>
<td>42 ± 15</td>
<td>86 ± 53</td>
<td>42 ± 15</td>
<td>80 ± 53</td>
<td>46 ± 13</td>
<td>52 ± 25</td>
<td>24 ± 10</td>
<td></td>
</tr>
<tr>
<td>AChE (U/g)</td>
<td>670 ± 180</td>
<td>759 ± 64</td>
<td>725 ± 182</td>
<td>730 ± 107</td>
<td>757 ± 193</td>
<td>604 ± 105</td>
<td>652 ± 109</td>
<td>639 ± 137</td>
</tr>
<tr>
<td>EROD (pmol/min)</td>
<td>5.33 ± 4.32</td>
<td>4.33 ± 3.16</td>
<td>4.30 ± 3.38</td>
<td>13.24 ± 14.71</td>
<td>5.16 ± 13.88</td>
<td>2.05 ± 2.11</td>
<td>2.97 ± 1.71</td>
<td>4.56 ± 2.72</td>
</tr>
<tr>
<td>GST (U/g)</td>
<td>7670 ± 1903</td>
<td>9452 ± 3854</td>
<td>7179 ± 2199</td>
<td>7281 ± 1960</td>
<td>8300 ± 2893</td>
<td>7334 ± 2090</td>
<td>7362 ± 1651</td>
<td>7767 ± 2271</td>
</tr>
<tr>
<td>TBARS (nmol/g)</td>
<td>85 ± 29</td>
<td>55 ± 32</td>
<td>78 ± 45</td>
<td>73 ± 50</td>
<td>96 ± 68</td>
<td>75 ± 35</td>
<td>67 ± 52</td>
<td>39 ± 13</td>
</tr>
<tr>
<td>AChE (U/g)</td>
<td>601 ± 165</td>
<td>671 ± 217</td>
<td>756 ± 242</td>
<td>797 ± 234</td>
<td>664 ± 235</td>
<td>760 ± 143</td>
<td>670 ± 158</td>
<td>689 ± 145</td>
</tr>
</tbody>
</table>

The observed induction of EROD activity in tilapia from Hann bay, may be attributed to the presence of PAH and PCB in these sites. GST activity was lowly different among sites, with decrease of GST activity observed in tilapia from Foundiougne. This low difference may probably due to the presence of mixture chemical at environment be able to cause both induction and inhibition of this enzyme. AChE activity was significantly different among sites. A significant difference in TBARS concentrations was found among sites.

A significant relationship was found between salinity and EROD activity, TBARS and CF. Positive correlations were also found for EROD activity and GST. The result obtained in this study showed that presence of contaminants change levels of biomarker and salinity change appears to have influence on biomarker in tilapia.

4. Conclusions

Integrated approach is very important to assess effect of environmental stressors on wild fish. The physiological and biochemical differences observed showed that both climate change and anthropogenic, industrial and agricultural activities affect these levels in the tilapia from area study. Salinity change is able to influence response of some biomarker and tilapia fitness and complex salinity-pollution affect physiological and biochemical responses in tilapia. Result suggests that environmental factor such as salinity can be considered in biomarker data analysis in estuarine environment.

5. References

Interactive effects of multiple-stressors resulting from habitat degradation and climate change in a model reptile species.

Kurt A Gust¹, Craig A McFarland², Mitchell S Wilbanks¹, Xianfeng Chen¹, Larry Talent³, Michael J Quinn Jr.³, Edward J Perkins¹

¹US Army, Engineer Research and Development Center, Vicksburg, MS, USA. ²US Army, Public Health Command, Edgewood, MD USA. ³Oklahoma State University, Stillwater, OK, USA.

E-mail contact: kurt.a.gust@usace.army.mil

1. Introduction

The ability to assess multiple-stressor effects that result from habitat degradation and climate change is crucial for maintaining viable populations and overall ecosystem health. Habitat degradation may result from a number of causes both as a result of point-source chemical contamination and via diffuse impacts resulting from general human activity and development (ie. urban encroachment and climate change). Chemical stressor effects have been relatively well studied but effects have seldom been investigated in conjunction with common stressors that wild populations must endure. For example, field studies have indicated that malarial infections in reptiles positively correlate with increased temperature (a concern regarding global climate change) leading to infection incidence as high as 50% in some populations[1], and the interactive potential of this projected increase in parasitism in conjunction with additional anthropogenic stressors has yet to be considered.

To approach this question, we have utilized the Western fence lizard (WFL, Sceloporus occidentalis) as a model species to characterize multiple-stressor effects likely to be caused by climate change and habitat degradation including: increased malarial parasitism, decreased basic resource (food) availability and exposure to chemical contamination (trinitrotoluene, TNT) to determine the overall interactive-potential of these stressors. To accomplish this, we are testing the null hypotheses: (1) Multiple ecosystem-level stressors characteristic of habitat degradation and climate change have no interactive effects on lizard health and fitness, and (2) Environmental stressors are uniquely identifiable via genomic signatures and these signatures can be used to identify predominant stressors in multiple-stressor scenarios.

2. Materials and methods

(Methods utilized to test proposed hypotheses):

1. Single-Stressor Effects: The effects of single stressors on WFL was characterized in conjunction with Pairwise-Stressor Effects to establish the clinical effects of each stressor, for development of a next-generation DNA sequencing-enabled WFL bioinformatics knowledgebase used in microarray construction and for characterization of the genomic fingerprint of each individual stressor (Table 1). A total of 29 clinical endpoints were investigated including: survivorship, reproductive condition, hormone tests, sperm test, immune system condition, multiple hematology measures, various blood chemistry parameters and histology. Genomic techniques leveraged clinical results to establish signatures of stressor effects.

<table>
<thead>
<tr>
<th>Food Limitation</th>
<th>MEC Exposure (TNT)</th>
<th>Total Lizards</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 mg/kg/d</td>
<td>5 mg/kg/d</td>
</tr>
<tr>
<td><strong>ad libitum</strong></td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td><strong>Effective Ration</strong></td>
<td>10</td>
<td>10</td>
</tr>
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</table>

<table>
<thead>
<tr>
<th>Malarial Infection</th>
<th>Food Limitation</th>
<th>Total Lizards</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected</td>
<td><strong>ad libitum</strong></td>
<td>10</td>
</tr>
<tr>
<td>MEC Exposure (TNT)</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>Infected</td>
<td>10</td>
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<tr>
<th>Malarial Infection</th>
<th>MEC Exposure (TNT)</th>
<th>Total Lizards</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-infected</td>
<td>0 mg/kg/d</td>
<td>5 mg/kg/d</td>
</tr>
<tr>
<td>Infected</td>
<td>10</td>
<td>10</td>
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</tbody>
</table>

Table 1. Sub-tables A-C represent experimental designs for 3 independent stressor-assessment bioassays investigating the effects of 3 individual stressors and the 3 pairwise comparisons among stressors in Western Fence Lizard.
2. **Pairwise-Stressor Effects:** To minimize animal exposures and eliminate batch effects, pairwise-stressor bioassays were conducted in conjunction with Single-Stressor tested using a completely randomized experimental design and factorial treatment arrangement (Table 1). This provided a statistically robust test of interactive potential among paired stressors in clinical trials and microarray analyses.

3. **Multiple-Stressor Effects:** Leveraging genomic signatures of single-contaminant exposures and the characterized pairwise-stressor effects, the effects of a complex stressor matrix will be used to test if the accumulated genomic database for WFL can be used to identify predominant stressors / stressor combinations in a single blind test.

### 3. Results and discussion

The first hypothesis was tested by evaluating stressor impacts on the 29 toxicological endpoints investigated in clinical toxicology bioassays characterizing single-stressor and pairwise-stressor effects. Although few interactive effects were identified, three interactive effects of potential ecological concern were observed: (1) food limitation may eliminate hormesis of TNT on animal weights (Figure 1), (2) combined food limitation and malarial infection may impact immune response (Figure 2) as well as (3) impact testes size and quality (not shown).

To test the second hypothesis, we utilized next-generation DNA sequencing to develop a de novo bioinformatics infrastructure and high-density microarray to assess transcript expression for Western fence lizard. GS-FLX pyrosequencing yielded 329 megabases in 929K reads with 354bp average read length. A genome-scale transcriptome for WFL was used for EST-based clustering and assembly via The Gene Indices Clustering Tools (TGICL). In all, 53,897 contigs and 5,065 singlets totaling 58,962 unigenes were identified. Approximately 44 % of unigenes were annotated for protein-coding potential via homology-based annotation. The WFL transcriptome was transitioned to Agilent G3 Custom 60K oligonucleotide microarray for transcript expression analysis (Figure 3). The tools are being used to determine the genomic signature for each stressor and stressor interaction utilizing observed mechanistic and metabolic impacts as the foundation for signature development.

### 4. References


**Acknowledgement** – Fundng from US Army 6.1 Environmental Quality on Installations Research Program
Climate change damage functions in LCA – (1) from global warming potential to natural environment damages

Ingeborg Callesen1,2, Hauschild, M.Z.1, Bagger Jørgensen R.2, Olsen, S.I.1 and Beier, C.2

1DTU Management Engineering, Section for Quantitative Sustainability Assessment, Productionstorvet, Building 426, DTU, DK-2800 Lyngby, Denmark
2Risø-DTU, Biosystems Division, Frederiksborgvej 399, DK-4000 Roskilde, Denmark
E-mail contact: inca@man.dtu.dk

1. Introduction

Climate change will alter the environmental conditions for terrestrial as well as aquatic ecosystems. Individual species and populations will experience new and stronger stress impacts, in some cases beyond their adaptive capacity. This may lead to loss of fitness, die-back and extinctions (1). Other plants and organisms may acquire competitive strength outside their current geographic distribution range and become successful invaders. Significant changes in ecosystems across current bioclimatic vegetation zones are foreseen e.g. in Dynamic Global Vegetation Models (2). Ecosystem services (i.e. provisioning, supporting and regulating services) may depend on stable, undisturbed ecosystems, but some of these services may also be robust in a changing and disturbed environment.

In this paper we address this ambiguity by introducing differently shaped hypothetical climate change damage functions in life cycle impact assessment (LCIA) for terrestrial ecosystems. Here, ‘damage’ is not a definitive term, but vaguely described as environmental change or rate of environmental change referring to the Area of Protection “Natural environment” considered in LCIA. Changes (and damages) to our natural environment are a result of industrialisation including homogenization and intensification of agriculture and forestry, mining and fishing industry. These changes are directly and indirectly driven by global population and wealth increases.

From this multitude of interacting drivers, the direct effects of climate change expressed as damages to the natural environment cannot easily be singled out. For a start, scenarios are required that build on assumptions of socio-economic and technological developments such as the Millennium Ecosystem Assessment (3) and IPCC’s Special Report on Emission Scenarios (4), or (now more freely defined) as representative concentration pathways for atmospheric greenhouse gases (5). Fig. 1 shows the route from product-related GHG emissions to future climate damages. In the life cycle inventory (A), emissions of greenhouse gases are assessed and converted into the midpoint indicator ‘global warming potential’. Scenarios of cumulative GHG emissions (B) over the next 100 years range from about 700 to 2500 Gt C (~2.6 to 9.1 Tt CO2) depending on scenario assumptions (4).

The resulting climate changes from global atmosphere-ocean general circulation models followed by regional climate models depict changing means and variances of temperature, precipitation, and climate extreme events. The climate change related environmental damages (C) depend on specific sensitivities of organisms and ecosystems. Climate – carbon cycle feedbacks are an integrated part of C (Fig. 1).

The responses of terrestrial ecosystems to multi-factorial changes in the growth environment for plants and other organisms are widely unknown. So is their ability to adapt to these changes. They are therefore studied theoretically, e.g. (2,6), but also experimentally. Effects may be additive, or include antagonistic or synergistic interactions. State-changing tipping points may be surpassed. It is thus clear that the impacts from a product system being studied in an LCA must be seen on the background of the changing future background situation.

Figure 1: From GWP (midpoint) to damage on the natural environment (endpoint). CV ~ coefficient of variation ($\sigma/\mu$) see Fig 2.

$$CV^2(A*B*C)=CV^2(A)+CV^2(B)+CV^2(C),$$ assuming (incorrectly) that A,B and C are not correlated.
Here, we study the influence of the uncertainties of the LCI itself, of future human development scenarios, and of climate-biosphere responses in climate damage functions.

2. Results

2.1. A set of differently shaped climate damage functions

At this point in time the magnitude of the future emissions and their effects are unknown. The uncertainties in the biological and biogeochemical responses to climate change may be illustrated by differently shaped damage functions, where relative damage is a function of cumulative CO$_2$ emission up to the year 2100 (Fig. 2). Next to a linear response (F1), also regressive (F2, F3) and partly progressive (F4) damage functions are sketched and two more extreme cases showing a very sensitive (F5) and a very robust (F6) response are shown. The CV (vertical variation, $\sigma/\mu$) of the relative damages varies at different levels of emission (138%, 64%, 16% and 15% for the cumulative emission scenarios of respectively 700, 1000, 1500 and 2500 Gt C in year 2100) (4). The range represents the total emission range of the 40 SRES scenarios from the lowest (B1) to the highest cumulative CO$_2$ emission (A1F1) (4).

![Figure 2: Climate damage functions expressing sensitivity (of a species, process, or other) as a function of total CO$_2$-C emission (methane and nitrous oxide not included)](image)

2.2. Sensitivity of the climate damage characterisation factor

The relative variation ($CV^2$) in characterisation factors for the impact category global warming will be the sum of the squared CV’s in Fig. 1 assuming (incorrectly) that A, B, and C are not correlated. The emissions estimate for the individual product (depending on the system, typically 30% on the LCI), the global emissions (55% on the emission scenarios) and the uncertainty in Fig. 2 in estimating ecosystem damage (15% - 100%) yields a CV of at least 64%-152%. The global warming potential (GWP) of a product life cycle is extremely small in comparison with the cumulative GHG emissions of different global future scenarios, Fig. 1. The GWP at the midpoint constitutes a fraction of $10^{-18}$ to $10^{-12}$ of the cumulative emissions in 2100 for a midpoint GWP in the range 1 g to 10$^5$ g CO$_2$-eq. The differently shaped damage functions will be linked with the currently established evidence for climate change damages to particular biomes, and one or more common metrics for this will be developed. With this approach no incorrect precision or detailed cause-effect chain is postulated. The characterisation factors will depend on the shape of the chosen damage function.

3. References


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Ship based oil spill monitoring, a new integrated system for thickness estimation and operational overview

Håkon Skjelten¹, Christian Gutvik² and Lars André Solberg³

¹Aptomar AS, Ulstadlekkveien 22, 7042 Trondheim, Norway
²Aptomar AS, Paul Fjermstads veg 15, 7052 Trondheim, Norway
³Aptomar AS, Fridtjof Nansens vei 21B, 7020 Trondheim, Norway
E-mail contact: hakon.skjelten@aptomar.com

1. Introduction

The increasing number of offshore oil wells entails challenges for protection of marine life and environment as well as safety of personnel working in the field. History has shown us the serious consequences of uncontrolled oil spills at sea, and it is an important responsibility for the industry to reduce this risk. Oil spill detection and monitoring have proven to be a difficult task. A rule-of-thumb is that approximately 90% of the oil is located at 10% of the area. The rest of the area is a thin oil film that is usually non-recoverable, implying that thickness estimation is crucial information for an efficient recovery.

Different oil spill sensor types have their own strengths and weaknesses. Up to now the most common sensor platform has been oil spill radar, which uses signal processing to evaluate reduced signal clutter. They are fairly easy to operate as the image is referenced to the ship and its orientation. The presentation appears similar to conventional radars which have been standard equipment on ships for decades. Oil spill radars needs certain wind speeds / sea waves to get a good image and can currently only measure the complete oil spill area and not the spill’s thickness.

Infrared (IR) sensors, combined with signal post-processing, can sense oil spills on sea water. This sensor has the advantage of measuring relative thickness of the oil spill, and has thus been valued for having complementary properties to the radar. Neither is it dependent on waves. Traditionally such sensors have been airborne, giving difficulties in interpretation of the information at the vessels executing the operation. Airborne assistance is normally also a limited resource. Additionally, all sensor types are subject to noise and mis-interpretation when used as standalone equipment.

The presented system is an oil spill monitoring system which is built around a motion-stabilized, ship mounted sensor system. The intention of the system is to integrate all available oil spill sensors in a common reference frame, namely in an Electronic Chart System (ECS), combined with a camera angled perspective, for improved operational overview. The main purposes of the system are to:

- Give an overview of oil spills in the sea chart, including thickness/volume estimation, position, area and drift
- Show boom formation and possible boom leakage in the sea chart for improved formation analysis
- Document all events for later evaluation

2. Methods

The system consists of:

- A motion stabilized pointing unit with a cooled IR camera, algorithm database for IR measurement analysis, daylight camera and Xenon search light.
- Integration interface towards radar based oil spill sensors
- Bridge console based on an ECS where the sensor information is displayed directly in the chart and/or projected through the camera view

The system combines motion and position information from the vessel's navigation instrumentation and its own orientation sensors to determine the exact position and direction of the cameras in real time. This allows the system to have continuous motion stabilization in 3D and calculate a geo-referenced video stream, which again allow sensor information to be transposed from camera view to chart and vice versa.

The crew of a ship is familiar with ECS and uses it regularly for navigation and everyday operations. Several other sensor sources can already be displayed in the ECS, like Automatic Identification System (AIS) and Automatic Radar Plotting Aid (ARPA). In other industrial applications it has been successfully demonstrated how sensor data or other computer generated graphics can be displayed as overlays in camera perspective.
video. The combination of camera views and sea chart is believed to greatly increase the operational overview and improve interpretation of complementary sensor systems in oil spill monitoring.

3. Results

Figure 1 shows an image taken with the IR camera at NOFO's Oil-On-Water 2009 exercise. While any blue shine or metallic oil films covering the area are invisible for the IR sensor, thicker parts of the oil spill is visible as a darker area. As the oil thickens closer to the boom it is displayed as a brighter area.

![Figure 1 Oil skimmer in boom with remaining oil. Image taken at night in complete darkness](image1)

![Figure 2 Radar sensor complementing IR in both video view and sea chart](image2)

Figure 2 shows oil spill radar detections integrated into the user interface, here displayed as pink areas. These areas are then directly comparable to the oil spill detected by the IR based sensor, both from top view in sea chart and through perspective in the video view. Overlay comparison between radar and IR greatly helps classifying recoverable from non-recoverable oil spill, and the combined view helps the information interpretation needed for operational decisions.

Figure 3 shows how a third ship surveyed two other ship exercising boom formation. Notice how the video is transposed onto the sea chart for easy evaluation. This video where used for documentation and quality control while the boom equipment where tested. Figure 4 shows distinct dark areas behind the boom indicating oil leakage. The system was in this scenario used to conclude that the vessels dragging the boom maintained too high speed.

![Figure 3 Boom formation in video and sea chart](image3)

![Figure 4 Oil leaking from boom due to high speed](image4)

4. Discussion

The system is demonstrated to help identification and localization of recoverable oil. Traditionally, air borne cameras and other non-ship based sensors have been used as complementary information to radars. The drawback of these systems is that the information is not available in real-time or directly comparable and is thus not suited for on-scene guidance. With the presented system, sensor data are displayed directly in the combined ECS/camera interface in real-time. The crew can now prioritize and manage the resources at sea more efficiently for oil spill responses, independent of sea and light conditions.
Effects of acute oil spills on the Norwegian marine environment

Stepan Boitsov¹, Bjørn Einar Grøsvik¹, Sonnich Meier¹, Jarle Klungsøyr¹

¹Institute of Marine Research, PB 1870 Nordnes, N-5817 Bergen, Norway
E-mail contact: stepan@imr.no

1. Introduction

A number of small-to-medium size acute oil spills have occurred in Norwegian waters in the latter years. The Institute of Marine Research (IMR) has performed damage assessment studies after several recent oil spills. Measurements of the levels of oil-related organic contaminants have been carried out in seawater and various biota samples after the spills, with follow-up investigations undertaken later when necessary. The objectives of the studies were to assess the degree of contamination in the marine environment, to monitor the contamination with time, and to quantify the possible damage inflicted on shellfish and commercially important fish stocks.

Oil spills taking place at three locations in Norway in the period 2007-2009 will be discussed:

1. Cargo vessel Server, ran aground near Fedje, Hordaland in Western Norway in January 2007. The total amount of oil spilled to the environment was 380 tons.
2. The discharge of 4000 tons crude oil at the Statfjord A oil platform in the Tampen region of the North Sea was the second largest discharge till now in Norway after 40 years of exploration. A second, minor spill of 70 m³ oil occurred at the same platform in May 2008.
3. Cargo vessel Full City ran aground near Såstein, Telemark in Southern Norway in July 2009, spilling 300 tons of oil. The spill has caused a considerable contamination of the nearby coastal area. The study of the consequences of the spill was one of the most detailed undertaken in Norway.

2. Materials and methods

Sampling was carried out at several locations in the vicinity of the oil spills and at reference locations unaffected by oil 1 to 5 weeks after the spills. Follow-up samplings were carried out 4 and 8 months after the accident in case of Full City spill. Geographical location of the spills is shown in Figure 1.

![Figure 1: Geographical location of the oil spills.](image)

Samples of seawater and fish were collected after each accident, while samples of shellfish, crabs and/or shrimps were collected in coastal areas. Seawater was analysed for total hydrocarbon contents (THC). Fish muscle and liver and shellfish/crab/shrimp meat were analysed for polycyclic aromatic hydrocarbons (PAH). Fish bile was analysed for PAH metabolites. Biomarker analyses were carried out on fish samples in the case of the Staffjord A spill.
3. Results and discussion

3.1. Server

Levels of THC in water and PAH in fish liver were only slightly elevated in the samples from the contaminated area, while fish muscle and bile, crab and shellfish exhibited no apparent contamination. Poor weather may have contributed to the rapid weathering of the oil. However, codfish trapped in the net in the contaminated area for several days have shown strong contamination in liver and bile, indicating a high degree of exposure.

3.2. Statfjord A

Levels of THC in water were elevated right after the accident but were back to normal within a month. Levels of PAH in fish liver was found to be elevated in some species after the accident. Levels of PAH metabolites in codfish bile as well as the biomarker response were studied in fish sampled one month after the accident and no significant differences were found. The condition of the environment thus seemed to be back to normal within a short time after the discharge, possibly aided by the strong storms in the area at the time of the spill. No negative effects were found after the second spill in May 2008.

3.3. Full City

Fish liver and water samples from the contaminated area exhibited moderately elevated levels of PAH and THC. No significant contamination was found in crab meat or in fish from outside the immediate vicinity of the shipwreck. However, the oil contaminated a large part of the coast nearby. Mussels collected at the most contaminated coastal site had strongly elevated PAH levels, above 10 μg/kg wet weight benzo[a]pyren, which is the food safety limit set by EU authorities for mussels [3]. A consequent study of the area 4 months after the spill has revealed a return to background levels in fish, shrimp and water, but not in mussel samples from the same area. A more detailed study of mussel samples, and a new sampling of mussels along the coast of South-Eastern Norway 8 months after the spill, demonstrated the contamination to be largely due to non-oil related sources.

4. Conclusions

The results of these studies largely lead to the same conclusions. In all cases, an increase in oil-related contaminants was demonstrated in various compartments of the environment. The levels in the water and mobile biota were not alarmingly high and went relatively quickly back to background levels. The highest levels were found in the organisms that could not escape from the contaminated area (trapped fish, mussels). The degree of contamination caused by the spill to the marine organisms further depended on the external factors such as the weather during the spill or the pre-existing contamination from other sources.

5. References


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Effects of oil and oil treated with dispergent on the arctic amphipod *Gammarus setosus*

Jan Fredrik Børseth\(^1\), Thierry Baussant\(^1\), Liv-Guri Faksness\(^2\), Bjørn Henrik Hansen\(^2\) Anna Ingvarsdottr\(^1\), Claudia Lucas\(^1\), and Anne Helene S. Tandberg\(^1\)

\(^1\)IRIS Biomiljø, Randaberg, Norway
\(^2\)SINTEF Materials and Chemistry, Trondheim, Norway
E-mail contact: jan.fredrik.borseth@iris.no

1. Introduction

When oil is released in an area with ice we will face a complex interaction between oil, water and ice. The oil will be absorbed by snow on the ice edges, it may be trapped in the ice in brine channels and it may be moved underneath the ice. The ice field as such will also be under constant transformation driven by wind, currents and temperature. Some ice floes may be transported relatively far from their original position and relatively far from their original neighbors. Altogether this may be a strong driving force for drift and spread of oil after oil has been released in an ice field.

These processes have been followed during an offshore field experiment in the marginal ice zone East of Svalbard in May 2009 [1] and basic field data have been used to accomplish a realistic and relevant exposure study in the laboratory. The results on body burden and biological effects of oil and oil treated with a dispergent on arctic amphipods are reported here.

2. Materials and methods

Our test species was *Gammarus setosus* Dementieva 1931, a gammarid amphipod very common in the intertidal zone of Svalbard [2]. The specimens were collected from Adventsfjorden in Longyearbyen Svalbard. All specimens were handpicked and transported to Stavanger in cooled thermoses in filtered and cooled seawater from the UNIS lab in Longyearbyen.

A crude oil from Troll (North Sea field) was used since much information exists from previous studies with this oil. The Corexit 9500A was selected as dispersant since it is much used (e.g. Gulf of Mexico). The intention of the study was to examine the effects of the water soluble fraction of oil versus oil chemically treated with a dispergent.

The different exposures were achieved by carefully adding oil, oil and dispergent or nothing (control) to the header tank (Figure 1). The oil was added in such a manner hat it formed a slick on the entire surface in the header tank. Experiments were run with 2°C seawater. At T12 all aquaria from the exposure were cleaned externally, and placed in a clean water bath and with continuous clean seawater supply for recovery.

![Figure 1: General view of the experimental set-up. A total of three header tanks was used and each header tank fed three exposure aquaria.](image)

Seawater was sampled at T0, T6, T12 and T25 for chemical analyses. Animals were sampled at T0, T12 and T25. All specimens were wet-weighed and measured dorsal length before retrieving other samples. Analytical methods were body burden (PAH), lysosomal stability (NRRT) [3], gene expression, histopathology and malondialdehyde (MDA; antioxidant).

3. Results and discussion

Chemical results show that there were less oil droplets in the water phase of the oil+dispergent treatment since levels of napthalenes are high and levels of 2 to 3 ring polycyclic aromates are low.
One of the more clear biological effect pictures is provided by the lysosomal stability (Figure 2). The combination oil+ dispergent is much worse for the animals than both oil alone and clean seawater. Between oil and clean water there are no significant differences on this parameter, but there is a significant difference between the control group and the oil+dispergent.

![Figure 2: NRRT for Gammarus setosus in control (green), oil (yellow) and oil+dispergent (orange) from the start (T0), the end of the exposure (T12) and the end of the recovery in clean water (T25). Error bars give standard deviation for the measurement, columns give average NRRT for the group, 10 individuals on all occasions except T0 (15 individuals).](image)

Chemical analyses show that the concentrations of oil components are relative low compared to concentrations tested in similar experiments, but the lethality registered during this exposure experiment is surprisingly high. However, the amphipods used in this study are taken directly from their natural cold water habitat in Svalbard and thus probably well acclimated for managing a relative high metabolism. This means they might be efficiently producing toxic metabolic intermediates of PAHs which could explain the lethality observed.

4. Conclusions

Chemical results from analyses of water samples of the laboratory exposure experiment showed good correlation with identical data monitored during the offshore field experiment with oil in ice infested seawater. Due to the low input of energy during the exposure period in the laboratory study, there were no indications of oil droplet formation in the water fraction caused by the dispergent. However, levels of naphtalenes were relative high. Among the effect markers, general stress was indicated by a significant decrease in lysosomal stability of amphipod haemocytes in the oil+dispergent treatment.

5. References


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Ecotoxicology and risk assessment of crude oil spill in a palm oil plantation

Ikechukwu N.E. Onwurah1, Olawale Otitoju2, Samuel Ubani1 and Mary Iroegbu3

1Pollution Control and Biotechnology Unit, Department of Biochemistry
University of Nigeria, Nsukka, Enugu State, Nigeria.
2Department of Biochemistry, University of Uyo, Akwa Ibom State, Nigeria
3Department of Plant Science and Biotechnology, Abia State University, Uturu, Imo State, Nigeria
E-mail contact: ikechukwu.onwurah@unn.edu.ng

1. Introduction
Crude oil pollution on land affects both the biophysical and biochemical properties of agricultural soil [1] which results into a shift in nutrient cycle and balance. Microbial population and other soil fauna in the soil that provide adequate nutrients and stored energy for primary productivity are equally affected. Hence the ecological effect of the massive crude oil spill that occurred in October 03, 2006 on a parcel of land having nine thousand, five hundred matured palm oil trees was assessed using relevant valued ecosystem components (VECs).

Biochemical end point parameters of the VECs were analysed from both laboratory and field study of the spill environment. The risk assessment was based on the toxicity of the spilled oil on lumbricus species and Azotobacter vinelandii in the soil ecosystem.

Biomass in terms of the concentration of chlorophyll \(a\) is one of the most widely accepted methods in the study of biological production as it indicates total plant material available at primary level of food chain [2], as well as toxicity [3]. Hence the loss in productivity of the palm oil plantation was based on the spatial and temporal variation of chlorophyll \(a\).

Objective of this study:
This study was carried out to ascertain the long term effect of the massive oil spill that occurred at Owasa in October 2006 on the productive capacity of the affected palm oil plantation.

2. Methodology

2.1. Study area
The study area, a parcel of land at Umuololo, Owasa-Asa, Ukwa Local Government Area of Abia State, Nigeria is located within latitudes 7°28‘ E and 7°32‘ E, and longitudes 6°26‘ N and 6°29‘ N and measuring 156.31 acres. It contains nine thousand, five hundred (9,500) palm oil trees at maturity.

2.2. Soil sampling and characterization

The palm oil plantation was divided into four (4) transects (A, B, C and D) from where the soil samples were collected in February 15, 2007. At each sampling location, a quantity of soil was taken at four (4) different depths (0 - 0.5; 0.5 - 1.0; 1.0 - 1.5 and 1.5 - 2.0 m), using hand augur. A total of 100 soil samples (4 × 25) were collected. Unpolluted soil samples were also collected from an adjacent palm oil plantation, about 100 metres away. All the soil samples were delivered to the laboratory in sterile labelled black polyethylene bags and subsequently dried under mild condition in the laboratory. Stones and debris were removed, ground with mortar and pestle, and then sieved through a 2.0 mm diameter sieve. Soil parameters were determined, using standard methods.

2.2.1. Azotobacter isolation and distribution

Average number distributions of Azotobacter \(spp\) present in the crude oil-polluted palm oil plantation were determined in the four transects (A, B, C and D) as described by Dicker and Smith [4] with the view of predicting the effect of the crude oil spill on the nitrogen fixing capacity of the soil cores when compared with control.

2.2.2. Crude oil concentration and distribution pattern in the palm oil plantation
Soil samples randomly taken from the four transects (A, B, C and D) of the crude oil-contaminated palm oil plantation and reference (control) uncontaminated palm oil plantation were analysed for total petroleum hydrocarbons (TPHC), using solvent extraction method developed by the Department of Toxic Substance and Control, California, USA [5].

2.2.3. Earthworm distribution pattern in the palm oil plantation

During soil sample collection from the crude oil contaminated palm oil plantation, five randomly selected one square metre (areas) of soil samples were escarvated to a dept of 5.0 cm and put into metal trays and native oligochaetes searched for. The total number of oligochaetes isolated from the crude oil contaminated transects and control site were counted and weighed.

2.3. Bioassays

2.3.1. TPHC bioaccumulation in isolated earthworms

All the isolated native oligochaetes from the transects and control sites were transported in different wet earthen pots, each containing soil at 20% water saturation. Some of the oligochaetes were homogenized and the TPHC extracted to determine bioaccumulation pattern.

2.3.2. Nitrogen Fixation in Soil Cores

*Azotobacter* cell-free extracts from soil cores were incubated under nitrogen for 60 minutes after the addition of sodium pyruvate. The total nitrogen fixed as ammonia was assayed by the indophenol reagent method, against a calibration curve.

2.3.3. Chlorophyll content and other growth parameters of plants:

The chlorophyll content analysis of definite weights of fresh palm oil leaves taken from the transients (A, B, C and D) of the oil-contaminated palm plantation and control were extracted with 90% acetone and estimated using an adapted spectrophotometric method described elsewhere [2].

3. Conclusions

The crude oil spill incident at Owasa resulted in a very significant negative impact on the entire ecosystem as was demonstrated in the selected/relevant valued ecosystem components, including the palm trees. One year after the oil spill incident in the palm oil plantation, the following lines-of-evidence demonstrated that there was and is still a significant impact of the spill on the palm oil plantation.

- Fresh earthworms exposed to the contaminated soil samples still bioaccumulate PHC in their body tissues even after one year of the spill incidence. This shows that the residual PHCs are still negatively impacting the ecosystem as a whole.
- As at November 2007, the chlorophyll content of the palm trees was still observed to be significantly low relative to control.
- Defoliation and continuous chlorosis of the leaves of the palm trees.
- Deficiency in nitrogen content in soil core relative to control sites.

References

Deriving disaster impact distances for ‘Seveso’-companies in relation to protected nature areas

Ellen Brand¹, Leo. Posthuma¹, Dik van de Meent¹, Nico van den Brink², Henri den Hollander¹ and Dick de Zwart¹

¹National Institute for Public Health and the Environment (RIVM), P.O.box 1 3720 BA Bilthoven, Netherlands.  
²Alterra, P.O. Box 47, 6700 AA Wageningen, Netherlands  
E-mail contact: ellen.brand@rivm.nl

1. Introduction

After the disaster at Seveso Italy in 1976 legislation was developed to protect humans and the environment from industrial accidents. This ultimately resulted in the development of the so called Seveso II directive (Council Directive 96/82/EC on the control of major-accident hazards). EU-member states are obliged to bring this directive into force in the national laws. In the Netherlands this resulted in the Regulation to assess distances to nature areas. This regulation is in short called Reban and became active in 2006.

Reban states that there needs to be a “sufficient distance between heavy industry and vulnerable nature areas in case of an industrial disaster”. To determine this distance there “needs to be an appropriate tool that can determine distances between the industry and nature” [1]. The Dutch government would provide such a tool to execute risk analyses and the National Institute for Public Health and the Environment (RIVM) was asked to develop this tool.

2. Modelling and Vulnerability analyses

The so called Seveso companies working with the most hazardous chemical compounds require a permit for new installations or expansions of facilities when positioned in the vicinity of protected nature areas. These nature areas are specifically protected under the Nature protection act and include Natura 2000 nature areas.

A risk analyses must make clear, that sufficient distance is kept between the facilities and the nature areas in case of an industrial disaster. A tool to assess the distance between industry and nature has to contain fate and effect assessment, to deliver critical distances when combined with regulatory acceptance criteria on maximum allowable exposures. Comparison of a critical distance with the geographical distance between facility and nature area provides insight in the matter of sufficient distance.

The RIVM was asked to develop an appropriate tool to comply with Reban because of its previous experience with the development of a similar tool called FEAT. FEAT, which stands for “Flash environmental Assessment Tool”, is a tool that identifies acute environmental effects immediately following (industrial) disasters [2]. In view of its scientific principles and its pragmatism for use in the field, FEAT is now used by the UN around the world as a first tier assessment tool for environmental disasters (e.g., landslides, tsunami’s) involving secondary release of toxic compounds.

The newly developed Reban tool uses the same basic knowledge and principles as used in FEAT. The conceptual model of the Reban tool consist of the international recognized source-path-receptor model. The Reban model is needed in the format of a modeling tool (and not as a simple decision matrix), because the Seveso companies in the Netherlands are all situated within approximately 15 km distances from protected Nature areas.

2.1. Fate and effect modelling

The fate and effect modelling within the Reban tool exists out of three separate models to simulate the fate of chemicals in air, water and soil. The model for soil is still under development and will not be discussed here.

2.1.1. Air

The air compartment is modelled by use of the Gaussian plume model. By use of preset worst case meteo conditions, substance specific parameters and regulatory acceptance criteria, air concentration patterns can be calculated, as illustrated in Figure 1. The model predicts which concentration of compounds can be expected at which site, after a certain release of the compound.
2.1.2. Water

For the water compartment the model Proteus II is used. Proteus II is also a Gaussian plume model. It predicts how an initial cloud of contaminated water is transported in surface waters, like rivers, channels and lakes, by dispersal of the compounds both in longitudinal as well as transversal ways. Sensitivity data for the species are available for a subset of compounds, and pertain to the well-known ecotoxicity test data for algae, daphnids and fish, or they are derived in a similar way for other Seveso compounds. By combining exposure plume data, sensitivity data, and the regulatory criteria, we were able to calculate critical effect distances for exposure through water.

2.2. Vulnerability analyses

The vulnerability analyses within the Reban tool were developed by the Research institute Alterra. They consist of a decision tree for aquatic and terrestrial animals. With use of these trees it can be determined if flora and/or fauna is actually vulnerable for the chemical that is released in case of an industrial accident. The vulnerability analysis consists of two types of exposure. The first exposure is short term exposure via non-persistent compounds in which effects are accepted as long as rehabilitation can occur within one year. The second exposure is long-term exposure which always leads to environmental risk. Further aspects of the vulnerability analyses are the possibility of an organism to repopulate the area from a neighboring population within one generation and the reproduction strategy of the organism.

3. Results

Exposure patterns in water and air were predicted for > 100 compounds, so that distributions of critical distances could be explored for realistic disaster scenarios. This was necessary, because – in the Netherlands – environmental regulations must be ‘tested’ before they are implemented. That is: RIVM must show what implementation of Reban would imply both for Nature protection as well as economically (refusal of permits to expand facilities); RIVM itself does not choose, however, for policy criteria – RIVM quantifies consequences.

4. Conclusions

In view of the small distances between Seveso facilities and protected Nature areas in the Netherlands, the Dutch national government requires a refined tool for disaster risk assessment. The tool should enable governments to decide on requests for permits, asked for establishment or expansion of such facilities.

A tool has been developed, and tested in part, for a set of > 100 compounds, and resulting in critical effect distances between facilities and protected Nature areas. As yet, the regulatory decision criteria have not yet been established at the time of writing. However, a suite of results will be shown, illustrating that there is an intricate balance between nature protection and stored volumes of dangerous goods.

By Reban, moreover, it is clear that environmental protection does not only require consideration of chronic emissions. It also requires an appropriate assessment of disaster scenario’s. This in order to avoid that a very succesful policy in reducing chronic emissions is accidentally ‘overruled’ by a major disaster which is harmful to (almost) all species acutely.

5. References

Sensitivity shift in *Daphnia magna* population response caused by size selective predation

André Gergs, Stefan Riehm and Thomas G. Preuss

RWTH Aachen University, Institute for Environmental Research, Worringer Weg 1, 52074 Aachen, Germany

E-mail contact: andre.gergs@bio5.rwth-aachen.de

1. Introduction

The quest for more realism in ecological risk assessment demands for looking beyond ecotoxicological standard tests by additionally considering e.g. population structure, intra- and interspecific competition or predation. To tackle this task, effects observed on individual level (e.g. survival, growth, fecundity) have to be extrapolated to population level, which is the primary aim of protection [1]. Moreover, we have to take the interaction of populations into account.

Predation is considered to be a major factor controlling diversity, dynamics and structure of freshwater communities [2]. The outcome of predation varies with both prey size and predator size, thus mostly appears to be size selective. For example early instars of the insect predator *Notonecta maculata* mainly feed on small crustaceans, whereas later instars prefer larger prey [3], thus it can be expected, that instars differ in the way they alter prey populations.

*Daphnia magna* is one of the most important test organisms in ecological risk assessment of pesticides, biocides, and industrial chemicals. Sensitivity to toxicants of individual *D. magna* varies with body size as shown e.g. for Nonylphenol (Np) [4]. Consequently, in population tests small daphnids will be more severely affected than larger individuals.

In order to analyse combined effects, we simulated the population dynamics of *D. magna* under toxic stress (p353-Np) and predation by the backswimmer *N. maculata*. The model output was subsequently compared to laboratory data. Thereby, special attention was drawn to the size selectivity of *Notonecta* and the size structure of *Daphnia* populations.

2. Materials and methods

For modelling *D. magna* population dynamics we used an individual based population model (IDamP) which includes food availability, crowding and a size depending dose response relationship for p353-NP [4, 5]. Predation of individual *N. maculata* was modelled using a mechanistic model including prey size selectivity of different backswimmer instars, satiation of predators, light regime and growth [3, 6]. An overview on processes is given in Fig 1. Both of the models were already shown to adequately predict different scenarios.

![Figure 1: Conceptual diagram of the asexual life cycle of Daphnia magna. Rectangles indicate processes on the individual level and queries are expressed in rhombi (left); and processes of the Notonecta maculata foraging model on the base of a general predation cycle. The rectangles illustrate the partition of time available to forage, rhombi indicate individual level queries and rounded rectangles demonstrate partitioning of the gut. Solid lines mark the decision tree whereas dotted lines indicate the passage of food extracted from prey items, modelled as a background process(right).](image-url)

In the laboratory, we tested the effect of p353-Np, in a concentration of the EC50 value for neonates, on populations of *Daphnia magna* under predation pressure. Experiments were carried out in 20 liter aquaria during a period of 11 weeks. Population dynamics of *D. magna*, including size structure of populations, were recorded for 4 different treatments: {1} control populations, {2} populations under predation (N), {3}
populations exposed to two peaks of Nonylphenol (Np) and (4) populations under predation and exposed to two Np peaks (Np-N). Single larvae of the backswimmer Notonecta maculata were placed in prepared aquaria after D. magna populations reached peak abundance (day 34). During test backswimmers grew from first to fith instar. Two-day peaks of Nonylphenol were given on day 41 and 69.

3. Results and discussion

In both, modelling approach and laboratory study predation was found to significantly alter Daphnia population size and structure (Fig 2). Since size selectivity differs in Notonecta instars, the impact on Daphnia populations changed during development of the predator and was highest in large backswimmers. Under predation of larger backswimmers, size structure of Daphnia population shifted towards higher number of neonates (<1.4 mm) and lower numbers of adults (>2.6 mm) compared to control. Within a period of 14 days after first Np-treatment Daphnia total abundance exceeded control level due to higher number of neonates. The combined effect of predation and Np finally led to the extinction of D. magna populations after the second Np peak, whereas total abundances of other treatments were similar to control.

4. Conclusions

Predation significantly affects population structure of Daphnia magna. The shift in population structure towards smaller Daphnia caused by predation subsequently led to a shift in the sensitivity of populations.

By this example we demonstrated that multiple stressors may result in severe effects on population level, which are not predictable from single stressors. Mechanistic modelling appears to be an appropriate tool for predicting the combined effects in environmental risk assessment.

5. References

Will climate change uncover low-dose effects of pesticides? A multigenerational study with the midge *Chironomus riparius*

Ruth Müller¹, Anne Seeland¹,², Lucas Jagodzinski², Joao Diogo¹,³, Carsten Nowak¹,³, Jörg Oehlmann¹,²

¹LOEWE Biodiversity and Climate Research Centre, Senckenberganlage 25, D-60325 Frankfurt am Main, Germany
²Goethe University, Department Aquatic Ecotoxicology, Siesmayerstr. 70, D-60323 Frankfurt am Main, Germany
³Senckenberg Research Institute and Natural History Museum, Clamecystraße 12, Gelnhausen, D-63571 Gelnhausen, Germany

E-mail contact: ruthmueller@bio.uni-frankfurt.de

1. Introduction

The potential risk of pesticides under climate change conditions is not yet accurately assessable for aquatic wildlife (WENNING ET AL. 2010). Several ecotoxicological studies hint to an increase of xenobiotic ecotoxicity on aquatic organisms at higher temperature. But none of these studies considered a near-natural situation. We hypothesize that near-natural dynamic temperature regimes may uncover the ecotoxicity of low dosed pesticides due to the concomitant successive exposure to suboptimal and optimal temperatures, in particular under impact of climate change. The hypotheses will be tested by performing a bifactorial multigenerational study with the aquatic model organism *Chironomus riparius*. *C. riparius* becomes chronically exposed to a low dose of the fungicide pyrimethanil and to dynamic spring and summer temperature regimes under two present-day simulations and under one expected for the near future.

2. Materials and methods

To define a low dose of the fungicide pyrimethanil, a ‘no-effect-concentration’ (NOEC) was derived from the dose-response-relationship of *Chironomus riparius* tested in a standard test (OECD, 2004) at 20°C. In the sediment-water chironomid toxicity test, effects on survival and emergence were assessed in the range of 0, 2, 4, 8, 16, 24, and 32 mg pyrimethanil L⁻¹ (*Pestanal®*) in accordance to the guideline. Reproductive endpoints were additionally determined.

In the following, multigenerational chronic effects of the NOEC of pyrimethanil on *C. riparius* were examined under a temperature situation typical for cold-temperate watercourses (slow-flowing or non-stratified) in spring and summer in either (a) a cold year in 1990–2005 (CY: 11.0 to 22.7 to 18.6°C), (b) a warm year in 1990–2005 (WY: 14.0 to 25.2 to 21.7°C) or (c) a warm year expected in 2050–2080 (WYF: 16.5 to 28.1 to 24.1°C). During the 140-days-lasting multigenerational study, parameters related to survival, emergence, reproduction, population growth and genetic diversity of *C. riparius* were analysed.

3. Results and discussion

3.1. NOEC of pyrimethanil derived from standard test

Pyrimethanil exposure of 2 mg L⁻¹ causes no adverse effect on *Chironomus riparius*, rather a slight hormesis of egg production and fertility is observed. Consequently, the derived NOEC of 2 mg L⁻¹ of pyrimethanil should act as reproductive stimulant during consecutive generations without causing detrimental effects.

3.2. Multigenerational effects of pyrimethanil under impact of climate change

In general, life-history-traits of *C. riparius* are highly dependent on temperature and generational time. In simulated spring, additional exposure to low dosed pyrimethanil provokes slightly adverse or hormetic effects on *C. riparius* in all temperature scenarios (P- to F1-generations, Fig. 1) as expected from the standard test. But an exposure of *C. riparius* to a NOEC of pyrimethanil at a thermal situation likely for a present-day or future warm summer (F2-generations onwards) uncovers considerable adverse effects on mortality and genetic diversity (Figs. 1, 2). Particularly in the F3-generation of the future scenario, pyrimethanil-treated *C. riparius* disclose the highest mortality (Fig. 1), the most reduced population growth rate and the utmost loss of genetic diversity (Fig. 2). Once the enfeebled F3-generation has been finished, both WYF-subpopulations of *C. riparius* break down due to long-lasting high temperature (Fig. 1). In two present-day scenarios,
however, adverse pyrimethanil effects on life-history of *C. riparius* vanish with decreasing temperature until F4-generation, regardless genetic diversity becomes reduced by 18–22% compared to controls (Figs. 1, 2).

![Figure 1: Mortality of Chironomus riparius (mean ± SD, n=5) after exposure to control conditions (uniform bars) or NOEC of pyrimethanil (striped bars) during consecutive generations (P to F3 or F4) at three dynamic temperature regimes. CY – thermal simulation of growing season in a cold year in 1995–2005, WY – thermal simulation of growing season in a warm year in 1995–2005, WYF – thermal simulation of growing season in a warm year in 2050–2080, C – control, PYR – exposure to NOEC of pyrimethanil, n.c. – not calculable as not enough agile larvae were available to establish five replicates for F4-generation in the WYF scenario.](image)

![Figure 2: Genetic diversity (observed heterozygosity) of Chironomus riparius imagines in the source population (grey bar, n=24) and final generations (n=18) completed in the multigenerational study. Percentage inhibition of heterozygosity of pyrimethanil-treated midges compared to controls is specified. For abbreviations see Fig. 1.](image)

4. Conclusions
The impact of climate change may augment the ecotoxicity of environmentally released pesticides, at least that of the tested fungicide in this study. Even under nowadays climate conditions, the NOEC of pyrimethanil provokes transiently adverse effects on the life-history of *Chironomus riparius* as well as a moderate reduction of its genetic diversity. Under the simulated climate change situation and if midges are additionally exposed to low levels of pyrimethanil, *C. riparius* reveals the highest mortality, a substantially reduced population growth rate and a sizeable loss of genetic diversity. Thus not only climate change, but also considered safe concentrations of pesticides might pose a reasonable risk for aquatic insects in future. The results gained so far highlight the importance of near-natural climate impact research to better understand and manage the ecotoxicological risk of agrochemicals in future.

5. References

Acknowledgement - The present study was was conducted at the Biodiversity and Climate Research Centre (BiK²), Frankfurt am Main and funded by the research funding programme ‘LOEWE – Landes-Offensive zur Entwicklung Wissenschaftlich-ökonomischer Exzellenz’ of Hesse’s Ministry of Higher Education, Research, and the Arts.
1. Introduction

The neonicotinoid imidacloprid is an active ingredient in many systemic insecticides. By acting on nicotinic acetylcholine receptors (nAChR), which are the pre-eminent receptors in insects, imidacloprid affects a broad spectrum of target pest insects [1]. It is used worldwide in agricultural and urban areas, and in domestic households.

Because of its persistence in soil and high solubility in water, imidacloprid has the potential to be transported by runoff [2]. Nevertheless, the use of imidacloprid was almost unrestricted due to its rapid photodegradation (<1h by 24°C) [2,3]. Periods of peak agricultural application followed by rainfall events can lead to pulse contaminations in surface water, where concentrations can reach toxic levels for non-target invertebrates. It should be taken into account that most of the tests conducted in the risk assessment are standardised single-species laboratory tests that run under highly controlled conditions, using continuous toxicant exposure [4]. They represent therefore an oversimplified reality that in turn generates uncertainty in the extrapolation from the laboratory to the field [5]. In the assessment of potential effects on ecosystems less attention was given to short-term exposures and in general to freshwater invertebrates.

To achieve realistic exposure conditions this study was carried out using in-field microcosms [6]. This method was designed to simulate natural lentic ecosystems and enables the comparison of the effects on various indigenous invertebrates.

The aims of this study were to evaluate the potential impact of imidacloprid pulses on several aquatic benthic macroinvertebrates and to determine its fate in the microcosms.

2. Material and methods

56 polypropylene containers of 20 L (microcosms) were filled with filtered water of a reference reservoir pond. The bottom of each microcosm was covered with ca. 1 cm of filtered fine sediment (the fine sediment was chosen to ensure homogeneity between the replicates). The microcosms were distributed on seven rafts next to the bank of the reservoir pond and left open to allow the colonization with macrozoobenthos. The microcosms were covered with a coarse mesh net of 2 cm in order to exclude large predators like dragonflies.

The experiment ran for nine weeks. Imidacloprid (six nominal concentrations, ranging from the NOEC 0.6 g/L to 40 g/L) was added to the water in three pulses at intervals of one week. Each raft contained two controls and all six concentrations, meaning seven replicates for each concentration. After the third pulse, the microcosms were covered with a fine elastic net, which stopped the colonization and prevented macroinvertebrates from escaping.

Emerging insects were collected with the aid of an aspirator over six weeks. At the end of the experiment, the larvae in the sediment were also collected. All the macroinvertebrates were counted and identified. Abundance, occurrence and emergence of main taxa were recorded. Particular emphasis was given to the pioneer species of the Chironomidae (non-biting midges).

Water and sediment samples of eight extra microcosms were analysed to determine the fate of imidacloprid.

3. Results

-Abiotic factors: Imidacloprid concentrations fell below detection limits in water phase within a few days after application. A rapid initial decrease was observed within six hours mainly due to photo-degradation. Imidacloprid was found in low concentrations in the pore-water.
- Biotic factors: In term of abundance, the fauna was dominated by Gastropoda, Chironomidae, Ephemeroptera, and Ceratopogonidae. According to preliminary results, measurements on structural aspects of the macrozoobenthos showed some evident responses especially at the highest concentration tested (40 µg/L): With regard to diversity the number of Chironomidae taxa declined significantly (Fig. 1). The abundance of Ephemeroptera, Gastropoda and of some chironomid taxa was also significantly affected. Emergence rate of some chironomid species (e.g. Ablabesmyia) and Ephemeroptera declined significantly.

![Graph showing comparison of the chironomid taxa between treatments. Asterisk indicates the imidacloprid treatment, which was significantly different from the control.](image)

**Figure. 1: Comparison of the chironomid taxa between treatments. Asterisk indicates the imidacloprid treatment, which was significantly different from the control.**

4. Discussion and conclusions

During the experiment atmospheric and geogenic conditions for imidacloprid degradation were optimal, therefore this experiment has to be considered a ‘best case’ rather than a ‘worst case’ exposure scenario. Despite the rapid dissipation imidacloprid had direct effects on abundance, occurrence and emergence of non-target species, whereas the increase in the number of the Gastropoda is presumably due to indirect effects, as a release from competition (nominal concentration: 40 µg/L).

The results of this experiment indicate that short-term exposures to imidacloprid, with limited recovery time, can significantly affect the structure of freshwater ecosystems at concentrations below those established in worst-case scenario laboratory tests. Moreover, the observation of indirect effects should be also considered in the risk assessment. The microcosms method proved to be a valuable tool in the evaluation of potential effects of pesticides under ecologically realistic conditions.

5. References


Assessment of soil contaminants bioavailability using a multi-marker approach in laboratory and field experiments

Pierre Yves Robidoux1,2, Virginie Bérubé1, Yann Berthelot1, Matthias Blanchard1,2, Kathleen Savard1 and Geoffrey I. Sunahara1

1Biotechnology Research Institute, National Research Council of Canada, Montreal, QC, Canada.  2Université de Quebec at Montréal, Montreal, QC, Canada

E-mail contact: Pierre-Yves.Robidoux@cnrc-nrc.gc.ca

1. Introduction

Simultaneous contamination of soil by various substances (contaminant mixtures) presents a challenge for risk assessment. Chemistry analyses give the level and type of contaminants. Toxicity tests assess the effects of bioavailable compounds to selected species using standard important endpoints (survival, growth, fertility). Bioavailability can be defined as “the fraction of a substance that will exert an effect in an organism”. This fraction is “toxicologically bioavailable”. Conversely, bioaccessibility is referred to as “the fraction of a substance which is readily mobilizable by an organism in a soil” (see Figure 1). Bioavailability is critical for understanding effects that might result from exposure of biota to contaminated soils and sediments. Soils and sediments from military range and training areas (RTAs) as well as Munitions Experimental Test Center (METC) are contaminated principally by energetic materials (EM) and metals. Their chemical characteristics are relatively well known and toxicity assessment of soils from RTAs and METC are in some cases available. However, bioavailability on these sites needs to be comprehensively characterized.

Conventional toxicity tests give limited toxicological information and do not consider variability (e.g., temperature, humidity) of current and future field conditions. Improvement of standard assays by adding alternative biological endpoints (cellular, biochemical, molecular biomarkers) can help to understand the toxicity observed and give the appropriate information for the selection of biological parameters for other tier assessment levels (e.g., field mesocosm assays, field studies) where standard chronic endpoints can not be used. Use of selected biomarkers alone gives appropriate information on chemical stress, range and class of contaminants, and health status. This paper presents case studies using laboratory and field bioassays and an integrated approach including earthworm (Eisenia andrei) toxicity tests and a suite of biomarkers and chemical analyses. Parameters such as lysosomal membrane fragility of coelomocytes (neutral red retention time) can be used to assess the chemical stress whereas the antioxidant system (catalase and superoxide dismutase activity) and detoxification metabolism (glutathione S-transferase activity, metallothionein), as well as the immune activity and the contaminant uptake are used to assess the bioavailability of contaminants and the health status of the exposed organisms.

Figure 1: Schematic model of bioavailability for soil/sediments.

2. Materials and methods

Experiments were carried out under laboratory and field conditions. Earthworms were exposed under laboratory conditions to different single contaminants, including metals (e.g., Cr, Pb Cu) and EM (e.g.,TNT, RDX, HMX). In addition to standard acute (lethality) and chronic (growth, reproduction), sublethal parameters (biomarkers) were measured. These biomarkers included lysosomal membrane fragility of coelomocytes (neutral red retention time [NRRT]), enzymes of the antioxidant system (catalase and superoxide dismutase [SOD] activity) and detoxification metabolism (glutathione S-transferase [GST] activity, metallothionein), as well as the immune activity (cell viability, phagocytic activity/efficiency). Experiments were also carried out using binary mixtures (e.g., HMX and Pb). Earthworm mesocosms studies were carried out on a explosives-contaminated site at different antitank firing ranges. Survival of earthworms and the selected biomarkers were used in these studies to assess the effect of explosives-contaminated soils on the earthworms Lumbricus terrestris and E. andrei under field conditions. Toxicity of the soils samples for E. andrei was also assessed under laboratory conditions using the earthworms reproduction test and the selected biomarkers.
3. Results and discussion

3.1. Effects of HMX-lead mixtures on reproduction of the earthworm

The response-addition model was used to predict the response of earthworms and to test for interaction between the two contaminants. The predicted toxicity was not significantly different than the observed toxicity, implying that Pb and HMX were considered noninteractive compounds. The combined action of Pb-HMX may be described, therefore, as dissimilar-noninteractive joint action in a sandy soil.

3.2. Integration of toxicological and chemical tools to assess the bioavailability

HMX and some metals (Zn, Pb, Bi and Cd), though not consistently the prevailing toxicants, were the most accessible to earthworms. Some metals (notably Cu, Zn, Cr and Bi) were also accumulated in earthworm tissue. These results were not necessarily expected given their bioaccessibility (i.e., the chemical availability of contaminants in the environment for the organisms) at the beginning of the exposure. The tested soils impaired earthworm reproduction and reduced adult growth. Measurement of selected sublethal parameters indicated that NRRT was decreased, while elevated SOD activity suggested that earthworms experienced oxidative stress. The correspondence between the NRRT and metal contamination pattern suggested that metals may be the main cause of lysosomal disruption in EM-contaminated soils.

3.3. Assessment of soil toxicity using mesocosms and laboratory studies

Survival was reduced in certain EM-contaminated soil mesocosms following 2 to 14 days of exposure under field conditions, whereas survival was reduced following 28 days of exposure under laboratory conditions. Reproduction parameters such as number of cocoons and number of juveniles were reduced in many of the selected contaminated soils. Compared to the reference, NRRT and immune activity was significantly reduced for earthworms exposed to EM-contaminated soils under both field and laboratory conditions. Analyses showed that HMX is generally the major polynitro-organic compound in soils. HMX is also detected in earthworm tissues. Thus, results from both field mesocosms and laboratory studies, showed lethal and sub-lethal effects associated to soil from the contaminated area of the antitank firing range.

4. Conclusions

HMX is more toxic than Pb in a sandy soil but Pb toxicity increased more rapidly as a function of dose. Because of the lack of parallelism of their dose-response curves, we consider that these two compounds have dissimilar modes of action on E. andrei reproduction. In addition, effects of Pb-HMX mixtures can be predicted by a response-addition model based on the toxicity of individual compounds. The results are consistent with a dissimilar and noninteractive joint action with respect to reproductive effects on E. andrei. The integrated approach applied in our studies is useful and relevant to provide a synthetic and more relevant picture of bioavailability and toxicity in EM-contaminated soils, due to the multiplicity of factors, processes involved and pattern complexity. Field studies are extremely useful in the validation of results derived from the laboratory. Mesocosm experiments may be used to assess the toxicity on a site and to characterize the effects of contaminants

5. References

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New developments in passive air sampling for current-use pesticides and other priority chemicals

Martina Koblizkova, Tom Harner, Sum Chi Lee, Susan Genuaidi

Environment Canada, Science and Technology Branch, 4905 Dufferin Street, Toronto, ON M3H 5T4
E-mail contacts: martina.koblizkova@ec.gc.ca; tom.harner@ec.gc.ca

1. Introduction

Passive air samplers (PAS) are cost effective and simple tools that are invaluable for assessing risks associated with pesticide use. They can be used to monitor and evaluate regional and long range transport of pesticides and provide spatially resolved data that is required for developing transport and fate models. Under the Global Atmospheric Passive Sampling (GAPS) network, passive air samplers comprising polyurethane foam (PUF) disks are being used to deliver air concentrations of several organochlorine pesticides at more than 50 global sites [1]. The value of these samplers has been recognized and there is a need to extend their application to more volatile and polar pesticides that are typically used these days.

The study investigates a modified PUF disk sampler, the SIP (or sorbent-impregnated PUF) disk which has XAD powder impregnated into the PUF to enhance sorptive capacity [2]. A field calibration study was conducted over 8 months to yield information on the sampling rate (R, m⁻³/day) and also on the capacity of the samplers for the various target compounds. This information is needed to determine air concentration values for SIP disk samplers. The results are applied to SIP disks deployed during a pilot study which provides the first global-scale survey of several classes of priority chemicals, including CUPs.

2. Experiments

During March-June 2009, a pilot study was conducted to investigate the operation of a new sorbent-impregnated PUF (or SIP) disk quarterly sampler for capturing CUPs and other priority chemicals. The SIP disk sampler was previously deployed at a subset of 20 GAPS sites alongside the conventional PUF disk sampler. This study provides the first global-scale survey of several classes of priority chemicals, including current-use pesticides (CUPs). The data will be used to assess long-range transport in air and transboundary inputs of CUPs to the Canadian environment.

In order to better interpret results derived from SIP and PUF disks, a calibration study was undertaken during March-October 2010 at an urban background field site in Downsview, Ontario. The study establishes sampling rates and sorptive capacities for CUPs and other priority chemicals so that reliable air concentrations can be derived from time-integrated passive samples. The field calibration study included the collection of high- and low-volume samples that differentiate the gas- and particle-associated compounds so that sampling of particle-associated compounds could also be investigated.

During preliminary screening, the pilot study SIP disk samples were analyzed for 7 target CUPs (chlorothalonil, malathion, metribuzin, dacthal, chlorpyrifos, pendimethalin, and trifluralin).

3. Results and discussion

The SIP disk passive air sampler is effective at collecting the more volatile chemicals such as FTOHs, FOSAs and FOSEs, and has comparable derived air concentrations to that of the PUF disk sampler for legacy POPs [3].

The SIP disk is also effective in collecting CUPs. Figure 1 shows accumulated amounts of selected CUPs in SIP disk samplers deployed for approximately 3 months during the pilot study. Of the targeted CUPs, the most frequently detected was dacthal (18/20 sites) while malathion was the least frequently detected (1/20 sites).
4. Conclusions

Several CUPs were successfully detected by the SIP disk samplers deployed at 20 global sites in 2009. Results from the field calibration will confirm sampling rates and capacities for the various target compounds. This will allow the results to be converted from accumulated amounts (ng/filter) in the SIP disk to air concentration units (e.g. pg/m$^3$). The calibration results will also provide guidance on which CUPs can be effectively sampled using conventional PUF disks and for which CUPs the SIP disk is preferred. In future work, the calibration study samples will be analyzed for a larger suite of priority chemicals in order to extend the application of the PUF and SIP disk samplers to a broader range of compound classes.

5. References


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EU-wide environmental monitoring of Persistent Organic Pollutants using butter as biomonitoring matrix

Jana Weiss1, Giulio Mariani, Lara Amalfitano, Ingrid Vives, Anne Mueller, Helle Skejo and Gunther Umlauf

1Institute for Environment and Sustainability (IES) Joint Research Centre (JRC), Via E. Fermi 2749, 21027 Ispra (VA), Italy
E-mail contact: jana.weiss@jrc.ec.europa.eu

1. Introduction

The Stockholm Convention (SC) was created due to the fact that persistent organic pollutants (POPs) have the properties for long-range atmospheric transport (LRAT) and hence no government alone can protect their citizens and the environment to exposure of these pollutants. POPs can accumulate to hazardous levels in living organisms and pose environmental and health risks. Members of the SC are requested to take measures to reduce or eliminate the release of the POPs on the SC priority list to the environment. The current priority list includes polychlorinated dibenzo-p-dioxins and furans (PCDD/Fs), polychlorinated biphenyls (PCBs), polybrominated flame retardants (PBDEs) and organochlorine pesticides (OCPs), 21 chemical groups in total and 3 compounds are under revision (i.e. endosulfane, HBCDD and chlorinated paraffin). The Global Monitoring Plan of POPs is an important component of the SC to effectively evaluate and collect comparable monitoring data or information on the presence of the POPs from all regions, in order to identify changes in levels over time, as well as to provide information on their regional and global environmental transport.

Within the EU the "Community Strategy for Dioxins, Furans and Polychlorinated Biphenyls" sets up the objective to reduce dioxins and PCBs in the environment, animal feed and the food chain (European Commission 2001). More recently the Regulation on POPs entered into force (European Commission 2004), providing the basis for implementing the provisions of the Stockholm Convention. To measure the temporal and spatial distribution of the priority POPs, which are both intentional and unintentionally released to the environment, suitable sample matrix for POPs are needed. Passive sample techniques have been applied to the GMP monitoring activities to assess the global LRAT of POPs. The data produced by passive samplers have been shown to correspond to the more advanced active air sampler. The advantage with passive air sampling is that no advanced equipment which demands electricity is needed. The samplers can therefore be set up everywhere, even at remote areas. But still some education of the personnel is. Aspects such as wind direction, temperature and sampling time are crucial to register and incorporate into the calculations.

In this study we demonstrate the feasibility to use another passive sample approach for screening purposes, i.e. animal lipids such as butter. The advantages to use butter as biomonitoring matrix are several; due to the lipophilic and persistent properties of POPs they are bioaccumulated in lipid rich matrix; butter reflects the contamination level of the environmental compartment from which they derive due to well known transfer factors; butter is available in almost all geographic regions and is cheap and easily accessible; moreover, this biomonitor is well buffered against temporal variations. The air-grass-cow transfer factors are available from controlled experiments. Hence it remains to verify the correlation in the field in order to assess ambient air quality. The POP levels of the samples will be analyzed and evaluated together with available air data to establish a correlation between air contamination and dairy products (assuming minimal commercial feed contamination influence).

2. Materials and methods

The target compounds of this study are PCDD/Fs, PCBs, PBDEs, and OCPs subject to the Stockholm Convention as by 2009. Analyses are executed with high resolution GC/MS according to the requirements of CEN and USEPA and described elsewhere [1]. Quantifications will be carried out based on labelled surrogate standards. The analytical protocols for all analytes have been validated through successful participation to international intercalibration studies.
The experimental approach is based on dairy products sampled at 3 different occasions all over Europe (Figure 1). In 2001 milk was sampled (n=88) at a campaign aiming at evaluating the levels of dioxin-like PCBs in European food and feedstuff\(^1\). All samples were taken directly from farms to have control over the coordinates of the grazing cows. In 2007 organic diary products (n=85) from biocertified (European Commission 1991)\(^2\) farms were targeted, both purchased commercially and directly from farm. Some additional samples were collected in 2009 from missing areas (n=6).

Available air data, reviewed from reported studies together with JRC owned data will be correlated to concentrations in the lipid samples of the corresponding areas. One of the largest sources of seasonal, congener specific data on POPs will be retrieved from the European Monitoring and Evaluation Programme (EMEP). EMEP has the largest pan-European coverage of air levels of priority pollutants.

3. Expectations of the project

The project is ongoing at the time for submission of this abstract. 80% of the samples have been analysed and the available air levels have been collected into a database. The data will be interpreted and reported within the next months. We expect of this project to find similar correlations as the one presented in Figure 2, where high milk levels were analysed in the same areas as reported mean annual concentration in air (EMEP).

4. References


Acknowledgement – we acknowledge all the people who kindly supplied us with butter samples.

\(^1\) conducted by BiPROM on behalf of DG ENVIRONMENT

\(^2\) Regulation EEC N° 2092/91 requests production conditions according Annex 1, e.g. the fertility and the biological activity of the soil must be maintained or increased by using appropriate multiannual rotation program. Pests, diseases and weeds shall be controlled by a combination of suggested methods which do not include the use of pesticides.
Determination of deployment specific chemical uptake rates for SDB-RPS Empore disk using a passive flow monitor (PFM).

Dominique O’Briena, Michael Bartkow, Karen Kennedy and Jochen F. Muellera

aThe University of Queensland, The National Research Centre for Environmental Toxicology, (Entox, 39 Kessels Rd., Coopers Plains QLD 4108, Australia
dQueensland Bulk Water Supply Authority trading as Seqwater Level 3, 240 Margaret St, Brisbane City QLD 4000 Australia
E-mail contact: d.obrien2@uq.edu.au

1. Introduction
The use of the adsorbed SDB-RPS-Empore™ disk (ED) in a Chemcatcher type passive sampler is routinely applied in Australia when monitoring herbicides in aquatic environments. One key challenge in the use of this passive sampling device is in mitigating the potentially confounding effects of varying flow conditions on chemical uptake by the passive sampler. While performance reference compounds (PRCs) may be applied to correct sampling rates ($R_s$) for site specific changes in flow and temperature, evidence suggests the use of PRCs is unreliable when applied to adsorbent passive samplers, such as the ED employed.

The use of the passive flow monitor (PFM) has been introduced for the assessment of site specific changes in water flow [1]. In the present study we have undertaken a laboratory based calibration where the uptake of the hydrophilic organic compounds atrazine and prometryn into the ED, when exposed to different water velocities, was correlated against the daily mass lost from the PFM ($r_{PFM}$) when exposed to fresh water. Furthermore the assessment of this alternative in situ calibration method under field conditions was undertaken through the deployment of ED passive sampler and PFM devices at a number of fresh water sites across South East Queensland, Australia.

2. Materials and methods
Five laboratory based experiments, each consisting of up to ten days of deployment, were carried out. During each deployment, PFM (in triplicate) and ED (in duplicate) samplers were exposed within a 1400 L tank and exposed to either negligible flow or simulated flow conditions, which equated to a water flow velocity of “0”-negligible flow, 0.034, 0.060, 0.16, 0.24 m s$^{-1}$ relative to the movement of the samplers through the water. During the deployment periods grab samples (duplicate 2 L) were collected at t=0 and consequently during each sample removal. Flow rate temperature and salinity were recorded with each grab sample collection. Samples were analysed according to method mentioned in Shaw et al. [2]. Two blank passive samplers and field and laboratory blank grab samples were incorporated.

Field deployment of PFM and ED samplers (in duplicate), exposed for a total of 28 days, occurred at 8 freshwater sites in South East Queensland. Over the deployment period, duplicate grab water samples (2 L each) were obtained when the samplers were first exposed, 14 days into the deployment and at the time of retrieval. Sites were selected to be used in the assessment of the performance of PFM correction of $R_s$ when atrazine or prometryn were detected in both the ED extracts and all grab samples taken over the deployment period.

3. Results and discussion
3.1. Correlation of SDB-RPS Empore™ disk $R_s$ with water velocity and $r_{PFM}$
A linear increase in $R_s$ was observed when the water velocity was increased from 0 to 0.16 m s$^{-1}$ (figure 1). However, a further increase in velocity up to 0.24 m s$^{-1}$ did not result in a corresponding linear increase in the $R_s$ of atrazine or prometryn. Instead it appears that the sampling rates are approaching a maximum that would correspond with a shift in the uptake limiting resistance from the WBL to the diffusion through the membrane ([3]). The relationship between $R_s$ and water velocity was described by an exponential relationship where:

$$R_s (\text{Atrazine}) = 0.01093 + (0.1756-0.01093)*(1-\exp(-6.33 v))$$

$$R_s (\text{Prometryn}) = 0.007296 + (0.1871-0.007296)*(1-\exp(-4.638v))$$
A plot of the $R_s$ for the uptake of both herbicides into the ED sampler showed a similar exponential relationship between $R_s$ and the $r_{PFM}$. As such we can assume that, for an $r_{PFM}$ of $\geq 2.74$ g d$^{-1}$, the resistance to uptake is controlled by the membrane and there is a maximum sampling rate of $0.142$ (SD = 0.0019) and $0.113$ (SD = 0.0058) L d$^{-1}$ for atrazine and prometryn respectively. Hence the use of the PFM will allow a direct estimation of the sampling rate of the ED sampler for atrazine and prometryn to be made from a $r_{PFM}$ of $0.57$ to $2.74$ g d$^{-1}$ or when exposed to a water velocity of between $0.034$ and $0.16$ m s$^{-1}$.

**Figure 1:** Empore Disk sampling rates ($R_s$) for the uptake of atrazine and prometryn relative to the water flow velocity.

### 3.1. Calculation of $R_s$ under field conditions

Only atrazine was detected in the EDs exposed under field conditions. Using the $r_{PFM}$, average flow velocities at these sites were estimated and ranged between $\leq 0.034$–$0.148$ m s$^{-1}$. All sites except Site 3 were found to have average water velocities in the range where the PFM allows estimation of sampling rate. The range of water velocities measured at these sites corresponds to a difference in $R_s$ (atrazine) of between $0.044$ and $0.135$ L d$^{-1}$.

A comparison of passive sampling based water concentrations with grab sampling data indicated that the passive sampling technique was able to detect atrazine present below the detection limit used for grab samples (0.01 µg L$^{-1}$). Most importantly, the application of the PFM based sampling rates provided results that compare very well with grab sampling data where grab samples detected atrazine and also where concentrations within the grab samples were below the LOD of the analytical method employed.

### 4. Conclusions

In the presented study we have demonstrated that the $R_s$ at which both atrazine and prometryn are accumulated within the SDB-RPS-Empore$^{TM}$ disk depend on the flow conditions. Further, the calibration of the measured $R_s$ for chemical uptake by the SDB-RPS-Empore$^{TM}$ disk to the mass lost from the PFM has shown that the PFM provides an accurate measure of $R_s$ for flow velocities from 0–0.16 m s$^{-1}$. Notably, for flow velocities > 16 cm s$^{-1}$, a non linear increase in the $R_s$ of both herbicides was observed which indicates that the key resistance to uptake into the SDB-RPS Empore$^{TM}$ disk is associated with the diffusion through the overlying diffusion limiting membrane. Overall the greatest uncertainty remains at very low flow conditions that may be observed in ground water and/or small ponds but are not typical for major surface water bodies. Validation of the application of the PFM was also achieved in a limited field study which further illustrated the need for an *in situ* calibration tool to ensure that the accurate assessment of $C_w$ is achieved.

### 5. References


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Effective in-situ measurement of pore water PCB concentrations in sediment profiles using passive samplers

Amy M.P. Oen1, Elisabeth M.L. Janssen2, Gerard Cornelissen1, Richard G. Luthy2

1Dept. of Environmental Engineering, Norwegian Geotechnical Institute, Sognsvæien 72, 0806 Oslo, Norway
2Dept. of Civil and Environmental Engineering, Stanford University, Stanford, CA 94305, USA
E-mail contact: amy.oen@ngi.no

1. Introduction
Since their introduction [1], the use of passive samplers to measure freely dissolved concentrations of hydrophobic organic compounds (HOCs) in aquatic environments has become more prevalent [2-4]. However, there are limited studies that have utilised passive samplers to assess in-situ pore water concentrations. Measurements of pore water concentrations are important as they are a more appropriate indicator of the bioavailable contamination in an ecosystem [5].

Recently, Tomaszewski and Luthy [6] determined sediment pore water concentrations of polychlorinated biphenyls (PCBs) in a contaminated mudflat in San Francisco Bay, CA using field-deployed polyethylene devices (PEDs). This study builds on the work of Tomaszewski and Luthy [6] by: (i) measuring vertical pore water profiles of PCBs from 0-50 cm using both PEDs and polyoxymethylene (POM), (ii) dosing the passive samplers with a larger suite of performance reference compounds (PRCs), and (iii) interpreting PRC depletion to assess the development towards equilibrium.

2. Materials and methods
The study site was located at the tidal mudflat area of the South Basin in San Francisco Bay in Parcel F, a treatment plot where 2 - 3 % activated carbon (AC) was amended in January 2006 as described previously by Cho et al. [7]. In August 2008, a total of five sampling rods with ultra thin POM (17 µm) and two sampling rods with polyethylene (PE) were placed in the sediment at three locations in Parcel F. The sampling rods were constructed such that the passive samplers were attached at approximately 5 cm intervals from 0 to 50 cm (Figure 1). This ensured measurement of pore water concentrations at the sediment surface, through the AC amended layer and down into the untreated sediment. The sampling rods were deployed in a radial pattern at each location and were retrieved over time (14, 28, 60, 90 and 150 days for POM and 60 and 150 days for PE). In order to assess the approach towards equilibrium, the PE and POM passive samplers had been impregnated with PRCs. Tomaszewski and Luthy [7] used a tri- and a tetrachlorobiphenyl (PCB 29, 69) as PRCs. In addition to these, we have included a penta-, a hexa- and a heptachlorobiphenyl (PCB 103, 155, 192) to indicate the samplers' time to equilibrium with the surrounding pore water.

As shown in Figure 1, sediment cores were also taken at each of the three locations in Parcel F. The cores were sliced (~ 2 cm) and analysed for AC content.

Figure 1: Pictures from the deployment of vertical passive samplers and sediment cores at Hunters Point.

3. Results and discussion
As shown in Fig 2, three different methods were used to estimate the pore water concentrations. Average pore water concentrations in the top 30 cm of sediment range from 0.4-2.2 ng/l assuming equilibrium has
been achieved in the POM, 0.6-5.2 ng/l using depletion of PRCs from the POM and 2.4-3 ng/l using depletion of PRCs from the POM. Average pore water concentrations in the deeper sediment layers (30-40 cm) range from 0.9-5.0 ng/l, 1.8-10.5 ng/l and 5.2-9.2 ng/l, respectively. The differences in PCB concentrations in the POM and the PE after 5 months of deployment reflect the degree to which the two different passive samplers approach equilibrium. Impregnated PRCs show 60 – 70 % depletion from the POM and 2 – 90 % depletion from the PE, depending on the degree of chlorination of the PRC. These results also reflect the distribution of AC where percentages of total organic carbon (TOC) ranged from 3 to 6 % in the top 30 cm (amended with AC) and from 1 to 1.5 % in the deeper sediment (untreated).

![Figure 2: Vertical pore water concentration profiles calculated based on (a) assuming equilibrium has been achieved in the POM after 100 days of exposure, (b) depletion of PRCs from the PE and assuming a first-order process uptake model and (c) depletion of the PRCs from the POM assuming a first-order process uptake model.](image)

4. Conclusions

The close agreements of pore water estimates for the two sampler materials (PE and POM) and the different methods used to derive pore water concentrations demonstrate the robustness and suitability of the passive sampling approach. Better understanding of the performance of different sampler materials and uniformity in methodologies to calculate pore water concentrations considering the dissipation of PRCs will allow sediment managers and researchers to make a well informed choice for monitoring and study designs.

5. References

Development and use of polyethylene passive samplers to detect triclosans and alkylphenols in an urban estuary

Victoria Paris Sacks¹, Rainer Lohmann¹

¹Graduate School of Oceanography, University of Rhode Island, Narragansett, RI, USA
E-mail contact: vpsacks@gso.uri.edu

1. Introduction
Polyethylene plastic drop cloth, conventionally used for protecting furniture during painting, can also be used to make inexpensive diffusive passive sampling devices. Hydrophobic organic contaminants are taken up by diffusion into the PE until equilibrium is reached, and measuring the concentration in the PE enables one to determine the dissolved concentration in the environment. To be able to use polyethylene passive samplers (PE) in the field, the partitioning constants between PE and water (K_{PEw}) of the compounds examined must be known. In this study, the K_{PEw}s of triclosan (TCS), methyl triclosan (MTCS), n-nonylphenol (n-NP), nonylphenol technical mixture (NP-tech), n-octylphenol (n-OP), and t-octylphenol (t-OP) into PE were measured as a function of pH, temperature, and salinity, and a salt effect was calculated for TCS, n-OP and t-OP. PE was then deployed in Narragansett Bay, RI to determine environmental concentrations and explore concentration gradients.

2. Materials and methods

2.1 Partitioning experiments
Low density PE (25.4 µm thickness, a plastic drop cloth from Covalence Plastics, Minneapolis, MN) was used throughout. Each sample was spiked with 1000 ng of TCS, MTCS, n-NP, NP-tech, n-OP, and t-OP. Triplicate samples and blanks using PRC loaded PE were shaken in climate controlled chambers under four conditions: 5°C/0 psu, 5°C/93 psu, 20°C/0 psu, and 20°C/93 psu for 6 weeks on an orbital shaker table. Replicates at pH 7-12 (1 sample for each pH) were prepared with PE and organic compounds as above using Tris (National Diagnostics), HCl (J.T. Baker), NaHCO₃, KCl (Fischer Scientific), and NaOH (Mallinckrodt) to adjust to a given pH.

2.2 Field sampler preparation and field collection and analysis
Two spatial deployments of Narragansett Bay were executed at eight water quality buoys which make up the Narragansett Bay Fixed-Site Monitoring Network [2]. At each sampling location, PE samplers were attached each to a line 1 m below the surface and 1 m above the sediment and allowed to equilibrate for four weeks in the early fall of 2009.

3. Results and discussion
For 6 week partitioning experiments, equilibrium was confirmed with both PRCs and by comparison to the mid range pH replicates. Log K_{PEw} (20°C/0 psu) ranged from 2.7 for t-OP to 4.5 for MTCS (SI Table 2). As expected, the more polar compound, MTCS, displayed a higher K_{PEw} than TCS (3.3). Within the alkylphenols, the longer chained n-NP (4.1) showed higher K_{PEw} than n-OP (3.6), and the linear chain n-OP had a higher K_{PEw} than the branched t-OP (2.7).

The change of partitioning as a function of pH (n=1 for each pH) was examined experimentally (Figure 1). As expected, the K_{PEw} of hydroxyl-group containing compounds were strongly affected by pH, whereas MTCS, with its methylated hydroxyl-group, was not. For comparison to our measurements, we predicted partitioning as a function of pH (K_{PEw}(pH)) assuming that K_{PEw} only accumulates neutral molecules (equation 1):

\[ \log K_{PEw}(pH) = \log K_{PEw}(pH = 7) + \log \left( \frac{1}{1+10^{(pH-pK_a)}} \right) \]

The change in K_{PEw} is consistent with predictions such that at the compound’s pKa values, K_{PEw} has decreased by 50% (Figure 1). For the hydroxyl-containing compounds (TCS, n-NP, NP-tech, and n-OP), the K_{PEw} of the protonated (neutral) molecules exceeded the deprotonated (charged) species by two orders of magnitude. This confirms that PE is primarily a sampling medium for neutral, apolar compounds, yet the
$K_{\text{PEw}}$ for TCS at higher pH suggests that to a small degree, charged species are accumulated, likely as charged species adsorbed to the surface.

Due to the interactions of the polar hydroxyl group with octanol in comparison to with PE, a correlation cannot be made between $K_{\text{PEw}}$ and $K_{\text{ow}}$ for the compounds studied. In contrast, hexadecane, a long chain hydrocarbon is a better choice to represent PE. A good correlation ($K_{\text{PEw}} = 0.679 \times K_{\text{hdw}} + 1.033$, $r^2=0.984$, $p=0.001$) was obtained with hexadecane-water partitioning constants ($K_{\text{hdw}}$) predicted from COSMOtherm (Figure 2).

During deployments in Narragansett Bay (RI) in the fall of 2009, concentrations of MTCS and t-OP in surface and bottom waters ranged from 50 – 270 pg L$^{-1}$ and 3.5 – 11 ng L$^{-1}$ respectively. These concentrations are far below EC$50$ values for rainbow trout. Surface/bottom and bottom/porewater activity ratios were calculated. These indicated surface waters as the main source of MTCS, while surface water as well as sediments were sources of t-OP.

4. Conclusions

It is evident from this work that PE can be used in the measurement of polar compounds such as TCS, MTCS, n-NP, NP-tech, n-OP, and t-OP. The goal of this research was to develop $K_{\text{PEw}}$ values for the emerging contaminants TCS, MTCS, n-NP, NP-tech, n-OP, and t-OP so that future researchers may use simple PE technology without measuring partitioning in the laboratory. Compared to traditional means of gaining water concentrations, PE is simpler, easier, and less expensive and makes a very useful tool for first order risk assessment. The remaining challenge is to establish dependable tools to predict $K_{\text{PEw}}$ for emerging contaminants beyond those derived here.

5. References

2. NBFSMN Narragansett Bay fixed site monitoring network. [http://www.dem.ri.gov/bart/stations.htm](http://www.dem.ri.gov/bart/stations.htm)
Air concentrations of current use pesticides (CUPs) in Tuscany and Lazio region, Central Italy, using passive air sampler (PUF disk)

Karla Pozo¹, Victor H. Estellano¹, Tom Harner², Maria Laura Monti¹, Simonetta Corsolini¹, Julieth Banguera³, and Silvano Focardi¹

¹Department of Environmental Science, University of Siena, Via Mattioli 4, 53100, Siena-Italy
²University of Valle, Cali-Colombia
³Atmospheric Science & Technology Directorate, Toronto, ON, Canada.
E-mail contact: gallardokarla@gmail.com

1. Introduction

Pesticides less persistent than the legacy organochlorine pesticides (OCPs) continue to be used in large quantities in many countries around the world and are often referred to as current use pesticides (CUPs). In particular, CUPs such as organophosphorous insecticides and triazine herbicides are being used extensively in many countries throughout the world (Yao et al., 2006). However, very little is known regarding the use and levels of pesticides in Italian agricultural regions. This information is needed to assess deposition/emission, human exposure and best strategies for minimizing the risks to humans and the environment. Use of pesticides in agricultural regions of Italy may also be important as a source to other receptor regions in the country. As a result of these concerns, organic farming practices have become more popular in Italy. There is some concern regarding continued pesticide exposure for organic farms – either from emissions of current-use pesticides (CUPs) from nearby conventional farms or from soil residues of historically used, persistent pesticides. A pilot study was initiated during the spring 2009 in the Tuscany region to address these questions. An air survey was conducted in 2 different agricultural regions of central Italy. Air samples were collected using polyurethane foam (PUF) disk passive air samplers that were deployed over three months from April 2009 to January 2010 in Montalcino (Tuscany region) and Terracina (Lazio Region).

2. Material and Methods

Passive air samplers (PAS) comprised of polyurethane foam (PUF) disk were deployed at four sites in the agricultural areas of Montalcino (Tuscany region) and four sites in Terracina (Lazio region) (Figure 1). PUF disks were housed inside a stainless steel chamber [1]. PUF disks were, spiked with recovery standards, and extracted with 300 ml of petroleum ether for 20 hours. Extracts were volume reduced using a rotary evaporator and concentrated to around 1 ml using a gentle stream of nitrogen and then solvent-exchanged into iso-octane. The final volume of the extracts was 0.5 ml, and 100 ng of mirex was added to the samples for volume correction.

The extracts were analyzed for 12 pesticides including: Trifluralin, Chlorpyrifos (ethyl and methyl), Chlortal-dimethyl, Dimethoate, Metribuzin, Malathion, Pendimethalyn, Terbufos, Diazinon, Phorate and Disulfoton using GC/MS (ion trap detector) ThermoFinnigan (TraceTM GC 2000/GCQ plus) with a negative chemical ionization source in selected ion mode. A Rt-x5MS column (30 m × 0.25 mm d.i., 0.25 µm) was used for separation. Helium was used as the carrier gas at a flow rate of 1.2 ml min⁻¹. The GC oven temperature program was: 100°C held for 2 min then increased at 20°C min⁻¹ to 140°C, then increased at 4°C min⁻¹ to 200°C (held for 13min) and finally increased.

3. Results and discussion

Figure 2 shows air concentrations of CUPs at Montalcino agricultural area. From twelve CUPs screened only 6 were routinely detected in samples (Chlorpyrifos ethyl, Chlorpyrifos methyl, Trifluralin, Clorothalonil, Pendimethalyn, and Chlortal-dimethyl).

At Montalcino, air concentrations (pg m⁻³) of Chlorpyrifos (ethyl and methyl) ranged from 3-80, and Pendimethalyn ranged from 1-90 followed by Trifluralin 2-20, and Chlorotalonyl 1-5. Chlortal-dimethyl was not
detected at this sampling site. These results are lower than those reported in other agricultural areas of the world. For instance Yao et al., 2007 [2] reported levels of 670 pg m$^{-3}$ for Chlorpyrifos and Guoin et al., 2008 [3] detected air concentrations of 85 pg m$^{-3}$ of Trifluralin in agricultural areas of Canada. Figure 2 shows seasonal variations of CUPs at both sampling sites. Pendimethalyn was detected only during period 1 and 2 while Chlorpyrifos (ethyl and methyl) showed a uniform distribution during the whole year at all the sampling sites, with exception of site 3 during period 1. This pattern is consistent with pesticide use at the agricultural site of Montalcino. Pendimethalyn is a herbicide that is used in premergence and postemergence application to control annual grasses and certain broadleaf weeds. Pendimethalyn is approved in Europe, North America, South America, Africa, Asia and Oceania for different crops including cereals (wheat, barley, rye, triticale), corn, soybeans, rice, potato, legumes, fruits, vegetables, nuts as well as lawns and ornamental plants. Chlorpyrifos is a crystalline organophosphate insecticide that inhibits acetylcholinesterase and is used to control insect pests. Chlorpyrifos is moderately toxic and chronic exposure has been linked to neurological effects, developmental disorders, and autoimmune disorders [4].

At the site in Terracina, air concentrations of CUPs were higher compared to Montalcino. Air concentrations (pg m$^{-3}$) of Pendimethalyn ranged from 30-1000 and Chlorpyrifos ranged from 70-10 000 (~10 ng). The seasonal variation of CUPs were less defined at Terracina. Nevertheless, Trifluralin showed a similar pattern of pesticide use at both sampling sites with highest concentrations during period 3 (Figure 2).

The differences observed at the sites might be related to the different agricultural practices. At Terracina, there is a prevalence of conventional agriculture and the use of a wide range of pesticides. However at Montalcino there is a long tradition for organic farming activities.

![Figure 2: Air concentrations (pg m$^{-3}$) of CUPs in Montalcino (Tuscany) and Terracina (Lazio) during April – December 2009 (Period 1: April-June; Period 2: July-September; Period 3: September-November; Period 4: November – December).](image)

4. Conclusions

Air concentrations of CUPs at Montalcino (Tuscany region) were lower than those detected at Terracina (Lazio). These results highlight differences in agricultural practices at the two sampling sites. The PUF-PAS samplers provides useful temporal information that can be used to relate atmospheric levels of current-use pesticides to seasonal usage patterns. This study is an important contribution to the knowledge of pesticides levels in the Italian atmosphere.

5. References


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1. Introduction

The use of illicit drugs within populations is difficult to estimate from crime statistics, surveys and information from individuals who seek treatment. Recent studies have shown that residues of illicit drugs in untreated domestic wastewater can be used to estimate drug consumption for the population serviced by the wastewater treatment plant [1-4]. In these studies, sampling of untreated wastewater has typically been conducted by either spot sampling or by collecting composite samples over periods of 8-24 h. However, Ort et al. [5] recently demonstrated that these sampling approaches may introduce artifacts that introduce errors of “100% or more” for estimates of the mean levels of drugs. One approach for avoiding these sampling errors is to utilize passive sampling devices to estimate the time-weighted average concentrations of drugs [5]. We and other researchers have shown that the Polar Organic Contaminants Integrative Passive Sampler (POCIS) provides estimates of the concentrations of pharmaceuticals in water and wastewater that are consistent with the concentrations of these compounds determined in spot samples [6,7]. However, in order to estimate the ambient water concentrations of compounds from accumulated amounts in POCIS, calibration data are required for sampling rates ($R_s$) of the individual compounds in POCIS in litres per day [7]. In this study, we conducted an evaluation of the capacity of POCIS to monitor municipal wastewater for estimates of community consumption of illicit drugs and other drugs of abuse, including cocaine and its major metabolite, amphetamine drugs, and prescription and illicit opioids. The study included: i) estimating POCIS sampling rates for the analytes in laboratory studies, ii) deploying POCIS in untreated wastewater from two wastewater treatment plants (WWTPs) in Canada to estimate time-weighted average concentrations of the analytes, iii) comparing estimates of community drug consumption generated by monitoring with POCIS and 24-h composite samples of the wastewater.

2. Materials and methods

Text POCIS sampling rates were determined using a static method described previously [7]. Briefly, experiments were conducted in triplicate in glass bottles containing 3 L of de-ionized water in a temperature controlled chamber at 20°C. The water was spiked with a mixture of the model compounds and a single POCIS was placed in the water for a period of 8 days. A magnetic stirrer was used to gently mix the water. Aliquots of water (20 ml) were removed from the bottles every 24 h to monitor the decrease in water concentration and the sampling rates were calculated from the decrease in water concentrations over time.

The POCIS samplers were suspended in for a deployment period of 2 weeks in untreated wastewater after grit removal in WWTPs serving a large city (population 1.6 million) and a small city (population 75,000) in Canada. POCIS in the pharmaceutical (i.e. Oasis HLB sorbent) configuration were purchased from Environmental Sampling Technologies, Inc. (St. Joseph, MO, USA). At the time of deployment and at retrieval, 24-h composite samples of wastewater were also collected for analysis. Wastewater flow rates and operational parameters in the WWTPs were monitored over the deployment period.

The method used to extract samples of water and wastewater was described previously by Metcalfe et al [4]. Briefly, aliquots of water from the static exposure experiment (20 mL) or the 24-h composite samples of untreated wastewater (100 mL) were extracted by solid phase extraction (SPE) using Oasis MCX cation exchange cartridges. An internal standard mixture of stable-isotope labelled surrogates was spiked into water or wastewater samples prior to SPE. The extraction procedures for POCIS were previously described by Li et al [7]. Briefly, the sorbent powder from the POCIS was transferred into a glass chromatography column and eluted with 50 ml of methanol. The internal standard mixture was spiked onto the sorbent prior to elution. The eluate was reduced in volume and transferred into sample vials for analysis. The extracts prepared from samples of water and wastewater and from POCIS were analyzed basically as described by Metcalfe et al [4], although the analysis methods for opioid drugs required development. All target analytes were analyzed by liquid chromatography with electrospray ionization and tandem mass spectrometry (LC-ESI-MS/MS) using an Applied Biosystems – Sciex QTrap 5500 instrument operated in multiple reaction monitoring mode.
3. Results and discussion

The sampling rates for the illicit drugs and other drugs of abuse in POCIS determined at 20°C varied between 0.1 to 0.3 L/d. These sampling rates are consistent with the Rs for pharmaceuticals determined previously using identical laboratory methods [7]. Of course, the conditions of temperature, pH, flow, biofouling, etc. for POCIS deployed in a WWTP will be different from the conditions in the static experiments used to estimate sampling rates. However, our previous experiments [7-9] have shown that sampling rates for pharmaceuticals into POCIS vary by less than 3-fold over water temperatures between 5-25°C, flow rates between 2.6 – 37 cm/s, and pHs between 3-9. Biofouling appears to have relatively little impact on accumulation into POCIS [10]. Therefore, these uptake rates for POCIS may be used to estimate time-weighted average concentrations of illicit drugs and other drugs of abuse in untreated wastewater over the range of conditions in a WWTP.

The preliminary results of monitoring studies in two Canadian WWTPs using analysis of both POCIS and 24-h composite samples show that cocaine and its major metabolite, benzoylecgonine (BE) are present at the highest concentrations among all of the target analytes. The concentrations of these compounds determined in composite samples and estimated from amounts accumulated in POCIS are consistent with our previous studies in Canada that showed BE concentrations between 287 and 2,624 ng/L and cocaine concentrations between 209 and 823 ng/L [4]. Amphetamine, methamphetamine, MDMA (i.e. Ecstasy), oxycodone, codeine and ketamine were detected at lower concentrations in 24-h composite samples and in POCIS.

4. Conclusions

Analysis is currently underway to compare the estimates of community drug consumption generated using data on the target analytes in 24-h composite samples and time-weighted average concentrations estimated from uptake into POCIS. Overall, these data indicate that monitoring of wastewater with the POCIS passive sampler may be a practical solution for providing accurate estimates of the consumption of illicit drugs and other drugs of abuse in communities.

5. References

Pharmaceuticals, personal care products, and agrochemicals in a rural Canadian watershed via passive and active sampling

Charles S. Wong¹, Jules C. Carlson¹-², Jennifer E. Low¹, Jonathan K. Challis¹, and Mark L. Hanson²

¹Richardson College for the Environment, University of Winnipeg, Winnipeg MB Canada
²Department of Environment and Geography, University of Manitoba, Winnipeg MB Canada
E-mail contact: wong.charles.shiu@alum.mit.edu

1. Introduction

Emerging organic contaminants, such as pharmaceuticals, personal care products, and current-use agrochemicals are of increasing concern in the aquatic environment. These chemicals are released into receiving waters by both human-use, through wastewater discharge, and by agricultural and veterinary use, through runoff. Although acute effects from such chemicals, particularly drugs, are unlikely at environmentally relevant concentrations, sublethal chronic concentrations may affect benthic invertebrates, fish reproduction, and microbial communities. Thus, it is essential to characterize loadings, fluxes, and aquatic fate of such contaminants in receiving waters, in order to characterize exposure and potential effects of such chemicals to non-target aquatic organisms.

Substantial work has been done to date to understand the occurrence, fate, and transport of such chemicals from urban centers to surface waters via wastewater treatment [1], which is generally done by conventional means (e.g., primary settling, secondary activated sludge treatment). Many smaller communities in North America, such as those within the Canadian Prairies, use wastewater lagoons to store and treat sewage before releasing at defined intervals, typically once or twice a year, to receiving waters. Treatment by this means is often more limited than that of conventional wastewater treatment used in larger municipalities. As a result, a pulse input with high concentrations of nutrients and organic contaminants enters receiving waters over discharge periods. These releases result in anoxic conditions from high ammonia and biochemical oxygen demand levels, and potential increased exposure to organic pollutants downstream of such sewage lagoons. However, little work has been done to date to characterize the occurrence of these emerging organic contaminants in rural areas [2,3] to determine if such contaminants pose a significant risk to human and ecosystem health. In addition, it is also not known if temporally variable inputs resulting from defined interval release from sewage lagoons can be adequately sampled using traditional grab sampling, or if time-weighted-average continuous measurements by passive water samplers could be used instead to characterize exposure levels of these contaminants [3].

In this study, we characterize occurrence, loadings, inventory, and post-release attenuation of pharmaceuticals, personal care products, and agrochemicals in Dead Horse Creek, Manitoba, a small tributary to the Red River that drains into Lake Winnipeg. The watershed of Dead Horse Creek is predominantly rural, with major agricultural and livestock land-use. The communities of Morden and Winkler (combined population 18,000) discharge their sewage lagoons to the Creek twice a year in late June and late October. We use both active grab sampling as well as passive sampling with the Polar Organic Chemical Integrated Sampler (POCIS) [4], to determine if reliable measurements of these chemicals can be obtained using both means, and to determine the extent of human versus agricultural and veterinary input of the target chemicals to receiving waters in this watershed.

2. Materials and methods

Surface water samples at Dead Horse Creek were periodically taken between June and October 2010 at five sites: a reference site upstream of known wastewater inputs (UPS); immediately downstream of Morden’s lagoon outfall (MDS), immediately upstream (WUS) and downstream (WDS) of Winkler’s outfall; and at the confluence of Dead Horse Creek and the Plum River (PUS) which is just upstream of the confluence of these tributaries to the Red River. POCIS samplers (pharmaceutical configuration with Oasis HLB sorbent) were deployed in triplicate over the field season at these sites, over sequential periods of approximately 3-5 weeks each. Grab samples were analyzed for nutrients (e.g., nitrogen and phosphorus) by wet chemistry techniques, to characterize inputs from point sources (i.e., sewage) and non-point sources (i.e., agricultural runoff), and for a suite of 44 organic chemicals typically found in wastewater-impacted receiving waters using Oasis HLB solid phase extraction and instrumental analysis by liquid chromatography-tandem mass spectrometry (LC/MS/MS). POCIS were extracted as previously described [3] and analyzed by LC/MS/MS.
3. Results and discussion

Eight analytes were routinely found in the Creek: the agrochemicals atrazine (ATZ) and 2,4-D, and the pharmaceuticals carbamazepine (CBZ), diclofenac, gemfibrozil (GEM), trimethoprim (TRM), naproxen, and sulfamethazine. Both agrochemicals had similar concentrations in the Creek both before and after lagoon discharge (Figure 1). Concentrations decreased downstream as measured by POCIS, and were not present in lagoon waters. These observations indicate that the presence of atrazine and 2,4-D in the Creek was from agricultural uses, consistent with corn and other crop production in the watershed, and that inputs from urban use (e.g., 2,4-D from home lawn and ornamental use) were likely minimal. Trimethoprim was found at low concentrations at UPS, indicating non-source contributions likely from veterinary usage. Carbamazepine and gemfibrozil (Figure 1) were not observed in Creek waters before lagoon discharge commenced, but rapidly increased in concentration to up to 200 ng/L similar to that observed in other wastewater-impacted waters in other Canadian urban and rural surface waters [3]. Thus, current wastewater treatment practices are insufficient to remove such chemicals from waste streams. Both chemicals are prescription drugs; thus, their presence only in wastewater-impacted receiving waters is consistent with a human-use point source. Time-weighted-average concentrations in POCIS, via published sampling rates [5] were consistent with grab sample measurements, indicating that POCIS was capable of providing consistent measurements of exposure levels of polar organic contaminants compared to instantaneous grab samples, with the added advantage of continuous measurement to average out temporal variability.

4. Conclusions

The POCIS passive sampler was able to provide measurements of pharmaceuticals and agrochemicals in surface waters consistent with grab samples. Together, these results show that while the predominant source of human-use drugs to rural watersheds was from wastewater inputs, a significant amount of contamination results from agricultural and veterinary inputs. Concentrations in receiving waters were similar to that in urban watersheds, indicating that exposure to pharmaceuticals in rural areas may be an issue of concern similar to that in urban regions.

5. References

Sampling of organic pollutants in marine waters using continuous flow integrative samplers and semipermeable membrane devices

León, V.M.¹, Llorca-Pórcel, J.², Moreno, R.¹, Tortajada, R.², Valor, I.²

¹ Instituto Español de Oceanografía, Centro Oceanográfico de Murcia, Apdo. 22, C/ Varadero 1, 30740 San Pedro del Pinatar, Murcia (Spain).
² LABAQUA-INTERLAB. Polígono Industrial Las Atalayas, C/Dracma, 16-18 03114 Alicante, Spain

E-mail contact: victor.leon@mu.ieo.es

1. Introduction

The determination of organic pollutants in the environment is relevant for the evaluation of the environmental status. The presence of organic pollutants in the water column has great fluctuations as a result of a combination of natural and anthropogenic effects. Indeed, the hydrofobicity of these compounds favours their sorption on particulate material and their bioaccumulation. For these reasons bivalves are commonly used in the marine international monitoring programs to evaluate the water column contamination. However as an alternative tool, last decades multitude of passive sampling devices and set-ups are being developed to get a representative water sample. Passive sampler accumulates contaminants as an organism from the environment, being proportional to concentration and time of exposition. The most commonly used device is the semipermeable membrane device (SPMD), that use low density polyethylene membrane for the determination of organic pollutants in surface, ground and marine waters. Recently other innovative integrative samplers have been developed with high sampling rates unaffected by turbulences and with negligible lag values, such as the continuous flow integrative sampler (CFIS), which use polydimethylsiloxane (PDMS) for the determination of polycyclic aromatic hydrocarbons (PAHs) and organochlorine compounds [1].

In this study, the efficiency of two integrative samplers (SPMD and CFIS) has been tested in the marine environment in spring and autumn. Concretely the study has been performed in Mar Menor Lagoon (SE of Iberian Peninsula, Spain), that is a hypersaline (42 to 47 psu) coastal lagoon with a mean depth between 3 and 4 m, that is one of the largest ones (135 km²) of the Mediterranean basin. Albujón Wadi is the main surface watercourse that flows into this lagoon from Cartagena Field, which is one of the most relevant horticulture areas in Europe. This watercourse is constituted by the excess of irrigation water from Cartagena Field area and brackish waters. The samplers have been immersed in 4 sampling points for 1 week and the daily concentration of pollutants has been determined by SBSE/GC/MS. The specific objectives of this study were: a) to determine the daily concentration of organic pollutants in marine waters, b) to determine the mean concentration of organic pollutants for a week using SPMD and CFIS c) to compare repeatability and the efficiency of both passive samplers for the integration of marine samples in the considered period and e) to assess the seasonality of the bioavailable concentration for organisms in this area.

2. Materials and methods

The low density polyethylene membranes of SPMD were supplied by…

Commercial stir bars (20 mm×0.5 thick and 49 µL of PDMS) used in CFIS were supplied by Gerstel (Mülheim a/d Ruhr, Germany).

In this study four sampling points have been considered to evaluate the passive samplers: close to Lo Pagán Port (J1), at the south of Los Alcázares port (J2) and at increasing distance from the mouth of the Albujón Watercourse (J3 at 0.7 km and J4 at 2 km). SPMD and CFIS were fixed to a stainless steel cage on the seabed (2-3 m depth) for a week. The repeatability of both passive sampling systems has been evaluated installing 2-3 sampling devices in different sampling points. The analysis of commercial stir bars of CFIS was performed by thermodesorption on GC/MS [2]. The extraction and analysis of organic pollutants in SPMD membranes applying the previously validated method [3].

Figure 1: Sampling points in Mar Menor Lagoon and Albujón Watercourse.
The efficiency of passive samplers has been evaluated comparing the obtained integrative concentration with the real water one. With this goal, surface water was sampled daily with a Niskin bottle in the early morning and the late afternoon. The input of organic pollutants through Albujón Watercourse (AW) has been also simultaneously determined (concentration and flow).

In the 5 sampling points temperature, pH, conductivity and dissolved oxygen were determined in situ using a portable multiparametric probe (VTW). PAHs, polychlorinated biphenyls (PCBs), triazines, organophosphorous and organochlorinated pesticides were analysed by stir bar sorptive extraction and thermal desorption coupled to capillary gas chromatography-mass spectrometry in surface [2] and marine water samples[4].

3. Results and discussion

3.1. Input of organic pollutants from Albujón watercourse

The input of organic pollutant in Mar Menor lagoon take place through direct discharges, surface watercourses and air deposition. The organic pollutants present in the Albujón Watercourse mouth were mainly pesticides and surfactants, although other pollutants were also detected, such as PAHs. The higher concentrations correspond to triazines, organophosphorous pesticides and nonylphenols (NP), which were continuously present in the Albujón water. The presence of these compounds in the Albujón watercourse confirms that is a relevant input pathway. Significant daily, weekly and seasonally differences were observed in the organic pollutants concentrations. Herbicides were especially abundant in autumn and insecticides in spring. NP concentration varies from 70 to 900 ng/L in spring, with higher concentrations detected last exposition days.

3.2. Efficiency of SPMD and CFIS in marine water

CFIS and SPMD have been applied per duplicate and triplicate in several sampling points. The repeatability of the obtained results has been satisfactory (lower than 20%). The sampling points J3 and J4 were close to Albujón Watercourse, and consequently a high variation in the organic pollutants concentrations were detected, especially for nonylphenols, triazines and organophosphorus pesticides. The mean concentrations of NP detected with CFIS in spring were similar in the four sampling points (180-250 ng/L). Consequently different sources of this pollutant must be present around Mar Menor lagoon. PAHs and organophosphorus pesticides were also detected with passive samplers, and low concentrations of different organochlorinated compounds. Terbuthylazine and other triazines have been also detected in the CFIS but the use of PDMS is only adequate for compounds with log Kow higher than 4 because of the short t₁/₂ for “polar” compounds, and no linear zone for long sampling days.

4. Conclusions

CFIS and SPMD systems are two useful tools as integrative samplers in the marine environment and results were similar to those obtained with daily sampling method. Their potential application is limited by the hydrofobicity of the considered pollutants, because they are not adequate for the more hydrophilic compounds as triazines.

5. References


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Evaluation of non agricultural pesticides air contamination: a field study using Tenax passive samplers

Caroline RAEPPEL1,2, Brice M. R. APPENZELLER2, Olivier BRIAND3, Maurice MILLET1, Ludovic TUDURI4

1 LMSPC, Equipe de Physico-Chimie de l’Atmosphère (UMR 7515 CNRS - Université de Strasbourg), 1 rue Blessig, 67084 Strasbourg Cedex (France)
2 CRP Santé, Laboratoire de Toxicologie (Université du Luxembourg), Campus Limpertsberg, 162A avenue de la Faïencerie, 1511 Luxembourg (Luxembourg)
3 ANSES, 27-31 avenue du Général Leclerc, 94701 Maisons-Alfort Cedex (France)
4 Institut des Sciences Moléculaires (UMR-5255 CNRS-Université Bordeaux 1), LPTC, Site Universitaire, 24019 Périgueux cedex (France)
E-mail contact: craeppel@unistra.fr

1. Introduction

If the influence of agricultural activities on atmospheric contamination processes is now well documented, few studies are available about the impact of non-agricultural pesticides use on indoor and outdoor air quality. Actually pesticides are also used for different treatments like pest control or green way and public roads upkeep. These uses lead to an outside contamination as well as an indoor contamination once pesticides are applied in enclosed rooms or, once a transfer from outside to inside trough clothes, shoes or air exchange is existing [1]. To evaluate the influence of these non-agricultural activities on air contamination, the technique of passive air sampling has been chosen. The sampling principle is based on a flow formation induced by a potential difference between the sample and the sampling support until equilibrium is reached or until the sampling is stopped [2]. This simple method is easy to carry out and cost-efficient, and it allows large scale sampling, essential for providing a specific description of the spatial and temporal variations in the atmospheric contamination levels.

2. Materials and methods

The passive air samplers (PAS) used in this study have been developed in our laboratory and consist of a Tenax resin tube protected by a specially designed shelter allowing an air flow. This system enables diffusion through the Tenax resin tube. Tenax resin presents properties which are suitable for pesticides sampling but also for being extracted by thermal desorption, what is enormously advantageous as no further sample handling is needed [3].

Afterwards exposure, and as some pesticides need a derivatisation step prior to the analyse, a silylation agent (MBSFTA) have been added in the tubes [4]. In order to do an internal calibration, internal standards (4-Nitrophenol-d4 and Trifluralin-d14) have been added in the tubes too. Then the tubes have been extracted by thermal desorption using an Automatic Thermal Desorber (ATD). After this first step, molecules have been analysed by gas chromatography and mass spectrometry.

In this study, two sites of two companies and one of a private house located in eastern France were chosen to perform the sampling which occurred after a professional or non-professional pesticides application for the upkeep of the areas. In total, eight active substances (2,4 D, Dichlorprop, Diflufenican, Flazasulfuron, MCPA, Mecoprop-p, Picloram and Triclopyr) were used during the treatments and were therefore analysed. PAS were deployed outside but also inside, in the offices or in the house, in order to see if there is a possible contamination coming from outdoor.

3. Results and discussion

3.1. Field sampling on a company site: June 2010

These results correspond to a field sampling lead in June 2010 on a company site. Three active substances were applied during the treatment. 10 samples were collected per sampling point during 1 month. Results have shown that a part of applied pesticides were found mainly in the PAS corresponding to the day of the treatment. One pesticide was found, only outside, on the PAS put in place on the application day (Picloram: 1518 – 14326 pg.PAS−1.day−1). The second one was also detected on the treatment day but on following days too (Diflufenican: 1274 – 42941 pg.PAS−1.day−1), suggesting volatilisation process. 2,4 D was observed...
throughout the sampling, outside and inside (2,4 D: 200 – 19498 pg.PAS\(^{-1}\).day\(^{-1}\)). This pesticide was also found in a blank made before the treatment what could be explained by another source, knowing that the sampling site is located in an agricultural area. But the highest values were nevertheless observed on the treatment day.

3.2. Field sampling on a company site: October 2009

The results presented in Figure 1, shows the evolution of the mass of two pesticides applied on the same company site as the precedent. One sample is represented by a horizontal line marked by two points. The quantities measured are highest on the application day even if for 2,4 D outside, the difference between this value and the others is less important. 2,4 D was detected during the all period of sampling, inside and outside, like in the first example. Triclopyr was only observed on the day treatment and outside. This suggests that there is no volatilisation of the product after the application.

![Figure 1: Mass of active substances in pg measured per PAS.](image)

4. Conclusions

This work has allowed to highlight the Tenax PAS capacity to measure pesticide concentrations in air as shown in this two field sampling and in the others not presented here. The highest quantities of pesticides have been detected on the day application. Some active substances continued to be emitted into air by volatilisation. For some pesticides, a contamination of indoor air has been observed.

As passive air sampling is based on a diffusive sampling, it is not possible to define the analysed air volume, unless to calibrate the samplers. Therefore, a calibration of the Tenax PAS is in progress in order to determinate the sampling rate of this system and express the results in mass per air volume.

5. References


Acknowledgement - The authors thank ADEME for the PhD financial support.
Monitoring by LC-MS/MS of 28 Endocrine Disruptor Compounds in surface water using passive sampling devices: comparison of POCIS and Chemcatcher

Julien Camilleri¹⁺, Nicolas Morin²⁺, Cécile Cren¹, Marina Coquery², Cécile Miège²

¹ Service Central d’Analyses (SCA) du CNRS, USR59, Echangeur de Solaize, Chemin du Canal, 69360 Solaize, France
² Cemagref, UR MALY, 3 bis quai Chauveau, CP 220, F-69336 Lyon cedex 09, France
⁺ Both authors contributed equally to this work.

E-mail contact: j.camilleri@sca.cnrs.fr

1. Introduction

Regarding to the growth of interest concerning the presence and the identification of human made pollutants and xenobiotics in the environment, multi-residue analysis techniques and representative water sampling methods need to be developed.

Some of these micro pollutants of interest are known as Endocrine Disruptor Compounds (EDCs) due to their effects on the endocrine causing behavior disorders, decreased reproduction or birth malformations for example [1], [2]. As those molecules display large range of physico-chemical properties, most of the current studies are focused on one or two EDCs families.

This project is focused on the evaluation of the current pollution of surface water media threw the development of a multi-residue and multi-family of EDCs analysis method coupled to passive sampling devices.

2. Materials and methods

The EDCs of interest have been chosen for this project among emerging contaminants listed by the European Union [3], [4]. Thus 28 compounds (see Table 1) has been selected in order to get a mixture of different EDCs families representatives of both agricultural, pharmaceutical and human pollution, that can be found in surface water and analysed by liquid chromatography tandem mass spectrometry (LC-MS/MS).

### Table 1: List of selected EDCs.

<table>
<thead>
<tr>
<th>Pesticides</th>
<th>Hormones</th>
<th>Alkylphenols</th>
<th>Phenolic derivates</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-dichlorophenoxyacetic acid</td>
<td>Estrone</td>
<td>4-n-octylphenol</td>
<td>2,4-dichlorophenol</td>
</tr>
<tr>
<td>Acetochlore</td>
<td>17β-Estradiol</td>
<td>4-tert-octylphenol</td>
<td>Bisphenol A</td>
</tr>
<tr>
<td>Alachlore</td>
<td>Megestrol acetate</td>
<td>4-nonylphenol</td>
<td></td>
</tr>
<tr>
<td>Atrazine</td>
<td>Progesterone</td>
<td>4-para-nonylphenol</td>
<td></td>
</tr>
<tr>
<td>Carbendazim</td>
<td>Testosterone</td>
<td>4-tetr-butylphenol</td>
<td>Resorcine</td>
</tr>
<tr>
<td>Duron</td>
<td>Tamoxifen</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iprodion</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Linuron</td>
<td>3,4-dichloroaniline</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Prochloraz</td>
<td>Carbamazepine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thirame</td>
<td>Diclofenac</td>
<td></td>
<td></td>
</tr>
<tr>
<td>UV Filter</td>
<td>4-methylbenzyldien camphor</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

2.1. Sampling devices

Two passive sampling devices have been selected to monitor selected EDCs: the Polar Organic Chemical Integrative Samplers (POCIS in Pharm configuration) and the Chemcatcher (in polar configuration). Both are still in development but already showed good efficiency to monitor some of the compounds of interest [5], [6]. Calibration of those systems have been realised with the Cemagref Lyon in 50L aquariums with continuous renewal of doped solutions for 28 days.

2.2. Analytical method

Endocrine disruptors display a wide range of structural diversity and chemical properties. Almost all available analytical methods only target one or two families of EDCs, such as steroids, pesticides or alkylphenols [7], [8]. In consequence, a multi-residue methodology for surface water have been developed on an Agilent 1200
RRLC system coupled to a triple stage quadrupole spectrometer from ABSciex (3200 QTrap) using Phenomenx Kinetex C18 columns.

3. Results and discussion

3.1. Calibration and exposure of passive samplers

Chemcatchers and POCIS showed good linearity for most of the compounds of interest up to 21 or 28 days but seem to have reached equilibrium after 14 days for some of the molecules. Calibration experiment has been validated by Cemagref Lyon with controlled and stable temperature, conductivity and flow. Both systems showed same behaviour but calculated sampling rates were higher for the POCIS than for the Chemcatcher. The two systems have been exposed in the field for 4 weeks between June and July 2010.

3.2. Analytical method

The developed analytical method allow us to detect compounds of interest (IDL) at 1 to 5 ng/L for positive ionisable molecules and varying from 20 to 250 ng/L for the negative ones. Separation and quantitation are performed in two runs within 12 minutes for each mode.

4. Conclusions

Calibration of POCIS and Chemcatcher on 28 endocrine disruptors have been performed with well controlled and reproducible exposure conditions. Experiments confirmed linearity for some of the molecules and enable us to calculate sampling rates for some that hasn’t been tested yet. Those two passive samplers combined to an optimised multi-residue analytical method allow us to monitor a representative mixture of endocrine disruptors in surface waters. Further investigations should enable us to compare water sampling methods to toxicological in the field exposure.

5. References

[4] European commission DG ENV. Towards the establishment of a priority list of substances for further evaluation of their role in endocrine disruption, bkh-annex 15

Acknowledgement - The authors thank the MEEDM (Ministère de l’Ecologie, de l’Environnement, du Développement durable et de la Mer) for project’s funding including Julien Camilleri’s doctoral stage and also the Region Rhône Alpes for doctoral funding for N. Morin.
1. Introduction

Equilibrium sampling into various formats of the silicone polydimethylsiloxane (PDMS) is increasingly used to measure the exposure of hydrophobic organic chemicals in environmental matrices, and passive dosing from silicone is increasingly used to control and maintain their exposure in laboratory experiments. Both these equilibrium partitioning approaches are normally calibrated to freely dissolved aqueous concentrations (C\text{free}), which often are considered the effective concentration for partitioning, bioconcentration and toxicity.

We are presently exploring two possibilities to go one step further by extending the calibration of such methods towards equilibrium partitioning concentrations in lipids (C\text{lipid,partitioning}). The first approach proceeds in two steps; (i) the concentration in the PDMS (C\text{PDMS}) is determined and (ii) multiplied with recently determined lipid to PDMS partition coefficients (K\text{lipid,PDMS}) [1] A variation of this first approach is to (i) determine the chemical activity in the polymer and (ii) divide it by recently determined activity coefficients in lipids [2]. The second approach applies external partitioning standards in vegetable or fish oil for the complete calibration of equilibrium sampling techniques without additional steps [3]. Both calibration principles take the analyte specific lipid to PDMS partitioning into account. This is in contrast to many biomimetic sampling techniques, which often assume a generic relationship between partitioning to polymer and bioconcentration into biota.

2. Materials and methods

\textbf{In tissue sampling.} Silicone (PDMS) thin-films were used for equilibrium sampling of polychlorinated biphenyls (PCBs) in intact tissue of two eels and one salmon [4]. The PCB concentrations in the polymer (C\text{PDMS}) were then multiplied with lipid to PDMS distribution ratios (D\text{lipid,PDMS}) according to equation 1 to obtain concentrations in fish lipids (C\text{fish lipid,partitioning}):

\[ C_{\text{fish lipid,partitioning}} = C_{\text{PDMS}} \times D_{\text{lipid,PDMS}} \]  

A ‘classical’ exhaustive extraction technique was applied to determine lipid normalized PCB concentrations, which assign the total body burden of the chemical to the lipid fraction of the fish.

\textbf{Equilibrium sampling of PCBs in sediment.} Two equilibrium methods were applied to PCB-contaminated lake sediment, and calibrated with respect to C\text{lipid,partitioning}; (i) Equilibrium sampling with internally coated glass jars with varying thicknesses of PDMS resulted in proportionality between coating and analyte mass, which confirmed several validity criteria. C\text{lipid,partitioning} was here determined as product of C\text{PDMS} and PDMS to lipid partition coefficient. (ii) Solid phase microextraction fibers with a 7 µm PDMS coating were equilibrated in the headspace above the sample (HS-SPME) and then calibrated using SPME fibers equilibrated above external partitioning standards in olive oil. Native chironomidae larvae from the sediment were analysed for PCBs and lipid content, and the results of the equilibrium sampling methods were finally compared to the lipid normalized concentrations in the worms.

\textbf{Passive dosing of polycyclic aromatic hydrocarbons (PAHs) to soil invertebrates.} Passive dosing with silicone was used to maintain defined and constant chemical activities of several PAHs in 7 days toxicity and bioconcentration tests with the terrestrial springtail \textit{Folsomia candida}. The chemical activities were divided by recently determined activity coefficients in olive oil in order to determine C\text{lipid,partitioning}, which then were related to the observed bioconcentration and toxicity in the springtails.
3. Results and discussion

**In tissue sampling.**

Lipid based PCB concentrations obtained by equilibrium in tissue sampling in the three investigated fish were in good agreement with those determined using total extraction and lipid normalization. These results support the validity of the in tissue sampling technique, while at the same time confirming that the fugacity capacity of these lipid-rich fish tissues for PCBs was dominated by the lipid fraction.

**Equilibrium sampling of PCBs in sediment.**

Concentrations, obtained by two different equilibrium sampling methods and two different calibration principles were in good agreement, meaning that the two methods validate each other. The coated glass jar method was applied to field sediment containing invertebrates, which led to concentrations that were about a factor of two higher than measured lipid-normalized concentrations in the organisms.

**Passive dosing of PAHs to soil invertebrates.**

**Bioconcentration:** Lipid normalized concentrations of 2 - 3 ringed PAHs at the end of the test exceeded the predicted equilibrium partition concentrations in lipids. This might be explained by the composition of the invertebrates that beside the lipids also contain waxes with an expected high capacity for PAHs. To the contrary, lipid normalized concentrations of the heavier PAHs remained below the predicted equilibrium partitioning level. This indicates that the test duration was not sufficient to reach the internal equilibrium partitioning level for the heavier PAHs.

**Toxicity:** Lethal concentrations (LC-50) of naphthalene, phenanthrene and pyrene were determined on a lipid partitioning basis and were in good agreement with the typical range of lipid membrane burdens for baseline toxicity (40-160 mM) [5]. This demonstrates that these new calibration principles also can be applied within a toxicological context.

4. Conclusions

Several equilibrium partitioning methods have been combined with new calibration principles that are directed at equilibrium partition concentrations in lipids. The obtained results showed good agreement between (1) in tissue sampling results and lipid normalized concentrations in fish, (2) two equilibrium sampling methods for sediment and (3) lipid based LC-50 values and the typical range of lipid membrane burdens for baseline toxicity. This gives confidence to the validity of the partitioning methods and the applied calibration principles. However, it remains important to be aware of that express equilibrium partitioning concentrations, which in some cases will make a good prediction of actual concentrations in lipids while in other cases only being a well defined reference value.

5. References


Acknowledgement - This research was funded by the EU Commission (MODELPROBE, 213161; Marie Curie IEF, PIEF-GA-2008-219675), CEFIC-LRI (ECO 15, ECO 16), the Danish Council for Strategic Research (REMTEC), the Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS, 216-2005-1200) and the Academy of Finland (project no. 123587). We thank Margit Fernqvist, Kilian Smith and Martin Holmstrup for their technical and scientific support.
PWSDs-YES application as estrogenic chemicals screening and monitoring tool for STP effluents

Un-Jung Kim¹, Hee-young Kim¹, In-Seok Lee¹,², Jeong-Eun Oh*¹

¹Dept. of Civil and Environmental Engineering, Pusan National University, Busan 609-735, Rep.of Korea
²National Fisheries Research & Development Institute (NFRDI), Busan, Rep. of Korea
E-mail contact: jeoh@pusan.ac.kr

1. Introduction

Commonly, STPs are regarded as point source of the EDCs in water that have inhibited human and animal endocrine activities [1]. Conventionally to monitor these compounds in water from point source, grab water sampling was used to analyze pollutants but by this method, only water soluble compounds’ temporal level can be detected and long term effect caused by bioaccumulation cannot be predicted [2]. It is suggested that integrative passive water sampling devices (PWSDs) like semi permeable membrane device (SPMD) can detect organic compounds in water to monitor wide concentration variation with time and to conjecture bioaccumulation of organic pollutants for a longer period [3]. Also, with combining yeast estrogen screening (YES), SPMD can show estrogenicity of sample as a term of Estradiol equivalent quantity (EEQ) [2]. Passive samplers are used globally as monitoring tool for organic contaminants in water [4] but in Korea, grab sampling method has been used only. With the restoration project on four major Korean rivers these days, water quality, flood control, and ecosystem vitality are major issues in Korea. Therefore, improvement of current water monitoring system is needed to understand exact status of water quality and potential impacts to the ecosystem. The research goal of this study is to develop water quality monitoring system using SPMD-YES combining method for monitoring Korean waters to compensate limitations of current water grab sampling methodologies. As a preliminary study, in this paper, comparison between grab and passive sampling was conducted for estrogenic compounds to check possibility of SPMD-YES application on water quality monitoring. Also several sophisticated conditions including adsorbent type, deployment period were tested.

2. Materials and methods

Sampling and deployment: Preliminary studies were conducted on June and July, 2010 at domestic sewage treatment plant. The water samples were collected by using grab sampling method and passive sampling method together at the same sampling positions. For SPMD, to estimate efficiency and consistency of adsorbent, triolein and olive oil were used as SPMD adsorbents and triplicate SPMDs packed with each adsorbent were deployed for one week and two weeks, respectively. Each 1 SPMD among triplicates were PRC injected and corrected for potential loss of target compounds.

Analytical procedures: Main target compounds will cover EDCs compromising PBDEs, PCBs, PCDD/PCDFs, HBCDs and TBBPA. Labelled PAH internal standard was injected as performance reference compounds (PRC) to check possible loss of target compounds during sampling period. To check SPMD-YES application, both filtered water sample and SPMD extracts were pretreated based on EPA method.

3. Results and Discussion

3.1. SPMD Consistency in STP treatment water (Preliminary result)

PCBs and PBDEs concentration were measured in each triolein and olive oil packed SPMD and presented in Figure 1. Even though some variation was observed, all SPMD samples were shown similar range of concentration regardless of adsorbent types, indicating the consistency of SPMD and efficiency of both adsorbents in water monitoring. The total amount of PCBs and PBDEs were not so different between 1 week deployed SPMDs and 2 weeks deployed SPMD but the calculated concentration based on the result of PRC-SPMD sample in 2 weeks deployed SPMD was almost two times higher than 1 week sample, suggesting either sampling period is okay. However, regarding RSD and convenience of pretreatment process, deployment of triolein based SPMD for 2 weeks is better to apply for Korean STP system.

3.2. Comparison of SPMDs deployment and Grab water sampling

The STP treatment water samples were taken at four spots of STP compromising influent, 1st and 2nd settling basin effluent with using SPMD and grab sampling method. SPMD was deployed for one week and the water samples were collected by grab sampling method on the first day of sampling period of SPMD. The
concentrations analyzed from SPMD and grab samples at 2nd settling basin and effluent were presented in Table 1. In the case of total PCBs and PBDEs, the concentrations of samples collected at 2nd settling basin were similar on both sampling methods but for the other cases, a pretty big difference was observed. However, this is only one case study and the grab sampling was done for only one time during SPMD sampling period (1 week), so further investigation is needed.

Table 1 Concentration of target compounds

<table>
<thead>
<tr>
<th>Compounds</th>
<th>2nd sett-Spmd</th>
<th>2nd sett-Grab</th>
<th>Effluent-Spmd</th>
<th>Effluent-Grab</th>
</tr>
</thead>
<tbody>
<tr>
<td>total PCBs</td>
<td>4.03</td>
<td>2.80</td>
<td>15.2</td>
<td>1.87</td>
</tr>
<tr>
<td>total PBDEs</td>
<td>2.41</td>
<td>1.11</td>
<td>10.0</td>
<td>0.87</td>
</tr>
<tr>
<td>PCDD/PCDF</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
<td>&lt;LOQ</td>
</tr>
<tr>
<td>TBBPA</td>
<td>5.4</td>
<td>NA</td>
<td>5.5</td>
<td>NA</td>
</tr>
<tr>
<td>total HBCDs</td>
<td>0.16</td>
<td>NA</td>
<td>0.56</td>
<td>NA</td>
</tr>
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3.3. Yeast Estrogenic Screening

YES assay was performed on the extract samples taken from grab sampling and SPMD sampling method. Although there was positive responses from both grab water and SPMD extract but, no significant relationship between EEQ and target compounds was observed in grab water and SPMD samples (Figure 2 and 3), indicating presence of other pollutants that induce estrogencity in waste water.

4. Future Research

As confirmed in preliminary result, 1-2 weeks deployment with triolen based SPMD was suitable and efficient to detect target estrogenic compounds in STP. Based on this result, SPMDs were deployed in STPs and river near STP to assess the SPMD-YES sampling method as monitoring tool in Korea, so the result will be discussed more in the conference.

5. Reference


Acknowledgement - This work was supported by grant No. 331-2008-1-D00280 of the National Research Foundation (NRF) of Korea.
1. Introduction

In many areas of environmental sciences and engineering, partition coefficients (K) are widely used for the characterization of the fate of chemicals. Experimental determination of partition coefficients between two phases, however, becomes very difficult as the absolute value increases. In a conventional shaking method, time required for equilibration between two phases increases exponentially with increasing log K. This often results in many potential experimental artifacts.

According to a film diffusion theory in the boundary layer, the rate of diffusive mass transfer is a function of diffusion coefficient, the thickness of the boundary layer of a fluidic medium, and partition coefficient between the two phases. Partition coefficient can be correlated with experimentally determined mass transfer coefficient by a simple relationship because diffusion coefficients in a well-characterized medium such as air and water can be easily predicted and the thickness of the boundary layer is a dynamic property of the medium. Thus, an innovative method using the diffusion in the boundary layer can be used for the measurement of partition coefficients between two phases in a short time.

In this presentation, we will show three model systems applying this principle - partition coefficients between poly(dimethylsiloxane) and water (K_{PDMSw}), 1-octanol/air partition coefficient (K_{oa}), and Henry’s law constant (H). Polycyclic aromatic hydrocarbons were chosen for the model compounds for a proof of principle. In addition, phthalate esters, musks, and chlorinated benzenes were added for the evaluation of H.

2. Materials and methods

Figure 1 describes three model systems for the determination of partition coefficients using diffusive mass transfer. In all three systems, although the concentration change in the dosing phase was very small and cannot be easily differentiated from the initial concentration, the increase in concentration of accepting phase was noticeably higher than the initial concentration (C_0 = 0). Thus, the increase of chemical concentration in the accepting phase with time was used for the determination of a mass transfer rate constant. This constant was then converted into partition coefficient. For the determination of Henry’s constant, we performed the experiment using both air and water boundary layers.

*Figure 1: Description of experimental designs used for kinetic determination of (a) K_{PDMSw} (ref 1), (b) K_{oa} (ref 2), and (c) Henry’s law constant (slightly modified from ref 3).*
3. Results and discussion

3.1. Comparison with literature data

All partition coefficients in three model systems were measured in 30 h, much shorter than that of conventional methods used for determination of \( K \). However, the obtained values of \( K_{\text{PDMSw}} \), \( K_{\text{oa}} \), and \( H \) were in good agreement with those obtained using conventional methods in literature, confirming that these kinetic method of determining high \( K \) can be used reliably in many other areas when the diffusive mass transport is dominated by diffusion in the boundary layer.

3.2. Applicability domain of the method

The range of partition coefficients that could be determined within 30 h was \( 10^3 \sim 10^6 \) for \( K_{\text{PDMSw}} \), \( 10^5 \sim 10^{10} \) for \( K_{\text{oa}} \), and \( 10^{-7} \sim 10^{-4} \) atm \( \text{m}^3 \text{ mol}^{-1} \) for \( H \). A simple model calculation can be made to know the change in the ratio of the acceptor concentration to the initial donor concentration with experimental time. Increasing experimental time is inefficient to extend the measurable range of partition coefficients and the solubility of chemicals in dosing phases (e.g., PDMS or n-octanol in the presentation) is limited. Thus, the reliable range of partition coefficients would be the range shown here. However, the limit of the upper \( K \) values might be extended by lowering the analytical detection limit of target chemicals.

4. Conclusions

In all three applications, kinetica partition coefficients were measured in a short time and were well correlated with literature data with only a few chemicals. This method can be applied for partitioning of many other organic chemicals between two phases when the overall mass transfer is dominated by diffusion in a stagnant film.

5. References


Acknowledgement - The authors thank 3R Research Foundation in Switzerland, Concawe, and National Research Foundation of Korea (NRF) for funding.
Does the kinetic resistance on the sampling medium side affect passive air sampling rates? Experimental evidence and modeling

Xianming Zhang\textsuperscript{1}, Ying D. Lei\textsuperscript{1}, Frank Wania\textsuperscript{1}, Masahiro Tsurukawa\textsuperscript{2}, Takeshi Nakano\textsuperscript{2}

\textsuperscript{1}Department of Chemistry and Department Physical and Environmental Sciences, University of Toronto Scarborough, 1265 Military Trail, Toronto, ON, M1C1A4, Canada
\textsuperscript{2}Hyogo Prefectural Institute of Environmental Sciences, 3-1-27, Yukihiro-cho, Suma-ku, Kobe 654-0037 Japan

E-mail contact: xianming.zhang@utoronto.ca

1. Introduction

Various types of dynamic-uptake based passive air samplers (PASs) have been widely applied to monitor semi-volatile organic compounds (SVOCs) in the atmosphere. The uptake of SVOCs in such PAS is assumed to occur by molecular diffusion from air to the passive sampling medium (PSM). So far, this process is described using the two-film model \cite{note1}, which was originally proposed to describe mass transfer across gas-liquid interfaces. Commonly made assumptions include (1) the resistance to chemical uptake posed by the PSM is negligible and the air-side resistance is rate-controlling; (2) chemical distribution within the PSM is uniform; (3) during the initial uptake stage (operationally defined as the linear uptake range) chemical concentration on the PSM are so low that surface evaporation is negligible. With these assumptions, chemical uptake in PAS can be quantified with a simple linear equation. Air concentration of the chemical can be determined from the amount sequestered on the PSM during the sampling period and the sampling rate ($R$, m\(^3\)/d). According to this approach, $R$ is proportional to air-phase molecular diffusivity, which is a weak function of temperature and molecular size as described by the Fuller-Schettler-Giddings Equation. However, more and more field calibration studies indicate that variations of $R$ with temperature and molecular size are larger than can be explained by the influence of temperature on diffusivity \cite{note2,note3}. This was attributed to a possible two-phase sorption process in some studies \cite{note4} but no further investigation has been done. In this study, we first critically review the current understanding of uptake in PAS. The difference of phase transfer between air and a liquid and between air and a porous medium is emphasized. Considering the observed dependence of $R$ on temperature and molecular size and the limited penetration of SVOCs into sampling media during active sampling (as revealed by break-through experiments), we hypothesize that SVOCs are not evenly distributed within porous PSM and thus the kinetic resistance within the PSM should not be ignored. The objectives of this study are to (1) test the hypothesis by experimentally investigating the penetration of SVOCs into interior layers of the PSM and (2) propose a model that considers the kinetic resistance within the PSM.

2. Materials and methods

2.1. Experiment on the uptake kinetics within PSM

In this study, we investigated the passive uptake of polychlorinated biphenyls (PCBs) in two of the most widely used PSM: XAD-resin and polyurethane foam (PUF). In order to assess the kinetics of chemical transfer within the PSM, we segmented the cylindrical PSM into three concentrical layers, which can be separated easily and analyzed individually after sample retrieval. The layered PSM and their dimensions are shown in Figure 1. An office previously identified as having very high PCB concentrations was selected as the sampling site. Duplicated XAD (PUF) samples were retrieved after being deployed for 0, 1 (0.5), 2 (1), 4 (2), 8 (4), 12 (8) and 24 (12) weeks. Upon retrieval, the layered PSM were separated. Each layer was spiked with 100µL 25 pg/µL \textsuperscript{13}C-labeled PCB-77, -101, -141 and -178 as surrogate standards and then Soxhlet extracted for 20 h with 1:1 acetone/n-hexane. Extracts were roto-evaporated to ~2 mL and eluted through dehydrated sodium sulphate. The eluent was blown down with N\textsubscript{2}, solvent exchanged to iso-octane and reduced to ~0.5 mL in GC vials, to which 100 ng of mirex was added for volume correction. PCBs in the samples were analyzed with an Agilent 5890 gas chromatograph (HT8-PCB column) coupled with a JMS-800 double focusing high resolution mass spectrometer.
2.2. A diffusive penetration model integrating chemical uptake from air and transfer within PSM

The PSM is composed of the sorbent material and the air-filled pores, which is quite similar to a soil except that there is no water phase in the PSM. Therefore, we modeled chemical transfer within the PSM analogous to chemical transfer within soil. Unlike soil, which is modeled as a plane sheet, the PSM used in this study have a cylindrical geometry and chemicals diffuse into the PSM in the radial direction. The modeling approach is schematically shown in Figure 2. The model considers the transfer kinetics within the PSM via the effective diffusivity which is controlled by diffusion in the pores, sorption to the solid PSM and tortuosity. The model only considers chemical transfer within the PSM via diffusion in the pores, i.e. no surface/solid phase diffusion, advection or reaction occur. Chemicals in the sorbed phase and in the air pores are assumed to be in local equilibrium.

3. Results and discussion

3.1. PCB Uptake and Distribution within the PSM

- PCBs sequestered within the PSM are not uniformly distributed
- Penetration of the PCBs into the PSM is kinetically limited
- PCB uptake in the outer layer shows a linear relationship with deployment time
- The lighter PCB congeners (mono- and di-) can be detected in the mid-layer only after 4 weeks; after 12 weeks the amount in the middle layer is only 5 to 20% of that in the outer layer
- In the inner layer, only the mono-PCB can be detected after 12 weeks
- Compounds with higher volatility (lower sampling medium-air partitioning coefficient) penetrate into the PSM at a higher rate, indicating diffusion within the porous air phase dominates the transfer process within the PSM

3.2. Model description of chemical uptake and penetration into the PSM

- In accord with the experimental results, the diffusive penetration model predicts that PCBs are mostly sequestered in the outer layer of the PSM; negligible amounts of less volatile congeners (e.g. Penta-PCBs) penetrate to the middle layer
- Compared with the current PAS model that ignores the PSM side resistance, the model presented here predicts a lower uptake rate.
- Predicted sampling rates of volatile compounds (Mono-PCB) decrease faster due to higher surface concentrations and thus higher loss rates via surface evaporation

4. References


Acknowledgement - The authors are grateful for research funding from the Canadian Foundation for Climate and Atmospheric Sciences and the Natural Sciences and Engineering Research Council of Canada. XZ acknowledges scholarship support from the Centre for Global Change Science, University of Toronto.
Global Cycling of PCBs - Intercontinental and Northward Migration of Distributions Predicted by Multicompartimental Modelling

Irene Stemmler¹, Gerhard Lammel¹,²

¹ Max Planck Institute for Chemistry, Mainz, Germany
² Research Centre for Toxic Compounds in the Environment, Brno, Czech Republic
E-mail contact: irene.stemmler@zmaw.de

1. Introduction

Global distribution and fate of contaminants depend on physical-chemical properties, geo-spheric transports, effectiveness of exchange between the atmosphere and ground compartments and degradation kinetics. A comprehensive global multicompartimental model is used to study how substance properties together with environmental conditions propagate into PCBs global distributions. This is the first time a fully coupled global atmosphere ocean general circulation model is used for studying PCBs. A special focus is given to intercontinental transports.

2. Materials and methods

The global multicompartiment chemistry-transport model MPI-MCTM was used, which encompasses coupled ocean and atmosphere general circulation models with embedded dynamic marine biogeochemistry and atmospheric aerosol sub-models [1-2]. To discern the impact of the substances physico-chemical properties on fate, identical primary emissions (i.e. PCB 153 emissions [3], high emission scenario) were input to study four PCB congeners, i.e. 28, 101, 153, and 180.

The period 1950-1995 was simulated under present-day model-generated climate with spatial resolutions of 3.75° (T31) in the atmosphere and ≈3° (GR30) in the ocean.

The substance large-scale mobility in meridional direction is compared with the predictions of a global, zonally averaging model, GloboPOP [4], run with similar input parameters (emissions, besides others).

3. Results and discussion

Up to between 2% (PCB 28) and 14% (PCB 153) of the global accumulated emissions have been stored in the Arctic. These fractions are decreasing since the 1970s, fastest for PCB 28. Substance distributions migrations are tracked expressed by the migration of the centre of gravity (COG) of environmental burdens [5]. Most of the PCBs environmental burden is stored in soils (> 80%).

The COGs of the PCB distributions in Eurasian top soils and vegetation surfaces move north-eastward, reflecting the prevailing wind direction (westerlies) in the mid latitudes of the northern hemisphere. While PCB 28, PCB 101, and PCB 153 are moving readily to the north and east, PCB 180 stays almost at the same latitude over the whole simulated period of time (Fig 1a).

Figure 1: Migration of the Centre of Gravity (COG) in soil and vegetation for North America (a) and Eurasia (b).
For North American soils a prominent shift of the COGs towards northwest is observed during 1950-90 (Fig. 1b). This is due to soils in Alaska and at the west coast of Canada increasingly storing PCBs, thereby building up a secondary storage maximum in North America. Principally, this can be caused by two processes, PCB primary emissions from local sources and subsequent deposition adjacent to them or long-range atmospheric transport either from other American sources or across the Pacific Ocean. The significance of the different sources was assessed for two PCB congeners of largely varying mobility, PCB 28 and PCB 180.

For 1950-1974, strongest atmospheric intercontinental transport was found in boreal winter (DJF). The analysis of PCB transport to Alaska shows, that Alaska gains PCBs from the south and west and looses them to the north and east. The net transport (sum of all the transports across these four lines; imports counted positive, exports negative) thereby exceeds PCB primary emissions, with 3.1 t PCB 28 and 4.7 t PCB 180 transported until 1974 vs. only 0.9 t emitted. Despite the higher net transport for PCB 180, import and export fluxes are higher for PCB 28 by approximately 50%.

Trans-Pacific transport of PCBs was budgeted. The detrended time series of trans-Pacific contaminant transport shows high correlations with the Pacific/ North American pattern index [6]. This indicates that events of effective trans-Pacific transport are linked to an amplified wave pattern in the North Pacific region. Correlations with sea level pressure winter anomalies reveal that these events are mainly associated with a lower than usual Aleutian low and higher than usual pressure over the North American continent.

3. Conclusions

Atmospheric transport is very effective for large-scale re-distribution of PCBs. The meridional re-distribution is more effective, but relaxes slower to long-term changes in emissions than predicted by a zonally averaging model. On the decadal time scale trans-Pacific transport constitutes a significant secondary PCB source to North America.

4. References


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Can clouds enhance long-range transport of low volatile, ionizable and surface-active chemicals?

Antonio Franco¹, Stefan Trapp¹

¹Technical University of Denmark, Miljøvej b.113, 2800 Kongens Lyngby, Denmark
E-mail contact: afra@env.dtu.dk

1. Introduction

Atmospheric partitioning and transport of low volatile organic compounds is strongly influenced by the presence of water (e.g. clouds) and its deposition velocity (e.g. rainfall, snow). Modeling the concentration and the deposition pattern of water is essential to predict the atmospheric fate of such chemicals. It was identified that the assumption of continuous rainfall underestimates the residence time and the transport potential of non-volatile substances [1]. The liquid water content of clouds [2, 3] and the high specific surface of frozen or liquid cloud droplets [4] can significantly contribute to the total activity capacity (i.e. the capacity to sorb chemicals) of the atmosphere for non-volatile, ionizable and surface-active substances.

A modified version of the regional Multimedia Activity Model for Iocs MAMI [2], including two-layered atmosphere, interface partitioning, intermittent rainfall and variable cloud coverage was applied to ten low volatile or ionizable chemicals, methomyl (MET), propoxur (PRO), 2,4-D, perfluorooctanoic acid (PFOA), pentachlorophenol (PCP), thiophenol (THP), diazinon (DIA), 4-chloroaniline (CHA), quinoline (QIN) and trimethylamine (TMA), to investigate the potential of clouds to enhance the atmospheric transport potential.

2. Materials and methods

In this new version of MAMI, the atmosphere is described by two compartments, the atmospheric boundary layer (ABL) with gas, aerosol solids and an aerosol-associated aqueous phase and the lower/middle troposphere (LMT), extending from 1000 m to 5000 m height, including gas, aerosol and cloud liquid water (Fig 1). Low- and middle level cumulus and stratus cloud are typically within the elevation range of the LMT. The model includes species-specific, temperature- and pH-dependent bulk and interface partitioning to aerosol, cloud droplets, rain or snow. Mixing between the ABL and the LMT is described by a constant vertical eddy diffusion term and wet deposition by the algorithm for intermittent rain proposed by Jolliet and Hauschild [1] for steady-state multimedia models.

Probabilistic simulations at steady state were run with the software Crystal Ball® on the spreadsheet version of MAMI, for a constant emission to the ABL. Probability density functions were derived for input substance properties and environmental parameters to quantify uncertainty and variability and to identify the key model inputs.

Figure 1: Mass transport and removal processes in the atmospheric boundary layer (ABL) and in the lower/middle troposphere (LMT) of MAMI.
3. Results and discussion

3.1. Calculated residence times

The residence times of the ten test chemicals range between a few hours for reactive compounds to >10 days (PFOA, PCP). In some cases, the residence time and its variability range is similar in the two compartments, while some compounds (e.g. DIA, 2,4-D and PFOA) are more persistent in the LMT (Fig. 2).

![Figure 2: Calculated median and variability range (5- and 95%-ile) of the residence time (days) in the lower/middle troposphere (●) and in the atmospheric boundary layer (▲).](image)

3.2. Dominant removal processes and parameters

On a regional scale, the fraction of a chemical transported the upper layer is small, i.e. maximum 7%. Wet deposition is the dominant removal process for all substances during the wet period. On a longer time scale including dry and wet periods, however, degradation is the dominant removal process for five substances (PRO, THP, DIA, CHA, TMA), wet deposition is the dominant removal process for MET, 2,4-D and QIN and dry gaseous deposition for PFOA and PCP.

The degradation rate, the duration of dry and wet periods and the parameters describing air-water bulk partitioning ($K_{AW}$ and $T$) and ionization ($pK_a$ and pH) determine the residence time in the ABL. In the LMT, however, the residence time depends also on the water content of clouds (MET, 2,4-D, PFOA, DIA) and on interface partitioning (PCP, DIA). The longer residence time predicted for some compounds in the LMT (Fig. 2) is due to the capacity of clouds to sorb non-volatile molecules in the liquid water and at the interface of cloud droplets. This limits the efficiency of oxidation by OH radicals and wet deposition and increases the transport potential of non-volatile (MET, 2,4-D) and surface active chemicals (PFOA, DIA).

4. Conclusions

The efficiency of wet deposition to remove low volatile organic pollutants from the atmosphere is limited primarily by the duration of the dry interval between precipitation events.

Persistent non-volatile chemicals can be transported to the troposphere during dry periods. Here, the high capacity of tropospheric clouds to sorb non-volatile and surface active chemicals limits the oxidation and wet deposition rates and increases the potential for long-range transport.

5. References


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Organic tracer compounds of ambient air particulate matter in the Western Mediterranean Basin and influence of natural and anthropogenic emission sources.

B.L. Van Drooge¹, J. Lopez¹, M. Aceves², X. Querol¹, J.O. Grimalt¹.

¹Institute of Environmental Diagnostics and Water Research (IDÆA-CSIC), Jordi Girona 18, 08034 Barcelona, Catalonia, Spain
²EMA. Air Quality and Mobility, carrer 62, 16-18, 08040 Barcelona, Catalonia, Spain

E-mail contact: barend.vandrooge@idaea.csic.es

1. Introduction

Monitoring and chemical analysis of atmospheric particulate matter (PM) is important due to its health impact and influence on climate change (1,2). The air quality in the urban area of Barcelona in the Western Mediterranean Basin is dominated by traffic related emissions and characterized by high levels of particulate matter and reactive chemical species due to emissions, the weak synoptic conditions and high solar radiation (3,4). Nevertheless, occasionally emissions from natural sources, such Saharan dust from Northern Africa and wildfires on the Iberian Peninsula, substantially influence the levels of the PM (5). Selected filter samples from 2009, representing different 'scenarios', were analyzed on organic tracer compounds, e.g. polycyclic aromatic hydrocarbons, hopanes, alkanes, anhydrosugars and primary sugars, as well as secondary organic aerosols tracer compounds, e.g. carboxylic acids. The results are discussed in terms of their relation to emission sources and influence of meteorological conditions in order to get an insight on the complex organic aerosol.

2. Materials and methods

PM1 and/or PM10 filter samples were collected with 12 or 24 hour resolution in background sites in Barcelona (Spain 41°22"N; 2°11"E) by a Hivol-sampler and analyzed on polycyclic aromatic hydrocarbons, hopanes, alkanes, anhydrosugars and primary sugars, as well as secondary organic aerosols (SOA) tracer compounds, e.g. carboxylic acids, using GC-MS with derivation of the polar compounds beforehand. Complementary data on OC/EC content of PM, concentration levels of daily registered O₃ and NOₓ as well as meteorological conditions, satellite images and air-mass trajectories for the sampled days were collected.

3. Results and discussion

Organic tracer compound levels and their relation with meteorological conditions and emission sources

Highest levels of generally all organic tracer compounds are observed in winter under calm meteorological conditions in the presence of a temperature inversion. This situation causes the accumulation of atmospheric contaminants emitted in the urban area, mainly related to traffic. Also secondary organic species have their highest concentrations under these conditions. The background concentrations are about 2-3 times lower in the presence of active sea breeze conditions. Sea-breezes are more active in summer, resulting in moderate background levels of PM and the organic species. Analyzed filter samples before, during and after a substantial PM event (PM tripled its average value) shows a 3 to 7 times increase of the organic tracer species (see figure 2 for levoglucosan and hopanes). Largest increases are observed for levoglucosan (as marker for biomass burning) and glucose (as marker for soil dust). These increases are related to a Saharan Dust intrusion and the presence of wildfires in the Iberian Peninsula, which is supported by the satellite images, air-mass trajectories and atmospheric dust load models (figure 2). Nevertheless, the increase of traffic related compounds, such as hopanes, during this event suggest that also the influence of traffic emissions increases, which can be related to a change in wind direction.
4. Conclusions

The concentration variability of PM in an urban background area in the Western Mediterranean Basin is supported by the concentration trends of organic tracer compounds and can partially be explained by changes of meteorological conditions and the strength of anthropogenic and natural emission sources. The complex mixture, and often simultaneous concentration trends of the different tracers, complicates the interpretation of results and future research should focus on the improvement of source apportionment of organics in the atmosphere.

5. References


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Sources, Transport and Deposition of Polycyclic Aromatic Hydrocarbons: China and the Western U.S.

Staci L. Massey Simonich

1Department of Environmental and Molecular Toxicology and Department of Chemistry, Oregon State University, Corvallis, OR 97331, USA
E-mail contact: staci.simonich@oregonstate.edu

1. Introduction

While U.S. emissions of seven of the most carcinogenic, PM-bound polycyclic aromatic hydrocarbons (PAHs) decreased from 2000 tons in 1990 to 1400 tons in 1996, Chinese emissions of these same PAHs were estimated at 3460 tons in 2003 [1]. This makes China the world’s largest emitter of PAHs. PAH concentrations in both indoor and outdoor air in rural and urban China are some of the highest in the world [2] and the lung cancer and asthma rates in Chinese cities have increased in recent years [3,4]. At the same time, the trans-Pacific atmospheric transport of other incomplete combustion derived air pollutants to the western U.S. has been measured.

The objectives for this research were to:

• Determine the significance of combustion source control measures in reducing PAH concentrations during the 2008 Beijing Olympics as a model for air pollution control in other Chinese megacities.
• Study the atmospheric transport and deposition of PAHs to remote sites in the Western U.S. and identify their regional and long-range sources.

2. Materials and methods

Size fractionated particulate matter (PM) samples (including PM$_{2.5}$ and PM$_{10}$) were collected in Beijing before, during and after the 2008 Olympics, during both source control and non-source control periods, and analyzed for a wide range of PAHs, nitro-PAHs, oxy-PAHs, and high molecular weight (MW 302) PAHs [5]. In addition, air samples were collected from three different remote sites in the Pacific Northwestern U.S. and analyzed for parent PAHs [6,7]. In addition, lake sediment cores, lichen, and seasonal snowpack were collected from remote, high elevational sites in western U.S. national parks and analyzed for parent PAHs [8,9].

3. Results and discussion

3.1. Effect of Source Control Measures on PAH Concentrations

The mean black carbon (BC), organic carbon (OC), and PAH concentrations were significantly reduced between non-source control and source control periods, as well as non-Olympic and Olympic periods, by ~45%, ~31%, and ~53%, respectively. The OC/BC ratio was high (5.88 ± 1.28) during all periods and increased during the source control and Olympic periods, indicating that the source control measures were more effective in reducing BC emissions than OC emission, likely because of the heavy restrictions on diesel truck traffic. Like PM$_{2.5}$ concentrations, BC and OC concentrations were significantly positively correlated to source regions south of Beijing and significantly negatively correlated to precipitation due to wet deposition of PM. However, PAH concentrations were not correlated with any source region outside of Beijing, indicating the importance of local (Beijing) PAH emissions and photodegradation of regional PAH emissions enroute to Beijing. A multiple linear regression model indicated that source control measures accounted for more of the variation in BC, OC, and PAH concentrations than meteorological parameters (20 to 51%).

3.2. Trans-Pacific Atmospheric Transport of PAHs

The trans-Pacific and regional North American atmospheric transport of PAHs in biomass burning emissions were measured in air masses from April to September 2003 at two remote sites in western North America [6]. Mary’s Peak Observatory (MPO) is located in Oregon’s Coast Range and Cheeka Peak Observatory (CPO) is located on the tip of the Olympic Peninsula in Washington State. During this time period, both remote sites were influenced by PAH and pesticide emissions from forest fires in Siberia and regional fires in
Oregon and Washington State. Concurrent samples were taken at both sites on June 2 and August 4, 2003. On these dates, CPO had elevated gas phase PAH and retene concentrations (p<0.05) and MPO had elevated retene and particulate phase PAH concentrations due to trans-Pacific transport of emissions from fires in Siberia [6]. In addition, during the April to September 2003 sampling period, CPO and MPO were influenced by emissions from regional fires that resulted in elevated gas phase PAH concentrations [6]. These data suggest that the trans-Pacific and regional atmospheric transport of biomass burning emissions results in elevated PAH concentrations in western North America.

3.3. Atmospheric Deposition of PAHs in U.S. National Parks

The fourteen remote lake catchments in western U.S. national parks ranged from low-latitude catchments (36.6° N) at high elevation (2900 masl) in Sequoia National Park, CA to high-latitude catchments (68.4° N) at low elevation (427 masl) in the Alaskan Arctic [8]. Over 75% of the catchments demonstrated statistically significant temporal trends in total PAH sediment flux, depending on catchment proximity to source regions and topographic barriers [8]. The total PAH and fluxes in seasonal snowpack, lichens, and surficial sediment were 3.6 to 60,000 times greater in the Snyder Lake catchment of Glacier National Park than the other 13 lake catchments [8]. The PAH ratios measured in snow, lichen, and sediment were used to identify a local aluminum smelter as a major source of PAHs to the Snyder Lake catchment [8]. These results suggest that topographic barriers influence the atmospheric transport and deposition of PAHs in high-elevation ecosystems and that PAH sources to these national park ecosystems range from local point sources to diffuse regional and global sources.

4. Conclusions

• PAH concentrations can be reduced in Chinese megacities by 25 to 75% if strict combustion source emissions controls are implemented.

• PAHs undergo both regional atmospheric transport and, episodic, trans-Pacific atmospheric transport to the western U.S.

• PAHs are deposited to remote ecosystems in U.S. national parks and sources include regional urban areas and industrial activities.

5. References

1. Introduction

The primary input of Persistent Organic Pollutant (POP) contamination to the Antarctic is expected to be via Long Range Atmospheric Transport (LRAT) from emissions in neighboring Southern hemisphere nations\(^1,2\). In addition to LRAT, system input of POPs must increasingly consider alternate pathways. Human activity in the Antarctic represents a potential direct source of both legacy and current-use chemicals.

It has been two decades since the organic chemical composition of air masses arriving in the Australian Antarctic Territory (AAT), which spans the majority of the eastern Antarctic sector, was last investigated\(^3\). The results presented here are the first atmospheric measurements made as part of a new continuous monitoring effort at Casey station (66°17' S 110°31' E), one of Australia's all-year research stations. These results are evaluated alongside POP contamination data of soil samples collected around the Casey station perimeter. Here we assess contaminant profiles for clues as to local and distant contamination sources.

2. Materials and methods

A high-volume flow-through atmospheric sampler\(^4\) was installed throughout 2010 in a remote location, 3km upwind from Casey station\(^1\). In addition, four soil samples were collected at various locations around Casey station (Fig. 1). Air samples were analysed for organochlorine pesticides, including dichlorodiphenyltrichloroethane (DDT) and hexachlorohexane (HCH) isomers; polybrominated diphenyl ethers (PBDEs) and polychlorinated biphenyls (PCBs). Soil samples were analysed for dioxins and furans, PCBs, PBDEs and DDT and HCH isomers. All analyses were conducted at the Norwegian Institute for Air Research (NILU), Norway.

3. Results and discussion

The most varied contaminant profile and the highest levels of contamination where detected in the “Antenna farm” soil sample. This sampling site is in close proximity to the Thala Valley historical waste disposal site of Old Casey station, decommissioned in 1986. Local PCB contamination of abiotic and biotic matrices has
previously been linked to historical waste disposal at McMurdo Station in the Ross Sea region\textsuperscript{5-7} and must also be considered in evaluation of chemical profiles of the current study. However, intermediately chlorinated PCB homologues, expected to carry the greatest potential for LRAT, dominated the PCB profiles observed in all samples, supporting LRAT as the major input pathway.

PBDEs profiles on the other hand provide preliminary evidence of a local source with the involatile, currently produced deca-brominated congener 209 contributing significantly to both air and soil profiles (24\% of ∑\textsubscript{16}PBDE contamination in the air sample < 41\% of “Transmitter Hut Site 2” < 58\% of “Line to Jack’s” < 60\% of “Antenna Farm” and 65\% of “Transmitter Hut site 1”) (Figure 2). Tetra-brominated congeners BDE-66 and -47 are predicted to be the major photodegradation products of BDE-209 and were detected in all samples.\textsuperscript{9} These levels are likely to be the product of both \textit{in-situ} processes as well as LRAT supplementation of atmospherically “aged” PBDE profiles.

Consistent profiles and contaminant levels of DDT and HCH isomers between all samples suggests long distance delivery of these agricultural chemicals whilst a dominance of Endosulfan-I in air evidences its ongoing application in the southern hemisphere.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{PBDE_Homologue_Profiles.png}
\caption{PBDE Homologue Profiles}
\end{figure}

4. Conclusions

Data presented here are the first results of the chemical composition of air masses of the AAT measured in over two decades. Here we present preliminary air results alongside soil concentration data. Results suggest a potential local source of deca-brominated PBDE and evidences the continued input of agricultural chemicals via LRAT.

5. References


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\textsuperscript{1} Only the first three months of sampling data were ready at the time of abstract submission, however a further 9 months of data is anticipated shortly and will be incorporated into the ensuing presentation.
A trace element approach for determining the geographic sources of semi-volatile organic contaminants in an alpine ecosystem in New Zealand

Karen Lavin\textsuperscript{1}, Kimberly Hageman\textsuperscript{1}, Samuel Marx\textsuperscript{2}, Balz Kamber\textsuperscript{3} and Peter Dillingham\textsuperscript{4}

\textsuperscript{1}Department of Chemistry, University of Otago, Dunedin 9054, NEW ZEALAND
\textsuperscript{2}School of Geography, Planning and Environmental Management, University of Queensland, Brisbane 4072, AUSTRALIA
\textsuperscript{3}Department of Earth Sciences, Laurentian University, Sudbury, Ontario P3E 2C6, CANADA
\textsuperscript{4}Department of Mathematics and Statistics, University of Otago, Dunedin 9054, NEW ZEALAND

E-mail contact: klavin@chemistry.otago.ac.nz

1. Introduction

Semi-volatile organic contaminants (SOCs) can be transported long distances through the atmosphere [1], and can ultimately accumulate in cold remote ecosystems far from where they have been used [1,2]. This is problematic because many of these SOCs are considered toxic and therefore have the potential to cause harm to the unique wildlife that live in these ecosystems [3]. In order to manage the transport and accumulation of SOCs to a specific remote site, it is necessary to understand how different geographic sources contribute to the sites' SOC burden.

Previous studies have used local wind observations [4] and/or air-mass back-trajectory modelling [5] to gain information about the geographic sources of SOCs to remote sites. However, these approaches rely on predicting the movement of the air mass and therefore have limitations. Local winds can be highly variable, especially in complex mountainous terrain, and do not adequately describe long-range sources. Similarly, air-mass back-trajectories often fail to accurately model the movement of spatially discrete synoptic-scale weather systems, such as those experienced in mountainous terrain.

The objective of this study was to investigate a new geographic source apportionment method for SOCs that uses trace element profiles in atmospheric particulate matter (PM) to determine SOC origin.

2. Methods

2.1. Sample Collection and Analysis

Atmospheric SOCs and PM were simultaneously collected using two high-volume air samplers. Samples were collected daily from 16 January to 16 February 2009 at Temple Basin, New Zealand. In addition, a weather station was set-up at the site to record local weather observations every two minutes.

SOCs, including current- and historic-use pesticides and polycyclic aromatic hydrocarbons (PAHs), were extracted from the sampling media using accelerated solvent extraction. The extracts were concentrated to 300 \(\mu\)L and analysed by gas chromatography-mass spectrometry in both negative chemical ionisation and electron impact ionisation modes.

PM was digested using nitric and hydrofluoric acids, and analysed by inductively coupled plasma-mass spectrometry.

2.2. Determination of Source Indicators

Due to the geography of New Zealand and Australia, only the Canterbury Plains and Australia were considered to act as sources of SOCs to Temple Basin. For this reason, source indicators have been defined for these two regions only using each of the three source apportionment approaches.

2.2.1. Local Wind Observations

Two source indicators were defined based on local wind observations. \(\%\text{Northwest}\) is the percent of time the wind came from the northwest direction (275° - 355°), representing sources from Australia; while \(\%\text{South}\) is the percent of time the wind came from the south (125° - 235°), representing sources from the Canterbury Plains of New Zealand.
2.2.2. Air-Mass Back-Trajectories

Two source indicators were defined based on air-mass back-trajectories following an approach used previously by Primbs et al. Source region boxes were defined for Australia and Canterbury. HYSPLIT back-trajectories were generated at three altitudes at six different times across the sampling day. Hourly back-trajectory points were plotted and the number that fell into each source region box was counted. \( %Aust \) is the percent of time the air mass spent in the Australian source region box and \( %Cant \) the percent of time the air mass spent in the Canterbury box before reaching Temple Basin.

2.2.3. Trace Elements in PM

The relative contribution of regional New Zealand sources versus longer-range Australian sources was determined using a binary mixing model of the source trace element profiles. \( %AustPM \) is the percent of Australian PM in the atmosphere at Temple Basin.

3. Results and Discussion

3.1. Current- and Historic-Use Pesticides

Endosulfan I was found to be correlated \(( p < 0.05)\) with \( %AustPM \), indicating that the endosulfan I present at Temple Basin tends to come from Australian sources. In addition, further evidence suggests that the endosulfan I present at Temple Basin is a direct effect of current spraying. Firstly, endosulfan I was not detected until part-way through the study. Secondly, the technical mixture of endosulfan was sprayed in Australia in late January. Thirdly, there is no temperature dependence ruling out volatilisation from endosulfan I present in soils from previous spraying events. Lastly, the degradation products of endosulfan I were not detected.

Chlorpyrifos was well-correlated \(( p < 0.01)\) with \( %South \), indicating that the chlorpyrifos present at Temple Basin tends to come from the Canterbury Plains, New Zealand.

3.2. Polycyclic Aromatic Hydrocarbons

The higher molecular mass PAHs (benzo[g,h,i]perylene and indeno[1,2,3-cd]pyrene) were correlated with \( %AustPM \), indicating that these PAHs are from longer-range Australian sources. Conversely, the lower molecular mass PAHs (fluorene and phenanthrene) were correlated with \( %South \), indicating more localised New Zealand sources.

4. Conclusions

The approaches based on trace elements in PM and local wind observations were useful in determining sources of pesticides and PAHs to Temple Basin. Endosulfan I and the higher molecular mass PAHs tended to come from longer-range Australian sources. In particular, endosulfan I appears to be from current spraying events during this time. Chlorpyrifos and the lower molecular mass PAHs tended to be sourced from the more localised Canterbury Plains. No correlations were found for the source indicators based on air-mass back trajectories.

5. References


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Comparison of lichen, conifer needles, passive air sampling devices, and snowpack as passive sampling media to measure semi-organic volatile organic compounds in the atmosphere

Jill E. Schrlau¹, Linda Geiser², Kimberly J. Hageman³, Dixon Landers⁴, and Staci Massey Simonich¹,⁵

¹Department of Environmental and Molecular Toxicology, Oregon State University, Corvallis, OR 97331, USA
²USDA Forest Service, Corvallis, OR 97339, USA
³Department of Chemistry, University of Otago, Dunedin, New Zealand
⁴Environmental Protection Agency-Western Ecology Division, Corvallis, OR 97331, USA
⁵Department of Chemistry, Oregon State University, Corvallis, OR 97331
E-mail contact: jill.schrlau@oregonstate.edu

1. Introduction

The magnitude of accumulation and relative sorption affinities for semi-volatile organic compounds (SOCs) in different passive sampling media is dependent on the sampler properties and the physical chemical properties of the SOC. Different types of passive sampling media have been used to monitoring SOCs in high elevation ecosystems including vegetation [1-3], precipitation [4, 5], and passive air sampling devices (PASDs) [6, 7].

In this study, magnitude of accumulation and sorption affinities for pesticide and polycyclic aromatic hydrocarbon (PAHs) accumulation in lichen, two-year old conifer needles, XAD resin PASDs, and snowpack were compared at high elevation sites located within federally-protected parks in the western United States.

The objectives for this work were:

- To determine which SOCs were preferentially accumulated in the different passive sampling media, at the same sites.
- To determine the role SOC physical chemical properties played in the accumulation of SOCs in the different passive sampling media.

2. Materials and methods

Lichen, two-year old conifer needles, and snowpack were sampled from five parks in 2004. In 2005, lichen and two-year old conifer needles were also sampled from 12 parks. PASDs were deployed at all 17 parks in 2005 for one year. The sample preparation steps varied between the passive sampling media. In general, the samples were spiked with labeled surrogates and extracted using pressurized liquid extraction (PLE) and purified using solid phase liquid extraction (SPE) [8]. Conifer needles required further clean-up using gel-permeation chromatography (GPC). The extracts were spiked with internal standards and analyzed with gas chromatography mass spectrometry (GC/MS) in both electron impact and chemical ionization modes for a total of 58 pesticides and PAHs.

3. Results and discussion

3.1. Preferential Accumulation

Preferential accumulation in the four passive sampling media was assessed by converting the actual concentration of each SOC to a percent of the total pesticide or PAH concentration at five parks. Statistically significant differences were resolved using Tukey Kramer Honestly Significance Difference test. The accumulation profiles for the media showed some preferential accumulation. Lichen and conifer needles preferentially accumulated endosulfan sulphate, PASDs preferentially accumulated HCB, and snowpack preferentially accumulated daetral, pyrene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, indeno[1,2,3-cd]pyrene, dibenzo[a]anthracene, and benzo[g,h,i]perylene. Using regression analysis, the profiles for lichen and snowpack were significantly correlated indicating that conifer needles and PASDs showed a different atmospheric composition compared to lichen and snowpack.
3.2. SOC Properties Governing Accumulation

The physical chemical properties of the SOCs included temperature-adjusted air-water partition coefficient, $K_{AW}$, log octanol-air partition coefficient, log $K_{OA}$, and the estimated fraction of an SOC in the atmospheric particulate phase ($\Phi$) [9-11]. The frequency of detection was evaluated for each physical chemical property in the four passive sampling media. The detection frequency of SOCs with $K_{AW}$ values ranging up to 0.05 was 80% for lichen, conifer needles, and PASDs, and 98% in snowpack. In all media, the highest detection frequency (~25 to 54%) occurred for SOCs with log $K_{OA}$ values ranging from 8 to 10. Snowpack also accumulated SOCs with log $K_{OA}$ values between 11 and 12. For $\Phi$, the highest frequency of SOCs that accumulated in the media had values up to 20%. The percent of SOCs with $\Phi > 60\%$ out of the total measurements made in lichens, conifer needles, PASDs, and snowpack were 4, 0.7, 0, and 22% indicating that snowpack and lichens had a greater affinity to particulate-phase SOCs compared to conifer needles and PASDs.

4. Conclusions

- Preferential accumulation was observed for endosulfan sulphate (lichen and conifer needles), HCB (PASDS), and dacthal, pyrene, benzo[a]anthracene, benzo[b]fluoranthene, benzo[k]fluoranthene, indeno[1,2,3-cd]pyrene, dibenzo[ah]anthracene, and benzo[ghi]perylene (snowpack).
- Lichen, conifer needles, PASDs, and snowpack accumulated more SOCs with lower $K_{AW}$ and $\Phi$ values and middle-range log $K_{OA}$ values; however SOCs with higher log $K_{OA}$ and $\Phi$ values were also accumulated in snowpack and lichen.

5. References


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Polycyclic aromatic hydrocarbons in air over Central Europe: what can we learn from diagnostic ratios for source apportionment and reactivity?

Alice Dvorská¹, Gerhard Lammel¹,², Jana Klánová¹ and Kateřina Hošková¹

¹Research Centre for Toxic Compounds in the Environment (RECETOX), Masaryk University, Kamenice 3, CZ – 62500 Brno
²Max Planck Institute for Chemistry, J.-J.-Becher-Weg 27, D – 55128 Mainz
E-mail contact: dvorska@recetox.muni.cz

1. Introduction

Despite numerous uncertainties [1], diagnostic ratios (DRs) of parent polycyclic aromatic hydrocarbons (PAHs) are frequently used for source apportionment of these pollutants. This is of special doubt when applied on data obtained from sampling at background sites, because the PAH pattern determined far from sources can be altered due to the different reactivity of species forming a ratio. Additional uncertainties include e.g. overlaps of PAH DR values for different source categories. However, due to the simplicity of the method, it is attractive especially for countries, where (e.g. due to lack of measurement data) sophisticated source apportionment methods cannot be applied.

2. Materials and methods

The following five commonly used PAH DRs were investigated: anthracene / (anthracene + phenanthrene) ANT/(ANT+PHE), fluoranthene / (fluoranthene + pyrene) FLT/(FLT+PYR), benzo(a)anthracene / (benzo(a)anthracene + chrysene) BAA/(BAA+CHR), indeno(123cd)pyrene / (indeno(123cd)pyrene + benzo(ghi)perylene) IPY/(IPY+BPE) and retene / (retene + chrysene) RET/(RET+CHR) [2].

PAH DR values derived from a literature survey on suitable PAH emission factors were applied to study their ability to distinguish between PAH sources at sites with well described source categories (road traffic, residential heating, industry) in the Czech Republic, Serbia and Bosnia and Hercegovina. Later, seasonal changes in source characteristics at the background receptor site Košetice, Czech Republic, were examined using ambient PAH data from long term monitoring (1996-2008). Then a mass balance model of PAHs in air was applied and uncertainties of PAH reaction rate coefficients were narrowed down [2]. The suitability of PAH DRs for distinguishing between various characters of sampling sites (urban, industrial, rural, background) in regions with very limited information on ambient PAHs (15 countries in Africa) was also studied using data from a passive air sampling campaign conducted in 2008.

3. Results and discussion

3.1. Source apportionment

Measurements carried out close to sources were in accordance with literature based DR values especially for BAA/(BAA+CHR) and IPY/(IPY+BPE). These DRs were suggested to be capable of distinguishing between traffic and residential heating. ANT/(ANT+PHE) was identified as the least useful DR due to its low variability in both the literature based DRs and the ambient measurements at sites with well defined sources.

Source apportionment of PAHs based on DRs did not lead to clear results at the background site. The well known sensitivity of RET/(RET+CHR) to softwood burning [3] was confirmed. Also BAA/(BAA+CHR) and especially IPY/(IPY+BPE) (Fig. 1) exhibited a significant seasonality which could indicate the capability of PAH DRs to reflect changes in source strengths in different seasons. This hypothesis was studied by applying a mass balance model.
3.2. PAH reactivities

The results of the mass balance study could not confirm nor reject the hypothesis that PAH DRs are able to reflect changes in source strengths as the modelling was hampered by the insufficient knowledge on PAH reactivities in air. However, DRs were used to narrow down ozone and OH radical reaction rate coefficients’ uncertainties: $k_{\text{O}_3}{(2)}$ of PYR and BPE in the particulate phase seem to be $\leq 10\%$ of the highest rate coefficient measured using model aerosols in the laboratory and $k_{\text{O}_3}{(2)}$ IPY in the gas phase could be higher than previously estimated by three orders of magnitude.

3.3. Application to PAH DRs to data from passive air samplers

As passive air samplers do not sample the whole particulate content in air, additional uncertainties are added to DR based source apportionment if such data are used. Still, RET/(RET+CHR) seems to be able to distinguish between sites of agricultural and other character in Africa.

4. Conclusions

A good agreement between some literature based PAH DRs and ambient DRs from sites with a dominant influence of a local source was observed. However, the current knowledge on PAH reactivity was found to be insufficient for source apportionment of atmospheric PAHs at receptor sites, especially when these are located far from sources. The sensitivity analysis of the mass balance model indicated that some PAH degradation rates need to be revised.

5. References


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Background atmospheric concentrations of PAHs are controlled by ubiquitous emissions from soils

Ana Cabrerizo, Jordi Dachs 1*, Claudia Moeckel2, María-José Ojeda1, Gemma Caballero1, Damià Barceló1, Kevin C. Jones2

1 Department of Environmental Chemistry, IDAEA-CSIC, Jordi Girona 18-26, Barcelona, Spain
2 Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK
E-mail contact: ana.cabrerizo@idaea.csic.es

1. Introduction
Soils are the major reservoir and one of the major sinks for persistent organic pollutants (POPs) due to their strong affinity to organic matter. The fate and toxic effects of POPs are strongly affected by the quality and quantity of soil organic matter (SOM) which controls POPs availability for biological degradation, burial or re-volatilization from soil. Combustion of fossil are major anthropogenic sources of polycyclic aromatic hydrocarbon (PAHs) to the environment but recent studies have highlighted the importance of biogenic sources for biological PAH formation. The recent development of a soil fugacity sampler makes possible to unequivocally determine soil-air gradients and partition coefficients under field conditions, so it is possible to study the relevant variables affecting them. The objectives of this study are to determine the direction of soil-air exchange of PAHs and its influence on background occurrence of the different PAHs.

2. Materials and methods
Ten sampling sites were selected; six rural: Borau, Alfaro, Nájera, Lasieso, Uruñuela located along the Ebro river basin (N-NE Spain) and Langden (near Lancaster, UK); three semi-rural: Tudela, Sabiñanigo (Ebro river basin-Spain) and Hazelrigg (near Lancaster, UK) and one urban in Barcelona.

PAH fugacity in surface soils was measured by analyzing the PAH concentrations in air that had been equilibrated in-situ with the soil surface using the soil fugacity sampler described by Cabrerizo et al, 2009. Ambient air concentrations were sampled with a low volume sampler with glass fiber filters and polyurethane foams identical to those used to determine soil fugacities. For this study, a total of 48 soil fugacity measurements, 47 ambient air concentrations, and 23 top surface soils were sampled and analyzed.

3. Results and discussion
3.1. Ubiquitous soil to air volatilization of PAHs
Figure 1 shows, large soil fugacities compared with ambient air fugacities for PAHs with 2-3 aromatic rings and their alkyl derivatives at all the sites. These gradients were much smaller or close to unity for PAHs with...
4-5 aromatic rings. It is also relevant to evaluate if these gradients are maintained during different sampling periods (diurnal variability) or seasons (figure 2).

![Figure 2: Seasonality of Ln fs/fa versus LogKOA in rural and semi-rural Ebro river sites](image)

The general trend was that values of fs/fa decreased with increasing KOA, confirming that the soil is an important source of lighter PAHs to the atmosphere at the rural, semi-rural and urban sites. Seasonal variation of soil/air fugacity quotients for individual PAHs were observed in the Ebro river watershed. The values of fs/fa were larger in early and late summer (June 2006 and September 2007) than in early winter (November 2006). The ubiquitous large soil to air fugacity ratios suggests the occurrence of biogenic sources of 2-3 ring PAHs in rural soils.

3.2. Factors affecting soil-air fugacity of PAHs

In order to study the influence of soil characteristics and temperature on gradients, the soil to ambient air fugacity ratios (fs/fa) were regressed against the different parameters describing the soil organic matter quality and quality, being the best fit given by equation: fs/fa = a + b (1/T) + c (log redox), thus indicating that the fugacity ratios increase at higher temperatures, and higher soil redox potential. The influence of the temperature is consistent with the seasonal variability (figure 2) and demonstrates that during warm periods soils increment their strength as a source. The influence of redox potential can be related to soil organic matter quality, more oxidized soils have lower capacity to retain PAHs. However, redox potential is also closely related to soil microbiology, an important factor that could be related to in-situ production of PAHs from degradation of organic matter.

3.3. Implications of ubiquitous net volatilization of PAHs from soils and biogenic sources

Our data suggest an important entry of biogenic phenanthrene into the atmosphere that could have important effects on atmospheric chemistry and air quality since it is ubiquitous. The potential implications for these large source of soil PAHs as a driver of PAH occurrence in background air will be discussed.

4. References


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Atmospheric deposition fluxes of contaminants close to a municipal solid waste incinerator

Elisa Venturini\textsuperscript{1}, Ivano Vassura\textsuperscript{1}, Fabrizio Passarini\textsuperscript{1}, Laura Ferroni\textsuperscript{2} and Luciano Morselli\textsuperscript{1}

\textsuperscript{1}University of Bologna, Department of Industrial Chemistry and Materials, Viale Risorgimento 4, I-40146 Bologna (Italy).
\textsuperscript{2}University of Bologna, Rimini Branch, Via Angherà 22, I-47900, Rimini (Italy).

E-mail contact: elisa.venturini6@unibo.it

1. Introduction

In Europe – 27, about 20\% in weight of Municipal Solid Waste (MSW) is incinerated [1]. Up until the 1990s, many incinerators had considerable impact on the environment and health, because of their inefficient flue gas treatment, which entailed a high exposure of people to pollution agents [2]. Among these, there are major concerns over persistent organic pollutants (POPs), such as polychlorodibenzo-p-dioxins (PCDDs), polychlorodibenzo furans (PDCFs) and polychlorinated biphenyl (PCBs), and heavy metals (HM), like Cd, Cr, Cu, Ni, Pb, Zn and As.

New generation plants, built after European Directive 2000/76/EC and the implementation of Best Available Techniques (according to IPPC Directive 96/61/EC), reduced the emissions of pollutants. However the locally contribution of POPs and HM in the flux depositions could be not negligible.

The main goal of this study is to assess the temporal trend of atmospheric depositions of PCDD/Fs, PCBs and HM in the vicinity of a medium-sized incineration plant (according to Italian standards), located near Rimini, in the Emilia-Romagna Region (Northern Italy). Atmospheric depositions are collected by bulk samplers, according to, for HM, the new standard method CEN EN 15841/2009. In order to estimate the contribution of the incinerator with respect to other potential sources, a comparison was performed with the concentration of the same compounds monitored in a control site.

2. Materials and methods

The incineration plant studied is situated in a suburban area, not far from a tourist town (Riccione), an important Italian highway (A14), and the Adriatic coast. This plant is authorized to burn 127,600 t per year of urban, hospital, and cemetery solid waste. From February 2008, the plant was revamped, with the construction of a new incineration line and the dismantling of the 3 oldest ones; for this reason in this year, the incinerator shut down its activity for about 6 months, while for another 6 months it operated at reduced capacity.

Atmospheric depositions were collected by means of a bulk sampler consisting of a funnel directly connected to a collection bottle. The device is made of Pyrex glass for POPs and PET for HM, as indicated by the above-mentioned European standard, and is placed in a polymer structure support hanging from a pole 2 m from the ground. From 2006 to 2010, bulk atmospheric deposition samples were collected monthly for heavy metal analysis and at intervals of about 6 months for PCDD/Fs and PCBs analysis.

Sampling net was drawn on the basis of the dispersion map calculated by the atmospheric dispersion model Calpuff, applied to incinerator emissions. Sampling sites (5 sites) were located in zones affected by different emission deposition amounts, due to their different positions with respect to the incineration plant.

3. Results and discussion

From the comparison of deposition flows of PCDD/Fs, PCB and HM in this area and in other urban and industrial ones, it results that values obtained in this study are low [1,2,3,4]. The area is generally subject to low contamination.

Some differences appear among the various sampling periods. They can be even of an order of magnitude for PCB. (Figure 1a and 1b). This is in particular due to seasonal differences and in particular to mean temperature and rainfall of sampling periods. On the contrary, comparing deposition flows in the various sampling sites in the same period, no differences appear, or they are very low. In particular flows are greater or not very different in site 4 (which is considered as a reference because it is outside the area most affected by plant emissions), compared to the most affected or urban sites.
The relative mean distributions of different congeners of PCDD/Fs and PCB in the different sampling sites are very similar; so the distribution does not change significantly in space.

![Graph](image)

Figure 1 – Total deposition flows of dioxins (PCDD+PCDF) (a) and PCB (b) in different sites and sampling periods.

The analysis of heavy metals deposition flows leads to similar conclusions. The study area doesn’t show an high burden of contaminants and is similar to other urban and suburban areas [5,6]; moreover, a part for seasonal variations, there are no differences among sites.

4. Conclusions

The relative contribution of incinerator to the total pollution load seems to be negligible compared to the high background concentration, which could be ascribed to the nearby urban area. This is confirmed by the observation that deposition flows are not significantly lower than in the other years, even though the plant was shut down for 6 months.

5. References


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Assessing bacterial diversity and its influence on biodegradation potential

Russell Davenport¹, Andrew Goodhead¹, Jason Snape², Timothy Martin¹,², Jon Ericson³, Torben Madsen⁴, Anne Pedersen⁴

¹School of Civil Engineering & Geosciences, Newcastle University, Newcastle upon Tyne, NE1 7RU, UK
²Brixham Environmental Laboratory, AstraZeneca, Freshwater Quarry, Brixham, Devon, TQ5 8BA.
³Pfizer Global Research and Development, Pfizer Inc., 235 East 42nd St., New York, NY 10017-5703 USA.
⁴DHI, Agern Allé 5, DK-2970 Hørsholm, Denmark
E-mail contact: r.j.davenport@ncl.ac.uk

1. Introduction

Mitigating the risks that manufactured chemicals pose to the environment and human health is a major global concern and one of the greatest challenges for the 21st Century. There are more than 143,000 registered chemicals in Europe alone (EINECS), an estimated 300 Mt per annum of which find their way into receiving water courses mainly through sewage treatment works (STW) and industrial effluents, with additional input from run-off [1]. In the EU, REACH, the Integrated Pollution Prevention and Control (IPPC), and Water Framework Directives (WFD) are designed to mitigate the risk of chemicals in the environment either at manufacturing source or by the treatment of wastewaters in STW and run-off. Under REACH there have been a particular shift in emphasis towards identifying persistent chemicals.

Biodegradation - and its corollary, persistence - is an important but poorly understood fate process that is central to all mitigation strategies. It represents one of the greatest scientific uncertainties in assessments underpinning the above-mentioned directives (e.g. [2]), and is often measured experimentally by observing the degradation of a chemical substance in the presence of a bacterial inoculum. Ready biodegradability tests (RBTs) have been the central foundation for understanding the biodegradation of chemicals in regulatory frameworks for 2-3 decades. They are simple, highly prescribed, standardised and conservative tests that measure the relative biodegradability of chemicals (e.g. OECD 301 tests). RBTs rely on the probabilistic inclusion of specific degraders in the test system, which can be high variable due to low inoculum concentrations, and together with their short duration, make them unsuitable for persistence assessments.

REACH guidance now advocates the use of a new tier of tests that includes enhanced tests (containing environmentally-relevant concentrations of inocula) and quantitative structure-activity relationships (QSARs) to enable efficient and effective identification of persistent chemicals [3]. Both methods have yet to be rigorously validated using suitable experimental biodegradation tests.

Many studies are confined to one inoculum-one chemical paradigm, and rarely relate biodegradation to the bacterial community structure and diversity. We have been attempting to escape this paradigm to investigate how variations in inocula concentration, community composition, diversity, and the factors that impact it, relate to biodegradation outcome. To achieve this we have been conducting inocula sample surveys, applying the latest molecular methods to characterise their diversity and subjecting them to novel miniaturised high-throughput biodegradation tests. One of our aims is a better understanding of biodegradation for the development scientifically sound screening tests for persistence.

2. Materials and methods

- Multiple locations from different environmental compartments (activated sludge, rivers, estuarine, and sea waters) were sampled and prepared for use as inocula in miniaturised high-throughput biodegradation tests (BTs). Locations were chosen to represent a broad range conditions likely to have an impact on microbial diversity (e.g. pristine versus eutrophic sites).
- Inocula were concentrated by filtration and a range of cell densities were prepared by serial dilution to give final concentrations of $10^2$ - $10^9$ cells ml⁻¹. Replicates of each dilution were inoculated into 96-well plates (one plate per dilution) containing the test compound (diluted to 10mg/l carbon in sterile mineral medium) and incubated at 20°C 60 days.
- Test compounds included 4-hydroxybenzoic acid (4-HBA), 4-nitrophenol (4-NP), and 4-fluorphenol (4-FP) to represent a range of intrinsically different biodegradableities. Parent compound disappearance was evaluated at the end of the test using a novel colorimetric assay based on azo-dye coupling [4].
Those showing greater than 70% parent compound disappearance were scored as positive providing a probability of biodegradation for each inoculum dilution, which was also used to determine specific degrader abundances by the most probable number method; MPN.

- Samples were also taken for bacterial community analysis using denaturing gradient gel electrophoresis (DGGE), which briefly incorporates; DNA extraction, PCR amplification of the 'universal' evolutionary biomarker - 16S rRNA gene fragments, and separation of the fragments based on sequence on a denaturing fingerprint gel. Each band in the resulting pattern represents a taxon and the number of bands (band richness) is indicative of the diversity of the predominant members of the community. This data was used to statistically compare bacterial community structure with biodegradation outcomes and the prevailing environmental conditions.

3. Results and discussion

The equivalent of approximately 150,000 individual Ready Biodegradation tests have been completed so far from 44 locations in 5 compartments. The order of relative biodegradabilities of test compounds was generally 4-HBA>4-NP≈4-FP (e.g. Figure 1).

Generally, enhanced inocula concentrations result in greater probabilities of biodegradation for all test compounds. A two-fold increase is sufficient to increase the probability of 4-NP to 100% from <10% at OECD-recommended inocula concentrations.

There was little correlation between the number of specific degraders and taxa richness. However, those environmental compartments with greatest dissimilarities in community structure between samples were those that had highest variation with respect to the probability of biodgradation for test compounds.

4. Conclusions

OECD-recommended inocula concentrations result in low probabilities of biodegradation for all chemicals tested (i.e. a high number of false negatives). Enhanced biomass studies do appear to offer a robust tool to prioritise chemicals based on relative biodegradability or persistence. Inocula source, concentration and bacterial community composition and diversity play an important role in biodegradation outcome and its variation. Whereas taxa diversity per se does not appear to correlate with biodegradation outcome, variation in community similarity does correspond to variations in biodegradation.

5. References


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1. Introduction

The Organisation for Economic Cooperation and Development (OECD) has established standardised laboratory methods to estimate the rate of chemical biodegradation for potential pollutants in air, water, and soil. Current OECD biodegradation tests are conducted under dark conditions in order to eliminate interference from algal photosynthesis and metabolism, and therefore the indirect effect of light on the biodegradation is not considered. The development of more environmentally realistic test systems for measuring chemical biodegradation could enable the Chemical Industry to better assess a chemicals persistence and potential risk to the environment.

Historically, the use and emissions of nitroaromatics has been widespread, adding to environmental pollution. Among these chemicals, para-nitrophenol (PNP) is one of the most important both in terms of quantities used and potential environmental impacts (Qiu et al., 2007) and the US EPA (1980) lists PNP as a priority pollutant. PNP is also commonly used as a model compound in biodegradation tests and PNP biodegradation pathways have been studied along with PNP degradative genes (Perry and Zylstra, 2007; Zhang et al., 2009).

The aim of this study was to investigate the effect of light and compound concentration on the biodegradation rate of PNP and the presence of PNP degradative genes in river water. The PNP degradative gene targeted was: pnpA and npdA2, encoding PNP 4-monooxygenase and PNP 2-monooxygenase in Gram (-) and Gram (+) bacteria (Chauchan et al., 2004), respectively.

2. Experimental design

Surface water was collected from River Dene (Wellesbourne, UK), downstream of Wellesbourne Sewage Treatment Plant (STP). Aliquots of river water were used as inoculum incubated in amber and light bottles. The test compound was para-nitrophenol (PNP) at an initial concentration of 2mg/L. Chemical analysis was carried out using High Performance Liquid Chromatography (HPLC). Terminal Restriction Fragment Length Polymorphism (TRFLP) of the 16S rRNA gene was used to determine the structure of the bacterial communities before and after incubation. Specific primers were designed to detect PNP degradative genes.

3. Results and discussion

3.1. Influence of light on the rate of PNP biodegradation

Total mineralisation of PNP was observed during incubation in the dark. After a lag phase of 5 days (adaptation phase) all three replicates degraded PNP within 2 days. Under light conditions replicates did not produce consistent results. A single replicate degraded PNP, whereas the other two remained unchanged. In the replicate which degraded the period of adaptation was longer than in the dark (20 days) and PNP degradation rate was slower with 93% of PNP degraded within 21 days.

3.2. Influence of light on the bacterial community profile

A strain AKDH2 which degraded PNP was isolated from dark incubated bottles, and 16S rRNA sequence demonstrated that it had 98% homology to Pseudomonas syringae.
The analysis of TRFLP peaks revealed that a single fragment appeared in dark bottles after PNP degradation. This was absent in light incubated samples. The fragment at 451 nucleotides matched fragment produced by *P. syringae* AKDH2 (red circle). *P. syringae* was the dominant strain responsible for PNP degradation (Figure 1).

### 3.3. Detection of PNP degradative genes

The PNP degradative gene *pnpA* was present in the dark incubated treatments as well as in the isolate AKDH2. No *npdA2* gene was detected in the treatments incubated under light or dark conditions.

### 4. Conclusions

- Light increased the length of the adaptation phase and reduced the rate of PNP biodegradation due to intensive algal growth.
- Light and dark conditions differentially shaped the bacterial community profile, and dark conditions stimulated the proliferation of PNP degrading bacteria.
- The strain AKDH2 isolated from dark bottles was related to *Pseudomonas syringae* and was found to proliferate during degradation of PNP.
- Detection of gene *pnpA* in both strain AKDH2 and river water incubated in the dark suggests that *pnpA* contributed to PNP degradation.

### References


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Impact of cell concentration methods on the performance of enhanced biodegradation tests within REACH

Timothy J Martin¹ ², Russell J Davenport¹, Andrew K Goodhead¹ Maggie Daniel², Abigail Bartram² and Jason R Snape²

¹Newcastle University, School of Civil Engineering and Geosciences, Cassie Building, Newcastle-upon-Tyne, NE1 7RU, UK
²Brixham Environmental Laboratory, AstraZeneca UK Ltd., Freshwater Quarry, Brixham, Devon, TQ5 8BA
E-mail contact: Tim.Martin@astrazeneca.com

1. Introduction

Exponential growth of the human population has been accompanied by increased manufacture, application and disposal of numerous chemicals from sources including industry, agriculture and medicines [1]. There are concerns over the impact of chemicals, both natural and synthetic, on aquatic environments with potential impacts on physiological processes of aquatic organisms, increased cancer incidence and evolution of antibiotic-resistant bacteria [1, 2].

Regulatory emphasis has shifted recently towards hazard identification and identifying chemicals which are persistent, liable to bioaccumulate and are toxic, since chemicals with these properties have previously been shown to be most harmful to human health and the environment. Ready biodegradability tests have formed the core protocol for developing regulatory guidelines for persistency and environmental exposure assessments. They are highly prescribed, very stringent standardised tests that measure the biodegradability of chemicals. Due to the stringent nature of these tests and their high false negative rate, they offer little potential for prioritising on the basis of environmental persistence. To offer more effective tools for prioritisation, enhanced biodegradation screening tests were identified in the REACH technical guidance [3].

Reliable extrapolations from small-scale systems to predict effects at local and regional levels are dependent upon test systems being true representations of the real environment, including the nature of the microbial populations present. Enhanced tests allow increases in inoculum density to environmentally-equivalent concentrations. The present study assessed several concentration methods for enhancing the microbial inoculum density for biodegradation testing with suitability assessed based on criteria including: community similarity with the original sample, cell number, community diversity, method practicality and probability of degradation using inocula derived from the respective methods.

2. Materials and methods

Inocula from a range of environmental sources were concentrated using membrane filtration (MF) [4] tangential flow filtration (TFF) [5], glass bead colonisation [6] and centrifugation. Concentrated inocula were then combined with the test chemical and OECD mineral medium solution and used in enhanced screening tests similar to the OECD 301 B ready biodegradation test [7]. The feasibility of use, introduction of community bias and probability of degradation using the enhanced inocula were used to assess the cell concentration methods.

Total DNA was extracted from 250µL of the concentrated samples using FastDNA® spin kit for soil (Q-Biogene, Cambridge, UK). Polymerase Chain Reaction (PCR) was then performed using universal bacterial primers and Denaturing Gradient Gel Electrophoresis (DGGE) was performed as described by Muyzer et al. [8]. DGGE analysis was executed using the D-code system (Bio-Rad, Hemel Hempsted, UK) with 10% polyacrylamide gels at a denaturant gradient of 30-60% run at 60ºC for 900 volt hours. Gel images produced were analysed using Bionumerics and Primer software packages.

3. Results and discussion

Increasing the cell density of the inoculum used in the ready biodegradation tests is accompanied by the possibility of introducing a diversity bias, arising from the cell concentration method chosen. Using Bionumerics and Primer software, the similarity of the concentrated samples to the original sample was assessed; preliminary results suggesting that MF and TFF produce communities most similar to the original sample, therefore introducing less diversity bias as compared to other cell concentration techniques, as visualised in Figure 1.
Highest cell concentrations were consistently achieved using MF, followed by TFF. The greatest band richness, or microbial diversity of the sample, was obtained using different cell concentration methods for all the environmental compartments. Probability of a positive biodegradation outcome from the ready test was typically found to be most likely using centrifugation, followed by TFF and MF. The greatest cell bias, with respect to reduction in diversity and community shift, was observed with methods to concentrate bacterial cells on glass beads i.e. biofilm formation.

4. Conclusions

The biological judgement criteria used to assess the cell concentration methods, such as community similarity, cell concentration achieved and band richness, indicate varied performance across the environmental compartments. Whilst similarity of the bacterial community with the original sample should be given preferential weighting within the judgement criteria, the practicality of implementing the procedures within the laboratory must also be considered. For the two best concentration methods, a ten litre sample could be processed in approximately 5 hours using MF compared to a vastly reduced time in the region of tens of minutes using TFF. The operational costs of a TFF unit are also lower than alternative methods, such as centrifugation, although initial equipment costs must be considered.

Preliminary findings suggest that there is no one method which can be used for all scenarios across the different compartments, although MF typically performed best, followed by TFF. The selection of a cell concentration method should rather be chosen on a case by case basis; when using small volume tests MF is the recommended method, however due to the impracticality of concentrating large volumes of inoculum using this method, an alternative procedure, such as TFF would be championed.

5. References


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Monitoring the transport and degradation of triclosan in field soils receiving sewage sludge

E. Butler¹, M.J. Whelan¹, R. Sakrabani¹ and R. van Egmond²

¹ Department of Natural Resources, School of Applied Sciences, Cranfield University, Cranfield, Bedfordshire, UK, MK43 0AL
² Safety and Environmental Assurance Centre, Unilever, Colworth Science Park, Sharnbrook, Bedfordshire, UK, MK44 1LQ
E-mail contact: e.butler@cranfield.ac.uk

1. Introduction

The anti-microbial substance triclosan has widespread use in home and personal care products. It is estimated that over 350 tonnes are produced annually for the European market [1]. It is moderately hydrophobic and, therefore, tends to partition to sewage sludge during waste water treatment. If sludge is applied to land, soils can be exposed. Triclosan fate and effects have been extensively studied in the aquatic environment [2]. However, relatively little research has focussed on triclosan fate in soils, although a few recent studies have considered triclosan transfers from soil in runoff [3] and leachate [4], as well as triclosan dissipation and, in the case of multiple applications, triclosan accumulation [5]. Methyl-triclosan is a known methylated metabolite of triclosan which is produced as a result of biodegradation during waste water treatment and in receiving environments. Methyl-triclosan is usually present in sewage sludge in relatively small quantities and an increase in its concentration in soil is, therefore, indicative of triclosan biodegradation. It is more lipophilic and more environmentally persistent than the parent compound [6]. Unlike triclosan, methyl-triclosan has no known anti-microbial properties. Here we present results from a twelve month field study in which triclosan movement and degradation was followed after the application of pelletised sewage sludge as a fertiliser. Three different soils with a range of physical and chemical properties were studied in order to evaluate the role of soil type on triclosan behaviour.

2. Methods

Field experiments were conducted at Silsoe farm (120 ha), Bedfordshire, in the south east of England, which has a range of different soil types. Three fields with contrasting textures (a sand, a sandy loam and a clay) and differing pH and organic matter contents were selected. All fields have been under the same arable rotation for the past 20 years and none of them had received sewage sludge prior to this study. Three replicate plots of 3 x 3 metres were set up in each field and pelletised sewage sludge was applied manually and incorporated into the top 10 cm at an application rate equivalent to 50 tonnes sludge per hectare. A fourth plot in each field was established as a control which received no sludge. Each field was planted with winter wheat which was harvested in August 2010. Monthly soil samples were taken in triplicate from three depths (0-10, 10-20 and 20-30 cm) in each plot between November 2009 and October 2010. Samples were subjected to accelerated solvent extraction (ASE) followed by solid phase extraction (SPE) and analysed for triclosan and methyl-triclosan concentrations using gas chromatography-mass spectrometry (GC-MS).

3. Results and discussion

Concentrations of triclosan in the top 10 cm were initially very high in all plots (850-900 µg kg⁻¹) but decreased progressively over time (Figure 1). Appearance of methyl-triclosan in the upper horizon was slow over the winter period but concentrations increased noticeably in the sand and the loam soils between March and June, suggesting that triclosan biodegradation accelerated in this period. There was also a very marked decrease in triclosan concentration in all three soils in August, which was accompanied by an increase in methyl-triclosan concentrations. Triclosan was translocated to the 10-20 cm horizon between January and March and appeared in the 20-30 cm layer from February. Concentrations in the 20-30 cm horizon were highest in the sandy soil and lowest in the clay – probably reflecting differences in triclosan mobility due to differences in drainage and soil organic matter content. Towards the end of the monitoring period, concentrations of methyl-triclosan exceeded concentrations of triclosan in all three layers of all three soils.
Figure 1: Measured concentrations of triclosan (TCS) and methyl-triclosan (Me-TCS) in each of soil at three depths (0-10 cm, 10-20 cm and 20-30 cm) between November 2009 and October 2010. Error bars signify the standard error of the mean. Note different concentration scales for each layer.

4. References

Occurrence, Cycling and Transport Pathways of Chlorinated Persistent Organic Pollutants to The North Atlantic and Arctic Ocean

Cristóbal Galbán-Malagón¹, Naiara Berrojálbiz¹, Maria José Ojeda¹ and Jordi Dachs¹

¹Department of Environmental Chemistry, IDAEA-CSIC. Jordi Girona 18-26, Barcelona 08034, Catalunya, Spain.
E-mail contact: cgmqam@cid.csic.es

1. Introduction
Occurrence of Persistent Organic Pollutants (POPs) in the North Atlantic and Arctic Ocean is one to most important issues in the actual Global Change Scenario. Transport and subsequent deposition of POPs to the Arctic has been studied extensively in the literature (Gioia et al., 2008; Sobek et al., 2004 and 2006). Several studies reported smaller latitudinal gradients and decreasing concentrations, from Europe to Arctic. Published results in this area did not report particulate, aerosol and Phytoplankton concentrations. Our study is the first showing this results together. No residence times and Fugacity ratios were published for pollutants in this area, this work is the first showing that results compared to published models (Jurado and Dachs, 2008).

2. Materials and methods

2.1. Sampling Area
Sampling was carried out during ATOS-ARCTIC cruise on board R/V Hespérides during July 2007, our sampling area was divided in two different scenarios Greenland Current (GC) and Arctic Ocean (AO).

2.2. Sampling Devices
Air sampling was done using High-Volume sampler as described in Bruhn et al., 2003 using Polyurethane Foam (PUF) for the Gas Phase and QMA for the aerosol. Seawater was sampled using XAD-2 packed columns as described in Dachs and Bayona, (año). For the Phytoplankton a net trawl was used and them filtered biomass using a GFF filter.

2.3. Sample extraction and Chemical analysis
PUFs, aerosol and particulate filters were soxhlet extracted for 24 hours (Acetone:N-Hexane 3:1 PUFs and Dichloromethane: Methanol 2:1 filters), later samples were purificated using a filled column with 0.5 g of NaSO4 over 3g of 3% deactivated neutral alumina, each extract was eluted with 5 mL of N-Hexane and finally samples were concentrated under a N2 gentle stream to 0.5 mL of Isooctane. Phytoplankton Samples were soxhlet extracted with N-Hexane:Dichloromethane(1:2) and purified by elution in a column filled with 1-2 g of NaSO4, 5 g with activated silica (250°C 12 h) and finally 3 g of 3% deactivated neutral alumina followed by a elution of 25 mL of N-Hexane.

Seawater dissolved Phase was extracted following the method published in Dachs and Bayona in 200 after the extraction samples followed the same purification process described above. Them the extract was eluted and purified as described above for the aerosol and particulate phases. Prior to extraction PCBs 65 and 200 were added as surrogate. Chemical analysis was done with an Agilent 7890 GC copled to uECD detector (Agilent Technologies) using PCB 30 and 142 as internal standards.

3. Results and discussion

3.1. Concentration Results
Resume concentrations PCBs are shown on fig 1. Concentrations ranges of HCHs, HCB and PCBs agree with published bibliography for similar areas. North Atlantic Ocean concentrations presented in this study for HCHs are the first presented for the Greenland Current showing a decrease in the Gas Phase concentrations along the transect in direction to the Arctic and Sub-Arctic areas, the same occurs with the
PCBs concentration and no clear tendency was found for the HCB. In the case of the dissolved seawater concentrations the same tendency was found for the HCHs, HCBs and PCBs. No clear tendency was found for the Phytoplankton concentrations and the Particulate phase of the different studied compounds. In the case of Aerosols no clear tendency was found in the data distribution.

![Fig 1. Shows concentrations in the Gas (pg m⁻³), Aerosol (pg m⁻³), Dissolved (pg L⁻¹), Particulate (pg L⁻¹) and Phytoplankton (ng g⁻¹) plotted against Latitude °N](image)

### 3.2. Residence times

Our results are useful in order to study latitudinal transects, especially the gas phase samples studied over the Greenland Current, air masses backtrajectories were tracked using HYSPLIT model from the NOAA. In order to study the residence times we choose those samples originated in southern areas.

The decreasing tendencies and low residence times agrees with published models, giving lower values than predicted (Jurado and Dachs, 2008).

### 3.3. Fugacity calculations and fugacity ratios calculations

Fugacity was calculated for the Gas and Dissolved phases, using Gas and Dissolved concentrations during the transects. Fugacity results gives us information about the direction of the net flux of compounds between the two studied phases. According to published Henry’s Law Constant values reported there are big uncertainties in given values affecting to fugacity ratio values. For the present work a range from 0.3 to 3 was chosen for equilibrium between water and air. According to published values of equilibrium ranges in the bibliography. Other authors described wider ranges for equilibrium (Axelman et al., 2001 and Wania et al., 2001…). A-HCH and HCB were always in equilibrium during all the sampling transect. For the PCBs one less chlorinated PCB was chosen (PCB 28) which shows volatilization for the first sampling point according to predictions published by Jurado and Dachs, 2008 and the more chlorinated (PCB 180) shows net deposition or equilibrium during all the sampling period.

### 4. References


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LC-MS/MS analysis for the identification and quantification of endocrine disrupters in surface water and in water for human consumption: monitoring of italian water supplies

Sara Bogialli¹, Laura Achene¹, Luca Lucentini¹, Emanuele Ferretti¹ Federica Nigro Di Gregorio¹, Franca Palumbo², Enrico Raffo² and Massimo Ottaviani¹

¹Italian National Health Institute, Department of Environment and Primary Prevention - Section of Inland Water Hygiene, Viale Regina Elena, 299 - 00161 Rome, Italy.
²AMGA Foundation, Via Piacenza, 54 16138 Genova, Italy
E-mail contact: sara.bogialli@gmail.com

1. Introduction

Many chemical substances of natural or anthropogenic origin are suspected or known to be endocrine disruptors (EDs), which can influence the endocrine system. This observation has led to an increasing interest by consumers and mass-media, as well as to a steep rise of research activities in the scientific community.

EDs are emerging as a major concern for inland water, also used in the production of water intended for human consumption. The presence of EDs in influent and effluent water samples from six Italian waterworks was studied within a national research project coordinated by the AMGA foundation and the University of Genova (Department of Health Science), involving different water supply systems and laboratories (Acquedotto Pugliese S.p.A.– Bari, ACSM S.p.A – Como, Hera S.p.A – Bologna, Iride Acqua Gas S.p.A – Genova, Publiaqua S.p.A – Firenze, SMAT S.p.A. – Torino).

We developed an advanced analytical method based on solid-phase extraction (SPE) followed by liquid chromatography–tandem mass spectrometry (LC-MS/MS) for the simultaneous identification and quantification of a number of EDs selected as relevant in surface water by the European Union [1]. Water samples before and after treatment obtained from 5 different sampling campaigns were analysed for natural and synthetic estrogens (17β-estradiol, estrone, 17α-ethinylestradiol), bisphenol A, alkylphenols (4-octylphenol, nonylphenol) and several perfluorinated surfactants (PFPeA, PFHxA, PFHpA, PFOA, PFNA, PFDA, PFUnDA, PFDoDA, PFBS, PFHxS, PFOS).

2. Materials and methods

1-L of the water samples (pH adjusted to 2.0) was spiked with the internal standard (17β-estradiol d₃). A SPE HLB column, 200 mg/6 cc, 60 µm (Supelco, Sigma Aldrich) with 200 mg solid-phase material (a copolymer of N-vinylpyrrolidone and divinylbenzene) was washed and conditioned with 5 mL of methanol and 5 mL of water. The water sample was passed through the cartridge at a flow rate of ca 10 mL/min. The elution was performed with a methanol/acetonitrile/ethyl acetate mixture (40:40:20, v/v/v) (2x3 mL). All extracts were reduced to dryness in a gentle nitrogen stream and then reconstituted with 0.5 mL water/methanol mixture (50/50, v/v). The analyses were carried out using an API 3000 triple-quadrupole mass spectrometer with turbo ion spray source (Applied Biosystems). The instrument was operated in negative ionization mode using Multiple Reaction Monitoring acquisition (MRM). Fig. 1 shows a representative MRM LC-MS/MS chromatogram of a standard solution (concentration 50 ng/L).
3. Results and discussion

After optimizing chromatographic and instrumental parameters, several experiments were conducted in order to developing a sample preparation protocol suitable for EDs determination in chlorine-disinfected water. All main experimental conditions and analytical performances were evaluated involving waterworks laboratories in a collaborative trial. With the aim of evaluating the reliability of the extraction protocol for analyses of surface and treated water, an inter-lab validation was carried out among the waterworks in terms of sensitivity, specificity, trueness, repeatability and inter-laboratory precision, with LODs in the range of 0.02-10 ng/L for all the analytes. A study of systematic and non-systematic contaminations was performed. Limits of Report (LOR), as described in previous published papers, were also established on the basis of the instrument sensitivity and the unforeseen contamination [2]. The proposed LOR values ranged for all the analytes from 0.3 (estrone) to 50 ng/L (bisphenol A and alkylphenols).

Chemical analyses demonstrated that relatively low concentrations of the selected analytes are present in raw water samples (<Limit of Report-806 ng/L); these values are comparable with the concentrations occurred in European surface water, confirming the homogeneity of pollution processes in different industrial countries [1]. Analyses of drinking water samples has shown that treatment processes are generally effective in removing these compounds (<Limit of Report-621 ng/L);

4. Conclusions

With the growing attention to EDs presence in the environment, there is a need for sensitive and reliable analytical methods that can simultaneously detect a wide variety of these compounds. This method has proved to be suited for analyzing several EDs compounds in raw water as well as treated water, with a reduced sample preparation. A deep validation was conducted during a 2-years collaborative trial assuring that this analytical protocol is robust and can be easily transferred to the stakeholders laboratories. Finally, an extensive monitoring of representative Italian water supplies was conducted in order to collect data, useful for the evaluation of exposure and risk assessment related to EDs contamination in drinking water.

5. References


1. Introduction

Atmospheric deposition, and particularly diffusive air-water exchange, is the main entrance and driver of the concentrations of many persistent organic pollutants (POPs) in the surface waters of open oceans and Lakes. Once in the water, partitioning processes influence the transport pathways, degradation processes, residence times and the final fate of the compounds. In fact, the occurrence and impact of pollutants in the aquatic environment are the result of the interplay of numerous trophic and physical drivers. Nevertheless there still are major gaps in our knowledge regarding to the relative importance of each of the factors controlling the occurrence of those pollutants in marine waters.

Indeed, it is known that biogeochemical cycles, especially those related to organic carbon, affect the POP transport and sinks in the water column. The biological pump has received a lot of attention during the last decade, but it is not clear the role of biodegradation on air water exchange and accumulation of POPs in planktonic food webs. Therefore, the main objective of this study is to clarify the interactions of atmospheric inputs of POPs and the biogeochemical processes occurring in the surface ocean mixed layer emphasizing the implications of the biological pump and degradation in such processes.

2. Materials and methods

Air-water-plankton coupled model has been developed modifying the approach proposed by Dachs and coworkers (1) to calculate air-water, water-plankton and settling fluxes (fig.1, left). Additional modeling exercise has been used to include the potential degradation process occurring in the water column (fig.1 right).

Field measurements of air \((C_A)\), seawater \((C_W)\) and plankton \((C_P)\) POP concentrations (fig.1) have been used in the validation of the model. All samples were obtained on board of RV-Garcia del Cid research vessel during two Mediterranean sampling cruises on June 2006 and May 2007. Both transects from Barcelona to Istanbul and from Barcelona to Alexandria respectively allowed the covering of many different Mediterranean regions including Marmara Sea and Black Sea. The used sampling strategy has allowed the simultaneous collection of different types of matrices to enable the parametrization of atmosphere-ocean-plankton interactions. All samples were analyzed for PCBs, PAHs, HCHs and HCB covering a wide range of physical-chemical properties.

3. Results and discussion

The traditional model (fig.1 left) has been used to account for atmosphere-water interactions of selected POPs in the mixed surface ocean layer and predict compounds concentrations in plankton. This approach
has been used effectively for more persistent compounds not only to predict $C_P$ concentrations from gas phase concentrations, but also to reproduce the seen dilution effect in $C_P$ (fig.2) in field samples. Air-water exchange is one of the main pathways for entry and loss of POPs in the ocean compartment. If POPs in gas phase are equilibrated with the dissolved phase which in turn is equilibrated with plankton, for a given gas phase concentration, POP concentrations ($C_P$) will be independent of biomass. This is the case of the less hydrophobic PCBs and HCB for which any potential depletion of dissolved phase is compensated due to a faster air-water exchange. On the contrary, for more hydrophobic PCBs, losses associated to bioaccumulation processes are important and thus $C_P$ will decrease at higher biomass due to air-water dis-equilibria driven by bioaccumulation process and settling.

![Figure 2: In general, planktonic POP concentrations were higher when lower concentrations of biomass (B) occurred, but the intensity of the response varies upon the chemical and their physical chemical properties ($K_{OW}$). $C_P$ correlates with planktonic biomass (B) following a power function ($C_P = a B^b$ where $a$ and $b$ are constants). The value of the slope of the correlation (b constant) is indicative of the influence of the mass dilution effect on the concentrations of a particular compound. In the case of PCB concentrations the dependence on biomass is significant for the more hydrophobic compounds while for PAHs this effect is reflected in less hydrophobic compounds.](image)

Conversely, for more labile compounds such as low molecular weigh PAH and HCHs, the vertical fluxes were not enough to explain the air-water net depositional fluxes found for all Mediterranean locations. Indeed, for these compounds, the dependence on biomass is significant for the less hydrophobic compounds, consistent with controls due to looses of the chemical associated with degradation processes in the photic zone. In this work we present the modeling of biodegradation and its coupling with phytoplankton uptake and air water exchange and the application of this model to the open Mediterranean Sea.

4. Conclusions

Air-water-planton model for more persistent POPs (PCBs and HCB) obtains not only good agreement for measured plankton concentrations, but also has been able to reproduce de seen dilution effect. On the contrary, more labile compounds such as low molecular weigh PAHs and HCHs require to integrate the biodegradation processes occurring in the water column to reproduce more accurately field measurements. The trends observed in the Mediterranean provide important clues on the processes driving POPs in other oceanic regions, where the gradients in biomass and other environmental variables can be larger than in the Mediterranean.

5. References


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Polycyclic aromatic hydrocarbons (PAHs) atmospheric concentrations and deposition over the open Mediterranean and Black Seas

J. Castro-Jiménez¹,², G. Mariani², G. Umlauf², J. Wollgast³, N. Berrojalbiz¹, J. Dachs¹,

¹ Institute of Environmental Assessment and Water Research (IDAE - CSIC), Barcelona, Spain.
² European Commission – Joint Research Centre, Institute for Environment and Sustainability, Ispra, Italy
³ European Commission - Joint Research Centre, Institute for Health and Consumer Protection; Ispra, Italy

Email contact: jvcastrojm@gmail.com

1. Introduction

The Mediterranean Sea, due to its nature as a semi-enclosed environment surrounded by highly populated areas, is a region of special ecological and commercial interest. Polycyclic aromatic hydrocarbons (PAHs) are a group of semi-volatile organic compounds (SOC) which are ubiquitous in the environment, bioaccumulate in planktonic food webs and may cause a wide range of toxic effects in biota and humans¹. Very limited information is available on ambient levels, occurrence and deposition of PAH in marine environments and in particular in the Mediterranean Sea far from the shore line (open seas). Existing data have been mainly acquired from coastal areas. The incorporation of PAH to marine open waters and possible toxic effects is driven by atmospheric deposition processes². It is therefore important to understand what the current ambient levels in the Mediterranean Sea airshed are in order to have a realistic idea of the potential inputs to this marine environment.

The overall objectives of this work were: (1) to obtain PAH ambient air concentrations and patterns along the open Mediterranean Sea and in the Black Sea; (2) To investigate the factors driving the atmospheric occurrence of PAHs in the open Mediterranean Sea and their day/night cycling in open waters; (3) to estimate the atmospheric deposition of PAHs in the Mediterranean and Black Seas.

2. Experimental

Air samples were collected during two sampling cruises performed on June 2006 and May 2007 on board of the oceanographic vessel B/O García del Cid (CSIC). In both campaigns, Barcelona was the initial and final port, with Istanbul and Alexandria being the intermediate stops respectively. The transects covered an extensive area within a year of difference allowing a good spatial coverage of different regions. Ten air transects were sampled along the Mediterranean and Marmara and Black seas. A total of 44 Integrated air samples (particulate + gas phase) were collected by using two high volume samplers operating contemporaneously and installed on the upper deck of the boat (around 6-7 m above the sea level) close to the bow. Quartz fibre filters (QFFs) were used for air particle phase collection whereas compounds in the gas phase were trapped by using polyurethane foam (PUF) plugs. Samples were Soxhlet extracted with a mixture acetone/hexane after being spiked with labelled internal standards, cleaned-up and analysed by gas chromatography–mass spectrometry (GC-MS). Isotopic dilution technique was used for quantification of target compounds.

3. Results and discussion

PAH atmospheric levels over the Mediterranean Sea were driven by air gas phase concentrations. ∑16 PAHs gas phase concentrations ranged from 2 to 4 ng m⁻³ whereas particulate phase concentrations varied from 0.1 to 0.3 ng m⁻³. The transect encompassing the Marmara and Black Seas exhibited a slightly highest PAH concentrations of 6 ng m⁻³ and 0.5 ng m⁻³ for gas and particular phases, respectively. Phenanthrene dominated the average gas phase congener pattern in the Mediterranean Sea accounting for 50 ± 15% of the sum of PAHs, whereas PAH congeners were more evenly distributed in the particulate phase being Benzo(b)fluoranthene and Benzo(e)pyrene the more abundant (12 ± 2% of the sum of PAHs) (Figure 1).

Dry atmospheric deposition fluxes in the Mediterranean Sea open waters ranged from 20 to 50 ng m⁻² d⁻¹, whereas in the Marmara and Black seas a value of 95 ng m⁻² d⁻¹ was calculated. Back trajectories analysis, diffusive air-water exchange and day and night concentration variations were also investigated in the present study.
4. Final remarks

The results from this campaign constitute a unique data set since few or no data on PAH ambient air concentration and depositions in the open Mediterranean Sea have been reported to date. PAH atmospheric concentration seems to be pretty homogenous across the Mediterranean Sea. In addition, ambient levels are in the rage of those measured over the Marmara and Black Seas. Back trajectories calculations and diffusive air-water exchange (result analysis from water samples, also collected during the campaign, are underway) will help better understand the occurrence of these pollutants in the Mediterranean region and to determine whether the atmosphere is the main source of PAHs for the Mediterranean.

5. References


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Fate of hydrophobic persistent chemicals in the pelagic system of Lake Maggiore during an algal bloom

Luca Nizzetto¹, Rosalinda Gioia², Jun Li³ and Kevin C. Jones²

¹Norwegian Institute for Water Research, Gaustadalléen, 21 NO-0349 Oslo, Norway
²Lancaster Environment Centre, Lancaster University, Lancaster, LA1 4YQ, UK
³State Key Laboratory of Organic Geochemistry, Guangzhou Institute of Geochemistry, Chinese Academy of Sciences, Guangzhou 510640, China
E-mail contact: luca.nizzetto@niva.no

1. Introduction

Several modelling studies have supported the hypothesis that carbon-rich/biogenic particle dynamics control environmental fate of hydrophobic chemicals such as POPs in pelagic ecosystems. For example they can affect the fugacity gradient between air and surface water and driving the air-water exchange toward net deposition [1-3], highlighting the control of biogeochemical cycles (e.g. C and nutrient cycles) over the environmental cycle of persistent chemicals [4-6].

Even though the interactions between air/surface water exchange of Chemicals and partitioning onto the organic carbon matrix in the water have received some attention in the last few years, these are still processes that are poorly understood especially considering the perspective of a highly dynamic scenario such as algal blooms. These are conditions in which the amount of particulate organic matter in water can change considerably in short time. Studying these unsteady systems can provide useful information on the dynamics of pollutants entrance into the pelagic food web, and their overall inter-compartment exchange.

Polychlorinated biphenyls (PCBs) (as a model for hydrophobic accumulative substances) were measured in the dissolved and different particulate matter fractions of the pelagic ecosystem of Lake Maggiore (Italy) during a spring algal bloom (between March and May 2009). In order to assess: i) the effects of biomass development on the air-surface exchange of chemicals; ii) the effects of biomass growth on the concentration of chemicals in the dissolved phase; iii) the distribution of PCBs across the different particle fractions (trophic levels); iv) The state and the evolution (with time) of the POC-water partitioning.

2. Materials and methods

Air, seawater and plankton samples were collected from a small boat in the middle of the lake in proximity of S. Caterina. Sampling occurred approximately every 1-2 weeks. Water samples were collected using a PTFE-Steelless steel-glass high volume filtration system equipped with a GF/F filter and a XAD2 cartridge. About 200 L of waters were collected at each sampling event. Plankton was collected and pre-concentrated using a 10 µm mesh nylon plankton net. Size fractionation was performed in lab using nylon screens in order to produce the following fractions: 10-95 µm, 95-200 µm and >200 µm.

3. Results and discussion

3.1. PCB concentrations and distribution

The measured dissolved phase concentrations were corrected for the colloidal effect using theoretical model as shown in Totten et al., 2001 and Garcia-Flor et al., 2005. DOC values did not changed significantly during the sampling period and ranged from 0.8 to 1 µg L⁻¹. The fractions of PCBs sorbed to the colloidal phase and bacteria (namely < 0.5 µm) predicted by this model are 4%, 8%, 18%, 45%, 52%, for the tri-, tetra-, penta-, hexa-, hepta-, respectively. The concentration of Σ26PCBs in the dissolved phase ranged from 34 to 164 pg L⁻¹ with Cl3Bs and Cl4Bs constituting 36% and 30% followed by Cl5Bs (25%), Cl6Bs (6%) and Cl7Bs (0.9%).

The analysis of PCB fingerprint and air-water fugacity ratio suggested atmospheric inputs as the main source of PCBs for the lake pelagic system.

The concentration of more hydrophobic chemicals in the truly dissolved phase tended to decline with time during the algal bloom.

The plankton concentrations were normalized to the organic carbon (OC) content for each. The concentration of Σ26PCBs in the plankton ranged from 3.6 to 115 ng g(C)⁻¹ from 220 to 728 ng g(C)⁻¹, from
120 to 2100 ng(C) g\(^{-1}\), from 163 to 2070 ng(C) g\(^{-1}\) for fraction total suspended particulate (TSP), 5-95 µm, 95-200 µm and >200 µm respectively.

Cl₅Bs and Cl₆Bs were the most abundant homologue groups for all size fractions with an average of 30±9% and 33±14 % respectively followed by Cl₃Bs, Cl₄Bs and Cl₇Bs in equal amount (approx. 10±6%). Figure 1 shows the distribution of selected PCB congeners in the different water fractions measured. The 0.5-10 um fraction is obtained by subtracting the sum of the distribution of the PCB congeners in fraction 10-95um, 95-200 um and > 200 um from the TSP.

3.2. POC-water partitioning \(K_P\)

The slope of the regression curve between log \(K_{ow}\) and Log \(K_p\) was close to 1 at the beginning and at the end of the sampling period for the different fractions. During the central part of the monitoring (likely the period with fastest algal biomass development) slopes values were significantly lower and ranged 0.40-0.70. This suggests either: i) the system was not at equilibrium partitioning for large part of the algal bloom or ii) variability in the properties controlling the partitioning.

4. Conclusions

The majority of PCBs are associated with the dissolved phase followed by the colloidal fraction and the 0.5-10 um fraction, while less than 1% of the PCBs was associated with fractions 10-95 um, 95-200um and >200um. PCB concentration in the dissolved phase decreased with increasing chlorination and with time during the algal bloom. POC and water phase likely were not at the partitioning equilibrium for large part of the algal bloom period. A possible explanation is that under conditions of fast organic matter synthesis and trophic transfer, exposure to PCBs of the different fractions was limited by the unbalanced atmospheric supplies.

5. References

Chiral signatures of selected pharmaceuticals as markers of biological attenuation processes in rivers

Serge Chiron\textsuperscript{1}, Zhi Li\textsuperscript{1,2}, Elena Gomez\textsuperscript{2}, Hélène Fenet\textsuperscript{2}, Claude Casellas\textsuperscript{2}

\textsuperscript{1}Laboratoire Chimie Provence, Aix-Marseille University, 3 place Victor Hugo 1331 Marseille cedex 3. \textsuperscript{2}UMR HydroScience, Montpellier University, 15 Avenue Ch. Flahault, 34093 Montpellier cedex 5.

E-mail contact: Serge.Chiron@univ-provence.fr

1. Introduction

The capacity of rivers for biological attenuation processes of trace polar organic pollutants are probably significant but poorly understood because the many factors (i.e., dilution, sorption, phototransformation, biotransformation) that control naturally attenuation are interrelated and difficult to study in isolation. Although it is possible to estimate in-stream attenuation rates from monitoring data, accurate estimates require large data sets [1]. To overcome these limitations, compound-specific isotope approaches have been suggested but these approaches are restricted to volatile chemicals and probably to sediment or soil studies where pollutant concentrations are high enough to be amenable by GC-IR/MS techniques. Chirality has been exploited in this work since only biological processes might change enantiomer composition of chemicals [2].

The main aim of this work was to investigate the chiral signature of the antidepressant venlafaxine (VEN) and the β-blocker metoprolol (MET) (Figure 1) to get insights into biological attenuation processes in river environment. VEN and MET were selected because (i) they are currently consistently detected worldwide in surface water, (ii) they undergo stereoselective metabolism in human through cytochrome P-450 catalytic reaction, (iii) Cytochrome P-450 enzymes are found virtually in every form of life supporting potential enantioselective biodegradation of VEN and MET in the environment.

![Figure 1: Investigated pharmaceuticals.](image)

2. Materials and methods

**Analytical methods:** LC-MS/MS using chiral column (chirobiotic V, 250 x 2 mm i.d.). LODs (< 10 ng/L) and precision <15% RSD after an extraction step on Oasis HLB cartridges.

**Sorption to river sediment:** Batch experiments were used which largely followed OECD guideline 106. The sorption data well fitted to the Freundlich isotherm.

**Photochemical fate:** 0.5 L immersion-type photo-reactor (Heraeus TQ 150 model) equipped with a medium-pressure mercury lamp and a filter to cut off the wavelengths shorter than 290 nm was used.

**Biodegradation experiments:** Batch experiments were used which largely followed the OECD 308 water/sediment system test. Spiking level of chemicals was 0.1 mg/L.

**Field observations:** Sampling took place at WWTPs outlets (n = 5) and at the Arc River (Southern France) at site 1 located just downstream the discharge of the WWTP of Aix en Provence (175 000 equivalent inhabitants) and at site 2 located at the mouth of the river. Between the two sampling sites, no continuous input of wastewater occurred. Sampling was carried out during dry conditions (May-June) and during rainfall events (October-November) which caused higher flow rate in the river.

3. Results and discussion

3.1 Laboratory-scale experiments

The aim of the laboratory experiment was to assess the contribution of sorption, phototransformation and biotransformation processes to the overall attenuation of VEN and MET.

**Sorption.** With $K_d$ values ranging from 5 to 10 LKg$^{-1}$, adsorption to suspended matter or sediment could not be excluded for both compounds but was not a stereoselective process. The sediment compartment with its high density of microorganisms with respect to the water column may be of significance for their biotransformation.
Phototransformation. Both compounds do not undergo significant direct photolysis in Arc river water. Indirect photolysis rates were very slow ($k_p$ in the 2-7 $10^{-4}$ h$^{-1}$ range) and the concentration of VEN and MET should not be affected by photolysis at least on the water residence time-scale relevant to this study (8-18 h according to the river flow rate).

Biotransformation. VEN and MET underwent stereoselective biotransformation in water/sediment systems (Figure 2). In spite of low kinetic rate constants biotransformation in biofilms in the hyporheic zone or on submerged macrophytes could not be ruled out.

3.2 Field observations

The laboratory data allowed a better understanding of field observations. Analysis of different WWTPs effluents ($n=5$). Although VEN and MET, which are administrated as racemates, are partially metabolized prior to excretion and are removed to a small extent in municipal WWTPs, they do not appear to undergo an apparent enantiomer shift during these processes probably due to a too short hydrological retention time (HRT). EF values in the 0.48-0.53 range were recorded in WWTPs influents and effluents.

Analysis along an Arc River stretch. Concentration decreased between the two sampling sites while dilution did not occur. EF of VEN remained constant during base flow conditions while EF values fluctuated during rainfall events (Figure 3), supporting biodegradation. Due to the fast downstream transport of water and solutes during precipitation events (8 h instead of 18 h during base flow conditions), the residence time should be too short for biotransformation processes to substantially reduce pharmaceutical concentrations. However, an increased exchange of water and solutes might entail the release of VEN accumulated during dry season in sediment, where enantioselective biotransformation probably took place. This assumption deserves further investigation to be fully validated.

4. Conclusion

Investigating chiral signature of selected pharmaceuticals is a promising tool not only to discriminate between abiotic and biotic transformation processes in river but also to assess the spatial and temporal variability in biological attenuation processes. Current researchs deal with chiral pharmaceutical metabolites (i.e., O-desmethyl venlafaxine and metoprolol acid) because these metabolites are usually detected in higher amounts than their parent compounds in river water samples and aim at quantitatively linking EF evolution of pollutants to their attenuation kinetics in river.

5. References

Deconstructing Complex Aquatic Communities; Identification of Active Components and the Metabolism of Fludioxonil by Phototrophs

Kevin A. Thomas¹ and Laurence H. Hand

¹Syngenta, Product Metabolism, Jealott’s Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, United Kingdom

Email contact: kevin.thomas@syngenta.com

1. Introduction

Assessment of the environmental fate of crop protection products (CPPs) incorporates a range of standardized laboratory studies designed to assess hydrolysis, photolysis, sorption and microbial degradation. In such studies complex, natural processes are separated in order to provide simple, standardized laboratory based systems that conform to legislative guidelines. In the case of freshwater microbial degradation, CPP degradation is examined in darkness. Clearly, such studies differ greatly from real world conditions. More complex study systems comprising biota more representative of natural water bodies are required to fully understand the fate of CPPs in freshwater ecosystems.

Our work is intended to investigate and uncover the role of aquatic macrophytes and algae in the transformation of CPPs in freshwater ecosystems. This work was divided into three distinct phases:

1. Comparison of the degradation rates of six CPPs in standard regulatory water-sediment test systems against the rates observed in systems in which macrophytes and algae are present. Results from this phase were presented at SETAC Europe 2010. Enhanced degradation was observed for all compounds tested, with the largest enhancement (20-fold reduction in the DegT₅₀) observed in the case of fludioxonil (Ref 1).

2. Isolation and culture of sub-communities from complex, phototroph inclusive, systems (planktonic algae, filamentous algae, heterotrophic bacteria, cyanobacteria, fungi, macrophyte and biofilms) and investigation of sub-community capacity to degrade fludioxonil. This included the identification of key metabolising species.

3. Comparison of environmental water samples against our inclusive laboratory test system and standard laboratory test system (by microscopic and molecular techniques) to establish relevance of communities to real world populations.

Results from Phase 2 of the project are presented herein.

2. Materials and Methods

The degradation of ¹⁴C-fludioxonil was initially investigated in natural water, obtained from a complex, phototroph inclusive system, as used in phase 1, both in the dark and under non-UV light, and in cultures of Elodea candensis (and its associated biofilm) under non-UV light. Both were performed without sediment to confirm that the degradation observed in Phase 1 was occurring in the water column itself. The following cultures were then prepared to deconstruct the complex community and assess the metabolic competence of sub-community factions by treatment with ¹⁴C-fludioxonil. In order to verify metabolic capability we quantified the level of parent remaining after a suitable elapsed time and compared this against negative controls in order to confirm significant loss of parent.

1. Fungal Community. Malt extract media was inoculated with the natural water and cultured in the dark (to prevent phototrophic growth) in the presence of antibiotics to prevent bacterial growth.

2. Total Bacterial Community. R2A media was inoculated with the natural water and cultured in the dark (to prevent phototrophic growth) in the presence of nystatin to prevent eukaryotic growth.

3. Total Algal Community. WC media (a minimal media to limit heterotrophic growth) was inoculated with the natural water and cultured under a 16h:8h light : dark cycle to allow phototrophic growth.

4. Algal Sub-communities. The total algal community was separated into planktonic green algae (filtration and addition of antibiotics), filamentous green algae (capturing and washing filtered matter on a 50 μm mesh and addition of antibiotics) and phototrophic cyanobacteria (culturing in nitrogen-free medium, at 35°C, with nystatin).
5. **Pure Algal Species.** Ten species were isolated from the natural water (from *Chlorophyta*, *Cyanophyta* and *Bacillariophyta* (diatoms)) and incubated in minimal medium. Three additional species of *Cyanophyta* were included, obtained from the CCAP, Cumbria, UK.

6. **Axenic Macrophyte.** Sections of *Elodea canadensis* were bleached with sodium hypochlorite and incubated in Hoagland’s medium with antibiotics under a 16h:8h light:dark cycle.

7. **Macrophyte Biofilm.** Biofilms were allowed to grow on inert substrate in the presence of *Elodea canadensis*, then removed and cultured in Hoagland’s medium under a 16h:8h light:dark cycle.

### 3. Results and Discussion

No degradation was observed in any of the heterotrophic communities, suggesting that fungal and heterotrophic bacterial metabolism are not significant in the degradation of fludioxonil. However, in all phototrophic inclusive cultures, incubated in a light:dark cycle, statistically significant enhancement of degradation was observed when compared to medium only negative controls (Figure 1). This was also the case for both the axenic macrophyte and the macrophyte biofilm. Furthermore, all thirteen pure species tested were shown to be metabolically competent (Figure 2).

**Figure 1: Degradation of fludioxonil in aquatic sub-communities**

**Figure 2: Degradation of fludioxonil in pure algae species**

### 4. Conclusions

This data conclusively demonstrates that phototrophic microorganisms, such as green algae and blue-green algae, are capable of metabolising fludioxonil. It is also clear that macrophytes, both with and without hosted biofilms, are metabolically competent. These results indicate that the enhanced degradation observed in complex water-sediment systems containing phototrophs (Reference 1) is not simply due to enhancement of indigenous heterotrophic communities, but due, at least in part, to direct metabolism by phototrophs. Though further work is required to confirm that this extends to other CPPs, it remains clear that exclusion of phototropic organisms from environmental fate studies will result in an incomplete understanding of the behaviour of CPPs under real environmental conditions.

### 5. References

1. Introduction

Iodinated X-ray contrast media (ICM) are among the most widely used drugs for intravascular administration. The worldwide consumption of the ICM is about \(3.5 \times 10^6\) kg per year [1]. They are used in human medicine for imaging of organs or blood vessels during diagnostic tests. They are metabolically stable in the human body and are rapidly eliminated via urine or faeces. Most of these radiographic contrast media are derivatives of 2,4,6-triiodobenzoic acid possessing polar carboxylic and hydroxyl moieties in their side chains (Figure 1). The potential adverse environmental impact of ICM has been considered since it was discovered that these compounds contribute substantially to organically bound halogens adsorbable on activated carbon (AOX) in hospital wastewater. Up to 90% of the AOX value could be traced back to the presence of ICM. [2]. They have been reported to occur in different water compartments including untreated and treated sewage, surface and ground waters. As consequence of the high dosages administered and the lack of human metabolism, ICM are frequently encountered in wastewaters at µg/L levels. In Germany [3], diatrizoate and iopromide were reported to be the dominant ICM present in sewage effluents with maximum concentrations of 15 and 21 µg/L, respectively. Iothalamic acid, ioxithalamic acid, iopamidol, iomeprol, and iohexol were also detected. Since ICM are not markedly eliminated during conventional wastewater treatment processes – both biodegradation and adsorption onto sludge play only minor roles - the high loads in the raw sewage eventually translate in their frequent detectability in surface waters. A monitoring program carried out in the river Danube to measure ICM levels [4] revealed that the maximum concentrations of ICM (over 500 ng/L for diatrizoate and iopamidol) were found in 2h-composite samples collected downstream of the metropolitan area of a major city. Concentration profiles recorded over a period of one month demonstrated that the highest ICM concentrations were observed on weekdays [4]. In another study [5] the concentrations of diatrizoate and iopromide in surface waters achieved maximum concentrations in the low µg/L range.

2. Objective

To assess the relevance of photodegradation and biodegradation on the environmental fate of ICM in sewage-impacted surface waters in Spanish rivers.

3. Approach

In the first stage, photodegradation and biodegradation products of the ICM were generated under controlled laboratory conditions simulating environmental settings in surface waters [6,7]. Following their structural elucidation, in the second stage these photoproducts were then measured in surface water samples collected from a number of rivers in Northeast Spain that received treated effluent discharges from municipal sewage treatment plants. The information on their presence was used to assess the relevance of photolytical and microbial degradation as means of natural contaminant attenuation.

4. Materials & Methods

To simulate the sunlight-induced photodegradation of the ICM, test solutions of the individual compounds, prepared in artificial freshwater and natural river water, were irradiated in a Suntest apparatus (Heraeus, Germany) equipped with a Xenon lamp. Formation and evolution of the photoproducts were monitored by liquid chromatography-mass spectrometry and their chemical structures were elucidated by high-resolution mass spectrometry (quadrupole-time of flight-mass analyzer) [7]. Degradates stemming from the microbial conversion of ICM in sediment-amended batch-reactors were identified using the same analytical protocol. For the screening of environmental samples, filtered river water samples were preconcentrated by solid-
5. Results and discussion

The photolysis of the ICM in aqueous solutions using the solar simulator was almost complete after a 120-min irradiation period. The breakdown of the ICM gave rise to a series of photoproducts. Their formation was the result of four principal photoreactions: (A) gradual, and eventually complete, deiodination of the aromatic ring; (B) substitution of the halogen by a hydroxyl group; (C) N-dealkylation of the amide in the hydroxylated side chain; and (D) oxidation of a methylene group in the hydroxylated side chain to the corresponding ketone. Regarding biodegradation of ICM in a sediment-water system, no hints on deiodination were obtained but a number of degradation products deriving from oxidative breakdown were observed [8].

![Structures of the ICM](image)

Figure 1. Structures of the ICM

6. References


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Stable isotopes and persistent organic pollutants patterns in an Antarctic food web

Caio V. Z.Cipro¹, Satie Taniguchi¹, Paco Bustamante² and Rosalinda C. Montone¹

¹ Universidade de São Paulo, Instituto Oceanográfico Praça do Oceanográfico 191, 05508-120, São Paulo/SP, Brasil
² Littoral Environnement et Sociétés (LIENSs), UMR 6250, CNRS-Université de La Rochelle, 2 rue Olympe de Gouges - F-17042 La Rochelle Cedex 01, France
E-mail contact: caiovzc@usp.br

1. Introduction

Antarctica continues to be one of the least polluted regions on Earth, which provides unique opportunities for studying environmental pollution processes at both local and global scales. Even though this territory has not had considerable direct exposure to POPs (Persistent Organic Pollutants), several exogenous factors can represent a contaminant source through long range transport processes, notably atmospheric, but also oceanic and biotic ones.

Organochlorine compounds (pesticides and polychlorinated biphenyls - PCBs) are chemicals that do not occur naturally in the environment and are not easily degraded by chemical or microbiological action. Some accidents and several studies have already reported their toxicity, persistence and biomagnification, which led them to be classified as POPs by the United Nations Environmental Programme (UNEP). For similar reasons, several, but not all, polybrominated diphenyl ethers (PBDEs) types were given the same status in 2009. Within this regard, samples of several Antarctic organisms, in different trophic levels (invertebrates, fishes, birds and pinnipeds) were analysed for organochlorine compounds and PBDEs as well as carbon and nitrogen stable isotopes, largely used as, respectively, organic matter origin and trophic level indicators.

The aim of the present work was to determine the concentrations and distribution patterns of these organic pollutants throughout an Antarctic food web and correlate these findings with stable isotope ratios, which can greatly enlighten the understanding of bioaccumulation and biomagnification, increasing the bulk of available pollution data for the Antarctic environment.

2. Materials and methods

Samples were collected in King George Island (62°05'S, 058°23'W), Antarctica in the austral summers of 2004/05 and 2005/06. Krill (whole Euphausia superba) was collected from the shore with manual nets, limpets (soft tissue from Nacella concinna) were collected manually as well. Fishes (Notothenia rossii and coriiceps) were collected by line and hook or midwater nets. All birds (egg and liver from Pygoscelis antarctica, papua and adeliae, Catharacta sp and Larus dominicanus; only liver from Daption capense and Macronecles giganteus and only egg from Sterna vittata) and pinnipeds (fat from Lobodon carcinophagus, Leptonichotes weddelli and Arctocephalus gazella) samples were opportunistically collected, i.e., from animals that were already dead. Bird eggs and invertebrate samples were analyzed according to the methodology described in [1], and all of the other ones with a methodology resulting from the modification of [2].

3. Results and discussion

In a general way the PCBs, HCB and DDTs were the prevailing compounds within the very heterogeneous set of samples. Preliminary results were consistent with diets, ranges and trophic levels of the organisms. Organic pollutants concentrations are shown in Table 1, as well as stable isotope ratios. Limpets presented results about three times quantitatively higher than Krill, which could be mainly due to different life spans. Fishes showed similar results in comparison to literature, which are reflected quantitatively and qualitatively on birds and pinnipeds in a superior position in the trophic web. Bird samples, on the other hand, could show how several factors influence the qualitative and quantitative results: in a general way, the northernmost the species reaches and the more opportunistic/scavenger feeding habit it has, the quantitatively higher concentrations and qualitatively heavier PCBs profile it shows. The pinnipeds results can be interpreted in a similar way: A. gazella reaches the northernmost within these pinnipeds species, and L. carcinophagus feeds almost exclusively on krill, whence comes the stratification presented for this group.
Carbon stable isotopes allowed the identification of two clearly distinct origins for organic matter, which could be attributed to primary producers: marine phytoplankton more depleted in $^{13}$C, as seen in its predator, the krill and the more $^{13}$C enriched microphytobenthos, as seen in N. concinna. The relative enrichment of the intermediate sample results could be explained by diversified diet: for Notothenia spp and L. dominicanus (the more enriched of the birds and the only one with a significant diet influence of N. concinna); by the δ$^{13}$C latitudinal variation for migrating species and by the trophic level (an evidently lower enrichment than nitrogen, but yet existent). Nitrogen isotopes showed patterns in accordance to the scientific literature in regard to trophic levels. Preliminary results showed significant Pearson correlation between δ$^{15}$N and several pollutants concentrations, which reinforces the biomagnification processes of such compounds.

### 4. Conclusions

Invertebrate samples presented results of persistent organic pollutants in accordance to their trophic levels, life cycles and diets, as well as the fishes. The birds group, due to its variety in diets, ranges, and ecological niches could show how each of these factors may influence the accumulation of organic pollutants. Stratification in the pinnipeds group can be explained by similar reasons. Carbon and nitrogen stable isotope ratio provide a deeper insight on the origin and biomagnification of contaminants throughout the food web, by making continuous and individual trophic position measurements.

### 5. References


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### Table 1: Organic pollutants average concentrations, ng g$^{-1}$ wet weight, followed by stable isotope ratio in ‰

<table>
<thead>
<tr>
<th>Species</th>
<th>Tissue</th>
<th>δC$_{HCHs}$</th>
<th>δC$_{HCB}$</th>
<th>δC$_{Drins}$</th>
<th>δC$_{Chlorodane}$</th>
<th>Endosulfan (I/III)</th>
<th>δC$_{DDTs}$</th>
<th>δC$_{Mirex}$</th>
<th>δC$_{PCBs}$</th>
<th>δC$_{PBDEs}$</th>
<th>δ$^{13}$C</th>
<th>δ$^{15}$N</th>
</tr>
</thead>
<tbody>
<tr>
<td>N. concinna (n=8)</td>
<td>soft p</td>
<td>2.59</td>
<td>0.560</td>
<td>0.311</td>
<td>2.81</td>
<td>N/D</td>
<td>0.742</td>
<td>N/D</td>
<td>41.26</td>
<td>N/D</td>
<td>-16.10</td>
<td>7.27</td>
</tr>
<tr>
<td>E. superba (n=4)</td>
<td>whole</td>
<td>0.250</td>
<td>0.060</td>
<td>0.440</td>
<td>0.130</td>
<td>N/D</td>
<td>0.410</td>
<td>N/D</td>
<td>12.3</td>
<td>N/D</td>
<td>-25.66</td>
<td>4.51</td>
</tr>
<tr>
<td>Notochthys spp. (n=33)</td>
<td>muscle</td>
<td>0.303</td>
<td>0.656</td>
<td>N/D</td>
<td>0.601</td>
<td>0.195</td>
<td>3.04</td>
<td>N/D</td>
<td>6.82</td>
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<td>-21.53</td>
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Study of bioaccumulation and metabolism of fluoxetine in benthic invertebrates by Micro-QuEChERS-NanoLC-Nano-ESI-MS/MS

Cécile Cren-Olive,1 Audrey Bulete,1 Robert Baudot,1 Laure Wiest,1 Marion Gust,2 Jeanne Garric2

1 Service Central d’Analyses (SCA) du CNRS, USR59, Echangeur de Solaize, Chemin du Canal, 69360 Solaize, France
2 Cemagref, 3 bis quai Chauveau, CP 220, F-69336 Lyon cedex 09, France
E-mail contact: c.cren@sca.cnrs.fr

1. Introduction

Fluoxetine is a widely used antidepressant, frequently found in aquatic ecosystems, which presented dissimilar effects on the reproduction of two gastropod species, P. antipodarum and V. piscinalis. Recently it has also been measured in fish downstream of effluent discharges indicating a high bioaccumulation capacity in vertebrate aquatic wildlife. However no data is available on fate and metabolism of pharmaceuticals in invertebrates. To better understand the interspecific sensitivity the bioaccumulation of fluoxetine and its in vivo metabolism into norfluoxetine need to be explored in P. antipodarum and V. piscinalis.

This kind of study requires the development of analytical tools for extraction and analysis of traces of fluoxetine and norfluoxetine in biotic environmental matrices like water's benthic invertebrates. These gastropods weigh just a few milligrams. With such a small sample size, extraction step and analysis are more difficult. So, advanced technologies are required to seek drugs traces in complex matrices like gastropods.

In this aim, we have established an analytical strategy as which consists in one single extraction for fluoxetine and norfluoxetine based on QuEChERS method, followed by a nano-LC-MSMS analysis. Indeed, nano-chromatography coupled with mass spectrometry (nano-LC-nano-ESI MS/MS) increases sensitivity, reduces the required initial sample amount and is a good tool to answer this ecotoxicological issue.

The aims of this study were (i) to determine if fluoxetine was bioaccumulated in snails after waterborne exposure to fluoxetine; (ii) to appraise its metabolism in vivo into norfluoxetine; (iii) to assess the interspecific variability for bioaccumulation and biotransformation of fluoxetine, in order to better understand the differences of sensitivity of P. antipodarum and V. piscinalis.

2. Materials and methods

Adults V. piscinalis and P. antipodarum were exposed to fluoxetine (11-100µg/L) during 7 to 14 days and both fluoxetine and norfluoxetine were measured in the organisms. Both snails are gastropods, living in running waters from small creeks to steams and lakes. However, P. antipodarum is an invasive parthenogenetic ovoviviparous freshwater mudsnail, whereas V. piscinalis is hermaphrodite oviparous.

In order to quantify traces of these two substances, we developed a new methodology using nano-LC-nano-ESI MS/MS in two gastropods (10 to 20mg): Potamopyrgus antipodarum and Valvata piscinalis prosobranch gastropods. An easy and quick extraction method was developed. The procedure involves an extraction of about 10 milligrams of matrix by 500µL of a mix of acetonitrile:water:hexane (50/20/30) and 100mg of buffer salt. Recoveries were 87% for fluoxetine. Nano-LC-nano-ESI-MS/MS analysis was performed with a nano Ultimate3000 (Dionex®) coupled with a Qtrap3200 detector (AB Sciex®). MS/MS detection was performed in the Multiple Reaction Monitoring (MRM) mode using a NanoSpray ® II (AB Sciex®) in the positive mode.

3. Results and discussion

The results show that the developed method presents a good robustness and obtained limits of detection and quantification are enough low to detect contaminants in the environment: for carbamazepine and fluoxetine, LOD was respectively of 4ng/g and 30ng/g with only 10 milligrams of matrix. If we compare instrumental detection limit obtained with Nano-LC-nano-ESI-MS/MS to instrumental detection limit obtained with LC-ESI-MS/MS during a previous study with the same detector, we can note an Increase of sensibility by a factor 125 for fluoxetine. Such sensibility allows the study of a unique gastroper
The validation was carried out over three days: within-day precision, inter-day variation and recoveries were determined by analysing three extracts of three different concentrations of analytes (low, intermediate and high concentration level of the linearity range).

Fluoxetine was measured in both *P. antipodarum* and *V. piscinalis*. Concentrations of fluoxetine in *P. antipodarum* were significantly higher (*P*<0.05) than in *V. piscinalis*. The estimate BCF tot were also significantly higher (*P*<0.05) in *P. antipodarum* than in *V. piscinalis*. They were about twice higher in *P. antipodarum*, implying a higher bioaccumulation of the parent compound in *P. antipodarum*. Interestingly, BCF tot estimates in *P. antipodarum* were significantly higher (*P*<0.05) at 11 and 100 µg fluoxetine/L than at 33µg/L both on days 7 and 14. Contrary to parental compound, norfluoxetine concentrations measured in both species were not significantly different (*P*>0.05). Thus the norfluoxetine/fluoxetine ratio was higher in *V. piscinalis* compared to *P. antipodarum* (respectively 353 and 608 at 33µg/L and 239 and 328 at 100µg/L). If their persistence in both snails is comparable, our results indicate that metabolism of fluoxetine into norfluoxetine is higher in *V. piscinalis* than in *P. antipodarum*. *V. piscinalis* seems to present higher metabolic capacities than *P. antipodarum*.

However, *P. antipodarum* was more sensitive to fluoxetine than *V. piscinalis* (Gust et al., 2009), whereas the presumed active metabolite is measured in comparable quantities in both species. It implies that metabolite activation does not explain the interspecific differences previously observed. It also seems that norfluoxetine is not the major metabolite of fluoxetine in gastropods.

4. Conclusions

This study is the first to investigate bioaccumulation and metabolization of a pharmaceutical in gastropod mollusks. Both fluoxetine and norfluoxetine were measured in *P. antipodarum* and *V. piscinalis*, implying that gastropod are able to metabolize fluoxetine. However, norfluoxetine concentration were low compared to fish and did not explain the difference of sensitivity to fluoxetine of both species. However, fluoxetine bioaccumulation was higher in *P. antipodarum* than in *V. piscinalis*, at least partially enlightening their response profile to this compound. More work is still needed to assess depuration capacities of both species, or the influence a physiological status (as starvation) on bioaccumulation.

Acknowledgement - The present work was funded by a CNRS_exploratory project funding. The authors wish to thank the BEMT of the National Veterinary School of Lyon for funding the biochemical analysis (Prof. F. Garnier). C. Dussard, R Mons, and C. Noel, are gratefully acknowledged for their technical help,
Analytical approaches for the investigation of emerging contaminants in sources and treatment systems for drinking water production

Marcel W.M. Tielemans1, Rob ten Broek1, Karin Lekkerkerker-Teunissen2,3, Jan-Peter van der Hoek4, Corine J. Houtman1

1 The Water Laboratory, P.O. Box 734, 2003 RS Haarlem, The Netherlands
2 Dunea Dune and Water, P.O. Box 34, 2270 AA Voorburg, The Netherlands
3 Delft University of Technology, P.O.Box 5048, 2600 GA, Delft, The Netherlands
4 Waternet, P.O. Box 94370, 1090 GJ, Amsterdam, The Netherlands
E-mail contact: corinehoutman@hetwaterlaboratorium.nl

1. Introduction
For their need of drinking water, millions of Europeans depend on surface waters, such as the rivers Danube, Meuse, Po and Rhine. In these waters, rapid improvements in chemical and bioanalytical techniques have led to the discovery of all kinds of so-called emerging contaminants at very low concentrations, including pharmaceuticals, illicit drugs, sweeteners, endocrine disrupting compounds and perfluorinated compounds[1]. Some studies also reported the presence of traces of emerging contaminants in drinking water samples. Dutch drinking water companies therefore intensively investigate their water sources for the presence of emerging contaminants and their fate during treatment processes. A combination of analytical approaches is applied for this purpose, including chemical screening techniques, hyphenated target analyses, biological early warning systems, bioassays and effect-directed analysis (EDA) approaches. This presentation gives an overview of chemical and bioanalytical approaches applied to investigate Dutch drinking water sources and treatment systems. Examples are discussed of the use of sensitive and specific techniques such as CALUX reporter gene bioassays for the analysis of hormone-like activities and the development and application of a UPLC-MS/MS method for the determination of (sub) ng/L concentrations of new pharmaceuticals.

2. Materials and methods
Bioassay analysis of glucocorticoid and estrogenic activity: Surface water samples were collected at locations along the Dutch parts of the rivers Rhine and Meuse, in treatment systems and drinking water samples for the analysis of glucocorticoid and estrogenic activity. Additional samples were taken for target analyses and for GC-screening. Samples were sand filtered and extracted with Oasis HLB SPE cartridges eluted with ethylacetate. Glucocorticoid and estrogenic activities were analysed with GR and ER CALUX bioassays, according to [2]. Glucocorticoid activities were expressed as dexamethasone equivalents (ng dex-eq/L), estrogenic activities as estradiol equivalents (pg E2-eq/L).

UPLC analysis of new pharmaceuticals: A very sensitive and specific UPLC-MS/MS method suitable for the determination of (sub) ng/L concentrations was developed for 46 pharmaceuticals, selected according to consumption volume, mechanism of action and literature data on their occurrence in the environment. SPE-extracts or samples without any pre-treatment were separated on a Waters Acquity UPLC Chromatograph and analysed on a Waters Micromass Xevo tQ mass spectrometer.

Loads were calculated by multiplying measured concentrations with the river flow at the sampling dates and expressed as gram per day. Results were used to investigate the occurrence of compounds in drinking water sources and their fate during treatment processes.

3. Results and discussion
Bioassays prove to be suitable tools for the sensitive assessment of compounds with biological activity in environmental samples, even if their chemical identity is unknown. In this study, CALUX bioassays were used for the analysis of compounds with glucocorticoid and estrogenic hormone-like activity in drinking water sources from the Rhine and Meuse. Activities were detected in concentrations between <LOD (2) and up to 19 ng dex-eq/L (glucocorticoid) and between <LOD (0.006) and 3.5 ng E2-eq/L (estrogenic). Both types of activities were detected at all surface water locations, although not at all sampling dates. Glucocorticoid activity was found with comparable maximum concentrations both in Rhine and Meuse. Estrogenic activity was found in higher concentrations in the Meuse than in the Rhine. This may be caused by the differences in
the way these drinking water sources are influenced by anthropogenic activities. Results indicated that concentrations of both activities follow a seasonal pattern; with maximum values for both activities in spring and autumn. Unintentional exposure to glucocorticoids is associated with impairment of the immune system, reproduction, and development. Therefore, the presence of compounds with glucocorticoid activity in surface waters might be an issue of concern regarding exposure of consumers depending on surface water for the provision of drinking water. However, results of treated water and drinking water samples indicated good removal of activity during purification.

As an example of recent developments in target analysis, our work on new pharmaceuticals in water sources for drinking water production and their fate in advanced water treatment will be discussed. The developed multi-component UPLC-MS/MS method proved successful for the analysis of SPE extracts of surface water sources and drinking water produced from surface water. Improved separation power and detection limits and reduced analysis times were achieved in comparison with conventional LC-MS. Almost all pharmaceuticals included in the method and representing various therapeutic classes were detected in water samples from the rivers Rhine (Figure 1) and Meuse in ng/L concentrations. These included previously detected compounds as carbamazepine, metoprolol and diclofenac, but also high consumption volume compounds such as the benzodiazepines oxazepam and temazepam and the very polar antidiabetic metformin, whose occurrence in the Dutch environment was not reported earlier or for which only very limited environmental data were available. As an example of pharmaceutical analysis employing direct injection a study will be presented investigating the removal efficiencies of several emerging contaminants in a pilot-scale advanced oxidation treatment process [3].

4. Conclusions

These studies show that quality assessment of drinking water sources requires the use of the most suitable techniques: biological, chemical or combinations thereof. Due to ongoing analytical improvement, our knowledge on contaminants in drinking waters sources steadily increases and also our responsibility to act accordingly.

References

Pharmaceuticals and personal care products in urban receiving waters

Andreas Musolf1, Gerhard Strauch1, Sebastian Leschik2, and Mario Schirmer3

1UFZ – Helmholtz Centre for Environmental Research, Department of Hydrogeology, Permoserstr. 15, 04318 Leipzig, Germany
2UFZ – Helmholtz Centre for Environmental Research, Department of Groundwater Remediation, Permoserstr. 15, 04318 Leipzig, Germany
3Eawag – Swiss Federal Institute of Aquatic Science and Technology, Department Water Resources and Drinking Water, Ueberlandstr, 133, 8600 Duebendorf, Switzerland

E-mail contact: andreas.musolf@ufz.de

1. Introduction
Pharmaceuticals and personal care products (PPCPs) and other organic micropollutant from wastewater sources pose a potential threat to aquatic ecosystems and the human health [1]. Urban areas are prone to PPCP contaminations since here large amounts of wastewater are produced transported and treated. Part of evaluating the environmental risk of PPCPs is an exposure assessment by providing measured and predicted environmental concentrations. However, urban water fluxes and wastewater inputs are very complex by nature since urban land use affects the entire water balance. Wastewater and thus PPCPs can enter urban receiving surface waters by different pathways: via the effluent of wastewater treatment plants and via combined sewer overflow (CSO). Moreover PPCPs may enter groundwater by losses from the sewage system.

In this paper we quantify the contribution of different PPCP pathways out of a mid-European urban drainage catchment. On the basis of a 13-months monitoring programme we moreover assess the temporal variability of this contamination.

2. Materials and methods

2.1. Study area
The study area is located in the city of Leipzig, Germany. Here, the catchment of shallow groundwater and wastewater (sewershed, 5.4 km²) are corresponding and therefore allow for a joint analysis of water and matter fluxes [2]. The sewershed is drained by a combined sewage system with a total length of 55 km. All the wastewater is pumped to the municipal wastewater treatment plant (WWTP), treated and released to the surface water. In the case of heavy rainfall, CSO from the sewershed discharges into the adjacent surface water.

2.2. Sampling and chemical analysis
A monitoring programme was conducted over the course of 13 months. Groundwater and surface water was sampled every three months. The influent and effluent of the WWTP, one observation well and one surface water sampling location was sampled every month.

The resulting samples were screened for six PPCPs and micropollutants: Bisphenol A (BPA), technical 4-nonylphenol (NP), caffeine (CAF), galaxolide (HHCB), tonalide (AHTN), and carbamazepine (CBZ) on the basis of solid phase extraction and gas chromatography-mass spectrometry.

2.3. Water flow and mass flow quantification
The annual water flow to and from the sewershed was quantified using the data of the wastewater flow (basis for the annual treated wastewater flow and CSO) and results from a numerical groundwater flow model (basis for groundwater discharge out of the sewershed boundaries). The combination of annual water flow with the median contaminant concentration yielded the mass flow out of the sewershed by treated wastewater (Mtww), CSO (Mcso) and by groundwater discharge (Mgw).
3. Results and discussion

3.1. Contaminant mass flow

The target compounds were present in all urban water compartments. The annual contaminant mass flow from the sewershed is given in Table 1.

<table>
<thead>
<tr>
<th>Micropollutant</th>
<th>$M_{\text{tww}}$ (g year$^{-1}$)</th>
<th>%</th>
<th>$M_{\text{csO}}$ (g year$^{-1}$)</th>
<th>%</th>
<th>$M_{\text{gw}}$ (g year$^{-1}$)</th>
<th>%</th>
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<td>302.9</td>
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<td>88.8</td>
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<td>97.7</td>
<td>29.9</td>
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<td>CBZ</td>
<td>128.4</td>
<td>98.3</td>
<td>0.8</td>
<td>0.6</td>
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<td>1.09</td>
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Table 1: Median annual mass flow of micropollutants from the sewershed and distribution of loads (100% for each substance), changed after [2]. $M_{\text{tww}}$: release by treated wastewater; $M_{\text{csO}}$: release by combined sewer overflows; $M_{\text{gw}}$: released by groundwater discharge out of the sewershed boundaries.

For the PPCPs HHCB, AHTN and CBZ most of the mass is released by WWTP effluents. On the other hand, most of the CAF and a substantial part of BPA and NP is released by CSO discharge. This is a consequence of different persistency in the wastewater treatment process. The polycyclic musks and CBZ are hardly retained in the WWTP and concentration contrast between untreated and treated wastewater is low. On the other hand, CAF is nearly completely removed in the WWTP and therefore the small portion of untreated wastewater release by CSO is of high importance for the overall release of this substance.

For BPA and NP a significant part of the total mass is released by groundwater discharge. These substances were found to be present in high concentrations in the groundwater (median concentration BPA: 362 ng/L; NP: 56 ng/L). Sewer leakages are the most likely reason for the presence of these substances in the groundwater [3].

3.2. Temporal distribution of concentrations and mass fluxes

The evaluation of PPCP concentrations and mass fluxes in the study sites surface water revealed characteristic temporal patterns [3, 4]. Concentrations and mass fluxes of CAF, HHCB and AHTN correlate negatively with the surface water temperature. Therefore, these substances are characterized by a pronounced seasonal occurrence with higher concentration and fluxes in the months when the water temperature was low. We relate these findings to temperature-enhanced degradation processes. As a consequence, in the surface water CAF was rapidly removed in the summer months although input by CSO events was highest in this period.

4. Conclusions

This work contributes to the knowledge of occurrence of PPCPs in urban receiving waters. The results of this study underline the complex interplay of release and fate of PPCPs in urban areas. The release of significant portions of CAF, BPA and NP by CSO and groundwater discharge point to the relevance of input pathways apart from the WWTP effluents. Here, the persistence of PPCPs in the different water compartments play a crucial role. The pronounced seasonality of CAF, HHCB and AHTN occurrence in the surface water stress the relevance of continuous monitoring programmes covering different temporal scales.

5. References

Attenuation and dynamics of pharmaceuticals in a small German stream

Uwe Kunkel¹, Michael Radke¹,2

¹ Department of Hydrology, BayCEER, University of Bayreuth, D-95440 Bayreuth
² Department of Applied Environmental Science (ITM), Stockholm University, S-10691 Stockholm
E-mail contact: uwe.kunkel@uni-bayreuth.de

1. Introduction
Pharmaceutical residues are commonly detected organic micropollutants in the aquatic environment. In rivers and streams, they are typically observed in the ng L⁻¹ – µg L⁻¹ range. Their environmental fate and the importance of individual elimination processes in rivers is still incompletely understood. Some substances like carbamazepine are reported to be very persistent in the aquatic environment [1]. Otherwise, laboratory experiments imply that biodegradation in the hyporheic zone or river sediments can be an effective elimination pathway (e.g. ibuprofen [2]), that some pharmaceuticals are susceptible to photodegradation (e.g. diclofenac [3]), and some might be removed by sorption to sediments (e.g. metoprolol [4]).

Previous studies of our group indicated that in rather deep and turbid rivers these potential processes did not always result in a significant attenuation of pharmaceuticals. Therefore, we performed experiments at a shallow stream with clearer water and an expected increased exchange of river water and sediments to check the attenuation in such streams.

2. Materials and methods
Experiments were carried out at the small river Gründlach, near Nuremberg, Germany. On average, the stream is about three meters wide and 30 centimeters deep; sandy sediments are prevailing. 6h-composite samples were taken with automatic water samplers at both ends of a river stretch of approximately 12 km downstream of a sewage treatment plant. Moreover, pore water samples were taken and in-situ photolysis experiments at several sites within the river stretch were performed. A set of 15 pharmaceuticals was monitored. Water samples were enriched by solid phase extraction and analyzed with HPLC-MS/MS.

3. Results and discussion
The temporal dynamics of carbamazepine and ibuprofen are shown in Figure 1. Carbamazepine was present in all samples at both sampling sites in the µg L⁻¹ range (Fig. 1a), while ibuprofen was only detected in periods with high river discharge (Fig. 1b). The sporadic occurrence of ibuprofen can either be caused by stormwater overflows or by a drastically reduced efficiency of the sewage treatment plant after heavy rainfalls. Carbamazepine can be assumed to be persistent at the timescale of the travel time between the two sampling sites. Hence, its decrease in concentration can be attributed to dilution processes or permanent loss of river water to the groundwater.

![Figure 1: Concentration of pharmaceuticals at the upstream and downstream sampling site together with the discharge of the stream Gründlach; a) carbamazepine, b) ibuprofen](image-url)
Diclofenac and metoprolol were determined in all samples at both sites in the high ng L\(^{-1}\) range (Fig. 2). However, compared to carbamazepine the concentration of these two substances decreased stronger between the two sampling sites. Thus, diclofenac and metoprolol were attenuated within the river stretch.

Figure 2: Concentration of pharmaceuticals at the upstream and downstream sampling site together with the discharge of the stream Gründlach; a) diclofenac, b) metoprolol

In in-situ photolysis experiments, diclofenac was rapidly removed from the river water (Fig. 3a). Half-life times of only a few hours were determined. Contrariwise, metoprolol was persistent to photolysis (Fig. 3b) and therefore has to be removed within the river stretch by biotransformation or sorption. Pore water samples still have to be processed. Sorption of pharmaceuticals to sediments and suspended matter also still have to be analyzed. These results combined with the time trends in surface water and the results of the photolysis experiments should further elucidate the importance of the individual elimination processes for each pharmaceutical.

Figure 3: Time trend of pharmaceutical concentration during photodegradation experiments; a) diclofenac, b) metoprolol

4. Conclusions

The results of this study suggest that photolysis and biotransformation can constitute relevant attenuation processes in small streams. Even within short river stretches and travel times, pharmaceuticals can be eliminated due to more intense exchange of river water with bed sediments and due to a higher efficiency of photolysis compared to large rivers.

5. References


Acknowledgement - The authors thank the Deutsche Forschungsgemeinschaft (DFG) for financial support of the study and Jutta Eckert and Klaus Kasparbauer for helping with field and laboratory work.
Mechanisms controlling the transport of carbamazepine and other trace organic compounds in a sand aquifer receiving wastewater discharge

Carol Ptacek¹, Michelle Sabourin¹, Laura Groza¹, Kelly McLagen¹, Will Robertson¹, David Blowes¹

¹Department of Earth and Environmental Sciences, University of Waterloo, Waterloo, ON, Canada, N2L 3G1
E-mail contact: ptacek@uwaterloo.ca

1. Introduction

In North America, approximately 35% of wastewater is discharged to the subsurface primarily through on-site wastewater disposal systems. As water shortages increase, managed aquifer recharge (MAR), where wastewater is intentionally discharged to the subsurface, also is expected to increase. Because of the ubiquitous presence of pharmaceutical compounds in wastewater, there is potential to use these compounds as indicators of wastewater contamination in the subsurface. Several studies conducted on the fate of pharmaceutical compounds in the subsurface have shown the potential for their persistence over many years to decades [1-5]. A comparative study on the behaviour of a suite of pharmaceutical compounds at three septic-system sites showed large differences in mobility and persistence among the sites [6]. Subsurface wastewater disposal often involves a series of biogeochemical reactions, with anaerobic digestion in holding tanks, enhanced oxidation in the unsaturated zone, and oxygen depleted conditions in the saturated zone. The purpose of this study is to gain an improved understanding of the influence geochemical gradients have on the fate of pharmaceutical compounds in the subsurface. The drugs included in this investigation, carbamazepine (CBZ), sulfamethoxazole (SMX), caffeine (CAF), ibuprofen (IBU), naproxen (NAP) and gemfibrozil (GEM), were selected based on their high prescription rates.

2. Materials and methods

Groundwater samples were collected from an existing piezometer network installed at a multi-user septic-system at Long Point Provincial Park, near Rowan, ON [6,7]. The Long Point septic system receives wastewater seasonally from May to November of each year. Samples were analyzed for pH, Eh, alkalinity, major and trace elements, anions and nutrients following previously described procedures [6]. Pharmaceutical compounds were analysed using solid-phase extraction followed by high-performance liquid chromatography tandem mass spectrometry. Isotope-labelled internal standards were added to the samples prior to the extraction step. The analyte responses were corrected based on the observed recoveries of the internal standards. Laboratory column experiments were conducted by packing columns with aquifer sand collected from the field site, and pumping simulated groundwater spiked with the pharmaceutical compounds and through the columns.

3. Results and discussion

3.1. Field studies

The pharmaceutical compounds selected for study cover a wide range of properties including solubility, medicinal use, acid-base dissociation constant (pKₐ), and octanol-water partitioning coefficient (K₀w). At the Long Point site, the highest concentrations of pharmaceuticals were observed for CBZ (3,050 ng L⁻¹), SMX (1,990 ng L⁻¹) and IBU (1,790 ng L⁻¹) near the infiltration bed. Further downgradient from the infiltration bed, concentrations of these compounds remained elevated, with the greatest persistence observed for CBZ. Concentrations of CAF, NAP and GEM were much lower than the other drugs and the plumes were approximately half of the size of the plumes for CBZ, SMX and IBU. The depth profiles for the pharmaceuticals differed from the major wastewater constituents such as chloride, nitrate and phosphate (Figure 1). For example, concentrations of chloride, assumed to exhibit conservative transport, were nearly constant versus depth, whereas the concentrations of pharmaceutical compounds generally were lower near the water table and the bottom of the plume. The lower concentrations observed near the water table are attributed to enhanced degradation due to oxygen entrainment during water table fluctuations. Predictions of pharmaceutical transport based on the physicochemical properties of the compounds generally were not in agreement with the observed field behaviour. The lack of agreement may be due to the rapid flow rates at the site because of the large volumes of wastewater disposal.
Groundwater samples collected in April after a 6 month hiatus in wastewater discharge showed slightly lower concentrations of pharmaceuticals, likely due to enhanced sorption reactions during the low flow conditions. After wastewater discharge resumed, the concentrations returned to those observed during the previous summer.

Figure 1: Depth profiles of pharmaceutical compounds below infiltration bed at Long Point septic system site.

3.2. Laboratory column experiments

Laboratory column studies conducted to delineate transport parameters under controlled flow conditions showed chromatographic separation of the compounds, consistent with the expected transport based on the physicochemical properties of the compounds.

4. Conclusions

The transport of pharmaceutical compounds in the field differed substantially from the transport observed under controlled laboratory conditions. Several drugs were transported long distances from the tile bed and were persistent after cessation of wastewater disposal during the winter months. There was little agreement between laboratory and field behaviour, possibly due to differences in degradation reactions.

5. References


Acknowledgement – This research was funded by the Natural Sciences and Engineering Research Council of Canada, and the Ontario Ministry of Research and Innovation ORF-RE Program. The authors would like to thank T. Scheytt and W. Woessner for helpful discussions.
1. Introduction

Dairy farms in the Golan Heights Region in Israel produce some 1.2 million m³ of sewage water annually. The Golan Heights is a significant segment of the watershed of the Sea of Galilee (Lake Kinneret), which comprises ca. 30% of the Israel’s water supply. The present alternatives for safe disposal of dairy farm sewage water are limited. Consequently, sewage water might overflow into local streams, resulting in the uncontrolled dispersal of sewage water. Furthermore, the reservoirs have to be emptied before the winter rains to prevent their flooding and subsequent uncontrolled overflow into the lake.

For the past 7 years, we have examined recycling of dairy effluents via irrigation of a mixed pasture – planted tree (Eucalyptus camaldulensis) ecosystem. The effluent is stored in lagoons and used following minimal treatment and contains very high concentrations of organic matter, plant nutrients, and an array of organic contaminants. Irrigation is maintained at ca. 400 mm/yr. Irrigation water was sampled at each irrigation event, the soil was sampled twice a year (after winter rains and at the end of summer irrigations), soil water was sampled via piezometers and the quantity of overland flow drainage water was monitored and the drainage water was sampled.

We hypothesize that under deficit irrigation the migration of the water constituents is greatly retarded. Plant uptake of nutrients (essential and nonessential elements) adsorption and precipitation of chemicals within the soil, microbial degradation of organic matter and organic micropollutants and pathogen die-off attenuate these wastewater pollutants in runoff water. This occurs as long as the capacities of the soil processes are not exceeded.

2. Materials and methods

We examined the partitioning of these contaminants between the soil, soil solution, run-off, woody plant parts and grasses. This was carried out in two similar size drainage basins for comparative purposes. One basin was irrigated with wastewater effluent from a reservoir that received both dairy and human wastewater, and another non-irrigated basin. The latter basin was still under forage by ca. 1000 cows.

Water samples were extracted using reversed phase extraction cartridges and analysis was performed using a triple quad mass selective detector equipped with an electrospray ionization ion source. LC-MS analysis of pharmaceuticals was performed in multiple reaction monitoring (MRM) mode.

3. Results and discussion

Data is presented with respect to PPCPs in samples monitored last winter (2009/2010; Table 1). Altogether 67 water samples were analyzed by LCMS. Carbamazepine was present in most of the water samples collected from the dairy wastewater effluent (DWWE) irrigated basin. Carbamazepine is one of the most ubiquitous PPCPs and is exceedingly stable in both sewage treatment plants and in soils [1-3]. It was also found in 6 samples collected from the non-irrigated basin. This is a bit surprising owing to the almost strict domestic use of this chemical (and furthermore, one inhabitant only was prescribed to take the medication). Caffeine was the second most abundant compound under DWWE irrigation, yet it was first (7 out of 24 samples) in the irrigation-free basin, which is again quite unexpected. These two products are rather resistant in the environment and ubiquitous. Most all other PPCPs tested occurred in a small number of samples. The veterinary antibiotics were either missing altogether or occurred in 1-4 water samples from the irrigated site. In any event, the concentrations of those products that were detected were in the low range of 0.5-35 µg/L.
Table 1: Number of water samples (piezometers and drainage) from the two basins studied, with dairy wastewater irrigation and without irrigation. The non-irrigated basin hosted forage cows during the preceding summers (Compounds in bold are known to be used in the local dairy farms).

<table>
<thead>
<tr>
<th>Studied compound</th>
<th>Number of appearances under DWE irrigation</th>
<th>Number of appearances under non-irrigation</th>
<th>Usage*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbamazepine</td>
<td>39</td>
<td>6</td>
<td>Anti-epileptic (D)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>18</td>
<td>7</td>
<td>Stimulant (D)</td>
</tr>
<tr>
<td>Codeine</td>
<td>8</td>
<td>0</td>
<td>Analgesic (D)</td>
</tr>
<tr>
<td>Acetyl salicylic acid</td>
<td>7</td>
<td>3</td>
<td>Pain reliever (D)</td>
</tr>
<tr>
<td>Doxycycline</td>
<td>6</td>
<td>5</td>
<td>Antibiotic (D/V)</td>
</tr>
<tr>
<td>Sulfadiazine</td>
<td>4</td>
<td>0</td>
<td>Antibiotic (V)</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>3</td>
<td>0</td>
<td>Lipid regulator (D)</td>
</tr>
<tr>
<td>Procaine</td>
<td>3</td>
<td>0</td>
<td>Antimicrobial (V)</td>
</tr>
<tr>
<td>Fexofenadine</td>
<td>2</td>
<td>0</td>
<td>Antihistamine (D)</td>
</tr>
<tr>
<td>Trimethoprim</td>
<td>2</td>
<td>0</td>
<td>Antibiotic (V)</td>
</tr>
<tr>
<td>Amoxycillin</td>
<td>1</td>
<td>0</td>
<td>Antimicrobial (V)</td>
</tr>
<tr>
<td>Fluvoxamine</td>
<td>1</td>
<td>0</td>
<td>Antidepressant (D)</td>
</tr>
<tr>
<td>Metoprolol</td>
<td>1</td>
<td>0</td>
<td>β-Blocker (D)</td>
</tr>
</tbody>
</table>

* D- Domestic, V- Veterinary

4. Conclusions

The research showed that recycling dairy wastewater through irrigation of a pasture – forest system under deficit irrigation poses little threat to PPCPs emission into water resources. Yet, this conclusion has to be reevaluated at this and other sites. In addition, it will be interesting to reveal the origin of PPCPs in the not-irrigated basin.

5. References

1. Introduction
The development and application of models to predict in-river concentrations of down-the-drain chemicals (i.e. those used in household and personal care products and pharmaceuticals) are important components of the environmental risk assessment process [1]. While such models (e.g. EUSES and GREAT-ER) exist in Europe, there are currently no available models to estimate exposure of personal care products in China. To this end, work was performed to create a GIS-based system that develops scenarios used to predict the fate of “down the drain” chemicals into freshwater ecosystems. Currently, many of the available inputs for the generation of environmental concentrations to be used in the exposure assessment are at a very coarse spatial scale, in some cases only a single value for the entire country is available. In this study, Predicted Environmental Concentrations in surface waters (PECsw) were generated at the county-level (ca. 3,000 counties in China) based on both user supplied product information, as well as other geographically-linked socio-economic and environmental information from official census and other data sources.

2. Materials and methods
Data used in the PEC calculation can be grouped into three types: product/ingredient information, socio-economic information, and environmental information. Product/ingredient information are generally proprietary and supplied by the study sponsor (Unilever). This includes the active ingredients (e.g., triclosan) that are contained in each product (e.g., toothpaste) and their related degradation rates in both secondary/tertiary treatments as well as direct degradation in surface water. Of particular importance is the “take off” value associated with each product. This value represents the minimum GDP generally considered to be needed for the product to be purchased. This allowed for variability in product use by county based on economic information, instead of assuming an even distribution of product use per person. Product consumption information was obtained from Euromonitor International (www.euromonitor.com), which reports volume of individual products used by region.

Socio-economic data included information about population at the county level and were primarily obtained from the 2000 Census [2]. These data included items such as urban and rural population, number of urban and rural households, percent of population with tap water, etc. GDP values for each county were derived from 1-km GDP [3] and population density grids [4], which were processed by Yang and Jiang [5] using the method described in [6]. GDP values were then updated using more current 2008 province level GDP data from the National Bureau of Statistics [7]. The fraction of population with different disposal mechanisms (e.g., STP, septic, direct discharge to river, etc.) was also accounted for. Figure 1 shows the varying scales of these data.

Figure 1: Varying scales of data: Product consumption data (Region); GDP (Province); and population (County)
Environmental information such as distance to the coast (for marine discharges) and dilution factor (for river discharges) were generated using GIS data layers on population density [4] and previously published work on dilution estimation [8].

Product information such as use category (e.g., hair, skin, etc.), inclusion level of ingredient in formulation and “take off” values were used to distribute total tonnes of individual ingredients used in China. These data were combined with county-level economic information, population density (including urban and rural separation), dilution factors, and disposal mechanism to estimate ingredient-level PECs in surface water based on user supplied tonnage of ingredient.

3. Results and discussion

The scenario development tool allows risk assessors to combine product/ingredient, socio-economic, and environmental data at a sub-country level to evaluate the variability in the final PEC distribution. Multiple ingredients were evaluated based on the supplied data. Results show that local PECsw varies considerably across the country and that economic information (“take off” values and population GDP) can have a significant influence on the resulting ingredient distributions. Figure 2 illustrates the spatial variability in PECsw for an example ingredient.

![Figure 2: Resulting map of PECsw based on model inputs](image.png)

4. Conclusions

The method presented incorporates the inherent spatial variability of the model inputs so that patterns can be identified and used in the risk assessment. In other words, the ability to identify areas where existing combinations of model inputs may yield greater exposure estimates, i.e., the identification of realistic “worst case” scenarios. In addition, an understanding of where these “worst case” scenarios fit within the overall country-wide distribution (i.e., 90th percentile) is achieved. The outputs of such a system can be used to better understand the nature of exposure by the risk assessor, as well as to help inform all stakeholders with maps, charts and tables of PEC distributions.

5. References

Predicting the fate and behaviour of cyclic volatile methyl siloxanes in two contrasting lakes

M.J. Whelan1*

1Department of Natural Resources, Cranfield University, Cranfield, Bedfordshire, UK, MK43 0AL
Email contact: m.j.whelan@cranfield.ac.uk

1. Introduction
Cyclic volatile methyl siloxanes (cVMS) are used in a range of applications. They possess a rather unusual combination of physico-chemical properties, including both hydrophobicity and volatility. Recently, concerns have been raised regarding their behaviour in the natural environment after their release [1]. In this paper, the behaviour of cVMS materials in lakes was explored using a fugacity-based steady-state non-equilibrium multimedia fate and transport model (a modified version of QWASI: [2]) in order to ascertain their likely environmental persistence and the relative importance of different loss processes (volatilisation, hydrolysis, burial in sediment and advection in outflow).

2. Modelling
Three substances were investigated: Octamethylcyclotetrasiloxane (D4), Decamethylcyclopentasiloxane (D5) and Dodecamethylcyclohexasiloxane (D6), in two contrasting North American lakes: Lake Ontario (one of the Laurentian Great Lakes) and Lake Pepin (a shallow lake on the Mississippi river on the border between the states of Minnesota and Wisconsin). Values for the principal partition coefficients [3] and the hydrolysis rate constant [4] were adjusted for the mean annual temperatures of each lake. Hydrolysis rate constants were also adjusted for the fraction of chemical calculated to be in the freely dissolved phase. Half lives in sediment were calculated from partitioning theory, assuming that hydrolysis would occur only in the dissolved phase of the interstitial water. Best estimates of substance-specific emissions were obtained by combining current per capita approximations of usage and fraction lost to domestic waste water, the population of the lake watershed and typical cVMS removal efficiency during waste water treatment. In addition, predicted concentrations in sediment were compared with available measured data (1).

3. Results and Discussion
Predicted concentrations were generally lower and chemical residence times longer in Lake Ontario than in Lake Pepin, owing to greater depth, a much longer water residence time and a higher degree of dilution. Overall persistence in Lake Pepin was significantly influenced by the high rate of sediment burial assumed in the model, as well as by a relatively high rate of water discharge. Despite the many similarities of the compounds considered, the dominant loss mechanisms varied significantly and were not the same in each lake system (Table 1).

The relative role of hydrolysis was much more important for Lake Ontario than for Lake Pepin, despite having a longer assumed reaction half life per chemical due to slightly lower mean annual water temperature (9 °C versus 14 °C). Although reaction rates for Lake Ontario were relatively low at these temperatures, particularly for D6, they were rapid compared with the water residence time of Lake Ontario. Hydrolysis was by far the most important loss mechanism for D4 in both systems and for D5 in Lake Ontario. Hydrolysis in sediment was not a significant loss process for any compound in either lake. Instead, the main net loss process for sediment was burial, which was especially important for D6. In the case of Lake Pepin, which had a high rate of net sedimentation, burial was the most important loss process of all for D6. Although all three cVMS compounds are very volatile (log $K_{AW} > 2.5$), the relative contribution of volatilisation to total losses was predicted to be limited in both lakes. This is partially due to the relative magnitude of other processes but it also reflects the fact that cVMS compounds are hydrophobic as well as volatile [5], and readily partition to organic phases in the water column. In addition, volatilisation will also be limited by lake depth. Nevertheless, volatilisation is still predicted to be the most important loss process for D6 in Lake Ontario and is also responsible for more than 20% of all losses of D5 in both lakes. In reality, limited epilimnetic circulation may also limit volatile losses in deep stratified lakes such as Lake Ontario. This is not accounted for in the QWASI predictions which may, therefore, overestimate the role of volatilisation.

No attempts have thus far been made to measure the concentrations of cVMS materials in water in either Lake System. None of the cVMS compounds considered were predicted to be detectable in the water
column of Lake Ontario and only D5 would be detectable in Lake Pepin, given current analytical method limits of detection (approx. 10 ng L⁻¹). Observed concentrations of D4, D5 and D6 in the four main sedimentary basins of Lake Ontario were all less than the analytical method limits of detection (range 1.7 to 3.6 ng g⁻¹ wet weight), which is in broad agreement with the model output. In Lake Pepin, average measured concentrations of D4, D5 and D6 in surface sediments were approximately 2, 166 and 50 ng g⁻¹ wet weight, respectively. The predicted concentration of D4 in Lake Pepin sediment was lower than the observed value, but the model appears to over-predict concentrations of D5 and D6 in sediment by factors of about 2.2 and 4.9, respectively. However, it should be noted that there is considerable uncertainty about both the emission rates and the true behaviour of all three materials in lake systems.

Table 1: Predicted flux estimates, expressed as a percentage of all loss processes, for D4, D5 and D6 in Lake Ontario and Lake Pepin. JQ is advective outflow; JRW is reaction in water; JRS is reaction in sediment; JB is sediment burial and JWA is the net flux from water to air. The most significant loss process for each compound in each lake is shown in bold.

<table>
<thead>
<tr>
<th>Season</th>
<th>Compound</th>
<th>JQ</th>
<th>JRW</th>
<th>JRS</th>
<th>JB</th>
<th>JWA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ontario</td>
<td>D4</td>
<td>0.16</td>
<td>98.64</td>
<td>0.03</td>
<td>0.00</td>
<td>1.18</td>
</tr>
<tr>
<td></td>
<td>D5</td>
<td>2.81</td>
<td>76.29</td>
<td>0.04</td>
<td>0.47</td>
<td>20.40</td>
</tr>
<tr>
<td></td>
<td>D6</td>
<td>6.80</td>
<td>33.70</td>
<td>0.72</td>
<td>12.58</td>
<td>46.21</td>
</tr>
<tr>
<td>Pepin</td>
<td>D4</td>
<td>14.78</td>
<td>76.84</td>
<td>0.39</td>
<td>0.53</td>
<td>7.45</td>
</tr>
<tr>
<td></td>
<td>D5</td>
<td>49.08</td>
<td>10.27</td>
<td>0.06</td>
<td>18.19</td>
<td>22.40</td>
</tr>
<tr>
<td></td>
<td>D6</td>
<td>35.10</td>
<td>0.96</td>
<td>0.13</td>
<td>53.60</td>
<td>10.21</td>
</tr>
</tbody>
</table>

This study highlights the pitfalls of subjective evaluation of chemical fate and illustrates the important role which models have to play in providing a quantitative framework for assessing chemical behaviour objectively under the influence of a complex and interacting set of factors.

4. Acknowledgements
The Centre Européen des Silicones (CES) is gratefully acknowledged for funding this work. Thanks are also due to the modelling task force, particularly Dave Powell, for their invaluable suggestions and comments.

5. References
Determination of nine native steroid hormones in biological (e.g. blood and tissues) and environmental (e.g. manure, soil and sediment) samples by GC-MS/MS

Martin Hansen, Naja W Jacobsen, Shelton T Mariga, Frederik K Nielsen, Erland Björklund, Kristine A Krogh, Bjarne Styrishave and Bent Halling-Sørensen

Section of Toxicology and Environmental Chemistry, Department of Pharmaceutics and Analytical Chemistry, Faculty of Pharmaceutical Sciences, University of Copenhagen, Universitetsparken 2, 2100 Copenhagen, Denmark
E-mail contact: mah@farma.ku.dk

1. Introduction

The vertebrate steroidogenesis is a metabolic pathway responsible for production of important biological process (e.g. growth and reproduction). Many substances are today known as endocrine disrupting chemicals (EDCs), i.e. interfering with hormone homeostatics and the steroidogenesis [1]. Key steroid hormones are; pregnenolone, progesterone, dehydroepiandrosterone, androstenedione, testosterone, dihydrotestosterone, estrone, 17α-estradiol and 17β-estradiol.

Many (eco)toxicologists seek to investigate the effects of EDCs on a range of vertebrates, and thereby identify the need for detailed and systematic studies on interactions between the individual steroid hormones expressed in the steroidogenesis. A prerequisite for this is a reliable analytical methodology for the simultaneous determination of several steroid hormones in the investigated biological sample (e.g. blood, gonads, brain).

When steroid hormones are released into the environment, predominant from animal wastes and human waste water, they have an environmental impact, e.g. the feminisation of fish, amphibians and others [2-3]. Consequently, there is a need among environmental chemists and engineers to measure steroid hormones in environmental samples (e.g. manure, soil, sediment and water) to describe the fate of these compounds in nature and in waste treatment optimization processes.

This presentation outlines for the first time the development and application of an analytical method to determine nine steroid hormones in biological and environmental samples. The methodology has been applied to toxicological studies (e.g. in-vitro studies, in-vivo frog studies, and seal and polar bears blood plasma) and environmental studies (e.g. soil sorption, manure content, manure separation technologies, activated sludge studies). Attempts will be made to present some of these cases.

2. Materials and methods

The developed method for steroid hormones relies on chemical analysis. Initially, solid phase extraction (SPE) for liquid matrices (e.g. serum) or pressurized liquid extraction (PLE) for solid matrices (e.g. soil) is performed. These extracts are further cleaned by two SPE clean-up steps and derivatized prior to 30 minutes GC-MS/MS analysis.
3. Results and discussion

3.1. Biological samples

Problems were quickly encountered with lipids during the method development of especially fatty tissues, e.g. brain and gonads. Therefore, some in-depth clean-up investigations were performed using surrogate interference chemicals (viz. cholesterol and coprostanols). Separation of steroid hormones from different lipid classes were achieved by modifying previous nicely evaluated lipid separations on amino propyl and silica SPE [4]. The methodology was validated on bovine serum, and absolute recoveries in the range 53-112% were obtained at 1.0 ng in 10 mL bovine serum. More important, the relative recoveries with deuterated steroid hormone analogues were close to 100% for all steroid hormones. The determination limits in biological samples are down to 0.8 ng per sample.

3.2. Environmental samples

Environmental solid samples were extracted utilizing the PLE system; however, huge amounts of lipids and other (unknown) interfering components were also encountered eluted here. Consequently, careful separation investigations of surrogate interference chemicals from the steroid hormones were investigated by inverse-PLE (i-PLE, described earlier [5]) and integrated clean-up PLE (ic-PLE). The latter step was also carefully evaluated with various layered adsorbents inside the PLE cell. Absolute PLE recoveries of the nine steroid hormones were between 67 and 107% at a 20 ng level in 10 g soil. Environmental water samples followed the same procedure as for biological liquid samples.

4. Conclusions

An analytical methodology to determine nine native steroid hormones in biological and environmental samples was developed and validated with determination limits down to 0.8 ng per sample. These low detection limits were obtainable due to the quantitative recoveries and an efficient two step clean-up for fractionation and elimination of interference components from the steroid hormones.

This methodology can be applied to a broad range of biological samples, toxicological studies, in-vitro assay media samples (e.g. cell assay) and environmental studies.

5. References


Acknowledgement - This work was supported by the PATHOS Centre (ENV 2104-07-0015) funded by the Danish Strategic Research Council.
The application of direct hollow fiber liquid phase microextraction for determining sludge adsorption of non-steroid anti-inflammatory drugs during sewage treatment

Estelle Larsson¹ and Jan Åke Jönsson¹

¹Center for Analysis and Synthesis, Department of Chemistry, Lund University, P.O.Box 124, 221 00 Lund
E-mail contact: estelle.larsson@organic.lu.se

1. Introduction

Non-steroid anti-inflammatory drugs (NSAIDs), including e.g. ketoprofen, naproxen, diclofenac and ibuprofen, are all consumed in large amounts in many countries and also belong to the pharmaceuticals most frequently detected in surface waters [1]. However, at a closer study, differences within the group are visible. For instance, although they possess great similarities in physicochemical properties, studies show that ketoprofen are removed to a much lesser extent than ibuprofen during sewage treatment: e.g. 65 and 96 % removal efficiency was shown for ketoprofen and ibuprofen, respectively, in a Swedish STP [2] and 22 and 88 %, respectively, in a Spanish plant [3]. This raises a number of interesting questions: Why do substances with such similar properties behave so differently under the same conditions? Ibuprofen is efficiently removed from the sewage water, but which are the main removal mechanisms? Is it totally degraded to carbon dioxide and water or non-toxic products or does a significant adsorption to the sewage sludge – which is often used as a fertilizer in crop production – take place during sewage treatment? How much of the total removal, in numbers, can be attributed to adsorption to sludge and where in the STP does the major adsorption in that case occur?

The main purpose of this study is to investigate and quantify the removal due to sludge adsorption of the four aforementioned NSAIDs throughout the STP. This will be performed by measurements of the NSAIDs in water as well as in sludge samples from the different steps of an STP (influent, pre-sedimentation, biological treatment, chemical precipitation and effluent). However, a study of this type poses great demands on the analytical procedures applied: Not only are the analyte concentrations low (ppb or sub-ppb level), but the samples are also very complex and diverse – from effluent water with an approximate suspended solids (SS) content of 5 mg/L to primary sludge where the SS content can be up to 20 000 mg/L.

Hollow fiber liquid phase microextraction (HF-LPME) is an upcoming extraction technique well suitable for this challenge. In this technique, the pH of the samples is adjusted to make ionisable analytes uncharged, whereby they can diffuse through the porous wall of a hollow fiber impregnated with an organic solvent and into the lumen of the fiber filled with an aqueous acceptor solution with a pH in which the analytes become charged. Due to their charge, the analytes can not diffuse back through the solvent but are trapped in the acceptor. The small acceptor volume (a few µL) yields very high enrichment and the fact that only substances which are uncharged at the pH of the sample and charged at the pH of the acceptor are enriched generate very efficient clean-up. This has been well demonstrated for aqueous samples in several cases [4] and lately also successfully applied for extraction of NSAIDs from digested sewage sludge [5].

2. Materials and methods

HF-LPME is performed using Q3/2 Accurel polypropylene hollow fiber membranes (Membrana, Wuppertal, Germany). To obtain total protonation of the analytes, the samples (volume 50 mL) are acidified to pH 2 by addition of sulfuric acid prior to analysis. The samples are extracted under stirring for 4 h whereafter the acceptor is removed from the fiber and analysed using HPLC (Agilent 1100 equipped with diode array and fluorescence detectors).

Initial analysis have been performed with HPLC-DAD. The fact that simple UV/DAD detection can be applied for identification and quantification of trace levels of organic contaminants in such complex samples as these (see Results and discussion below) gives us an insight in the the power of enrichment as well as selectivity associated with HF-LPME. However, to take full advantage of the potential of the method, further quantification is instead performed with high resolution quadrupole time of flight mass spectrometry (LC-MS Q-TOF). This also leads to decrease RSD values since these seem to be generated partly from imprecision in the HPLC analysis.
Sewage water and sludge samples are collected from Källby STP, city of Lund, Sweden. For method development experiments, grab samples are collected, preserved with sulfuric acid and stored cold and dark until extraction and analysis. For quantification experiments 24-hour weighted flow proportional samples are collected and a sampling regime to ensure that variations in influent concentrations will not distort the data from other parts of the plant is designed together with experts from the STP.

3. Results and discussion

3.1. Method development

The HF-LPME method, comprising simple equipment and only microlitres of solvent in the extraction step, yields high enrichment factors (3 000 – 4 000 times) for all four analytes in reagent water. In the presence of analyte matrix (influent water, diluted 50 %), a matrix effect (i.e. an apparent decrease in the enrichment factors due to analyte adsorption to the matrix) is observed, but enrichment factors are still very high (1 600 – 2 500 times). Matrix effects are analyte specific (i.e. the decrease in enrichment when matrix is present varies strongly between the analytes). Such experiments provide valuable information about the unique adsorption pattern for each analyte. This generates overall MDL values for influent samples of 0.04 µg/L for ketprofen to 0.11 µg/L for ibuprofen. Initial extractions of spiked biosludge samples indicate that the extraction is also successful for these samples, but with enrichment factors lowered approximately one order of magnitude compared to influent samples, most probably due to the much higher content of suspended solids. Also RSD values are higher (approx. 20 % compared to approx. 10 % for reagent water). However, such issues could to a great extent be reduced applying an optimal dilution scheme of such samples.

3.2. Occurrence in STP samples

In extraction and analysis of STP influent samples ketoprofen, naproxen and ibuprofen have been detected in concentrations around 1, 10 and 10 µg/L respectively. However, further experiments are needed to confirm and quantify the statistical uncertainty of these values. Also diclofenac was shown to be present but below the LOD of the existing method.

4. Conclusions

The proposed method has shown the capacity of selective and efficient extraction of four anti-inflammatory drugs from water as well as sludge samples taken from inside an STP. Further experiments are needed and will be performed during late 2010 and early 2011. The study is planned to be completed well before May 2011 and will result in a quantification of the sludge adsorption of four anti-inflammatory drugs throughout the sewage treatment process as well as a simple and environmentally friendly analytical method applicable for complex aqueous as well as semi-solid samples.

5. References


Acknowledgement - The authors thank RECETO (Research School of Environmental Chemistry, Microbiology and Toxicology) and Region Skåne, Sweden as well as Michael Cimbritz at Källby STP/VA Syd, Ayman Rabayah and Niklas Larsson.
The uptake of pharmaceuticals into aquatic organisms; the importance of species traits and exposure route

Melanie Netherton1,2, Richard Fussell1, Dave Raffaelli2, Alistair Boxall2

1 Food and Environment Research Agency, Sand Hutton, York, U.K.
2 Environment Department, University of York, Heslington, York, U.K.
E-mail contact: melanie.netherton@fera.gsi.gov.uk

1. Introduction

A range of pharmaceuticals, have been detected in soils, surface water, sediments and groundwaters across the world. While the reported concentrations are generally low (i.e. < µg L⁻¹ in surface waters), these substances have been observed throughout the year across a variety of hydrological, climatic and land-use settings [1]. A wealth of information is now available on the occurrence and effects of pharmaceuticals in the environment, but our understanding of the uptake dynamics of pharmaceuticals through aquatic and terrestrial food webs is limited. The present study aimed to address this by:

• Comparing the uptake of pharmaceuticals into aquatic invertebrates with different traits, including the freshwater shrimp (Gammarus pulex), the water boatman (Notonecta glauca) and the freshwater snail (Planobarius corneus)
• Exploring the relationship between pharmaceutical physico-chemical properties and pharmacological properties and uptake into aquatic invertebrates
• Assessing the importance of the exposure route (water/dermal or food) in the uptake of pharmaceuticals into aquatic invertebrates
• Studying the trophic transfer of pharmaceuticals through a simple aquatic food chain

2. Materials and methods

To compare differences in uptake between species, all organisms were exposed to water spiked with 0.1 mg L⁻¹ of radiolabelled pharmaceuticals (carvedilol, fluoxetine, 5-fluorouracil, moclobemide, diazepam and carbamazepine). The concentration was measured in the animals and in the water throughout the exposure period by liquid scintillation counting (LSC) [2]. A one-compartment model was fitted to the measured data and bioconcentration factors (BCFs) were calculated from the rate constants. The relationship between uptake to the pharmacological parameter Volume of Distribution (VD) and to the pH corrected liposome water coefficient (Log Dlipw) were analysed by regression.

To assess the importance of the route of exposure, G. pulex and N. glauca were fed food that had been previously exposed to pharmaceuticals. The food (leaf material was fed to G. pulex and G. pulex were fed to N. glauca), was exposed to an aqueous solution of 0.8 and 0.1 µg L⁻¹ of radiolabelled fluoxetine or carvedilol. G. pulex and N. glauca were then allowed to feed on the contaminated food for 72 hours. The uptake into G. pulex and N. glauca from the food was measured by LSC. This was then compared to uptake from the water alone and a combine exposure from the food and the water at the same concentrations of fluoxetine and carvedilol.

Finally, the trophic transfer of pharmaceuticals through a simple food chain was studied. Leaf material was exposed to 0.8 and 0.1 µg L⁻¹ of radiolabelled fluoxetine or carvedilol. The leaves were transferred to beakers containing ten G. pulex. The G. pulex was allowed to feed on the leaves for 72 hours. The concentration of pharmaceutical was measured in five animals and five were transferred to a beaker containing one N. glauca. N. glauca was allowed to feed before the pharmaceutical was measured in its tissues.
3. Results and discussion

In *G. pulex*, bioconcentration factors (BCFs) ranged from 4.55 – 185900 and increased in the order moclobemide < 5-fluorouracil < carbamazepine < diazepam < carvedilol < fluoxetine. In *N. glauca* BCFs ranged from 0.13 – 1.60 and increased in the order 5-fluorouracil < carbamazepine < moclobemide < diazepam < fluoxetine < carvedilol. For *P. corneus*, the BCF for carvedilol was 57.3. The differences in uptake of carvedilol between species are shown in figure 1.

![Figure 1: Uptake of carvedilol into three aquatic invertebrates](image1)

![Figure 2: Concentration of pharmaceuticals in *N. glauca* tissues when taken up from the food, the water or both food and water exposure routes.](image2)

The relationships between V_D and BCF were weak, strong correlations were found between Log D_lipw and BCF for both *G. pulex* and *N. glauca*.

When looking at the importance of exposure, the *G. pulex* data showed that the tissues concentrations in the *G. pulex* were significantly different for each exposure route for carvedilol and fluoxetine. The results indicate that uptake from dissolved chemicals from the water may be more important for accumulation in *G. pulex* than uptake from the food alone. However for *N. glauca*, the data show that the route of exposure of fluoxetine made a significant difference to the body burden of *N. glauca*. *N. glauca* took up less from the water compared to that assimilated from the food (figure 2).

The preliminary results show that fluoxetine can be transferred through three trophic levels of an aquatic food chain.

4. References


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Environmental fate of pharmaceuticals: Identification of fish bile metabolites and phototransformation products of anti-inflammatory drugs

Leif Kronberg¹, Jenny-Maria Brozinski¹, Jesper Svanfelt¹, Aimo Oikari², Marja Lahti² and Johan Eriksson³

¹Åbo Akademi University, Biskopsgatan 8 FI-20500 Turku/Åbo, Finland
²University of Jyväskylä, FI-40014 Jyväskylä, Finland
³Stockholm University, S-106 91, Stockholm, Sweden
E-mail contact: leif.kronberg@abo.fi

1. Introduction
The anti-inflammatory drugs diclofenac (DCF), naproxen (NPX) and ibuprofen (IBU) are among the most widely used pharmaceuticals worldwide. They are not fully eliminated during the wastewater treatment processes and consequently enters the recipient waters through discharges from the treatment plants [1]. Although detected at low concentrations (from ng to µg l⁻¹), the biologically active pharmaceutical may pose a risk to aquatic ecosystems, mainly due to the continuous discharge from the sewage treatments plants. Further, it is known that the compounds are not stable in the aquatic environment and phototransformation is one of the major reaction the compounds undergo [2].

The objectives of our work were
- to study the up-take and biotransformation of DCF, NPX and IBU in fish exposed to the drugs at environmental concentrations
- to determine the main phototransformation products of DCF and their formation pathways.

2. Materials and methods
For the study of the uptake of DCF, NPX and IBU (Figure 1) at environmentally realistic levels, rainbow trouts were exposed to the drugs at a concentration of about 1.5 µg L⁻¹ for ten days. Following the exposure, the bile samples were collected from immobilized fish and frozen in liquid nitrogen. The bile was analysed by LC-MS. The structural elucidation of the metabolites was based on the exact masses of the compounds generated by the Q-ToF mass analyzer and from the fragmentation pattern observed in the spectra recorded by the ion trap (IT) mass analyzer.

![Figure 1: Structures of the anti-inflammatory drugs](image)

The phototransformation of DCF was studied by irradiation of the compound with UV light that simulated the light from the sun. The transformation was followed by LC-UV analyses and the main products were identified by data collected from LC-MS analyses and from NMR data of isolated products.

3. Results and discussion
3.1. Bile metabolites
The mass spectrometric analysis of the trout bile showed the presence of several phase I and II metabolites of the drugs as well as the parent compounds. Thus it was evident that the drugs were taken up from water by the fish and that the compounds underwent biotransformation reactions in fish liver and produced several metabolites. Acyl (ester) glucuronides were the main metabolites. In the case of DCF, also an ether
glucuronide and sulphate conjugates (phase II metabolites) and two hydroxylated metabolites (phase I metabolites) could be detected [3]. IBU was found to give rise to relatively high concentration of taurine conjugates and to a phase I metabolite which has not been previously described, i.e. a novel metabolite. These results strongly indicate that when these drugs are present in natural water, they accumulate and undergo biotransformations in fish.

3.2. Phototransformation of diclofenac

The absorption spectrum of DCF has a maximum at 275nm and shows absorption up to about 340nm, which explains its tendency to undergo transformation in natural sunlight. Two transformation routes have been identified upon aqueous photolysis of the drug. The dominant one is the intramolecular cyclization to produce a chlorinated carbazole. However, this carbazole undergoes a rapid photochemical substitution of the chlorine with a hydroxyl group. Gradually, a second hydroxyl is attached, mainly para to the first one and following an oxidation step, the end product, a carbazole with a quinone ring, is obtained (Figure 2). The second, minor transformation pathway leads to the formation of a derivative of DCF where the acetic acid function is replaced by an aldehyde group (Figure 2) [4].

![Figure 2: Phototransformation products of DCF](image)

4. Conclusions

The work shows that at concentrations found in the environment, anti-inflammatory drugs may bioaccumulate and form metabolites in fish. The analyses of biliary metabolites could be a useful way of monitoring fish exposure to drugs. Further, it is shown that the loss of diclofenac in water downstream waste water treatment plant can in fact be due to phototransformation. The environmental significance of the transformation products is not known.

5. References

Investigating the Chlorination and By-Products of Pharmaceuticals by Liquid-Chromatography-Tandem Mass Spectrometry

Rosario Rodil¹, José Benito Quintana, Rafael Cela

Department of Analytical Chemistry, Nutrition and Food Sciences, Institute for Food Analysis and Research-IIAA, University of Santiago de Compostela, 15782 Santiago de Compostela, Spain
E-mail contact: rosario.rodil@usc.es

1. Introduction

The fate of pharmaceuticals in the water cycle has received a strong attention during the last few years and particularly during wastewater treatment and drinking water preparation. However, most studies have considered just the elimination of these compounds without accounting for possible transformation processes. Indeed, this may result in an underestimation of possible environmental and health hazards, as transformations may in some cases even lead to the production of more toxic compounds. An example is the chlorination of the widely used analgesic acetaminophen, which produces the toxic benzoquinone [1]. Thus, it is crucial to investigate the degradation routes of pharmaceuticals during chemical oxidations that are often employed for drinking water preparation. To identify the formed by-products, liquid chromatography-tandem mass spectrometry (LC-MS/MS) is particularly useful [2].

The aim of this work was to study the chlorination of pharmaceuticals and some metabolites, bearing in mind that these compounds are among the most frequently found in the environment [3, 4] and that, according to the European Federation of Chlor-alkali Producers, 98% of the DWTPs in Europe use chlorination as one of the main disinfection steps [5].

Thus, the reaction kinetics of pharmaceuticals were investigated in detail at different chlorine dose, pH and bromide concentrations by means of an experimental design methodology. Also, several transformation products were tentatively identified by liquid chromatography–(tandem) mass spectrometry (LC–MS(/MS)) with a quadrupole time-of-flight (Q-TOF) system and measured at different environmental conditions.

2. Materials and methods

Pharmaceuticals were obtained from Sigma–Aldrich (Steinheim, Germany). Chlorination of pharmaceuticals was performed on 22 mL amber closed vials that were maintained at room temperature (20 ± 2°C). Parallel control samples (without chlorine) were also measured.

Preliminary experiments to determine the stability of pharmaceuticals were done with 10 mL of Milli-Q water, adjusted to pH 7.1 with a phosphate buffer and spiked with the tested compounds at the 1 µg mL⁻¹ level and 10 mg L⁻¹ Cl₂. Seven aliquots of 1 mL each were taken at different reaction times and the reaction stopped with ascorbic acid (0.6 mg mL⁻¹). Moreover, the effect of the presence of a high concentration of bromide (100 µg L⁻¹) on the degradation was evaluated. These experiments were also used for identification of chlorination by-products.

Further experiments to study chlorination kinetics were performed in a similar way, but with lower pharmaceuticals concentrations (50 µg L⁻¹) and different concentrations of chlorine (1-10 mg L⁻¹), bromide (0-100 µg L⁻¹) and pH of sample (5.7-8.3) being considered. In these experiments, five aliquots were taken at different reaction times and the reaction stopped with ascorbic acid.

Degradation of pharmaceuticals was measured by LC–MS/MS (QqQ) in the multiple-reaction monitoring (MRM) mode of acquisition [6]. By-products were screened by LC–MS (scan) and LC–MS/MS (product ion scan) in a Q-TOF instrument.

The real samples were extracted by SPE and determined by LC–MS/MS as detailed elsewhere [6].

3. Results and discussion

3.1. Screening of degradable pharmaceuticals

A first chlorination test of the 15 pharmaceuticals was performed in order to assess their degradability upon chlorination. Thus, they were treated for 24 h with a 10 mg/L Cl₂ concentration at neutral pH (7.1). Under...
these relatively strong oxidation conditions, 6 of them (ibuprofen, ketoprofen, clofibric acid, fenoprofen, carbamazepine and bezafibrate) were found to be non-reactive.

Subsequently, the reaction kinetics of the 7 compounds fully removed with chlorine (phenazone, propyphenazone, salbutamol, propanolol, diclofenac, naproxen and indomethacine) and the 2 compounds degraded into a higher than 50% extend (atenolol and salicylic acid) were investigated in detail.

3.2. Influence of pH, chlorine and bromide concentrations on chlorination kinetics

In a deeper study, the factors pH (5.7–8.3), chlorine dose (1–10 mg/L as Cl₂) and bromide concentration (0–100 mg/L as Br⁻) were evaluated by means of a Box–Behnken experimental design. Empirical degradation half-lives (t₁/₂) were calculated from the pseudo-first order kinetic plots for each experiment and the design analysed for each pharmaceutical. Chlorine concentration was the overall most significant factor, except for salbutamol and diclofenac, with a negative value for all pharmaceuticals. The concentration of bromide into the solution was also significant and negative for naproxen and diclofenac, and close to significance level for salicylic acid. The other factor considered, pH, was only significant for salbutamol, with a negative effect.

3.3. By-products identification

The degradation path of salicylic acid, naproxen, phenazone, propyphenazone, propanolol, salbutamol and diclofenac consisted of aromatic substitution of one or two hydrogens by chlorine and/or bromide. Moreover, other by-products corresponding to a decarboxylation pathway of diclofenac from the monohalogenated products or hydroxylation pathway of phenazone and atenolal from the monohalogenated and/or dihalogenated products were also identified.

On the other hand, indomethacine, propranolol, atenolol and propyphenazone degradation lead to oxidation derivatives.

3.4. Application to samples

Finally, once the by-products were identified, they were screened, together with the parent compounds, for their occurrence in water samples by LC–MS/MS in MRM mode after the SPE of the samples [6]. Some of the parent compounds and by-products were found in tap water and wastewater samples. However the concentration of the by-products could not be determined because of the lack of standards.

4. Conclusions

Fifteen pharmaceuticals have been studied and among them nine (phenazone, propyphenazone, salbutamol, propanolol, atenolol, salicylic acid, diclofenac, naproxen and indomethacine) react with hypochlorous acid at significantly high reaction rates. The effect of pH, chlorine and bromide concentrations has been studied with a response surface methodology, showing the relevance of these factors in the degradation of the parent compounds and formation of by-products. Moreover, the by-products of these nine pharmaceuticals have been tentatively identified by LC–MS/MS experiments.

5. References


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Modelling approach to estimate emission of Plant Protection Products from protected crop systems to surface water in Mediterranean countries

Nicoleta A Suciu¹, Mark Egsmose², Alberto Pardoss³ and Ton dan der Linden⁴

¹ Università Cattolica del Sacro Cuore
   Istituto di Chimica Agraria ed Ambientale
   Via Emilia Parmense, 84 – 29100 Piacenza, Italy
² European Food Safety Authority
   Largo N. Palli 5/A, I-43121 Parma, Italy
³ Università degli studi di Pisa
   Dipartimento di Biologia delle Piante Agrarie
   Viale delle Piagge, 23 56124 Pisa, Italy
⁴ National Institute of Public Health and the Environment
   P.O. Box 1, N1-3720 BA Bilthoven, The Netherlands
   E-mail contact: nicoleta.suciu@unicatt.it

1. Introduction

The Scientific Panel on Plant Protection Products and their Residues (PPR) has been requested by the EFSA Executive Director to compile an inventory of protected crop systems and to estimate emissions of plant protection products (PPP) from these systems to relevant environmental receptors, for the support of the EU guidance document on the importance of emission routes including the circumstances under which they are relevant. These are important prerequisites for the process of developing risk assessment procedures which can be used in the framework of evaluation of PPPs in Europe.

With the present work we evaluate whether and how the existing models, used in the risk assessment for open field, particularly to calculate leaching and drainage of PPP to surface water, can be applied for estimating emissions from greenhouses to this specific receptor, under Mediterranean conditions.

2. Materials and methods

The FOCUS PEARL model was parameterized for an open field and greenhouse tomato cultivation in Pisa in the Tuscany region of Italy. The weather conditions for an open field were taken from a local weather station database for an 18 years period. The period was extended to 26 years by coping the last eight years, of which six years were used as a "warming-up period". For the greenhouse situation data were available only for one year of cultivation with one crop cycle. Correlations between daily indoor and outdoor temperature and ET were used to establish a long-term data set for the greenhouse simulations, based on the long-term open field dataset for Pisa.

Two scenarios were established for greenhouse cultivation, one with one crop cycle per year and one with two crop cycles per year, and one scenario was established for open field cultivation. The soil characteristics, PPP characteristics and PPP application scheme were the same for both growing systems. Calculations were performed to determine the concentrations of the "dummy" substance CC and its metabolite CC-M in water drained to surface water. The target concentration for comparing the scenarios was the 80th percentile of annual averaged drainage concentrations. For the greenhouse cultivation, additional scenarios were established to test the influence of irrigation volume given to the crop and indoor temperature on CC and CC-M emissions to surface water. For all scenarios the weather conditions for periods of 26 years were created by copying the available weather data to the desired period.

3. Results and discussion

The results show that there are potential PPP emissions to surface water associated with crop cultivation in greenhouses and the climatic conditions of the growing system have a major role on the PPP emissions. A more controlled climate in greenhouses than in open field determines a lower PPP emission from greenhouses when compared to the open field (Fig.1).
Concerning the greenhouse cultivation, it was noticed that the temperature has a higher influence on CC-M emission from greenhouse than the irrigation excess volume. A 50% increase of CC-M concentration in drained water was observed when the irrigation volume increased with 14%, whereas the same increase was reached when the temperature decrease from 21°C to 19.2°C. In greenhouse cultivation, over-irrigation is frequently employed to avoid soil salinisation. Several over-irrigation strategies can be applied. A comparison of 'continuous over-irrigation' with 'flushing the soil at the end of the growing cycle (same overall amount of irrigation water) showed that the flushing strategy leads to lower emissions. Lower areic masses of CC and CC-M in the soil profile at the end of the crop cycle and the lower CC and CC-M average concentrations in drained water were found for the situation in which the crop is irrigated with a minimum water excess during crop growth and flushing the soil after the crop cycle.

4. Conclusions

Finally it can be stated that the FOCUS PEARL model generally used for calculating leaching and drainage of PPP from open field cultivations to ground/surface water can also be parameterized to simulate PPP emissions from greenhouses to surface water. More simulations, also concerning other substances and scenarios (combinations of crops, soil and climatic conditions) are required to derive more generic conclusions.

5. References


1. Introduction

The FROGS (French Refinement Of Groundwater Scenarios) higher-tier national scenarios and interface were developed for field crops in order to support the registration of Plant Protection Products (PPPs) in France. Higher-tier evaluation is needed in case the limit for PPPs in groundwater (0.1 µg/L for parent substances and relevant metabolites; 10 µg/L for non-relevant metabolites) in one or more FOCUS scenarios [1]. In the past no national scenarios for France were available. The aim of the UIPP (Union des Industries de la Protection des Plantes) working group was to finalise the development of national scenarios which cover the most representative agricultural conditions of field crops in France and to implement them in a ready-to-use tool.

2. Scenario set up

The scenarios were implemented to represent average realistic agricultural conditions. France is divided into 31 Agricultural Units (AUs) defined based on their homogeneity regarding environmental conditions (hydrology, geomorphology, climate), and crop distribution and characteristics. The scenarios consist of combinations of weather, soil, and crop parameters.

Weather: One MARS-tile [2] is assigned to each AU, consisting of daily weather data from the years 1981 - 2006. The selected tile represents the largest agricultural area within the AU. Additionally irrigation is considered for grain maize, fodder maize, sugar beet, and potato.

Soil: Relevant soil types were identified and their area within each AU calculated by INRA Orléans [3]. In total 18 major representative soil types are considered with profile depths ranging from 40 cm to 140 cm.

Crop: The field crops sugar beet (sb), winter wheat (ww), winter oilseed rape (wosr), grain maize (gm), fodder maize (fm), winter barley (ba), potato (po), and sunflower (sf) are considered in the scenarios. They are not calculated as monoculture, but as realistic crop rotations (1-3 years), identified by French agricultural experts and confirmed by data from the agricultural census [4]. The selected rotations as well as the crop characteristics (such as emergence and harvest dates) are AU-dependent. BBCH codes (for application timing) for each crop in each AU are calculated based on temperature-sum.

Depending on the occurrences of the crops within the AUs and the possible combinations of soils and crops, different numbers of scenarios are available for the crops (Table 1), ranging from 49 scenarios for potatoes to 289 scenarios for fodder maize.

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<th>wosr</th>
<th>gm</th>
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<td>183</td>
<td>49</td>
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Table 1: Number of scenarios for the 8 field crops

3. Calculation of PECgw

As recommended by FOCUS 6 warm-up years are considered before 20 rotations are calculated for each scenario (i.e. in total 26 years for a monoculture and e.g. 66 years for 3 years rotation). The average
concentration in the groundwater during one rotation is calculated by dividing the mass of substance reaching the target depth with the water volume leached below the target depth. Target depth is the bottom of the soil profiles (ranging from 40 – 140 cm). The final endpoint used in the evaluation is the 90th overall percentile concentration, resulting from the combination of the 80th temporal percentile (17th value of 20 rotations) of each single scenario and the 80th spatial percentile from all scenarios together (Figure 1).

Figure 1: FROGS output graph showing the cumulative areal distribution and final PECgw value

4. The FROGS tools

The scenarios are implemented in an ACCESS® database, so that all parameters can be viewed easily and in all transparency. A FROGS user interface is also available in order to enter the substance parameters and application scenarios, prepare the relevant input files for PEARL 3.3.3 simulations, and process the PEARL 3.3.3 results for evaluation. All the available options of PEARL 3.3.3 regarding substance and application parameters are also applicable in the FROGS tool. In addition, FROGS allows for input of the applications relative to BBCH growth stage, in full adequation with the intended use of the PPP. Output summaries, distribution graphs (Figure 1), and the final PECgw value for the parent substance and all metabolites are derived automatically by the FROGS interface. Mitigation by location or soil properties is possible without the necessity of further scenario calculations. Free download of the tools and relevant documentation can be done upon registering on http://frogs.eclosion-share.net.

5. Conclusion

The FROGS national scenarios consider crop rotations of 8 field crops throughout France as higher-tier refinement of the European FOCUS scenarios for PECgw calculations. They represent realistic agricultural conditions and are in line with the standard FOCUS approach. The tools consist of an easy-to-use interface and ensures high transparency by storing the scenario data in an accessible database, which also allows the implementation of further user-defined scenarios. Main limitations are the coarse resolution of the 31 AUs, which is mostly due to the limited availability of soil data at a sufficient resolution, and the relatively long runtime of up to 289 PEARL 3.3.3 runs.

6. References

1. Introduction

During their lifetime in the global environment, semivolatile organic chemicals (SVOCs) may undergo several cycles of volatilization to air and deposition to environmental surfaces\(^1\). Recently, attention was devoted to the capability of variable air concentrations of SVOCs in determining a rapid response of such surfaces (e.g., foliar biomass or soil)\(^2\), which could accumulate or release SVOCs and influence their environmental fate. In order to detail the importance of such phenomena in the diverse environmental conditions, monitoring campaigns from different locations investigated the variability of air concentrations of SVOCs over short periods (less than 24 hours). The observed patterns were ascribed to different factors, such as the variability of the planetary boundary layer (PBL) height and structure\(^3\). The importance of PBL dynamics in determining the dispersion of pollutants is well documented in the literature\(^4,5\) and accurately accounted for in atmospheric dispersion (AD) models, which can also be used to describe single episodes of pollutant release and transport. On the other hand, AD models often ignore the interactions between atmosphere and the other environmental compartments; this aspect is usually accounted for in multimedia fate box (MFB) models, which are well suited to describe the behaviour of chemicals in a multimedia environment. In this context, an integrated model may allow to overcome some of the inherent limitations of the two modelling approaches and could offer important insights into the key processes that govern the short- and long-term behaviour of SVOCs.

1.1. Aim of the study

The aim of the present study is to investigate the ability of an integrated modelling approach recently developed by our research group\(^6,7\) to:

(1) capture the short-term variations in air concentrations of organic chemicals at a local scale;
(2) understand the influence of PBL dynamics and meteorology in determining such variations;
(3) assess the impact on concentrations of point emission sources such as incinerators;
(4) evaluate the response of the soil compartment to the high variability characterizing the atmospheric layers and its buffer capability with respect to air pollution.

2. Materials and methods

The integrated model\(^6,7\) combines the AD modelling system AERMET/AERMOD\(^8,9\), developed by the American Meteorological Society/Environmental Protection Agency Regulatory Model Improvement Committee, and a dynamic MFB model based on the fugacity approach\(^10\) developed for the integration purposes by our research group\(^6\). The new MFB model derives in part from the dynamic site-specific model SoilFug\(^11\) and consists of two atmospheric layers, representing the PBL and the atmospheric residual layer, and a surficial soil compartment. The atmospheric layers vary in height and average wind speed on an hourly basis, in order to account for the dynamic nature of the PBL. These values are provided by the integration with the meteorological preprocessor AERMET\(^8\). The further integration with the AD model AERMOD\(^9\) allows to simulate the contribution of a point source located outside the spatial domain of the MFB model, which is supplied with hourly values of inflow concentrations. The resulting integrated model, coded using Visual Basic 6, is available as a software for Windows.

The model performance was tested for two PCBs against a dataset of air concentrations measured in Zurich, Switzerland\(^3,6\). An illustration involving a chemical and particulate matter (PM) emission from a point source was then performed for two PAHs in a realistic scenario for Northern Italy\(^7\).
3. Results and discussion

The evaluation of the performance of the coupled AERMET-MFB model was assessed comparing the model output for the lower-air compartment with the air concentrations of two PCBs (PCB 52 and PCB 149) measured in Zurich during a three-day period\cite{Gasic2009}. Results showed the ability of the integrated model in reproducing the diel pattern observed in measured concentrations and pointed out the importance of the PBL dynamics in determining the observed oscillations. A good agreement between predicted and measured values, generally within a factor of 3 for PCB 52 and of 2 for PCB 149, was observed\cite{Morselli2010}.

The fully-integrated model, including the whole modelling system AERMET/AERMOD, was run for two PAHs using a realistic semi-urban scenario located near Milan (Po Valley, Northern Italy). The contribution a point source placed outside the MFB model spatial domain and emitting realistic amounts of chemical and PM for an incinerator was modelled by the AERMOD submodel. Results stressed the large influence exerted by PBL meteorology in determining the frequency and amplitude of the short-term variations in air concentrations and showed the magnitude of the potential increase in concentrations due to an incinerator-like point source\cite{Morselli2010}. Further simulations showed the increased contamination of the soil environment due to the combination of low PBL height, precipitation and point source contribution and the extent of the consequent degassing episodes due to favourable atmospheric conditions (such as increased PBL heights and wind speed), which have the effect of “recharging” the lower air compartment.

4. Conclusions

The obtained results highlighted the importance of integrating AD and MFB modelling approaches in investigating the high variability observed in air concentrations of semivolatile contaminants and elucidating their short- and long-term behaviour in the air/soil system. Moreover, a model coupling such as the one presented here could help in understanding the impact of specific sources on pollutant concentrations in air and soil and the assessment of the response times for mitigation/remediation measures for contaminated soils.

5. References

Modelling the cycling of persistent organic pollutants in shelf seas with a combined hydrodynamic and fate and transport ocean model: the North Sea system

Kieran O’Driscoll¹, Thomas Pohlmann¹, Bernhard Mayer¹, Tatiana Ilyina², Peter Damm¹

¹ Institut für Meereskunde, Universität Hamburg, Bundesstrasse 53, 20146 Hamburg, Germany
² Max Planck Institute for Meteorology, Bundesstrasse 53, 20146 Hamburg, Germany
E-mail contact: kieran.odriscoll@zmaw.de

1. Introduction

The environmental fate of selected persistent organic pollutants (POPs) in the North Sea is modelled with a combined hydrodynamic and Fate and Transport Ocean Model (FANTOM). Large amounts of POPs enter the North Sea system from the surrounding highly populated, industrialised and agricultural countries. Major entrance pathways of POPs to the North Sea are through atmospheric deposition and river inputs, with additional contributions coming from bottom sediments and adjacent seas, including the North Atlantic Ocean, the English Channel and the Baltic Sea (Figure 1). This is a continuation of the work of Ilyina et al., [1], who first used FANTOM in the southern North Sea. The model is undergoing further development.

POPs in the ocean are subject to a wide range of processes including mechanical, chemical, physical, and biological processes (Figure 2). The fate of POPs in the oceans is not yet completely understood, though oceans are generally considered to act as ultimate sinks for POPs. Budgets of POPs are calculated to determine whether the North Sea can act as a reservoir of POPs. In this study, the POPs PCB 153 (almost insoluble in water) and lindane (γ-HCH) (very soluble in water) are modelled for the period 1995-2005.

A 3-D hydrodynamic model is a necessary prerequisite tool for modelling the fate of POPs in the ocean. For this purpose, we have developed a very high resolution version of the Hamburg Shelf Ocean Model (HAMSOM) for the North Sea at the Institut für Meereskunde, Universität Hamburg, see [2] for details.

The impact of climate variability on POP levels in the North Sea is investigated by performing future scenario model runs in 10 year time slices to the year 2100 using plausible POP input levels.

2. Modelling approach

The modelling approach is outlined in Figure 3. Hydrodynamic variables, e.g., temperature, salinity, velocity, and mixing coefficients, are calculated in the HAMSOM which is forced at the surface and open boundaries. POP processes are calculated with the FANTOM model. Evolution of the total concentration of a pollutant at a fixed point is calculated with a simple Eulerian, advective-diffusive model with sources and sinks. Atmospheric concentrations of POPs are taken from the atmospheric model of the MSC-E, EMEP [3]. The net flux of a pollutant to the sea surface from the atmosphere is calculated as the net value of gaseous air-sea flux, dry particle deposition flux and wet deposition flux. POP concentrations in river estuaries have
been calculated from available datasets. Pollutants in the water column may be either freely dissolved or bound to suspended particulate matter which rapidly sink out of the water column into the sediment. POPs in sediment can be resuspended into the water column during storm events or during high tides. Degradation in seawater is represented by a 1st order rate decay coefficient. Model resolution for both models is 1.5' latitude x 2.5' longitude (~ 3 km horizontal resolution) with 30 vertical levels. Additionally, the FANTOM has 20 sediment layers. Time step for the FANTOM is 10 minutes.

3. Results

$\gamma$-HCH concentrations are highest in summer but PCB 153 concentrations appear to be greatest during the winter months. Concentrations of PCB 153 and $\gamma$-HCH decrease in the North Sea over the period 1995 - 2005. Preliminary results show that sediment concentrations also decrease during this period, suggesting that perhaps the North Sea cannot act as a reservoir of POPs for the oceans.

4. Future work and research needs

Further development of the FANTOM is required to:
- improve parameterisation of surface processes by including foam formation and breaking waves
- improve processes in the water column such as sorption of POPs to organic matter
- make model calculations more efficient

The models can be applied to various shelf regions of the ocean to model the fate of different POPs and other chemicals, both for the present and for future scenario runs. This will provide a valuable tool for future management decisions concerning control of POPs in the environment.

5. References

Development and validation of environmental fate model for herbicides of paddy fields using grid-catchment integrated multimedia modeling system (G-CIEMS) for all Japan area

Yoshitaka Imaizumi, Noriyuki Suzuki, Fujio Shiraishi, Daisuke Nakajima, Shigeko Serizawa, Ryo Kamata, Shihoko Kageyama, Jun Kobayashi, Takeo Sakurai and Hiroaki Shiraishi

National Institute for Environmental Studies, Tsukuba, Japan
E-mail contact: imaizumi@nies.go.jp

1. Introduction

Monitoring data were actively used for representative chemical concentrations in the environment for estimation of risk analysis. However, observed values have temporally-spatially specific information for limited numbers of chemicals. It is important to cover temporally-spatially variable concentration for many chemicals. So, model calculation should be applied, especially for ecological risk analysis.

Pesticides are absolutely necessary. However, pesticides should be carefully controlled because of its original purpose of herbicidal, insecticidal, or fungicidal actions. Rice is a most major crop plant in Japan. Since schedules of rice transplanting collect to a specific period in each region, pesticides are also intensively used and run off to rivers. Herbicides are especially need to be regarded because these are thrown in paddy water and that why have high run-off ratio. For ecological risk analysis, it is important to calculate daily variation of pesticide concentrations in all rivers in Japan for many kinds of pesticides.

We had developed the multimedia environmental fate model G-CIEMS (Grid-Catchment Integrated Multimedia Modeling System) and Japanese GIS data set used for this model. In this study, we developed the method to predict daily variation of concentrations of many herbicides to paddy fields in all area in Japan. For developing the method, we collected, analyzed, and make a database of various kinds of relative information. We calculated daily emission amount of 26 kinds of herbicides for each river segment and air mesh, which data suitable for G-CIEMS model. In order to validate this model, we investigate herbicide concentrations in seven rivers in Japan.

2. Materials and methods

The prediction method was divided into following three phases.

In the first phase, used amounts of pesticide formulation were calculated for each day for each prefecture. In order to predict used amounts of as many paddy herbicides as possible, we categorized all paddy herbicides to three usage groups based on a standard period of use. The database was made for all paddy herbicides which contained relative information such as the usage group, shipping volumes of pesticide formulations for each prefecture, contained ratios of active ingredients in pesticides, pesticide forms, and standard used methods of pesticide formulations. Used herbicide amounts were calculated from this pesticide database, usage records of pesticides by farmers, and transplanting schedules in each prefecture.

In the second phase, we predicted concentration variations for each herbicide in paddy fields and emission ratios to a river and air for each day and area. Although herbicide concentration variations in paddy field were calculated by the pesticide paddy field model (PADDY), runoff amount of herbicides to a river were corrected by total runoff ratio calculated using the prediction expression of runoff ratio suggested by Maru because of difference between runoff ratios from one paddy field to side ditch and that from paddy area to medium scale river. In the third phase, we calculated environmental concentration of each pesticide for all over Japan by the G-CIEMS.

3. Results and discussion

From results, predicted peak concentration maps were drawn for each herbicides concentrations for all over Japan as shown in Fig. 1. In total 182 pairs (= 7 river sites x 26 herbicides), herbicides were detected in 171 pairs that usable to the validation of this model. For each pair, we compare observed concentration with predicted concentration validation as shown in Fig.2. To evaluate the reliability of this model, peak concentration and peak days were compared between predicted variations and observed variations for 171 pairs of sites and herbicides as shown in Fig.3. Comparing among rivers, overestimated river is Usui, underestimated rivers are Kokai, Hanamuro, Asahina and Koutsuki. Peak concentration differences between
predictions and observations were less than one order of magnitude in 113 pairs which reached 66% of total 171 pairs. Peak day differences between predictions and observations were less than two weeks in 136 pairs which reached 80% of total 171 pairs.

Figure 1: Examples of predicted peak concentration maps of a) simetryn, b) daimuron, c) cumylron.

Figure 2: Examples of comparison between observed concentrations and predicted variation.

Figure 3: Relationships of a) peak concentrations and b) peak days between prediction and observation.

4. Conclusions

We developed high accuracy model which could calculate daily concentration variation for many herbicides in all rivers in Japan. We validated this model for 26 kinds of herbicides with river water surveys in 7 rivers. Further research is required for improving predictive accuracy of this model and expanding target pesticides.

5. References


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Transfer of PCB from sediment to biota: development of a bioaccumulation model in a risk assessment perspective

Lopes, C.1 ; Roy, A.1 ; Persat, H.2 ; Desmet, M.3 ; Perga, M.E.4 and Babut, M.1

1Cemagref, UR MALY, 3 bis quai Chauveau - CP 220, F-69336 Lyon, France
2UMR CNRS 5023, LEHF, Université Claude Bernard Lyon 1 69221 Villeurbanne, France
3UMR CNRS 6113, ISTO Orléans/Tours, Université François Rabelais 37200, Tours
4Station d’Hydrobiologie Lacustre INRA 75 avenue de Corzent, F-74203 Thonon les Bains, France
E-mail contact: christelle.lopes@cemagref.fr

1. Introduction

Many chemical, physiological and trophic factors are known to be important in the bioaccumulation processes and trophic transfer of PCB in the biota. Understanding the primary factors influencing PCB contamination of fishes is critical for predicting and assessing risks to upper-trophic levels consumers including humans. In fall 2005, fish contamination by PCBs was observed in the Rhone river. This incidental observation triggered a series of investigations on fish and sediment along the main stream, leading to fish consumption advisories along a major stretch of the river. Three freshwater river fish species (Barbel, Bream and Chub), invertebrates and sediment cores were sampled in three sites along the Rhone river in the vicinity of Lyon (France) in order to (1) identify PCB contamination pathways that could explain between and within species variability in fish concentration levels; and (2) describe PCB transfer from sediment to these fish species along the food chain.

2. Materials and methods

2.1. Study sites and sampling

Three sites along the Rhone river were sampled: a relative reference site (MTE), upstream Lyon and the first fish advisory area; a site close to Lyon (GDL) in the contaminated area; and a site downstream Lyon (BRE).

Collected fishes species were the barbel (Barbus barbus), the chub (Squalius cephalus), and the bream (Abramis brama). These all large and long-living cyprinids were chosen because they are prone to accumulate PCBs over many years, and despite their relative similar trophic positions, they have different diets and exploit different habitats. Invertebrates were also sampled: Chironomidae (CHI), Gammaridae (GAM), Ephemeroptera (EPH), Pisidium (PIS) and Corbicula (COR). All (except Corbicula) are known to be in these fish species diets [1]. Finally, sediment cores have been collected at each site.

2.2. Analyses

Fish length and weight were measured, sex determined and age estimated by scalimetry. Each invertebrate type was pooled and weighted. The prey species types present in fish stomachs were identified and counted. Isotopic analyses ($\delta^{15}N$ and $\delta^{13}C$) were performed on fish tissues and invertebrates pools to relate the Carbon source exploited ($\delta^{13}C$) and the trophic position ($\delta^{15}N$). Lipid contents were also analysed.

In each sediment core, radionuclides measurement was used to age the successive layers. PCB analysis was performed and measurements of $^{210}$Pb and $^{137}$Cs correlated with documented hydro-sedimentary events were used to estimate a mass accumulation rate for each interval in each core.

2.3. Statistical and modelling framework

Classical statistical tests were performed using R statistical computing program [2]. Stable isotope mixing models were performed using R software [3] in order to determine the carbon source exploitation of each fish species in each site by using two baselines: Pisidium and Corbicula characterizing autochtonous and detrital carbon sources respectively. Trophic position (TP) was estimated by Bayesian inference from the equation proposed by Post [4].

A log-linear regression model was developed to explain fish PCB concentration according to all available explanatory variables: size, TP, the percentage of detrital carbon exploited (estimated by mixing models), lipid content, sex and the maximal PCB concentration in the sediment at which fishes were exposed during their life. Backward stepwise regression was then realized.

The bioaccumulation model developed is derived from [5, 6]. The fish contamination kinetics is as followed:
\[
\frac{dC_{c,i}(t)}{dt} = U_i(t) \alpha_c C_{c,w}(t) + \beta_c F_i(t) \sum Q_{i,j} C_{c,j}(t) - (E_i(t) + G_i(t)) C_{c,i}(t) - R_i(t) C_{c,i}(t)
\]

where \(C_{c,i}(t)\), \(C_{c,w}(t)\) and \(C_{c,j}(t)\) are the concentrations in congener \(c\) in a fish species \(i\), in the water and in the prey \(j\) respectively. \(U_i(t)\) is the water filtration rate, \(\alpha_c\) and \(\beta_c\) the assimilation efficiencies of the dissolved and ingested particles respectively, \(F_i(t)\) the ingestion rate, \(Q_{i,j}\) the diet preference toward the prey \(j\) (gut contents data), \(E_i(t)\) the elimination rate, \(G_i(t)\) the growth rate and \(R_i(t)\) the reproduction rate. Variation of environmental conditions (water temperature), individual physiological traits (growth rate) and physico-chemical properties of PCBs \((K_{ow})\) are considered through their effects on several of these functions. This equation was coupled to an equation describing the contamination kinetics of each prey according to the part of detrital carbon exploited and the concentration in the sediment. Parameters involved in each of these functions are estimated by Bayesian Inference.

3. Results and discussion

The analysis of PCB contamination data shows that: (i) the contamination increases from upstream to downstream Lyon (MTE<GDL<BRE) and (ii) the chub is the less contaminated while the barbel is the more.

Bayesian inference was realized on \(\delta^{15}N\) and \(\delta^{13}C\) data for each species in each site in order to consider data variability and parameter uncertainty on species TP estimation. Thin posterior distributions were obtained for all parameters, meaning that data sets were informative enough to provide a good parameter estimation.

By using two endmembers, each representative of a feeding habitat, mixing models showed two habitat exploitation patterns: the first revealing that the chub at the three sites and the bream and the barbel at GDL exploit preferentially autochthonous carbon sources than detrital ones; the second pattern showing that the bream and the barbel at MTE and BRE exploit the two carbon sources (and then the two habitats) in a similar way. Finally, the use of mixing models at the individual level showed that PCB concentration seemed to increase, in a general way, with the proportion of detrital carbon exploited by fishes, especially at GDL.

Fish TP and PCB concentrations were found to be not related while the exploitation of detrital carbon as a food source appeared as an essential factor of fish contamination. The regression model showed that fish length, PCB concentration in sediment and individual fish foraging habitat (exploitation of detrital carbon sources) explained about 80% of within- and between- species variability observed in PCB concentrations.

The predictions of our bioaccumulation model describes seasonal variations in fish PCB concentrations (due to the variation of environmental conditions) and appeared particularly efficient in a risk assessment perspective. The use of Bayesian Inference to calibrate the functions involved allowed to pass on data variability and parameter uncertainty to model predictions and provided a credibility interval around them.

4. Conclusions

Sediment management needs raise concerns about the PCB range of concentration levels in sediment which could determine an exceedence of regulatory thresholds for fish consumption. The bioaccumulation model proposed here to describe the transfer of PCBs from sediment to fishes gives promising results in helping to determine sediment management guidelines in the future.

5. References


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Prioritising chemicals used in PCPs in China for environmental risk assessment: Application of the RAIDAR model

Todd Gouin¹, Roger van Egmond¹, Juliet Mortimer¹, and Oliver Price¹

¹Safety and Environmental Assurance Centre, Unilever, Colworth Science Park, Sharnbrook, U.K.
E-mail contact: todd.gouin@unilever.com

1. Introduction

Brazil, Russia, India, and China (BRIC) are expected to be the four biggest economies by 2050. Of these four countries, China is commonly recognized as the biggest potential market for home and personal care products. It is thus important that infrastructure and regulatory instruments are sufficient to reduce environmental impacts related to economic growth and use of such products.

Chemicals used in personal care products (PCPs) represent a significant fraction of chemicals used in commerce in China. The environmental fate and effect datasets of many cosmetic ingredients, however, are limited. Recently, there has been emerging concern regarding the use of a number of substances classified under this category, including the nitro- and polycyclic musks, UV blockers such as methylbenzylidene camphor, and preservatives such as the parabens. Given the continuous emission of chemicals used in PCPs to wastewater and the aquatic environment after regular use during showering or bathing, methods for prioritising the environmental risk assessment are thus needed.

In an effort to address this knowledge gap we have identified the chemical ingredients used in 2500 PCPs across China, and estimated the annual emission of these chemicals. The physical-chemical property data for these substances have been estimated and used as model inputs in the Risk Identification And Ranking (RAIDAR) model. A sensitivity analysis relating to model input parameters and environmental release scenarios is performed as a method for assessing the influence of input parameters with respect to chemical ranking and prioritisation.

2. Materials and methods

• Using a combination of information obtained from the Global New Products Database (www.gnpd.com) and Euromonitor (www.euromonitor.com) we have assembled a list of chemical ingredients that are used in personal care products sold in China between December 2008 and December 2009.

• Physical-chemical properties for chemical ingredients for which molecular structures are readily available have been estimated using a suite of estimation programmes, including EPIWIN, SPARC, and ACD.

• Emission estimates are based on information made available from the Cosmetic Ingredient Review, which publish data on the maximum inclusion level for a chemical ingredient in various categories of personal care products (www.cir-safety.org/index.shtml). For Chemicals for which inclusion levels were not available, a conservative estimate of 5 kt was assumed, representative of the mean value for substances with inclusion level data.

• Property data estimates combined with emissions estimates are then input to the RAIDAR model and prioritised using baseline narcosis as the model output criterion (1). Baseline narcosis is selected to represent the least sensitive toxicological endpoint, and a critical emission rate ($E_C$) needed to achieve this endpoint is estimated.

• A risk assessment factor (RAF) is thus estimated as the ratio of the actual emission rate ($E_A$) and $E_C$.

• Six scenarios investigated with and without biotransformation included: Direct discharge scenario, where total usage of chemical ingredient is emitted directly to the aquatic environment; Removal of chemical through waste water treatment (WWT) assuming perfectly persistent chemicals where removal is by sorption to biosolids only; and Removal of chemical through WWT assuming biodegradation estimated using BIOWIN output.
3. Results and discussion

3.1. Identifying chemical ingredient and estimation of physical-chemical properties

Molecular structures and SMILES code were readily available for 476 (39%) of chemical ingredients, with nearly 40% of chemicals being classified as botanical extracts (i.e. mixtures) having no molecular structure information. SMILES codes were used to estimate property data using the Estimation Property Interface (EPI) Suite of programmes. The list of 476 chemicals is then reduced to 254 chemicals, which are identified as having properties that fall in an applicable domain for multi-media modelling (i.e. log $K_{OA} <15$ and log $K_{AW}$ between -10 and 5.

3.2. Estimating emissions

Emission estimates for 254 chemical ingredients used in PCPs are based on market share data for different household and personal care product categories using the Euromonitor (www.euromonitor.com) marketing database. The Euromonitor database, for instance, provides volume estimates of product categories sold within a particular market. For instance, 375 million litres of shampoo were sold in China during 2009. Combining maximum inclusion levels (%Max_inclusion) defined for a chemical ingredient within a product category and total volume of product category sold in China ($V_{Product}$), a conservative emission estimate can be obtained, where:

$$Usage = \left(\frac{V_{Product} \times \%Max\_inclusion}{100}\right)$$

3.3. Results from RAIDAR model

![Figure 1](image1.png)  
**Figure 1:** Comparison of risk assessment factors (RAF) and risk identification bins for 254 chemicals under three different discharge scenarios: Direct discharge, with WWT assuming perfectly persistent chemicals that are removed via sorption only, and with WWT including estimates of biodegradation.

![Figure 2](image2.png)  
**Figure 2:** Comparison of risk assessment factors (RAF) and risk identification bins for 254 chemicals for which metabolic transformation has been estimated, under three different discharge scenarios: Direct discharge, with WWT assuming perfectly persistent chemicals that are removed via sorption only, and with WWT including estimates of biodegradation.

4. Conclusions

Results shown in Figures 1 and 2, illustrating the position of the risk assessment factors and risk identification bins, suggest that the majority of chemical ingredients used in PCPs in China will have relatively low environmental risk, where populations are connected to WWT. Risk assessment factors are shown to be influenced by estimates of emissions removal efficiencies, biodegradation rates, and biotransformation. Thus a combination of improved understanding of WWT removal processes, biodegradation, and biotransformation will strongly influence the risk characterisation.

5. References

Increase of contaminant levels in the Arctic due to future climate change.

Kaj M. Hansen¹, Jesper H. Christensen¹, Jørgen Brandt¹, Lise M. Frohn¹, Camilla Geels¹, Allan Gross¹, Gitte B. Hedegaard¹, Carsten A. Skjøth¹, Ayoe B. Hansen¹

¹ Department of Atmospheric Environment, National Environmental Research Institute, Aarhus University, Frederiksborgvej 399, P.O. Box 358, 4000 Roskilde
E-mail contact: kmh@dmu.dk

1. Introduction

We have investigated the impact of climate change on the environmental fate of Persistent Organic Pollutants (POPs) in the Arctic by using an atmospheric chemistry-transport and multimedia fate model to simulate the atmospheric transport and deposition of six POPs in the Arctic area under a ten year period under present (1990-1999) and future (2090-2099 and 2190-2199) climate scenarios.

2. Materials and methods

In this study we use the Danish Eulerian Hemispheric Model (DEHM) (1-5) - a 3-D atmospheric chemistry-transport model original developed in 1991 to study the long-range transport of SO₂, SO₄ and Pb to the Arctic. The model covers the entire Northern Hemisphere and in the model domain all important sources for the Arctic are included. This model has been developed further to include four chemical groups: a group related to ozone chemistry, a group related to primary particulates, a group with mercury species/chemistry, and finally a group with Persistent Organic Pollutants (POPs). The model has a spatially detailed 3-d atmosphere up to 15 km over the surface. In addition it has four surface compartments: a 75 m thick ocean layer, a 15 cm thick soil layer, and dynamic evolving vegetation cover and seasonal snow pack layer. DEHM performs well for studies of mercury and POPs with input of real meteorology from the MM5 numerical weather prediction model, which is using global analyzed meteorological data as input (2-4, 6). By using climate meteorological data from the ECHAM5/MPI-OM model (run with the SRES A1B scenario) for three different decades: 1990-1999, 2090-2099 and 2190-2199 instead of MM5 based data, it is possible to see how the predicted atmospheric concentrations and depositions of POPs in the Arctic responds to a changed climate in the DEHM model. One of the weaknesses of such model simulations is that it is questionable if the model has a proper response to a changed climate, because all models have parameterizations of processes that are tuned to the current climate in order to improve the model performance.

The model system was run initially for a period using input from the MM5 model in order to build-up concentrations of POPs in ocean water and soil. The concentrations in air, sea water and soil of all the species from this simulation where used as initial concentrations for three different model runs with constant emissions (from year 2000) for the decades: 1990-1999, 2090-2099 and 2190-2199 using input from the ECHAM5/MPI-OM model. The differences between the results for the three decades are only due the different meteorological climate input from the ECHAM5 model system.
3. Results and discussion

The atmospheric concentration of many POPs is expected to increase in a future warmer climate. The main reason for this is that the POPs will have a higher volatilization from the surface in a warmer climate. In figure 1 the mean surface air concentrations of gamma-HCH for the 1990-1999 decade and the difference in percentage between the decades 2090-2099 and 1990-1999 is shown. The gamma-HCH air concentrations increase almost everywhere in the model domain.

In figure 2 the average surface air concentrations for the arctic area north of the Arctic Circle (66.56°N) are shown for the three simulated decades. For all 6 studied POPs there is an increase of the average air concentrations in the Arctic in both the 21st century and the 22nd century. The effect of a future warmer climate in the simulations is thus to increase the atmospheric transport of POPs into the Arctic.

4. Conclusions

Using meteorological output from climate models have shown to be a useful tool to investigate the exposure levels of contaminants in a future changed climate. The results produced with the DEHM model system indicates that the totals deposition of mercury will decrease over the marine areas due to changed ice cover. The atmospheric concentrations of the six studied POPs are predicted to increase due to increased atmospheric transport to the Arctic. The change in surface concentrations depend on the initial concentrations and no firm conclusions can be drawn from this study.

5. References

Assessing the potential implications of global climate change on human exposure to contaminants in the Arctic: Opportunities & Limitations

James M. Armitage¹, Cristina Quinn¹, Frank Wania¹

¹University of Toronto at Scarborough, Department of Physical and Environmental Sciences, 1265 Military Trail, Toronto, ON, Canada M1C 1A4
E-mail contact: james.armitage@utoronto.ca

1. Introduction

The influence of global climate change on the transport of organic contaminants to the Arctic and the long-term implications for human exposure is a complex issue that presents many challenges to researchers seeking to estimate the importance of and interactions between different processes. There are at least five broad categories of change to consider [1]: i) changes in chemical use and emissions ii) changes in the extent of contaminant delivery to the Arctic environment iii) changes in the processing of contaminants in the physical environment iv) changes in the processing of contaminants in the human food chain and v) changes in exposure due to alterations in the lifestyle of Northern communities. Global-scale modeling simulations incorporating some aspects of the projected alterations to the physical environment (e.g. temperature, precipitation, atmospheric circulation, ocean currents) conducted for PCBs [2] reported increases in modelled air concentrations in the Arctic in the range of approximately 2 to 2.5-fold compared to the baseline. As demonstrated in a model sensitivity analysis however [3], responses to global climate change with respect to long-range transport and contamination of the Arctic environment are highly dependent on physical-chemical properties. More importantly, the concentration of contaminants in the atmosphere is only an indirect indicator of human exposure in the Arctic because exposure to contaminants of concern is typically dominated by dietary uptake and hence dependent on contaminant levels in other media, particularly the freely-dissolved concentration in surface ocean water. Therefore, the purpose of this study is to more broadly assess the potential implications of global climate change for human exposure with a specific focus on the marine environment due to its importance in this context [4].

2. Materials and methods

Climate model-based projections describing changes to the physical environment in the Arctic are available [5] and were used as guidance in scenario development. The main goal of the study was to estimate/constrain the factor of change associated with potential alterations to the physical environment and other aspects identified above (e.g. dietary transition). Quantitative approaches included generic calculations of temperature-dependencies, phase distribution (e.g. influence of temperature and particulate organic carbon in the water column) and Characteristic Travel Distance (CTD) [6] as well as compound-specific calculations on the magnitude of potential reservoirs (e.g. inventories associated with glaciers) and mass flows (e.g. forest fires and emissions of PCBs). Global-scale fate and transport simulations were also undertaken using Arctic Contamination Potential (eACP10), a model-derived output integrating long-range transport and accumulation in surface media (i.e. excludes mass in atmosphere), to estimate factors of change associated with different scenarios. Simulations coupling output from fate/transport models with human food web bioaccumulation models were also conducted to examine the influence of diet.

3. Results and discussion

Compensatory behaviour was found to be a recurrent theme throughout this investigation whereby a single parameter (e.g. temperature) or combination of parameters (e.g. temperature + primary productivity) can exert antagonistic effects that tend to dampen the response to alterations in the physical environment with respect to human exposure potential. For hydrophobic contaminants (i.e. octanol-water partition coefficient > 100 000), shifts in diet appear to represent the greatest potential for change in contaminant exposure, an example of which is illustrated in Figure 1.
Arctic Contamination Potential (eACP\textsubscript{10}) exhibits only a modest response (~10–35%) in a scenario incorporating warmer Arctic Fall/Winter (+8 °C) and no sea ice cover in summer in comparison to the baseline. On the other hand, the shift away from consumption of marine mammal blubber corresponds to a 20 ("Market food") to 40-fold (No seal) reduction in lifetime exposure to PCB153. Alterations to the base of the Arctic food web (i.e. additional zooplankton consumer) correspond to higher exposure potential but this effect is not sufficient to counteract a complete shift away from seal blubber consumption (mammalian biomagnification factors >> invertebrates). Consistent with [3], changes in primary emissions and particularly northward shifts in emissions of compounds with low CTD (e.g. many current-use pesticides) are also associated with relatively high factors of change.

4. Conclusions

Based on this assessment, the most important potential changes for human exposure are likely to be related to changes in how humans interact with and exploit the physical environment in the Arctic (e.g. dietary choices) in addition to the magnitude and spatial distribution of emissions globally (and possibly in the Arctic itself). While long-range atmospheric transport potential and air concentrations in the Arctic may be enhanced under global climate change [3], contaminant amplification and exposure potential in surface compartments most relevant for humans may actually be reduced in comparison to contemporary conditions. However, the realism/representativeness of the modeling tools used is a major uncertainty underlying these conclusions. A more detailed and dynamic treatment of the cryosphere would be a particularly useful undertaking in the overall context of this study.

5. References


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Effects-directed Analysis of Contaminated Sediments from the Sava River, Croatia

Marijan Ahel¹, Senka Terzić¹, Jovica Lončar¹, Iva Mikac¹, Roko Žaja¹, Ivan Senta¹, Marta Popović¹, Roberta Sauerborn Klobočar¹, Knut-Erik Tollefsen², Kevin V. Thomas², Tvrtko Smital¹

¹Division for Marine and Environmental Research, Rudjer Boskovic Institute, Bijenicka 54, HR-10000 Zagreb, Croatia; ²Norwegian Institute for Water Research (NIVA), Gaustadalléen 21, NO-0349 Oslo, Norway
E-mail contact: ahel@irb.hr

1. Introduction

The application of toxicity identification and evaluation (TIE) protocols for the toxicological characterisation of contaminated sediments showed that the majority of the observed adverse ecological effects were associated with toxic organic chemicals [1]. Despite the common understanding that contaminated sediments represent a major sink for numerous anthropogenic chemicals, routine monitoring activities are usually directed towards a very limited number of selected contaminants. However, considering enormous number as well as chemical and toxicological diversity of organic contaminants, effects-directed analysis (EDA) represents the best available tool for the comprehensive assessment of hazardous chemical contamination in aquatic sediments [2].

The aim of this paper is to present the results obtained in a case study carried out in the Sava River basin, Croatia, and to demonstrate advantages of the applied fractionation scheme.

2. Materials and methods

Sediment samples were collected in July 2009 from the Sava River at the location Crnac situated few kilometers downstream of the city of Sisak. Sub-samples of about 40 g dry sediment were extracted by accelerated solvent extraction (ASE) using methylene chloride and methanol as extracting solvents. The resulting extracts were reduced to a small volume under nitrogen using a TurboVap system and transferred into 4 ml screw-cap vials. The total extract was subjected to pre-fractionation on a simple column filled with deactivated silica gel (15% water), providing 3 distinct fractions: non-polar, medium-polar and polar.

The analytical approach used for screening included a detailed characterisation of the collected samples, encompassing a wide range of possible non-target and target contaminants. The first two silica gel fractions were analysed for identifiable compounds using gas chromatography/mass spectrometry (GC/MS), while liquid chromatography/time-of-flight mass spectrometry (LC/Q-TOF) was used for the identification and additional confirmation of the more polar compounds. Ecotoxicity profiling of the investigated samples was performed using a battery of bioassays, including cytotoxicity, chronic toxicity, EROD activity; inhibition of the multixenobiotic resistance (MXR), genotoxicity and estrogenic potential.

3. Results and discussion

The chronic toxicity bioassay using AlgalTox assay showed the highest toxic response in the medium polar fraction, which was even higher than the toxicity of the total extract, indicating that complex interactions between the numerous constituents present in the crude extract, can mask some specific effects. As to the estrogenic activity, the effect was associated with medium polar and polar fractions. It is interesting to note that sub-fractions C19 and C20 displayed higher estrogenic activity than the total extract itself, which indicated again the importance of simple fractionation procedures for the prevention of masking effects.

The most significant effect determined in the analysed sediment was a high CYP1A induction potential. The measurements of EROD activity in PLHC-1 fish hepatoma cells in three initial fractions showed that most of the activity was associated with the nonpolar fraction, followed by medium-polar fraction, while the polar fraction showed relatively low EROD activity. The detailed chemical analysis of the two highly active fractions revealed predominance of complex hydrocarbon mixtures in the nonpolar fraction and different sterol compounds in the medium polar fraction. Further fractionation by preparative HPLC showed relatively complex toxicological profiles in all of the three main fractions. The simplest profile, but with the highest activities in individual fractions was found in the nonpolar fraction (Fig. 1A). The detailed analysis of the highly active nonpolar sub-fractions (A7-A17) by GC-MS showed that these sub-fractions typically contained various polycyclic aromatic hydrocarbons (PAHs). The most active fraction (A14) was composed of 4- and 5-
ring PAHs, including both nonsubstituted species and a significant contribution by mono-, di- and trialkylated homologues. This clearly indicated predominately petroleum origin of PAHs as well as the oil refinery at Sisak as the most probable individual source of this type of contamination.

The CYP1A induction potential of the medium polar fraction was widely distributed in the preparative HPLC fractions, while the observed activities in individual sub-fractions were several times lower than in the nonpolar fraction (Fig 1B). As to the chemical composition of the medium-polar fraction, it was strongly dominated by a complex suite of sterols, including sewage markers cholesterol and coprostanol as the most abundant species. Among the compounds having predominately xenobiotic character, phthalate esters were the most prominent. As expected, fraction C containing polar compounds showed the lowest EROD/CYP1A induction potential. Nevertheless, further comparison of the subsequent 40 sub-fractions of the polar fraction (C) clearly revealed significant differences among individual sub-fractions. The highest EROD induction potential was identified in the relatively lipophilic sub-fractions C19-23, C27 and C28.

Figure 1: Toxicological profiles of EROD induction potential in nonpolar (A), medium polar (B) and polar (C) fractions obtained for the corresponding forty HPLC subfractions (A and B – NP-HPLC using NH₂ column; C – RP-HPLC using C18 column).

4. Conclusions

The most pronounced effect, detected in the Sava River sediment, was CYP1A induction potential. It was predominately associated with the nonpolar fraction and polycyclic aromatic hydrocarbons were indicated to be responsible for the observed effect. However, other endpoints, such as algal toxicity and estrogenic potential, indicated comparatively higher importance of polar contaminants.

5. References

Integrated bioassay and chemical analysis of glucocorticoid and estrogenic activities in the rivers Rhine and Meuse

Corine J. Houtman¹, Rob ten Broek¹, Merijn Schriks², Jan A. Van Leerdam², Peter Stoks³, Sander C. van der Linden⁴ and Ruud J.C.A. Steen¹

¹The Water Laboratory, P.O. Box 734, 2003 RS Haarlem, The Netherlands
²KWR Watercycle Research Institute, P.O. Box 1072, 3430 BB Nieuwegein, The Netherlands
³Association of River Waterworks RIWA, Groenendael 6, 3439 LV Nieuwegein, The Netherlands
⁴BioDetection Systems, Science Park 406, 1098 XH, Amsterdam, The Netherlands
E-mail contact: corinehoutman@hetwaterlaboratorium.nl

1. Introduction

The application of effect-directed analysis (EDA) has shown to be a valuable approach to investigate the nature of biologically active compounds in the environment. Various research groups have applied EDA approaches and thereby successfully identified compounds responsible for endocrine disrupting, especially estrogenic, effects. The majority of EDA studies for estrogens have addressed natural and synthetic steroid estrogens as the primary causative agents of estrogenic activity in the aquatic environment[1-3]. With the development of new bioassays, it has become possible to investigate also the activity of other classes of hormones and compounds with comparable or antagonistic activities. Many hormone-like compounds are excreted naturally or are used as pharmaceuticals and thus might enter the environment via similar routes as estrogens[4]. Indeed, using CALUX bioassays for progestagenic, androgenic estrogenic and glucocorticoid receptor activation, the presence of especially glucocorticoid activity in Dutch surface waters and glucocorticoids in waste water was recently reported [5,6]. However, data on glucocorticoid activity remains scarce and its chemical identity in surface water unknown. Unintentional exposure to glucocorticoids is associated with impairment of the immune system, reproduction, and development. Therefore, the presence of compounds with glucocorticoid activity in surface water might –like estrogens- be an issue of concern regarding exposure of aquatic organisms or humans depending on surface water for the provision of drinking water.

The present study was undertaken to investigate the presence and identity of glucocorticoid and estrogenic activities in the Dutch catchments of the main rivers Rhine and Meuse. GR and ER CALUX measurements were done throughout the year and at five different locations to investigate spatial and seasonal differences. Having experienced that natural and synthetic hormones often explain the majority of endocrine activities in aquatic samples, we decided not to perform a full EDA, but first to develop and apply specific and very sensitive target analysis methods on LC-LTQ-FT-orbitrap MS and UPLC-tQ-MS for a large number of natural and pharmaceutically used steroid hormones (estrogens, glucocorticoids, androgens, progestagens). The methods were used to analyze bioactive samples and to calculate the extent to which natural and synthetic hormones were responsible for the measured activity. GC-screening was applied to obtain a more integrated picture of the composition of non-hormonal (bioactive) contaminants in the samples.

2. Materials and methods

Surface water samples were collected at five locations along the Dutch parts of the catchments of the rivers Rhine and Meuse for the analysis of glucocorticoid and estrogenic activity. Every three months, a larger volume was sampled for the analysis of steroid hormones and for GC-screening. In addition, some waste water samples were collected as examples of samples with high glucocorticoid activity. Samples for CALUX-bioassay (1L) and hormone analysis (10L) were sand filtered and extracted with Oasis HLB SPE cartridges eluted with ethylacetate. The equivalent of 1L sample was used for CALUX bioassay analyses. The equivalent of 10L was used for steroid hormone analysis. Samples for gc-screening (4L) were extracted with XAD-4 resin and eluted with diethylether.

Glucocorticoid and estrogenic activities were analysed with GR and ER CALUX bioassays [6]. Glucocorticoid activities were expressed as dexamethasone equivalents (ng dex-eq/L), estrogenic activities as estradiol equivalents (pg E2-eq/L). Target analysis for steroid hormones was performed by injection of the extract on LC-LTQ-FT-orbitrap MS and on UPLC-tQ-MS. Screening was performed on a GC-MSD operated in full scan mode (m/z 40-500). Mass spectra were compared with reference spectra in the NIST MS and INFOSPEC databases. Loads were calculated by multiplying measured concentrations with the river flow at the sampling dates and expressed as grams per day. To calculate the contribution of hormones to the measured activity,
concentrations were multiplied with their relative glucocorticoid or estrogenic potencies in the assay to derive activities caused by the presence of each individual compound.

3. Results and discussion

Glucocorticoid activity was detected in concentrations between <LOD (2) and up to 19 ng dex-eq/L (Fig. 1, left). Estrogenic activity was detected at concentrations between <LOD (0.006) and 3.5 ng E2-eq/L (Fig. 1, right). Both types of activities were detected at all locations, although not at all sampling dates. Glucocorticoid activity was found with comparable maximum concentrations both in Rhine and Meuse. Estrogenic activity was found in higher concentrations in the Meuse than in the Rhine. This reflects the differences in the way both rivers are influenced by anthropogenic activities. Results indicate that concentrations of both activities follow a seasonal pattern; with maximum values for both activities in spring and autumn. This is even more clear when expressed as loads.

![Figure 1: Glucocorticoid and estrogenic activities measured with the GR and ER CALUX bioassays in five locations in the Dutch catchment of the rivers Rhine and Meuse](image)

Glucocorticoid activity in waste water ranged between 0.01 and 0.6 μg dex-eq/L. Analytical methods were developed for over 30 steroid hormones, such as estrogens, glucocorticoids, androgens and progestagens. These methods were used to correlate measured concentrations to bioassay activity. In waste waters, glucocorticoid activity was predominantly explained by cortisol, cortisone, prednisone, prednisolone and triamcinolone acetonide[5]. The results in surface waters will also be discussed. In addition, GC-screening of the bioactive samples showed that the water samples from Rhine and Meuse consisted of complex and sometimes location specific mixtures of contaminants, including pesticides, pharmaceuticals, industrial solvents and flame retardants.

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References


Characterisation of dioxin-like compounds in road-side snow

Martine Muusse¹,², Gerard Cornelissen³, Ketil Hylland¹,², Katherine Langford¹, Knut Erik Tollefsen¹, Peter Haglund⁴, Kevin V. Thomas¹

¹ Norwegian Institute for Water Research (NIVA), Oslo, Norway; ² Department of Biology, University of Oslo, Norway; ³ Norwegian Geotechnical Institute, Oslo, Norway; ⁴ University of Umeå, Umeå, Sweden

E-mail contact: mmu@niva.no

1. Introduction

Snow is a useful tool to measure the accumulation of contaminants because it scavenges pollutants from the air due to its large specific surface area. In many cities in Norway, large amounts of snow are each year collected from the city’s streets and placed in a snow depot or dumped into the sea. The street snow is routinely analysed for heavy metals, oil and organic pollution such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs). We hypothesised that street snow may contain other unknown contaminants in addition to those analysed using a targeted approach. In the current study volatile and non-volatile dioxin-like contaminants were identified in snow samples collected from Oslo using an effect-directed analysis (EDA) approach. This was achieved by measuring the aryl hydrocarbon receptor (AhR) agonist levels in the melted and filtered snow samples using the chemically activated luciferase expression (CALUX) assay and subsequently attempting to identify the compounds responsible for the activity with broad-spectrum analytical chemical methods. In addition the black carbon (BC) content of the particles was analyzed since a large fraction of many organic compounds is often present in BC and BC can reduce the bioavailability and effects of AhR-active compounds.

2. Materials and methods

Snow was collected in Oslo, the capital of Norway in February 2009 (Table 1). In addition one background sample was collected in a ‘pristine’ mountain area. The samples were filtered and extracted using solid phase extraction. The particles on the filters were extracted using accelerated solvent extraction, cleaned-up on a multilayer silica gel column and separated into two fractions: a hexane fraction (F1) and a DCM fraction (F2). A sub-sample of particles was analysed for BC. Both the water fraction and the two particle fractions were analysed for AhR agonists using the CALUX assay and by gas chromatography coupled to high resolution time of flight mass spectrometry (GC-HR-ToF-MS) for broad-spectrum analysis and to target for PAHs.

<table>
<thead>
<tr>
<th>Location</th>
<th>Coordinates</th>
<th>Amount (l)</th>
<th>Weight (g)</th>
<th>TOC (%)</th>
<th>BC (%)</th>
<th>BC/TOC (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Water fraction</td>
<td>Particle fraction</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Suburban street (Oslo)</td>
<td>59º95’N</td>
<td>5</td>
<td>2.88</td>
<td>8.19</td>
<td>0.13</td>
<td>1.61</td>
</tr>
<tr>
<td>Ring road 2, (Oslo)</td>
<td>59º92’N</td>
<td>4.4</td>
<td>3.93</td>
<td>8.93</td>
<td>0.15</td>
<td>1.67</td>
</tr>
<tr>
<td>Main highway (E 18, Oslo)</td>
<td>59º67’N</td>
<td>5.02</td>
<td>7.51</td>
<td>12.08</td>
<td>0.17</td>
<td>1.37</td>
</tr>
<tr>
<td>Ring road 3 (Oslo)</td>
<td>59º94’N</td>
<td>5.23</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
<td>n.a.</td>
</tr>
<tr>
<td>Norefjell (mountain area)</td>
<td>60º21’N</td>
<td>9º61’O</td>
<td>2.86</td>
<td>n.d.</td>
<td>n.d.</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

n.a.: not analysed, n.d.: no data
3. Results and discussion

The AhR agonists levels (expressed as TEQ_{CALUX}) in the total water fraction were between 15-387 pg/l (Figure 1). Sample 1 had the lowest concentration of AhR agonists (15pg/l TEQ_{CALUX}) reflecting the location as a quiet suburban street outside the city’s outer ring road. The highest concentration of AhR agonists was in sample 3 (387pg/l TEQ_{CALUX}), collected next to a highway outside Oslo. An elevated AhR activity of 221pg/l TEQ_{CALUX} was also detected in sample 5, the reference sample collected in a mountainous area with little human activity. In the F1 particle fraction, where dioxins, furans and PCBs elute, TEQ_{CALUX} was <LOD. In the F2 particle fraction, where PAHs elute, TEQ_{CALUX} was between 1354-7389 pg/l, where the lowest activity was measured in the sample collected on the suburban street and the highest in the highway sample.

Figure 1, AhR agonist activity (pg/l TEQ_{CALUX}) in the different snow fractions,

![AhR agonist activity (pg/l TEQ_{CALUX}) in the different snow fractions](image)

One possible explanation for the elevated levels in the water fraction of the mountain sample could be the occurrence of BC in the snow samples collected from the city. All samples except the mountain sample, which was pristine white snow, were filtered before analysis. Dioxins and other contaminants are known to bind strongly to BC [1] and BC could possibly trap part of the contamination originally present in the snow, this could result in lower AhR agonist levels in the water fractions of the filtered city samples. A BC percentage of 0.13-0.15% was measured and a BC/TOC ratio of between 1.61-1.67% (see table 1), which is low compared to the average BC content found in sediment or soil (9% and 4% respectively) [1].

Of the 19 PAHs analysed using the TargetLynx® software, 10 were detected in the water fractions. Of these 10 PAHs only 2 showed induction [2] in the CALUX assay and the calculated induction equivalent factor (IEF) of these 2 compounds could explain 0.0008-0.04% of the TEQ_{CALUX}. In the particle F2 fractions 9 PAHs were detected of which 5 showed induction, explaining 2-9% of the total TEQ_{CALUX}. This highlights the fact that there are other unknown AhR agonists present in the snow samples, both in the water as in the particle fractions. Preliminary results from the GC-HR ToF MS showed a large amount of aromatic unresolved complex mixture (UCM) in the particle F2 fraction and there might be AhR agonists present in this UCM. Ongoing work utilising comprehensive gas chromatography coupled to mass spectrometry (GCxGC ToF MS) is focused on teasing out AhR agonists in this aromatic UCM.

4. Conclusions

Both water and particle fractions showed elevated levels of AhR agonists, although some of the activity in the particle F2 fraction might not be bioavailable due to the BC content in these samples. In both water and particles PAHs could explain part of the activity. TCDD/F and PCB analysis of these samples is necessary to understand more of where the elevated TEQ_{CALUX} levels come from. Further work will focus on more advanced broad-spectrum analytical techniques.

5. References


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Sewage epidemiology: potential of a novel approach

Ettore Zuccato, Sara Castiglioni

Department of Environmental Health, “Mario Negri” Institute for Pharmacological Research, Via La Masa 19
20156 Milan, Italy.
e-mail: ettore.zuccato@marionegri.it

1. Introduction

Sewage epidemiology is a new approach to population studies, which use wastewater analysis for assessing collective voluntary and involuntary exposure of the members of a community to a wide range of chemicals. Chemicals that can be investigated by this approach include those that are voluntarily ingested (pharmaceuticals, licit and illicit drugs) and those to whom subjects are involuntarily exposed to (for instance, chemical contaminants ingested with food and water, atmospheric pollutants which are inspired, personal care products which can be absorbed transdermally, and others).

2. Experimental

The rationale of this approach is that traces of almost everything we eat, smoke, drink, ingest, or absorb, are excreted with our urine or stool and end up in the sewage system. This is the basis of what we called “sewage epidemiology”; illicit drugs were the first application of this new branch of environmental epidemiology [1]. Residues of the illicit drugs consumed by a collectivity are excreted in wastewater and their levels, knowing kinetic, metabolism, and behaviour in wastewater, and characteristics of the sewage system, such as flow rate and population size, can be used to back-calculate for the type and amount of illicit drugs collectively consumed by a population (Figure 1) [2].

![Image of a diagram](https://via.placeholder.com/150)

*Figure 1. The pathway of the illicit drugs: from the consumer to the wastewater treatment plant (WWTP).*

However, this approach has a much wider range of potential applications. Practically every chemical our body enter in contact with, can be absorbed, metabolized and excreted with urine and stool in wastewater of an urban treatment plant [3]. By monitoring metabolites and parent compounds we can back-calculate for the amount of a given substance which has been collectively taken and absorbed by the subjects of a community.

This include for instance pharmaceuticals. We know the amount of a given drug which is prescribed or sold in a given area, but we don’t know how much is really taken by patients. Frequently, patients do not comply to the treatment and this is an important medical issue in the study of the effect of a treatment. Wastewater
analysis for the metabolites of the pharmaceuticals can help in elucidating the compliance of the patients to the treatment, contributing to evaluate its efficacy.

Other examples include pesticides, PCBs and food contaminants. Monitoring of the metabolites of these substances in wastewater is a novel approach to estimate the real exposure of the population to these chemicals, that can be important in the study of environment-related diseases.

Sewage epidemiology needs an extensive knowledge of kinetic, metabolism and excretion of the substance we study, a knowledge in medicine to understand potential applications, and a wide expertise in analytical chemistry. Wastewater is a complex milieu of thousands of different substances, dissolved, mixed or suspended in water. The list of compounds in wastewater is long: chemicals, from industrial or agricultural activities, and remnants derived from an enormous number of production and house activities end up in wastewater and contribute to increase the complexity of its composition, and thus the difficulties to detect specific target substances [4].

3. Conclusion

Sewage epidemiology needs therefore a multidisciplinary approach, with the interaction of experts of several different fields, such as pharmacokinetic, medicine, and analytical chemistry. Monitoring wastewater has the potential to extract useful epidemiological information from qualitative and quantitative profiling of biological indicators entering the sewage system and might become a useful tool to be used in population studies.

4. References

The overlooked importance of sampling to advance wastewater analysis from a promising method to a useful and reliable tool for the estimation of illicit drug abuse

Christoph Ort\textsuperscript{1}, Alex John Brewer\textsuperscript{2}, Caleb Banta-Green\textsuperscript{3} and Jennifer Field\textsuperscript{2}

\textsuperscript{1}The University of Queensland, Advanced Water Management Centre (AWMC), QLD 4072, Australia
\textsuperscript{2}Department of Environmental Toxicology, Oregon State University, Corvallis, Oregon 97331
\textsuperscript{3}Alcohol and Drug Abuse Institute, University of Washington, Seattle, Washington 98105
E-mail contact: c.ort@awmc.uq.edu.au

1. Introduction

Sewers were not designed to assess illicit drug consumption. However, immense advances in analytical chemistry have propagated wastewater analysis as a promising tool to tackle this difficult task. Numerous wastewater studies have been published over the past decade. Illicit drug concentrations and loads are compared among different locations and trends over time are evaluated (e.g. \cite{1}). Recent review articles determine the need for future research (e.g. \cite{2}). Despite being considered as superior over - or at least complementary to - conventional approaches (i.e. a variety of surveys or interviews), several aspects of the wastewater epidemiology method are known to be uncertain. The following list covers some factors that can lead to the uncertainty in estimated illicit drug loads: 1) metabolism/excretion, 2) transformation in sewers, 3) sampling (collection and preparation), 4) precision and accuracy of the chemical analysis, 5) flow measurements (to determine mass loads), and 6) number of people in the catchment (to normalise mass loads).

To quantify the illicit drug consumption in an individual city or community and to avoid the uncertainty surrounding potential biodegradation in wastewater treatment plants (WWTPs), sample collection is not in rivers or effluents of WWTPs anymore but in influents, i.e. from sewers; in some cases even further ‘upstream’ within sewers, closer to the source, as in the effluents of specific facilities (e.g. prisons \cite{3}). In the review by van Nuijs et al. \cite{2} the actual, physical sample collection is not mentioned explicitly as a factor that determines uncertainty in the wastewater epidemiology method.

Relevant sampling guidelines, specifically to determine (waste)water quality (e.g. \cite{4-6}), have existed long before the idea of applying wastewater analysis to determine illicit drug loads. It is known that wastewater streams are not homogeneous and can vary quickly in quantity and quality. It is the experimenters’ responsibility to adapt recommendations to their specific cases, accounting for the site specific environment and dynamics. Yet, a review of papers applying the wastewater epidemiology method reveals that the published sampling recommendations are not heeded \cite{7}. Furthermore, insufficient details on i) the dynamics in the sewers under investigation (including influents to WWTPs), ii) the exact sampling location and iii) the sampling mode and frequency, make it difficult if not impossible to properly evaluate the trueness and precision of published data. It has been shown that sampling artefacts of 24-hour composite samples can be anywhere between “not significant” to “100% or more” for legal drugs \cite{8} and hence can exceed uncertainty due to chemical analysis by far. Excreted by humans, flushed down the drain and collected in the same sewers, it is expected that this also holds true for illicit drugs.

2. Materials and methods

To experimentally assess the dynamics in a complex drainage system, grab samples need to be collected at high temporal resolution (e.g., on the time scale of minutes since urine ‘pulses’ containing illicit drugs are only 2-5 minutes in duration). Corvallis WWTP (Oregon, USA) is fed by open channel gravity flow sections and also by intermittently-operated pump stations and pressurised mains. After combining all trunk sewers from different sub-catchments into one inlet pipe, the wastewater is being lifted at the WWTP’s head. The pump is controlled to keep a constant water level in the pump sump resulting in variable flow rates. Samples were collected after this first lift station and after the screens. Detailed investigations of the pump stations distributed in the catchment and the knowledge on the expected variability in the gravity flow sections \cite{8} revealed that no significant variations below five minutes are expected. A conservative sampling frequency of two minutes was therefore deemed appropriate. Over a time period of two hours, 120 samples were collected in 50 mL HDPE centrifuge tubes, kept on ice before and during transport and stored at -20°C. All samples were individually analysed according to the method described in Chiaia et al. \cite{9}.
3. Results and discussion

The 120-sample time series reveals a unique pattern for 11 legal and illicit drugs and select metabolites. To our knowledge it is the first data set at high temporal resolution for illicit drugs and select metabolites in sewers. It becomes evident that both concentrations and mass loads are subject to high short-term fluctuations. Consequently, to obtain a representative 24-hour composite sample, a flow-weighted sampling mode with a high sampling frequency is necessary. Obviously, only one pooled sample will require analysis, reducing the effort to determine a truly representative 24-hour load of illicit and legal drugs and their metabolites.

General recommendations to sample in other catchments: To avoid sampling biases the sampling mode should always take flow variations into account (ideally flow-proportional or as a second choice volume-proportional, see [7]). In a gravity sewer system, the appropriate sampling frequency can be determined according to [8] if the number of users in the catchment - and hence related toilet flushes containing the substances of interest - were known as for pharmaceuticals. One of the main points is that we do not know the number of users of illicit drugs and that is why we do wastewater analysis. Therefore, we recommend to apply a precautionary high sampling frequency in the range of five minutes or shorter (see sampling guide in [8]). In a pressurised sewer system, the on- and off-times of pumps distributed in the catchments will give you an idea of what a suitable sampling frequency is. As a rule of thumb, we recommend a sampling frequency to obtain at least two to three samples for the shortest wastewater pulse to be expected from any pump. In a mixed sewer system (gravity flow and pressurised sections), it is not straight forward to determine the optimal sampling frequency. We therefore recommend opting again for a precautionary high sampling frequency, or in the best case, for a continuous flow-proportional sampling setup (relatively inexpensive to implement for short sampling campaigns).

4. Conclusions

Besides chemical analysis and factors associated with the back-calculation, sampling is the first, and one of the most crucial factors for the estimation of illicit drug abuse at the community level. Sampling can be actively influenced and drastically improved at relatively low cost to maximise data quality. Traditional sampling equipment was designed to sample for bulk parameters such as suspended solids and nutrients etc., but not for illicit drugs. If these sampling methods are not appropriately adapted, the wastewater epidemiology method is prone to systematic biases (inappropriate sampling mode) and random errors (inappropriate sampling frequency); hence it may not be more accurate and reliable than traditional surveys/interviews. Sophisticated chemical and statistical analyses simply cannot make up for any deficiencies in sampling.

5. References


Acknowledgement - The authors thank Guy Allan from the WWTP Corvallis for his competent, friendly and prompt support.
1. Introduction

Most illicit drugs are chiral compounds. Among them are plant-derived substances (e.g. cannabis, cocaine and heroin) and synthetic drugs (e.g. amphetamine, methamphetamine and related designer drugs). Their enantiomers reveal different potency and are often characterised by stereoselective disposition in the body. R,R(+)-LSD is for example over 20 times more psychoactive than (-)-LSD. Cocaine, similarly to heroin, naturally occurs in the form of 1R,2R,3S,5S(-)-cocaine. (+)-Cocaine (the unnatural enantiomer) is inactive. Both the metabolism and toxicity of (+-) and (-)-cocaine were found to be stereoselective. In cannabinoids, the natural delta-1-THC and delta-6-THC have a (3R,4R) configuration and a negative rotation. Synthetic (+)-isomers are much less active, e.g. (+)-delta-1-THC is ca 13 to 230 times less active than the (-)-isomer in cannabinimetic activity. Amphetamines are characterised by one asymmetric carbon centre and exist in the form of two enantiomers, which significantly differ in potency, e.g. S(+)-amphetamine has twice as high stimulant activity as R(-)-amphetamine. However, R(-)-amphetamine has been reported to be as effective as the S(+)-enantiomer in the development of the psychotic syndrome. MDMA is used as a racemate, although similarly to amphetamine its S(+)-enantiomer is much more potent as a CNS agent than R(-)-MDMA. The phenomenon of the chirality of amphetamine is crucial in forensic identification of its illicit use. This is because amphetamine and methamphetamine have some limited therapeutic use in narcolepsy and attention deficit hyperactivity disorder, but most are manufactured in clandestine laboratories. Amphetamine is also formed as a metabolite of methamphetamine and several prescription drugs such as selegiline.

The aim of this presentation is to raise awareness of the importance of the phenomenon of chirality in forensic estimation of drugs abuse using the sewage epidemiology approach. The report will present results obtained during a ten month long monitoring programme of several WWTPs in the UK. To the authors' knowledge this is the first report tackling the phenomenon of chirality in the estimation of drugs use using a sewage forensics approach. Among the studied chiral drugs are: amphetamines (R/S(±)-amphetamine, R/S(-)-methamphetamine, R/S(-)-MDMA, R/S(-)-MDEA and R/S(-)-MDA) and ephedrines (1R,2S(-)-ephedrine, 1S,2R(+)-ephedrine 1S,2S(-)-pseudoephedrine, 1R,2R(-)-pseudoephedrine).

2. Materials and methods

The following reference standards were used: R(-)-amphetamine, S(+)-amphetamine, R/S(-)-amphetamine, R(-)-methamphetamine, S(+)-methamphetamine, 1R,2S(-)-ephedrine, 1S,2R(-)-ephedrine 1S,2S(-)-pseudoephedrine, 1R,2R(-)-pseudoephedrine, R/S(±)-MDMA, R/S(±)-MDEA and R/S(±)-MDA and R/S(±)-norephedrine. Deuterated surrogate/internal standards included: R/S(±)-amphetamine-d11, R/S(±)-methamphetamine-d14, R/S(±)-MDMA-d5, R/S(±)-MDEA-d5, R/S(±)-MDA-d5 All standards were purchased from LGC Standards (Teddington, UK) and Sigma-Aldrich (Gillingham, UK)

The analysis of chiral drugs of abuse was undertaken with the usage of chiral liquid chromatography coupled with tandem mass spectrometry. Waters ACQUITY UPLC™ (Waters, Manchester, UK) system was used for the separation of analytes. Chiral-CBH column, 100x2mm, 5µm and Chiral-CBH 10x2.0mm guard column (Chromtech, Congleton, UK) were used for the separation of enantiomers of chiral drugs. A TQD (triple quadrupole) mass spectrometer (Waters, Manchester, UK) equipped with an electrospray ionisation source was used for illicit drugs quantification. Mass spectrometry analyses were performed in the multiple reaction monitoring mode, measuring the fragmentation of the protonated pseudo-molecular ions of each chiral drug. Solid-phase extraction on Oasis HLB sorbents (60mg, 3mL, Waters, UK) was used for sample preparation.

Wastewater samples were collected from seven different wastewater treatment plants over the period of ten months (December – August). The relative concentration of enantiomers of chiral drugs was expressed as the enantiomeric fraction (EF) and was calculated with the following equation:

\[
EF = \frac{E1}{E1 + E2}
\]
where $E1$ and $E2$ are peak areas for the first ($E1$) and the last ($E2$) enantiomer of a chiral drug eluting from the CBH column (see Figure 1). $EF$ equals 1 or 0 in the case of single enantiomer form and 0.5 in the case of racemate. In the case of amphetamine and methamphetamine, $E1$ and $E2$-enantiomers were identified as $R$(-) and $S$ (+)-enantiomers respectively.

3. Results and discussion

The following chiral drugs were studied in wastewater: amphetamines ($R/S$-(±)-amphetamine, $R/S$-(±)-methamphetamine, $R/S$-(±)-MDMA, $R/S$-(±)-MDEA and $R/S$-(±)-MDA) and ephedrines ($1R,2S(-)$-ephedrine, $1S,2R(+)$-ephedrine $1S,2S(+)$-pseudoephedrine, $1R,2R(-)$-pseudoephedrine,). Among drugs commonly present in wastewater were: $1R,2S(-)$-ephedrine, $1S,2S(+)$-pseudoephedrine, amphetamine, MDMA, MDA.

The monitoring programme aiming at the verification of the enantiomeric composition of chiral drugs revealed that these chemicals are not released into wastewater in the form of racemic mixtures. It was for example observed that in the case of amphetamine, $R$(-)-enantiomer was dominant in all analysed wastewater samples (Fig. 1). It was also noted that enantiomeric ratios of amphetamine enantiomers differed significantly between sampling points and sampling times and varied from 0.53 to 0.84. Similar patterns were observed in the case of other studied chiral drugs. There are several possible reasons for this behaviour. Among them are: (i) different metabolism patterns of enantiomers of the same drug with preferential metabolism of one enantiomer only, (ii) formation of illicit drugs as a result of metabolism of legally prescribed drugs (e.g. selegiline leads to the formation of $R$(-)-amphetamine and $R$(-)-methamphetamine), or (iii) use of drugs in non-racemic forms of drugs (e.g. medical use of one active enantiomer of $S$ (+)-amphetamine only). Although a complex and demanding process, the verification of enantiomeric ratios can provide vital information about patterns of drugs usage and can help in the differentiation between their legal and illicit usage.

![Figure 1: Enantiomeric fractions of amphetamine in wastewater samples](image)

4. Conclusions

The main aim of this report is to increase understanding of the phenomenon of chirality and its possible application for the accurate estimation of the abuse of drugs in local communities using a sewage epidemiology approach.

5. References


Analysis and Interpretation of Specific Ethanol Metabolites in Sewage Effluent for the Quantitative Measurement of Regional Alcohol Consumption

Malcolm J. Reid¹, Katherine H. Langford¹, Jørg Mørland², Kevin V. Thomas¹

¹Norwegian Institute for Water Research, Gaustadalleen 21, Oslo, NO-0349 Norway
²Norwegian Institute of Public Health, Postbox 4404 Nydalen, 0403 Oslo, Norway
E-mail contact: malcolm.reid@niva.no

1. Introduction

Estimates of the average alcohol consumption in a regional population are an important public health measure as there is evidence to suggest that the percentage of heavy drinkers in a population is related to the average consumption of the general population [1, 2]. Consumption per capita can be deduced from alcohol sales statistics, but such data are not always available or reliable [3, 4]. Additional methods are therefore required to characterise the level of consumption in a population.

Ethyl sulphate (EtS) and ethyl glucuronide (EtG) are excreted in urine following the consumption of alcohol, and as such are useful biomarkers for the identification or confirmation of acute alcohol consumption [5, 6]. The present study reports a novel ion-exchange mediated chromatographic method for the quantitative measurement of ethyl sulphate (EtS) and ethyl glucuronide (EtG) in sewage effluent, and presents a novel calculation method for the purposes of relating the resulting sewage concentrations with rates of alcohol consumption in the region. Estimates are compared with alcohol related sales statistics in order to confirm if this sewage epidemiology technique can be used for the assessment and estimation of alcohol consumption in a large urban centre.

2. Materials and methods

Sampling was performed at the inlet to a sewage treatment plant in Oslo, Norway. This plant processes sewage from a metropolitan and suburban population of approximately 500 000 people. An Isco 6712 portable automatic wastewater sampler (Teledyne, Nebraska USA) was used to collect 6-hour composite sewage effluent samples with sample mid-points corresponding to 2am, 8am, 2pm and 8pm throughout the course of September, 2009. An aliquot (1mL) of each sample was transferred to 1.5mL microcentrifuge tubes and spiked with deuterated internal standards (5 µL of ethyl dulfate-d5 and ethyl-d5 glucuronide at 10 µg/mL). Sample cleanup was via centrifugation at 20,000 G for 10 minutes, and the supernatant was analysed by Liquid Chromatography (Tandem Quadrupole) Mass Spectrometry (LCMS/MS).

Chromatographic separation of the analytes was performed on an Acquity UPLC System (Waters Milford, USA) using an Acquity UPLC BEH C8 column (1.7 um, 50mm x 2.0mm) at 50°C. A dihexylammonium acetate (7mM) in a methanol-water gradient provided the optimum conditions since it offered adequate chromatographic retention without inhibiting the sensitivity of the mass spectrometer (Quattro Premier XE, Waters Corp. Milford, USA).

3. Results and discussion

Ethyl sulphate (EtS) and ethyl glucuronide (EtG) were detected in sewage effluent samples. EtS was present in all samples at concentrations ranging from 2 µg/L to 31 µg/L (Figure 1). EtG was found to be unstable in sewage effluent and is therefore unsuitable for use as an alcohol biomarker in sewage epidemiology studies.

Large variation in the concentration of EtS was observed over the course of the 25 day sampling campaign, and trends are easily identified (Figure 1). Results show that 61% of the weekly alcohol consumption occurs on Friday and Saturday. Alcohol consumption for Sunday, Monday, Tuesday, Wednesday and Thursday each only account for 6 - 11% of the weekly total.

Statistics from the sale of alcoholic beverages [7] indicate that consumption of alcohol in the Oslo region averages approximately 6750 kg/day (absolute alcohol, excluding illegally manufactured “home brew” and imported tax-free alcohol). Measured mass-flow of ethyl sulphate in sewage effluent is equivalent to an estimated alcohol consumption of 4900 - 7800 kg/day (absolute alcohol) and is therefore in excellent agreement with national alcohol sales statistics.
4. Conclusions

Sewage epidemiology assessments of urinary biomarkers for alcohol can provide valuable information on the scale of alcohol consumption, and investigation into the kinetics of the flow of these compounds in the sewage can also identify detailed trends in the consumption patterns over time. Such data could be useful in measuring the effects of campaigns to change alcohol intake in populations.

5. References


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Use of legal and illegal drugs in communities: Methodological considerations for generating annual estimates of drug excretion based upon municipal wastewater sampling

Caleb J. Banta-Green1, Alex John Brewer2, Christoph Ort3 and Jennifer Field2

1 Alcohol and Drug Abuse Institute, University of Washington, Seattle, Washington 98105
2 Department of Environmental Toxicology, Oregon State University, Corvallis, Oregon 97331
3 The University of Queensland, Advanced Water Management Centre (AWMC), QLD 4072, Australia
E-mail contact: calebbg@uw.edu

1. Introduction

Municipal sewage based testing for medications and drugs of abuse is in its infancy. The ability to document the presence of substances has been shown repeatedly around the world [1 2 3], however the utility of these data is limited until we understand how to reliably gather samples to generate estimates for a single day or for an entire year [4]. Current efforts to interpret results are very limited due to the lack of confidence bounds around estimates. Without these confidence bounds it is not possible to indicate whether excretion of a substance is: higher than another substance, higher than a prior estimate for the same substance, or different compared to another municipality.

The ability to utilize sewer derived data for practice and policy decision making is important as drug abuse continues to have major public health consequences. Differential impacts of drugs are evident among and within municipalities/regions. Current methods for drug surveillance are crude [5]. Drug abuse epidemiology currently relies on indicators that are limited by: 1) poor geographic detail, 2) an emphasis on major metropolitan areas 3) significant time lag in data availability, 4) detection and self-report biases, 5) measurement error, and 6) an over reliance on morbidity and mortality data.

2. Materials and methods

To address these methodological issues we undertook a year long sampling campaign in 18 cities in the Northwest region of the United States. Diverse municipalities were chosen for their variable population size, commuting flows, urban or rural location, weather, and resident characteristics. A time-based, stratified random-sampling approach was utilized that accounted for both seasonality and inter-week variation. A total of 13 samples each quarter were obtained on random days of the week, resulting in a total of 52 samples over the course of a year per location.

Each wastewater treatment provided 24 hour composite samples. The approach to compositing varied by wastewater treatment plant (WWTP) both in the time intervals as well as whether flow or volume proportional sampling was utilized. Samples were acidified and shipped for analysis. Large volume injection LC-ESI/MS/MS was used to identify and quantify illicit drugs and metabolites [6]. Compounds studied included methamphetamine, 3,4-methylenedioxymethamphetamine (i.e. MDMA, Ecstasy), caffeine, cocaine and its metabolite benzoylecgonine, nicotine and its metabolite cotinine. Additionally, several pharmaceutical opioids are reported including methadone, oxycodone, and hydrocodone.

Descriptive statistics were conducted and different methods for accounting for non-quantifiable results, e.g. “no detects” and below the level of quantification, were explored. Parametric and non-parametric test statistics were utilized to determine efficient sampling approaches for different compounds as well the most appropriate ways to validly account for variability.

3. Results and discussion

Data resulting from more than 900 tested samples provide insights into how to design sampling plans that account for day of the week, and longer periods, for stimulant drugs of abuse and opioid pharmaceuticals. The concentrations and index loads (accounting for wastewater flow and population size) of compounds varied substantially. Certain compounds, such as methamphetamine, were nearly ubiquitous with measurable levels in most places and on most days, while other compounds such as MDMA were measured at detectable levels on about half of all days, though the frequency of detection varied by city characteristics. Methadone, an opioid medication that is to be used daily for several medical indications, is an important compound as it is useful in understanding the impacts of sampling and population variability.
Compounds consistently measured at detectable levels without variability in index loads by day of the week, such as methamphetamine, may be sampled less than weekly and without regard to the day of the week. In contrast, compounds that are infrequently measured at detectable levels and that exhibit an association with weekend use, such as MDMA, may need to be sampled more frequently to obtain a reliable estimate of annual consumption or somewhat less frequently if the modal temporal period of use (e.g. weekend) is of greatest interest. An initial sampling campaign for a particular municipality may be necessary to determine the optimal sampling plan for specific substances. Variability in data, and resulting confidence in the results, is impacted by the specific compound of interest, the type of composite sampling utilized by WWTPs and the population characteristics of the municipality.

4. Conclusions
Determining sewer derived population estimates for drugs excreted appears to be a worthwhile endeavor and has the potential to provide data of sufficient quality to inform practice and policy decisions. However, it does have limitations due to variability that limits the precision of estimates. This variability differs for specific compounds based upon the chemical properties of the compound as well as the consumption pattern(s) for a community. Additionally, community characteristics and the WWTPs’ methods for generating 24 hour composite samples influence index loads and variability. Future sewer based community estimates of drug excretion should be explicit about how samples were obtained. Researchers should also be explicit about the variability of estimates and ensure that variability is accounted for when making conclusions about the level or trends in substance use.

5. References

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Illicit drugs in prisons: sewage epidemiology to evaluate use and trends.

Cristina Postigo¹, Nicola Mastroianni¹, Miren López de Alda¹, Damià Barceló¹,²

¹Institute of Environmental Assessment and Water Research, (IDAEA, CID-CSIC), Department of Environmental Chemistry, C/ Jordi Girona, 18-26, 08034 Barcelona, Spain.
²Catalan Institute for Water Research (ICRA), Parc Científic i Tecnològic de la Universitat de Girona, Edifici H₂O, 17003 Girona, Spain
E-mail contact: dbcqam@cid.csic.es

1. Introduction

The relationship between illegal drug use and offence commitment, although it is not direct and simple, is unquestionable. Different strategies for detoxification and rehabilitation and to avoid drug supply in prisons are carried out by prison authorities worldwide; however, drug consumption in prisons is still remarkable. Evaluation of the success of such programs requires fast and economic tools to monitor illicit drug use, such as the sewage epidemiology, which consists of backcalculating collective drug use from the levels of drug residues found in sewage water.

The present work describes for the first time ever the application of the sewage epidemiology approach in a prison to assess and quantify consumption of both illicit substances, such as cocaine, heroin, cannabis, amphetamine, ecstasy or MDMA, methamphetamine and LSD, and prescribed drugs, such as alprazolam, ephedrine and methadone. The main objective of the study was to evaluate the suitability of this tool to monitor trends of illicit drug usage in prisons or in similar facilities.

2. Materials and methods

Up to 19 drugs and metabolites belonging to 6 different chemical classes (coca inics, cannabinoids, opioids, amphetamine-like compounds, lysergic compounds, and benzodiazepines) were monitored in sewage waters. 42 sewage water samples in total were collected as 24-hour composite samples from June 2008 to January 2009 at the entrance of the sewage treatment plant (STP) that gives service exclusively to the penal complex under investigation. After reception, sewage water samples were vacuum filtered and stored at -20ºC in the dark until analysis.

Drug residues were measured in the collected samples by means of on-line solid phase extraction-liquid chromatography-tandem mass spectrometry (on-line SPE-LC-MS/MS). The methodology applied was a variation of a previously described and validated fully automated method [1].

Only a few of the investigated analytes were selected as drug consumption indicators, as it is shown in Table 1; whereas the rest were used to confirm drug usage. Levels (ng/L) of consumption indicators found in sewage water samples were normalized across the prison population (3500 people) and the STP effluent flow-rate measured each day (m³/day). These values were corrected by a factor that takes into account the average metabolic excretion rate of a dose after the most frequent route of intake and the molar mass ratio between the consumed drug and the selected consumption indicator (see Table 1).

Table 1. Drug residues used as drug consumption indicators in the back-calculation process, and respective average excretion rates, molar mass ratio between the drug and the consumption indicator, correction factor and average drug dose.

<table>
<thead>
<tr>
<th>Drug residue (Drug)</th>
<th>Drug residue excretion rate (%)</th>
<th>Molar mass ratio (Drug/Drug residue)</th>
<th>Correction factor: (molar mass ratio/excretion rate)*100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzoylecgonine (Cocaine)</td>
<td>45</td>
<td>1.05</td>
<td>2.3</td>
</tr>
<tr>
<td>THC-COOH (Cannabis)</td>
<td>2.5</td>
<td>0.91</td>
<td>36.4</td>
</tr>
<tr>
<td>Methadone (Methadone)</td>
<td>27.5</td>
<td>1.00</td>
<td>3.6</td>
</tr>
<tr>
<td>6-acetylmorphine (Heroin)</td>
<td>1.3</td>
<td>1.13</td>
<td>86.9</td>
</tr>
<tr>
<td>Ephedrine (Ephedrine)</td>
<td>75</td>
<td>1.00</td>
<td>1.3</td>
</tr>
<tr>
<td>MDMA (MDMA)</td>
<td>26</td>
<td>1.00</td>
<td>3.9</td>
</tr>
<tr>
<td>Amphetamine (Amphetamine)</td>
<td>30</td>
<td>1.00</td>
<td>3.3</td>
</tr>
<tr>
<td>Methamphetamine (Methamphetamine)</td>
<td>43</td>
<td>1.00</td>
<td>2.3</td>
</tr>
<tr>
<td>LSD (LSD)</td>
<td>&lt;1</td>
<td>1.00</td>
<td>&gt;100.0</td>
</tr>
<tr>
<td>Alprazolam (Alprazolam)</td>
<td>20</td>
<td>1.00</td>
<td>5</td>
</tr>
</tbody>
</table>
3. Results and discussion

Daily use was only observed for methadone (13.7 g.day\(^{-1}\) on average), ephedrine (4.0 g.day\(^{-1}\) on average), cannabis (3.4 g.day\(^{-1}\) on average), cocaine (1.1 g.day\(^{-1}\) on average) and alprazolam (0.5 g.day\(^{-1}\) on average), while only sporadic consumption of heroin, ecstasy, methamphetamine and amphetamine took place. LSD was occasionally found in the analysed sewage waters. Since LSD levels were considered to be too low to be derived from consumption, these findings were attributed to other reasons, but consumption.

![Figure 1](image-url)  
*Figure 1: Estimated use (g.day\(^{-1}\)) of cocaine, cannabis, methadone, alprazolam and ephedrine in the prison: a) during the studied period and b) throughout the week.*

Figure 1 shows the consumption trends of those drugs used on a daily basis. In the light of these results, different conclusions regarding the consumption of these drugs could be drawn, such as day of the week with the highest consumption and patterns of consumption throughout the week and during the whole period of study. Methadone and alprazolam showed a relatively stable consumption throughout the studied period and they were less consumed on Sunday, which may be linked to the weekend permits of prison inmates. An upward trend was observed in the consumption of cannabis, cocaine and ephedrine during the period of study, the latter probably linked to an increasing pharmaceutical treatment of asthma and bronchitis-related diseases.

The amount of doses/day/1000 people were also calculated by using average drug doses for each drug. Comparison of consumption data obtained in the prison with those obtained in a study performed in Barcelona [1] showed that all investigated drugs, but ephedrine and cannabis, are consumed to a higher extent in the city, by the general population. The same conclusion is achieved if the average cocaine use in the prison is compared with that obtained for other Spanish localities and for other European cities.

4. Conclusions

This study points out the sewage epidemiology approach as a useful, economic and fast tool to monitor drug usage in prisons or similar facilities having an STP associated or an accessible collector system. This approach still suffers from bias that need to be investigated and refined to achieve more accurate results. However, it is an irrefutable tool to test the efficiency of measures adopted to prevent drug use. The application of the sewage epidemiology approach to sewage waters from a prison showed that contrary to society perception, moderate or at least lower illicit drug consumption takes places among the prison inmates compared to the general population from a nearby city.

5. References


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Estimation of illicit drugs consumption by wastewater analysis: a five years-long investigation in Italy

Sara Castiglioni, Renzo Bagnati, Manuela Melis, Ettore Zuccato

Department of Environmental Health Sciences, “Mario Negri” Institute for Pharmacological Research, Via La Masa 19, 20156 Milan, Italy.

*Email contact: sara.castiglioni@marionegri.it

1. Introduction

The idea that wastewater analysis could be employed to estimate drug consumption in a community was tested for the first time by our group in 2005 [1] using cocaine as a case study. This novel method, being part of a wider approach called sewage epidemiology, is based on the direct measurement of illicit drugs residues (parent compounds or urine metabolites) excreted with the urine of the consumers in urban wastewater, and the subsequent back-calculation of the local drug consumption from the measured levels. The first investigation was later extended by our group to the most common drugs of abuse, i.e. cocaine, opioids, amphetamines, and cannabis [2] and reproducible and characteristic profiles of illicit drug consumption were obtained by the analysis of wastewater in three European cities [3]. In 2008, the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA) started to consider wastewater analysis as a new tool to monitor the use of illicit drugs in a population [4], since this method is able to provide objective estimates of drug consumption in real time. More recently, the same method has been applied in several European countries (Italy, Spain, Belgium, UK, Switzerland) and in the USA. Drug consumption estimates obtained by wastewater analysis were in agreement with the existing prevalence data from epidemiological studies [5], thus demonstrating the reliability and potential of this methodology.

The aim of this work was to investigate the pattern of drug consumption in several cities in Italy by wastewater analysis. A five years-long study allowed the identification of local differences and changing trends in drug consumption, providing reliable and updated estimates of cocaine, cannabis, amphetamines and heroin consumption.

2. Materials and methods

Illicit drugs were measured in urban wastewater using a specific multiresidue analytical method [2]. Twenty four-hours composite samples were collected at the entrance of wastewater treatment plants (WWTPs) in large and medium size cities in Italy during successive campaigns from 2006 to 2010. Water samples were filtered, enriched with internal standards, solid-phase extracted and analyzed by liquid chromatography-tandem mass spectrometry (LC-ESI-MS/MS).

Drug consumption was estimated directly from the levels measured in wastewater following the scheme reported in Figure 1. The specific target residues selected to estimate drug consumption were the main urinary metabolites for cocaine, heroin and cannabis and the unchanged parent drug for amphetamines. The target residues concentrations were multiplied by the wastewater flow rate to obtain the load of each target residue entering the WWTPs every day. The total amounts and the number of doses of drugs consumed collectively by the population served by a WWTP were then extrapolated from the loads considering first the human metabolism, and then the average dose of each substance.

![Figure 1: Scheme of the methodology used to estimate drug consumption in a population.](image)

3. Results and discussion

3.1. Estimates of drug consumption in Italy

Wastewater analysis was employed to estimate drug consumption in several cities in Italy located across the country. The cities selected for this investigation were: Milan, the largest city in northern Italy (>1.000.000
inhabitants), Como and Mozzanica, two relatively small (100,000 inhabitants) and closed cities in northern Italy, and four cities (20,000-300,000 inhabitants) in Sardinia, an island in central Italy.

The most used drug was cannabis and its estimated amounts entering the WWTPs daily ranged between 3-3.5 kg/d in Milan and between 100 g/d and 1 kg/d in the other cities. The estimated amounts of cocaine were of about 600 g/d in Milan and lower than 60 g/d in the other cities, while those of amphetamines and heroin were generally lower and ranged between few g/d in the small cities to about 100 g/d in Milan.

Seven days-long sampling campaigns were conducted in all the cities to study the weekly pattern of consumption of the selected drugs. The weekly consumption profiles were very similar in all the cities showing, as expected, an increase in cocaine and amphetamines consumption over the weekend, and a stable consumption of cannabis and heroin through the week.

The estimated amounts were then converted to the number of doses/day/1000 inhabitants allowing a comparison of drug consumption among the different cities investigated. Except cannabis, which was highly consumed in all the cities investigated (20-33 doses/d/1000 inhabitants), the other drugs presented peculiar patterns of consumption. For instance, cocaine use was high in Milan, Mozzanica and Como (4-6 doses/d/1000 inhabitants), and much lower in Sardinia (1.7 doses/d/1000 inhabitants). Methamphetamine was used mostly in Milan and Mozzanica (3 doses/d/1000 inhabitants), while its use was very low in Como (0.6 doses/d/1000 inhabitants) and Sardinia (0.01-0.3 doses/d/1000 inhabitants). Finally, the use of heroin was higher in Como (4 doses/d/1000 inhabitants) and in Sardinia (2.5 doses/d/1000 inhabitants). These estimates were generally in line with the national prevalence data from epidemiological studies, but provided also additional information on the local patterns of consumption.

### 3.2. Trends in drug consumption in a five years-long investigation

The trends of drug consumption estimated by wastewater analysis were studied in Milan in a five years-long investigation. The estimated amounts (g/day) of cocaine, methamphetamine, heroin and cannabis consumed in Milan in 2006-2010 are reported in table 1.

<table>
<thead>
<tr>
<th>Illicit drugs consumed (g/day)</th>
<th>Milan 2006 (two weeks monitoring)</th>
<th>Milan 2008 (five weeks monitoring)</th>
<th>Milan 2009 (five weeks monitoring)</th>
<th>Milan 2010 (two weeks monitoring)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cannabis</td>
<td>3533±480</td>
<td>3142±912</td>
<td>3343±961</td>
<td>3032±597</td>
</tr>
<tr>
<td>Cocaine</td>
<td>1094±183</td>
<td>1148±220</td>
<td>615±119</td>
<td>620±71</td>
</tr>
<tr>
<td>Heroin</td>
<td>78±32</td>
<td>80±31</td>
<td>30±20</td>
<td>48±16</td>
</tr>
<tr>
<td>Methamphetamine</td>
<td>15±5</td>
<td>31±12</td>
<td>54±23</td>
<td>127±24</td>
</tr>
</tbody>
</table>

Table 1: Illicit drugs consumption estimated by wastewater analysis in Milan from 2006 to 2010.

Daily cannabis consumption was stable along all the investigated period. Cocaine and heroin consumption did not change in Milan from 2006 to 2008, but fell in 2009-2010. The same result was obtained for these substances in the city of Como during a two years-long investigation conducted in 2008-2009. On the contrary, methamphetamine consumption, which had already risen in Milan from 2006 to 2008, rose further in 2009 and 2010. These results show that wastewater analysis is a suitable tool to identify rapidly changing habits or new trends of drug use in a population.

### 4. Conclusions

This investigation allowed to monitor the pattern of drug consumption in Italy and to highlight local differences. Moreover, successive investigations conducted from 2006 to 2010 in Milan allowed the identification of changing habits in drug consumption that might reflect a decrease in consumers’ money supply caused by the economic crisis. The extension of our investigation at a national level confirmed wastewater analysis as a suitable tool to produce objective and updated estimates of drug consumption in a defined population with the unique ability to identify local patterns of consumption and changing habits. Due to these peculiar features, wastewater analysis has the potential to be adopted to complement epidemiological studies, and to test the efficacy of different preventive interventions.

### 5. References

The consumption of illicit drugs in Brussels (Belgium) through sewage epidemiology

Alexander L.N. van Nuijs¹, Jean-François Mougel², Isabela Tarcomnicu¹, Lieven Bervoets³, Ronny Blust³, Philippe G. Jorens⁴, Hugo Neels¹,⁵, and Adrian Covaci¹,³

¹ Toxicological Centre, Department of Pharmaceutical Sciences, University of Antwerp (UA), Universiteitsplein 1, 2610 Antwerp, Belgium
² Aquiris Wastewater Treatment Plant Brussels-North, Vilvoordsealaan 450, 1130 Brussels, Belgium
³ Laboratory for Ecophysiology, Biochemistry and Toxicology, Department of Biology, University of Antwerp (UA), Groenenborgerlaan 171, 2020 Antwerp, Belgium
⁴ Department of Clinical Pharmacology/Clinical Toxicology, University of Antwerp (UA), University Hospital of Antwerp, Universiteitsplein 1, 2610 Antwerp, Belgium
⁵ Laboratory of Toxicology, ZNA Stuivenberg, Lange Beeldekensstraat 267, 2060, Antwerp, Belgium

E-mail contact: alexander.vannuijs@ua.ac.be

1. Introduction
In order to better counter the abuse of illicit drugs, the development of alternative approaches to estimate illicit drug consumption have been recently encouraged by the European Monitoring Centre for Drug and Drug Addiction [1]. Sewage epidemiology, which assumes that influent wastewater can be regarded as a pooled urine sample of a large population, could be one of those approaches. Recently, this methodology has gained in interest [2]. In the present study, an one-year sampling campaign (235 samples) was conducted in the Brussels-North wastewater treatment plant and sewage epidemiology was applied for cocaine (COC), amphetamine (AMP), methylenedioxymethamphetamine (MDMA), methamphetamine (METH), heroin (HER), and methadone (MTD). Back-calculations (from concentrations to amount of used illicit drug) were refined: 1) based on new insights in the stability of the measured compounds and on the excretion pattern of illicit drugs and 2) through the inclusion of real-time estimates of the number of inhabitants in the catchment area of the WWTP.

2. Materials and methods
COC and its metabolites benzoylecgonine (BE) and ecgonine methyl ester (EME), amphetamine-like stimulants (AMP, METH, MDMA), MTD and its metabolite 2-ethylidene-1,5-dimethyl-3,3-diphenylpyrrolidine (EDDP), 6-monoacetylmorphine (6-MAM) were measured in 24-hour composite influent wastewater samples with a validated method based on solid-phase extraction and liquid chromatography coupled to tandem mass spectrometry [3]. In first instance, concentrations in wastewater were corrected for occurring degradation, as demonstrated for EME, AMP, COC and 6-MAM. In a second step, the correction factors taking excretion patterns into account were updated for MDMA (20% excretion as parent compound instead of 65%) and for COC (taking co-consumption of alcohol and COC into account). For COC, back-calculations based on BE and EME alone, or on BE and EME together were generated. For HER, back-calculations were based on 6-MAM. In a last step, this study presents for the first time real-time calculations of the amount of served inhabitants for each sample based on concentrations of P, N, BOD and COD, measured in the samples.

3. Results and discussion
3.1. Amount of inhabitants served by the WWTP
Based on concentrations of N, P, COD and BOD, large variations in the amount of served inhabitants were observed in the sampling period with a range of 77,831 to 1,670,562 (Figure 1). Along the complete sampling period, calculated values of served amount of inhabitants were often very different from the design capacity of the Brussels-North WWTP (1.1 million inhabitants). These results demonstrate that the use of the design capacity of a WWTP as amount of served inhabitants in sewage epidemiology calculations does not reflect the real amount of served inhabitants and should be replaced by real-time calculations of this parameter.
3.2. Sewage epidemiology results

The use of METH was extremely low with an average consumption of only 2 mg/day per 1000 inhabitants; this corresponds with the official statistics, stating the METH use in Europe is negligible [1]. MTD consumption was on average 138 mg/day per 1000 inhabitants with small variations along the sampling campaign (RSD < 15%), which is a logical observation since this compound is mainly used in controlled opiate withdrawal programs. No significant variations in MTD consumption along the week was observed. HER showed large variations in its consumption with an average of 415 mg/day per 1000 inhabitants (RSD = 45%). This value is higher than other sewage epidemiology studies [2]. These studies used morphine (which can also be present in wastewater due to the use of other compounds) concentrations for back-calculations of HER consumption, while the present study uses 6-MAM concentrations. No significant variations along the week could be observed. The average value of consumed COC calculated in this study was 519 mg/day per 1000 inhabitants (RSD = 31%), which corresponds with approximately 5 doses/day per 1000 inhabitants. Statistical significant higher COC use was observed from Friday to Sunday compared with the use from Monday to Thursday. These findings also confirm the so called “recreational character” of COC. The three different back-calculations showed no significant differences. For AMP (mean: 76 mg/day per 1000 inhabitants) and MDMA (mean: 13 mg/day per 1000 inhabitants) also statistical higher consumption was observed during the weekend. An explanation for the higher use of these compounds is its presence in recreational settings, comparable with COC.

4. Conclusions

The sewage epidemiology methodology and temporal variations in the calculated amounts of used illicit drugs could be evaluated in a valid and reliable way because of the extensive sampling campaign. Back-calculations were updated to new information regarding the stability of illicit drugs in wastewater and the excretion pattern of the substances. For the first time, the amount of inhabitants served by a WWTP were calculated in a real-time way, based on concentrations of N, P, COD and BOD in the wastewater samples. This manuscript shows that sewage epidemiology delivers consistent and logical results and efforts should be made to further optimize the methodology.

5. References

1. Introduction

In 2008, the United Nations Office on Drugs and Crime (UNODC) reported that an estimated 4.9% of the world’s population aged 15-64 used some type of illicit drug on an annual basis and that in Canada alone, it was estimated that 2.3% of the same population group had used cocaine, 1.0% had used amphetamines and 1.3% had used ecstasy [1]. It is difficult to accurately estimate the use of illicit drug within communities using current indirect survey methods. However, as proposed by Zuccatto et al. [2], we determined the concentrations of selected illicit drugs in untreated wastewater from three Canadian municipalities that have widely different population sizes and used these data to estimate community consumption patterns for the drugs. Monitoring of the levels of drugs of abuse in the treated effluents also allowed the determination of the effectiveness of the treatment process for removing illicit drugs.

2. Materials and methods

Samples of treated and untreated wastewater were obtained from the WWTPs for three municipalities located in eastern Canada. The samples were 24 h composites, except for one grab sample of untreated effluent that was collected from WWTP3. Table 1 provides information on the WWTPs, including the populations served, the sampling dates and the wastewater flow rates over the sampling dates, the hydraulic retention times (HRTs) and the wastewater treatment technologies. The samples were stored in the dark at 4°C until they were extracted (within 48 h of collection). Each sample was extracted in triplicate using the extraction method described in Metcalfe et al. [3] (recoveries 83-101%) and analyzed by LC-MS/MS using the analytical method described in [3], with LOQs in the range of 3-22 ng/L.

<table>
<thead>
<tr>
<th>Location</th>
<th>Pop (x10⁶)</th>
<th>Treatment Technology</th>
<th>HRT (hours)</th>
<th>Average Flow (m³/d)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WWTP1</td>
<td>1.6</td>
<td>Primary</td>
<td>3</td>
<td>3155</td>
</tr>
<tr>
<td>WWTP2</td>
<td>0.5</td>
<td>Secondary (activated sludge)</td>
<td>14</td>
<td>398</td>
</tr>
<tr>
<td>WWTP3</td>
<td>0.075</td>
<td>Secondary (activated sludge)</td>
<td>15</td>
<td>41</td>
</tr>
</tbody>
</table>

Table 1: Description of WWTPs.

The method described by Zuccatto et. al. [2] was used to estimate community use of the target drugs. The mean (n=3) concentration of the target drug in the samples of untreated wastewater and the daily flow rate in the WWTP were used to calculate the amount of drug (grams) discharged per day. This value was then divided by the number of people served by the WWTP was normalized to a value of grams per day per 1,000 people. Using excretion rates and average doses available in the literature, community drug use (i.e. doses/day/1,000 people), was estimated.

3. Results and discussion

3.1. Concentration in wastewater and removal in WWTPs

All of the target drugs, with the exception of MDA and occasionally methamphetamine and amphetamine were detected at concentrations >LOQs. The concentrations of BE and cocaine varied between 287 and 2,624 ng/L and 209 and 823 ng/L, respectively in samples of the untreated wastewater. These concentrations are very similar to the range of concentrations reported for WWTPs in the USA [4] and in Europe [5, 6, 7, 8]. MDA couldn’t be quantified because of the relatively high LOQ of 22 ng/L and the maximum concentrations of the other amphetamine compounds in untreated wastewater were all <70 ng/L. In comparison to data reported for untreated wastewater from WWTPs in the USA [4], the concentrations of methamphetamine and amphetamine were lower but the levels of MDMA (i.e. ecstasy) were comparable to the present study. In Europe, methamphetamine is generally present at lower concentrations (i.e. <10 ng/L) in untreated wastewater, but the concentrations of MDMA and amphetamine are comparable to the levels reported in the present study [5, 6, 8].
BE and the amphetamine drugs were generally removed in WWTP2 and WWTP3 with efficiencies >50%. There was a much lower percent removal of all target analytes in WWTP1, with the possible exception of amphetamine, which can be attributed to the primary treatment technologies used in this plant.

3.2. Estimation of community drug usage

Figure 1 illustrates the estimates of community use of the illicit drugs within the Canadian cities served by the three WWTPs. As with estimates for other cities in continental Europe, Wales and Ireland [2, 5, 6, 8, 9, 10], cocaine is the most commonly used illicit drug. It also appears that consumption increased slightly on weekends, from a low of 8.1 doses per day per 1,000 people on a weekday in the city served by WWTP3 to a high of 56.7 doses per day per 1,000 people on a Friday in the city served by WWTP1. Similar trends over the weekdays were observed for amphetamine, methamphetamine and MDMA. The median estimates of community drug use determined here are consistent with the World Drug Report data on the prevalence of drug use in Canada [1]. These data comparing three cities of different sizes could be interpreted as evidence that there is greater drug use in large Canadian cities; possibly because of greater access to the distribution networks for illicit drugs. However, any interpretation of drug use patterns among cities in Canada, and elsewhere will require an evaluation of the population demographics and socioeconomic conditions.

4. Conclusions

This first estimation of North American communities consumption of illicit drugs based on data for untreated wastewater demonstrated the validity of this new approach. Community drug use estimates for the Canadian cities are generally consistent with the estimates of drug use that have been generated for European cities using this method, although methamphetamine use appears to be higher in Canada. Further work is underway to determine whether opioid drugs are present in the wastewater from these Canadian cities and these data will be reported in the session at SETAC Europe. There is evidence that prescription opioids are the fastest growing class of drugs of abuse in Canada.

5. References


Acknowledgement - The authors gratefully acknowledge the assistance of the staff of the wastewater treatment plants for collecting the samples. This work was supported by the Natural Sciences and Engineering Research Council (NSERC) of Canada.
Estimation of illicit drug consumption via wastewater analysis in South-East Queensland, Australia: uncertainty evaluation

Foon Yin Lai¹, Christoph Ort², Coral Gartner³, Steve Carter⁴, Jeremy Prichard⁵, Paul Kirkbride⁶, Raimondo Bruno⁷, Wayne Hall³, Geoff Eaglesham⁴ and Jochen F. Mueller¹

¹The University of Queensland, The National Research Centre for Environmental Toxicology, 39 Kessels Road, Coopers Plains, QLD 4108, Australia
²The University of Queensland, Advanced Water Management Centre, QLD 4072, Australia
³The University of Queensland, Centre for Clinical Research, Royal Brisbane and Women's Hospital, Herston QLD 4029, Australia
⁴Queensland Health Forensic Scientific Services, 39 Kessels Road, Coopers Plains, QLD 4108, Australia
⁵Faculty of Law, University of Tasmania, Private Bag 89, Hobart, TAS 7001, Australia
⁶Australian Federal Police, Forensic and Data Centers, GPO Box 401, Canberra, ACT 2601, Australia
⁷School of Psychology, University of Tasmania, Private Bag 30, Hobart, TAS 7001, Australia

E-mail contact: foon.lai@uqconnect.edu.au

1. Introduction
Illicit drug use is an important public health and social problem. Analysis of wastewater for estimating drug use has become a useful tool to estimate drug consumption at a community level¹⁻³. A key potential application of this tool is the assessment of changes in illicit drug consumption in a given population. However, the successful application relies on understanding the uncertainties associated with all aspects of the measurement. The uncertainties include sampling (Uₕ), flow measurement (Uₚ), chemical analysis (Uₙ), population size (Uₚ) and excretion fraction (Uₑ) and biodegradation in sewers (Uₚ) of a given drug. It is relatively easy to accurately estimate and thus reduce Uₕ and Uₙ compared to Uₚ and Uₑ. Researchers also need to rely on operators at sewage treatment plants (STPs) to obtain best available estimates for Uₚ.

The aims of our study were to
- Reduce the sampling uncertainty through an optimized sampling method
- Identify and evaluate the total uncertainty associated with our per capita drug consumption estimates
- Provide an estimation of illicit drug consumption over 12 days in a urban catchment from South East Queensland, Australia

2. Materials and methods
A continuous flow-proportional sampling mode was applied to ensure the collection of representative raw wastewater samples. Samples were collected from 20th November to 1st December 2009 at a municipal STP located in South-East Queensland. We measured a range of illicit drug residues (DRs), including cocaine (COC), benzoylecgonine (BE), ecgonine methyl ester (EME), amphetamine (AM), methamphetamine (MA), 3,4-methylenedioxyamphetamine (MDMA), 3,4-methylenedioxyethylamphetamine (MDEA), 3,4-methylenedioxyamphetamine (MDA), 9-tetrahydrocannabinol (THC) and 11-nor-9-carboxy-9-tetrahydrocannabinol (THC-COOH).

DRs in the samples were extracted by solid phase extraction method with Oasis® MCX cartridges (6 cc, 150 mg, 30 µm) and then analyzed by liquid chromatography (Shimadzu Prominence, Shimadzu Corp., Kyoto, Japan) coupled with tandem mass spectrometry (Applied Biosystem/Sciex API 4000Q). Procedural blanks, procedural recoveries and matrix spike recoveries are analyzed in every batch of sample extraction. None DR chemicals were detected in the blanks. The mean recovery of DR compounds spiked in Milli-Q water and wastewater samples are in a range of 89-102% and 83-113% respectively.

3. Results and discussion

3.1. Daily measured load and estimated consumption of illicit drugs
Figure 1 shows the daily measured load of the DRs in 12 monitoring days. Seven out of 10 DRs were detected in all the samples. COC, BE, MA and MDMA were measured at relatively higher levels and daily loads in the samples. AM, MDA and THC-COOH loaded at about one order of magnitude lower than these four DRs. EME, MDEA and THC were not found in the samples. The loads of the COC, BE, AM, MA, MDMA
and MDA were higher during weekends compared to week days. The weekly pattern of THC-COOH did not follow this pattern.

![Graphs showing daily load (g/day) of DRs and flow rate (ML/day)](image)

**Figure 1**: Measured daily load (g/day) of DRs (left: COC and BE; middle: AM, MA, MDMA and MDA; right: THC-COOH) and flow rate (ML/day)

We back calculated the daily consumption of four illicit drugs using conventional methods\(^1\). For the given study period, THC was estimated as the most prevalent illicit drug consumed (median: 964 mg/day/1000 people), followed by COC (158 mg/day/1000 people) and MA (170 mg/day/1000 people). In 1000 inhabitants, the estimated daily consumption of MDMA (34.5 mg) was much lower compared to the other prevalent DRs.

### 3.2. Components of uncertainty

We performed an uncertainty analysis that associates with the determination of chemical loads. The relevant total uncertainty (\(U_{\text{tot}}\)) comprise of four independent components, including sampling (\(U_S\)), chemical analysis (\(U_C\)), flow (\(U_F\)), and excretion (\(U_E\)). The propagation is shown in Eq.1:

\[
U_{\text{tot}} = \sqrt{U_S^2 + U_C^2 + U_F^2 + U_E^2} \quad (\text{Eq.} \ 1)
\]

The uncertainty value is conservatively proposed based on our experience in sewage engineering, complexity of wastewater sampling\(^4\) and chemical analysis. By applying a continuous flow-proportional sampling mode, we reduced sampling artefacts to a minimum\(^4\) and then conservatively estimated \(U_S\) to be 5%. We estimated \(U_C\) to be about 10 – 20% depending on the sensitivity of chemicals. The STP operators specified \(U_F\) as around 20%. We evaluated \(U_E\) (THC: 4.3%; COC: 5.5%; MA: 21%; MDMA: 11%) by transforming the range of excretion data (assumed this is uniform distribution) provided in the literature to a standard deviation of a normal distribution for the error propagation. The evaluated \(U_{\text{tot}}\) for estimated consumption of THC, COC, MA and MDMA was 29%, 24%, 31% and 26%.

### 4. Conclusions

We predicted our estimates having a remaining uncertainty in a range from 24 – 31% even with the best sampling practice and current chemical analysis. Apparently, the respective uncertainties, particularly \(U_S\) and \(U_F\), could be further reduced when there is a platform to normalise loads of DRs with those of other chemicals in wastewater. More effort is needed in the future study to refine the back estimation method so as to improve the confidence of the estimated data.

### 5. References


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Going Beyond Qualitative Assessment of Ecosystem Services

R.A. Pastorok¹ and D.V. Preziosi²

¹Integral Consulting, Inc, 23205 119th Avenue SE, Woodinville, WA 98077 USA
²Integral Consulting, Inc, 4D Bay Street, Berlin, MD 21811 USA
E-mail contact: rpastorok@integral-corp.com

1. Introduction
An understanding of ecosystem services is essential for responsible environmental management. A wide range of conceptual models and metrics have been developed for analyzing and measuring ecosystem services. Yet we know little about which metrics are most useful and cost-effective in the long-term, except in particular ecosystems that have been well studied. Past practices in natural resource management have generally led to overexploitation of species or in extreme cases, ecological catastrophes. While adaptive management strategies will help the current situation, we must increase our knowledge of quantitative relationships between ecological structure (e.g., biodiversity, species abundance, biomass, and habitat connectivity/fragmentation) and function (e.g., productivity, population persistence, ecosystem resilience, and nitrification) because these relationships are the underpinnings of service provisioning in ecosystems.

2. Materials and methods
We reviewed metrics for assessing ecosystem services. We also searched the literature for quantitative relationships between structural and functional variables for analysis of ecosystems. Examples of such relationships and their use in understanding ecological services were developed. We also evaluated how analysis of food webs and ecological modelling at various biological scales could be used to understand ecosystem services and prioritize metrics for measuring services.

3. Results and discussion

3.1. Understanding ecosystem services in relation to ecological structure and function within a socio-economic context
Ecosystem services depend on the existence of viable habitat, sustainable food webs, and functioning ecosystems. Ecological systems vary in their structural complexity, yet each structure supports some degree of function and provisioning of ecological services. Figure 1 illustrates relationships among ecological structure/habitat, processes and functions, socio-economic factors, and ecosystem services. Improving our knowledge of relationships between ecological structure and function is needed not only for understanding how ecosystems provide services but also for resolving conflicts among services by analyzing service tradeoffs and the benefits that accrue from particular functions.

Figure 1: Understanding ecosystem services within a socio-ecologic framework
3.2. Understanding ecological structure and function through food web analysis

Understanding ecosystem services and their flows to human populations depends on a reliable assessment of ecological structure and function. The basis for this understanding should be an analysis of food webs and interactions among species because together they provide the mechanisms by which biological communities and associated ecosystems persist.

Ecosystems are not in a state of constancy or stasis, but represent dynamic configurations of species, functional guilds, and abiotic components such as soil, water, and atmosphere. Despite the non-equilibrium nature of ecosystems, clear associations of functional components can be identified and prioritized. For example, the analysis of food webs supports the development of an understanding of ecosystem functions as well as potential alternative states. Moreover, the concepts of foundation species, keystone species, functional guilds, and engineer species clarify how food webs function and help to prioritize species for protection as part of managing for ecosystem sustainability.

3.3. Going beyond conceptual models and qualitative analysis of ecosystem services

The initial basis for representing key ecosystem components, their functions, and service flows is a conceptual model. While such models form the basis for quantitative analyses of services, the quantitative analyses are required to minimize the chance of excluding important components in conceptual models and the mathematical models that derive from them. Thus, it is necessary to develop both statistical and theoretical relationships between ecosystem structural metrics (e.g., species richness, biomass, population abundance, and habitat connectivity/fragmentation) and functional metrics (e.g., productivity, population persistence, resilience, and nitrification). Because there are few empirical data on basic relationships between ecological structure and function, ecological modelling will continue to play a central role in developing an understanding of ecosystem services in a general as well as site-specific sense. Examples of analytical tools for evaluating ecological structure and function relationships include models of: 1) single-species populations, 2) food webs (biological communities comprised of multiple species), 3) ecosystems (communities plus abiotic components of the environment), and 4) landscapes (including spatial scales encompassing multiple ecosystems).

4. Conclusions

Ecosystem services can be understood only through an analysis of ecological structure and function, including elucidating relationships among key species in food webs. Because empirical assessments of relationships among ecological variables are limited, we maintain that attempts to assess ecosystem services in the absence of modelling populations, food webs, and/or landscapes could fail. We have no shortage of metrics to assess ecological structure, function, and services. The pressing issue is which of these metrics are most useful and cost-effective. Ecological models can aid in identifying and prioritizing metrics for assessing ecological services.
Mapping soil biodiversity and ecosystem services in The Netherlands

Michiel Rutgers¹, Harm van Wijnen¹, Ton Schouten¹, Christian Mulder¹, Dick de Zwart¹, Ton Breure¹,²

¹National Institute for Public Health and the Environment, Box 1, 3720BA Bilthoven, The Netherlands
²Department of Environmental Science, Radboud University Nijmegen, Heyendaalseweg 135, 6525 AJ Nijmegen, the Netherlands
E-mail contact: michiel.rutgers@rivm.nl

1. Introduction

Soil policy in the Netherlands is in a transition phase - it is changing from a focus on the protection and restoration of soil from threats such as contamination and sealing, towards a focus on sustainable land use. A key aspect of the new focus is that soil quality should be assessed using chemical, biological and physical indicators in a systems approach. Furthermore, soil ecosystem services will be the basic concept in a framework for sustainable land use in order to split up the denominator ‘soil quality’ in meaningful aspects for society.

Ecosystem services play a major role in maintaining the environment in a condition where the human population can live healthy and happy. Examples of the products of such services are: protection against flooding, provision of clean drinking water, production of food and fibers. The economic value of these services becomes ever more clear [1,2].

There are no simple and scientifically accepted tools available to measure soil biodiversity and ecosystem services. The assumption is that ecosystem services and soil biodiversity can be quantified in a systemic approach using chemical, physical and biological characteristics, but there is no broad consensus on how this should be done. Certain soil characteristics or combination of features can be associated with soil biodiversity or ecosystem services on the basis of literature data and ecological knowledge, but without knowing the exact meaning.

Maps may play an important role in the process of spatial planning and may bring ecosystem services on the table during negotiations of stakeholders. As an example we show the quantification of the ecosystem service ‘purifying capacity’ of pollutants, which is a service necessary to keep the soil clean enough for, e.g. production of safe food and clean drinking water.

2. Materials and methods

We used data from the Netherlands Soil Monitoring Network (NMSN) [3], including the Biological Indicator for Soil Quality (BISQ), to produce habitat-response relationships as proxies for soil biodiversity. These proxies were used to map soil biodiversity. Parallel to habitat-response modeling, functions for the performance of ecosystem services were produced based on biological, chemical and physical parameters. These functions will also be used for mapping purposes and their usefulness discussed. Maps were produced showing 1) predicted soil biodiversity (for several proxies of soil biodiversity), 2) predicted performance of ecosystem services (for several ecosystem services) and 3) the difference between predicted and expected performance of ecosystem services when the land use is sustainable. The expected state of a soil with a sustainable management (good ecological status) was characterized by a panel of experts [4]. Maps were generated for both agricultural and nature areas. It is the aim of the maps to raise public awareness and to give support towards transition to sustainable land use. It is given acknowledgement that with current data, knowledge and consensus, the level of uncertainty of these maps is quite high. However, the general trends on a national scale may be helpful to show the potential for ecosystem services in The Netherlands.

The conceptual framework is based on the appreciation of environmental quality (e.g. soil quality) as a weighted sum of relevant ES. In a formula:

\[
\text{Environmental quality} = \Sigma (\text{weighted ES}_n)
\]

ESn is a specific ES, such as listed in [1,2] and [3]. ESn can be derived from a function of characteristic ecosystem attributes:

\[
\text{ES}_n = f (\text{biotic factors, abiotic factors, other factors})
\]
We explored these relationships by the construction of maps showing soil biodiversity and ecosystem services.

To quantify proxies for soil biodiversity, habitat response relationships were derived for the number of taxa and total number of species of Arthropods, Enchytraeids, Lumbricidae, Nematodes, Soil Functional activity and the potential C and N mineralization. These soil characteristics were used to quantify Ecosystem services. The Ecosystem Service ‘purifying capacity’ was derived from:

\[
E_{Sn} - \text{purifying capacity} = f(\text{Soil Functional activity, pH, SOM, Pal, potential C min., potential N min.}) \text{ relative to the reference values. The more the actual values differ from the references, the lower the purifying capacity.}
\]

3. Results and discussion

The soil purifying capacity in The Netherlands (ranging from 0 – 1) is relatively high in areas with a clay soil, whereas in nature areas on sandy soils the purifying capacity is relatively low (Fig 2).

4. Conclusions

The continuing growth of human population, and, to a lesser extent, that of its average prosperity, will further increase the dependency of society on the environment quality, whereas the same environment is facing increasing impacts from management practices. With sustainable management of the environment and its functioning, mankind can optimally profit from its capacity to provide Ecosystem Services (ES), for instance to support health, provide clean water, maintain clean groundwater and support production of food and fiber. A new focus on ES will provide the insight on where and how management can be made sustainable.

Figure 1: Soil purifying capacity in The Netherlands.

5. References


Acknowledgement - Activities within RIVM were commissioned by the Netherlands Ministry of Housing, Spatial Planning and the Environment (VROM) and took place within project M/607604, entitled ‘Soil ecosystems – monitoring, data management and integration’.
An Ecosystem Services Framework: A Case Study on Citrus Production and Insecticide Use. To What Extent are Specific Ecosystem Services Affected?

Samantha Deacon¹, Gregory Reub², Gretchen Greene² and Steve Norman³

¹ ENVIRON UK Limited, Box House, Box, Wiltshire, SN13 8AA, United Kingdom
² ENVIRON International Corporation, 605 First Avenue, Suite 300, Seattle WA 98104, USA
³ Dow AgroSciences, 3 Milton Park, Abingdon, OX14 4RN, United Kingdom
E-mail contact: sdeacon@environcorp.com

1. Introduction

The findings of this pioneering proof-of-concept study will deliver a case study to inform discussions between scientists and policy makers in pesticide regulation and stakeholder discussions with the European Food Safety Authority (EFSA) where an ecosystem services approach can inform risk management decisions.

In this study, an ecosystem services framework has been developed and is applied to a key insecticide for use in citrus. In south EU, citrus growing is particularly important both economically and culturally to the communities in these regions and nationally. The study focusses on Southern Spain where generations of farmers have grown citrus fruits with some regional landscapes being dominated by citrus groves. Spain has long been a leading producer and foremost exporter of oranges with nearly 6.5 million tonnes produced by the European Union in 2007/08 [1].

Recent scientific thinking is increasingly focussing on the ecosystems services approach. The concept, advanced by the Millennium Ecosystem Assessment [2], brings a fresh approach to managing identified ecological risks in a holistic manner. The European Commission has already introduced the concept into legislation under the Environmental Liabilities Directive and through the E.U. Sixth Framework Project Remede [3].

In addition, EFSA has recently developed and published a framework for deriving specific protection goals for the environmental risk assessment of pesticides. This EFSA framework is based on the ecosystem services approach [4]. The following scheme (Figure 1) is presented in the EFSA publication.

![Figure 1: Relation between problem formulation, protection goals, risk assessment framework, and risk management in the process of developing specific protection goals.](image-url)
The ecosystem services approach identifies and values the primary ecosystem services that a habitat or ‘property’ may provide to humans given different land uses and actions (e.g. food production; recreation; biodiversity). The type, quantity, and quality of ecosystem services provided by an area are influenced by the surrounding landscape and land uses. Human activity can affect the quality and quantity of each ecosystem service provided. Overall, some services may be improved, some services may not be affected, and some services may be harmed. A systematic evaluation of such changes in service flows is required to allow for consistent comparisons across alternatives, as well as to optimise the achievement of environmental objectives while maximising benefits and minimising costs to society.

The framework developed for this study builds on the EFSA specific protection goals and demonstrates how an ecosystem services framework is applied by identifying and valuing the primary environmental services that a habitat may provide given different land uses and actions.

The regulatory context is that EU legislation of use of plant protection products (‘Uniform Principles’ of Directive 91/414 EEC: Directive 97/57/EC [5]) requires that for non-target species it must be established that ‘no unacceptable impact occurs after use of the plant protection product according to the proposed conditions of use’.

2. Materials and methods

The framework allows for the formal quantification (semi-quantification) of ecosystem service values associated with different land cover types and the anthropogenic influences within each cover type. The approach relies on gathering existing data and development of an analytical framework. The approach governs the collection and analysis of data on a detailed land cover type scale, establishing existing capacities of ecosystem services or baseline conditions. The results produce a composite measurement of baseline conditions expressed in a standard metric or “currency” (e.g., service hectares) allowing ecosystem service levels to be compared. Results are then estimated for conditions after a change in a management action (such as the application of a pesticide) and the net ecosystem service gains and losses between scenarios or condition levels may be compared.

A number of management actions are compared in this study on citrus including (i) baseline ecological service conditions, (ii) existing ecological service conditions with continued use of the example insecticide, (iii) an alternative scenario without the application of the example insecticide (iv) a fourth alternative scenario (e.g., including practical habitat management measures to promote local biodiversity).

The relationships or pathways that link ecosystem services with these management actions are critical to estimating changes in ecosystem services. Initially GIS mapping is used to find the spatial overlap between management actions and land cover types or habitats. Then, the impact from an action in the overlapping habitat is analysed to estimate how it changed a specific ecosystem service. Finally, relative impacts and benefits from proposed management actions are evaluated.

3. Results and Conclusions

The project is in its early stages and preliminary results are awaited. The conclusions of the study will be available for presentation at the SETAC Europe 2011 conference when the project has completed. It is anticipated that this project will provide a proof-of-concept study for discussions between scientists and policy makers in pesticide regulation where an ecosystem services approach can inform risk management decisions.

4. References

Enhancing multiple ecosystem services in existing grass buffer strips

Robin J. Blake1, Ben A. Woodcock2, Duncan B. Westbury1, Peter Sutton3 and Simon G. Potts1

1Centre for Agri-Environmental Research, School of Agriculture, Policy and Development, University of Reading, Reading, Berkshire, RG6 6AR, UK
2NERC Centre for Ecology and Hydrology, Crowmarsh Gifford, Wallingford, Oxon, OX10 8BB, UK
3Syngenta, Jealott’s Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK
E-mail contact: r.blake@reading.ac.uk

1. Introduction

Plant and invertebrate diversity plays a key role in terrestrial agro-ecosystems through the provision of multiple ecosystem services. Plants are the primary producers, while bumblebees pollinate crops and wildflowers, and spiders can help control crop pests such as aphids [1]. Butterflies, particularly as larvae, and spiders also represent key food items for higher trophic levels including birds [1]. Despite their importance, agricultural intensification has led to widespread declines in invertebrates and other taxa in the UK and NW Europe [2]. In response to these biodiversity losses Agri-Environment Schemes (AES) were introduced and provide farmers with financial incentives in return for environmentally sensitive farming [3]. A popular option is the establishment of perennial grass buffer strips with over 29,000 ha currently under UK AES agreements [4]. However these strips tend to lack a wildflower component and as such are botanically species-poor [5]. Studies have demonstrated how scarification of the buffer strip surface to create bare ground and application of graminicide to suppress competitive grasses can promote the development of sown wildflowers, benefitting invertebrates [5, 6]. The aim of this study was to investigate how the enhancement of existing grass buffer strips on arable farmland can be used to promote the abundance of bumblebees, butterflies and spiders, thereby supporting multiple ecosystem services.

2. Materials and methods

The study was initiated in spring 2008 on two arable farms in Berkshire, UK. Three replicate blocks each consisting of three treatment plots were established on the outer 4 m of existing 6 m grass buffer strips to investigate two management practices: (a) scarification to create bare ground into which wildflower seeds were sown; (b) graminicide (fluazifop-P-butyl) application to reduce the competitive dominance of grasses. The three treatments were (T1) control (no scarification, seed or graminicide); (T2) scarification and seed only; and (T3) scarification, seed and graminicide. Scarification was applied once in March 2008 with a power harrow to a depth of approximately 5 cm to break up the existing sward. Following scarification, a perennial wildflower seed mixture containing nine species known to provide pollinator foraging resources and structural features for spiders was sown into the scarified plots at a total rate of 2.4 kg ha⁻¹. The graminicide fluazifop-P-butyl (Fusilade Max 125 g L⁻¹ EC) was sprayed once at a rate of 93.75 g ai ha⁻¹ in April 2008.

The buffer strip vegetation was assessed once per year from June 2008-2010 using 0.25 m² quadrats. All species were identified and assigned a percentage cover value based on an eight point scale. The abundance of bumblebees and butterflies was recorded from monthly transect walks during May-September 2008-2010. Spiders were collected twice per year in 2008 and 2009 using a Vortis suction sampler. Adults were identified to species and categorised according to their feeding specialisations (hunting; wandering; or orb-weaving spiders). Percentage cover values of sown wildflowers and abundance of bumblebees, butterflies and spiders were summed to provide total cover/ abundance counts. All data were log-transformed (LN+1) and analysed in SAS 9.2 using general linear mixed models with repeated-measures. Graphs show untransformed data, with treatment effects averaged across both farms and all sampling years. Between-treatment differences were tested using post hoc Tukey’s test. Treatments with the same letter do not differ significantly (P > 0.05).

3. Results and discussion

3.1. Effects of management treatments on vegetation

Significant effects of sown wildflower cover were observed (F2,9.93 = 17.79, P = 0.0005) (Figure 1), with Tukey’s test revealing a significantly greater sown wildflower cover in T3 compared to T1 and T2.
3.2. Effects of management treatments on invertebrates

Significant effects were observed for the abundance of bumblebees ($F_{2,13.4} = 12.94, P = 0.0007$) and butterflies ($F_{2,10.9} = 11.14, P = 0.0023$) (Figure 2). Tukey's test revealed higher abundances in T3 for both taxa. Although no significant treatment effects were observed for the spiders, orb-weaving spider abundance was correlated with sown wildflower cover (Figure 3).

4. Conclusions

The combination of scarification, sowing and graminicide resulted in the greatest abundance of sown wildflowers, and of bumblebees and butterflies reflecting a higher availability of foraging resource. Abundance of orb-weaving spiders responded to the sown wildflower cover, probably due to the utilisation of vegetation structures, e.g. inflorescences, to construct webs, and also higher prey densities [6]. Increasing the floristic diversity of existing grass buffer strips represents a potentially important conservation tool for enhancing the quantity and quality of invertebrate habitat on farmland. This study has highlighted how a combination of scarification, sowing with wildflowers, and graminicide use, can help achieve this. Incorporating these management tools into existing agri-environment options could benefit key invertebrate groups and support the delivery of multiple ecosystem services in arable landscapes.

5. References


Acknowledgement - The authors thank the farmers for allowing access to their land for data collection. This project was funded by the Biotechnology and Biological Sciences Research Council and Syngenta.
1. Introduction

There have been reports, in recent years, of declines in native and managed pollinators in several regions of the world [1][2][3][4][5] with important ecological and economical consequences [6]. Modern crop management practices, progressively implemented in order to allow the extension of cropped areas and to ensure a proper control of pest populations and diseases have often been pointed as a factor responsible of this decline [7]. Plant Protection Products (pesticides) are part of these management practices. Regulatory texts have been adopted all around the world, requiring an assessment of the impact of these products on the environment and ecosystem, including non-target arthropods and pollinators [8][9][10].

Concern regarding the extent to which the use of pesticides in crop protection affects pollinators varies among countries [11]. The primary concern for insect pollinators has typically been predicated on potential reductions in pollination services and temporary declines in the crops dependent on those services [1]. With regard to evaluating the potential impact of pesticides on pollinating species, these efforts have progressively focused on key indicator species, like the honey bee (Apis mellifera), primarily since the test guidelines for assessing the effects of pesticides on non-target terrestrial invertebrates have been developed mainly on this species because of their ecological importance and for practical reasons as they are easily reared [12]. In Europe as in North America, the risk assessment for pollinators is based on the honey bee as a surrogate. However, the extent to which these studies are used in risk assessment varies, which thus appears as the only species for which a dedicated risk assessment is performed, the other non target organisms being assessed at the level of higher taxonomic or trophic groups (i.e. fish, aquatic invertebrates, algae, aquatic plants, birds etc). The amount and nature of the information required for the risk assessment process is not limited to the ecological importance of pollination but also reflects the potential for the increased level of public concern resulting from effects in field, either when effects are noted through surveys or as the result of accidents and misuse. As an example, the accident that occurred in Germany following the sowing of seeds coated with an insecticide has highlighted the possible risks that may result from an exposure in the environment to dislodged seed coating “dusts” and sowing under certain circumstances reflecting poor agricultural practice [13]. As regulatory processes aiming at assessing the potential risks of pesticides to pollinators differ between countries, it is important to point out the gaps to account for some key potential routes of exposure that had been identified only in one country.

A global Pellston SETAC workshop on estimating the potential risks of plant protection products to insect pollinators was held in January 2011, bringing together the best available science regarding exposure and effects assessment methodologies for Apis and non-Apis bee species in order to harmonize the risk assessment approaches among North and South America, Europe, Asia and Australia for a global improvement of the protection of insect pollinators in the environment.

2. Materials and methods

The workshop gathered about 50 international experts in the assessment of effects, exposure and/or risks posed by pesticides to honey bees, other pollinating species, and/or other taxa. For a comprehensive review of the state of the art science to be performed during the workshop, the work was organized to cover four primary components of evaluating risk, i.e., (1) exposure of pollinators to pesticides, (2) assessment of
effects using laboratory tests, (3) assessment of effects in field tests and (4) strategies for integrating exposure and effects data to estimate the likelihood and magnitude of potential adverse effects [risk]. Non-
Apis bee experts were integrated into each group to consider how exposure, effects and risk could be evaluated for non-Apis bee pollinators to address uncertainties where honey bees do not serve as ideal surrogates.

Participants were provided an extensive overview and supporting documentation of the current regulations, risk assessment tools (testing guidelines and risk assessment guidance documents) and open literature in advance of the meeting. In addition, a list of “charge questions” was prepared to guide panelists through the background literature and to encourage written responses to specific challenges facing risk assessors and to which each of the workshop focus groups would be expected to contribute.

3. Results and discussion

This presentation focuses on recommendations which have emerged from the SETAC Pellston and how they might be adopted by international regulatory agencies within the context of their existing risk assessment policies and procedures. The outcome of the SETAC Pellston will be presented more extensively in the form of proceedings, aimed at providing the state of the art science-based process for evaluating the risk of pesticides to insect pollinators shared by all the countries and continents represented.

4. Conclusions

The SETAC Pellston workshop, focused on developing a risk assessment process for honey bees exposed to plant protection products and to identify the data needed to inform that process. The workshop was the first experience in the area of sharing science and regulatory expertise at a global scale toward addressing a common concern to further improve the relevance of regulatory recommendations. It is intended to assist in establishing a basis for a common understanding of the science and to encourage regular communication and work sharing among experts and regulators in this and other areas of ecotoxicological assessment of pesticides.

5. References

Use of ecosystem services concept to determine need for open soils in urban areas

Joke van Wensem¹

¹Soil Protection Technical Committee (TCB), PO Box 30947, 2500 GX The Hague, The Netherlands
E-mail contact: vanwensem@tcbodem.nl

1. Introduction
The draft Soil Framework Directive contains an obligation to limit the permanent covering of soil with impermeable material as far as possible and, where soil sealing is unavoidable, to mitigate the negative effects of doing so. This concerns new situations, in other words situations in which the soil has not yet been sealed. The main reason for the European Commission's decision to include soil sealing as a threat in its Soil Strategy is the increase in urbanisation. There is widespread concern about the large number of hectares of agricultural and natural land being used for urban building every year. There is also concern about the fact that preference is being given to using new land over the redevelopment of brown-field sites.

In order to be prepared for the possible implementation of the Soil Framework Directive, the Dutch Ministry of Environment requested TCB to report on the cases in which sealing needs to be limited and how this can be achieved (1). As a follow-up the Ministry requested to indicate a minimum percentage of open soil in each type of development area, in order to put a limit to negative effects of sealing on soil functions (2). This paper addresses the establishment of a minimum percentage of open soils in urban areas.

2. Materials and methods
There is limited data on the effects of soil sealing on soil functioning, and in most cases the data are qualitative in nature. As an alternative, the minimum percentage of open soils in urban areas was estimated by the space needed by soils to provide or support ecosystems services to the inhabitants. With good management and structuring, the open soil in an urban environment can, for example: retain, store and gradually evaporate water, or release it into groundwater, support vegetation, regulate temperature and humidity, capture airborne particles and gases, promote biodiversity, promote human health and wellbeing (green space in the city) and promote the local economy. Quantitative data on the required open soil or green area to provide these ecosystem services were collected by means of a literature search. Because of the applied nature of the research, reports from the ‘grey literature’ proved to be important information sources. Additionally, an inventory was made of projects in municipalities in which (characteristics of) soils were seen as providers of ecosystem services (3).

3. Results and discussion
Quantitative data from the literature search are summarized in table 1. For some ecosystem services it was not possible to find useable quantitative data. These were: supporting vegetation, capturing airborne particles and gases, and promoting biodiversity. For the latter it was concluded that the more open soil is available in urban areas for green, the better it will be for biodiversity, provided there is a good interconnectivity between the green areas and that the green areas are diverse in vegetation and design.

<table>
<thead>
<tr>
<th>Service</th>
<th>Character of, and necessary surface area for the service</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water regulation</td>
<td>Discharge of rainwater by infiltration in the soil: circa 50% of the sealed surface. Discharge of rainwater by infiltration via a “wadi”: circa 15% of the sealed surface. Halving the amount of sealing (estimate) reduces run off by circa 25-50% from clay and sand respectively. Run off is always reduced by open soil, even more when vegetated, and by green roofs, effect is dependent on local circumstances.</td>
</tr>
<tr>
<td>Temperature regulation</td>
<td>Area around cities is 3 to 8 degrees cooler than the city. Large parks cool urban areas up to a distance of 1 to 2 km. Cooling by a fews degrees Celsius by small green areas (0,1 ha) at regular distances requires 1,5% of the urban area.</td>
</tr>
</tbody>
</table>
An increase of green on open soil by 10% leads to cooling by a few degrees Celsius in urban areas. Locally a few degrees Celsius cooling by vegetation or open soil. Local cooling in green areas with a size from circa 1 ha and larger. Trees near buildings lead to reduction of energy demand up to 50%. Green roofs lead to at least halving the the amount of energy absorbed by buildings.

<table>
<thead>
<tr>
<th>Human well being and health</th>
<th>Green supports human well being and health. To realise and maintain green in cities, open soil is needed. There is no quantitative relationship between green and human well being and health, some guidelines and standard are available. National Spatial Strategy: 75 m² public green per house. City of Venlo: 47 m² public green per inhabitant. Standard distance for daily visits to public green and larger green areas: respectively 300 m and 2-10 km. City of Eindhoven: 1 tree per house.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Economical value</td>
<td>View on public green or water increases value of a house by 5-15%. Nearness of a park increases the value of a house with circa 7%. Presence of one or more trees near a house or in the street increases the value of a house.</td>
</tr>
<tr>
<td>Estimates for all services</td>
<td>City of Hengelo: 16 – 20 % public green and water. Recommendation from projects with soil: 40 % open soil and water (3).</td>
</tr>
</tbody>
</table>

Table 1: Summarized main findings from the literature search to quantify ecosystem services provided or supported by open soil in urban areas. All values are indicative; the benefits of open soil are site dependent. Data from TCB 2010.

4. Conclusions

The area necessary for temperature regulation and contribution to human health and well being is estimated at 5 to 10% of the urban area. Water regulation via soil needs much more space. ‘The more, the better’ can be concluded for biodiversity in urban areas, provided there is horizontal and vertical diversity in vegetation and good interconnectivity between areas with open soils. Surface water also contributes to certain urban ecosystem services. Based on all findings, it was concluded that 20 to 40% of the urban area should be reserved for open soil and water to provide all necessary ecosystem services. These values should be used at the scale of neighbourhoods. Not only the total area of open soil and water matters, the locations and good connections between these are crucial too.

5. References


Acknowledgement - The author thanks the committee members and colleagues for input and comments on various drafts of the reports.
1. Introduction

A comprehensive strategy is necessary to protect the ecology and characterise reference conditions in urban heavily modified areas and new towns. The importance of the quality components defined by the “good ecological status”, “good chemical status” and “quality of life” will be demonstrated in this study by “on site effects monitoring” tools to promote an environmentally sensitive and sustainable use of the resources in urban areas. The most successful strategy is to include bio-analytical and sensing systems as well as “real time bioassays” standardised by ISO and CEN into the approaches to define the quality components i.e. good ecological and chemical status. The study contributes to the resource management, evaluation of the interaction of ecosystems and urbanisation as well as quantification of risk. The environmental risk assessment in urban systems has two major elements: characterization of effects and characterization of exposure. Monitoring in support of Environmental Risk Assessment (ERA) will be demonstrated by a case study in urban systems. The data and investigations will be adequate for the characterization of exposure and effects indicating the sustainable development for ecosystem and human health protection in urban areas. The water quality norms of the different water sources concerning their possible use will be determined. The water quality objectives are for testing whether or not sufficient measures have been taken to protect the individual body of water and especially its valuable and exploitable resources from "dangerous substances". In order to secure the functioning and stability of a body of water (ecosystem) or to actually re-establish these qualities, concrete ecological knowledge is necessary concerning the interactivity of functions and structures of the living systems under these extreme conditions, as well as nutrient cycles and energy fluxes in their temporal succession. The water quality norms will serve as management tools for Environmental Quality protecting the waterways as a valuable natural source supporting drinking water, human health, outdoor recreation, and in general the ecosystem health. There is a need for unified strategies for the use of biomonitoring tools to assess the ecological health status in the context of environmental quality. The biomonitoring tools are used to characterise exposure data and potential effects and their use must be characterized and possibly tailored for different types of environmental media, adequate for the characterization of exposure and effects thus enabling the sustainable development for aquatic life and human health protection. Strategic Dimension Urban Development:

In 2008, at the beginning of the main phase of the demonstration project Young-City Hashtgerd (Iran) a Evaluation Group was founded. It aims to develop strategies that allow the evaluation of the project and that are applicable to other projects in the field of of urban development as well. The development of strategies that enable relevant, scientific monitoring to capture and assess qualitative and quantitative effects of Young City projects’ possible measures is the main purpose of a Evaluation-group.

2. Materials and method

One of the Evaluation-Group’s first steps was the development of matrices that were subdivided into the three Fields of Action that are provided and shown below. They serve as a presentation of the teams, their Dimensions and Workpackages in a clear and manageable way.

Field of Action 1: Energy / Climate (energy efficiency – mitigation of CO2);

Field of Action 2: Resources (except energy);

Field of Action 3: Sustainability (LCA, social aspects, adaptation to climate change) Initially the matrices’ structure consisted of following parameters:


At the end of March 2009 the column "Data of Initial Situations" was added to the matrices, in order to show the initial values. After that the scheme of the matrix-representation looked as follows:
To keep the matrices up-to-date the ongoing communication with the stakeholders, in this context is necessary. By this close collaboration, permanent adaptation and improvements were and are possible.

3. Results and discussion

The Evaluation Matrices are built after this scheme and form a complex picture that is not easy to grasp. This is a problem, as one function of these matrices is to serve as a means for representation. At the mid term of 2010 it was decided to mitigate this weakness by condensing the Evaluation Matrices. This compression aimed primarily for the simplification of the matrices to enable a better understanding and an improved overview. These matrices concerning sustainability will be demonstrated The European Green City Index will be used as a benchmark and used as an appropriate tool to evaluate the Young City project in the field of sustainability. The idea behind “Contribution of Sustainability” was to summarize and assess the measures and aims for a sustainable development, while the idea behind “Field of Sustainability” was to assign these aspects to one (or multiple) of the five columns of sustainability (Economy, Ecology, Social, Cultural, Governance).

4. Conclusion

This condensed representation in conjunction with the contribution to sustainability enables the viewer a quick and meaningful overview over the project, as well as the qualitative and quantitative effects and impacts of the proposed measures.

Unification of units for CO₂-Emissions. E.g. Grams per capita and year \([\text{g/(capita*year)}]\), sealed soil in square meters per day \([\text{m}^2/\text{d}]\) and noise in decibels \([\text{dB}]\).

In addition to the condensed matrices a Decision Support System will be demonstrated:
1. Introduction
To ensure sustainability integration into spatial planning, especially in urban areas, a reliable evaluation of benefits and cost of each planning alternative has to be set, taking into account also the ecosystem services provided by the natural environment, and their role in support to human living and socio-economic context. Lifestyles and relative household consumption play a relevant role in the total share of impacts caused by human activities in a given region [1], so it is important to consider the effects of household consumption during the planning process. It is necessary to include in the evaluation of planning procedure (e.g. within Strategic Environmental Assessment, SEA) some decision support tools that take into account also the private consumption component and its relative effects [2]. Therefore, the present work presents the result of the integration of two level of assessment to perform SEA. The typical evaluation made through a set of indicators (which results are compared with local limits and thresholds) is integrated with other sustainability assessment methodologies. The idea is to include in the evaluation some issues of global concern, such as resource depletion and climate change and to reinforce the use of the carrying capacity concept within the local planning. The evaluation includes Ecological Footprint (EF) assessment of citizens’ consumption and a carbon balance (CB) of the area. The case study presented refers to the implementation of this approach in the Strategic Environmental Assessment of a spatial planning plan of four municipalities in Northern Italy.

2. Methodology
Two composite indicators are used and considered as a preliminary proxy of the production and consumption pattern of the local authorities: 1. a methodology developed by Pennati and colleagues [3] is applied as a basis for estimating and mapping the CO2 balance (CB) at local scale, 2.the EF at local scale. The evaluation of CB (comparison between emission and uptake of CO2) can help to identify the role of spatial planning choices in determining the sustainability of the entire system both in the CO2 reduction strategy and in conservation of the uptake capacity of the territory. The methodology proposes to compare data of direct (emission related to activities that take place in the area under investigation) and indirect (related to emission that occur outside the area under investigation but related to production and consumption pattern in the area under investigation) emissions with data of uptake referred to specific land uses, which were collected from literature and optimised for local condition. The assessment of EF is based on an evaluation of consumption, clustered in five components: food, housing, transportation, goods and services and follows the bottom-up approach [4], that allows for sub-national EF assessment and permits to consider the changes in consumption patterns caused by the implementation of the Plans. To determine if the EF of a community is sustainable or not, it is necessary to compare local extension of bioproductive land (biocapacity, BC) with local demand of land (EF), defining an environmental balance of the local system.

3. Results and discussion
The first step needed for the evaluation of the two composite indicators is the analysis of land use in the area (using data coming based on Corine land cover classes); the extension of each land use class is then multiplied for uptake factors (derived from literature review) to calculate the uptake capacity of the area (145 kton CO2eq/year) and for bio-productivity factors to calculate biocapacity (39.090 gha). Table 1 and Table 2 report the results of the two balances in the area of investigation. EF from household consumption is 4.8 gha/person in Gordona and Samolaco and 4.9 gha/person in Novate Mezzola and Verceia. The result is in line with the average Italian EF (4.8 gha/person in 2008) and represents a high level of consumption that, if extended to the entire world, cannot be sustained by the Earth capacity. However at the local scale this result can be considered quite positive, because almost in all municipalities the EF doesn’t overshoot the local BC. Also the carbon balance is positive: the area uptake every year 145 kton and emit 99.88 kton.
This result is mainly due to the presence of a dense forested area. Nevertheless, even if the balance in this area is positive, the emission per capita are quite high (17 ton/year per capita). Hence, the spatial planning has to take into account policies focused on the reduction of CO₂ within the main drivers of emission (cattle, transport, heating system) and policies for natural capital conservation (to maintain the uptake capability of the area). The results presented refer to the current condition but can be useful also to develop scenarios for future evolution (i.e. measuring the variation in the balances due to the actions foreseen in the development plans), enabling local planners to predict not only direct environmental impacts of the interventions, but also indirect ones determining, for instance, a rise in consumption and waste production, (i.e. rise in EF and direct and indirect emissions) or a reduced BC or uptake capacity (due to land use change).

4. Conclusions

In the case of urban plans, especially if referred to small areas without significant industrial activities or infrastructures, household lifestyles can play a relevant role in determining the environmental impacts of the local community, i.e. the effects of the actions included in the plan. In many cases the absence of relevant actions doesn’t entail necessary the absence of any environmental impact, especially if considered in the long-term (not only the sustainability of the current condition, but also of future interventions). Therefore it is important to support the definition of spatial planning programs with suitable tools, such as sustainability composite indicators, that are able to consider a wider range of aspects, with reference to the carrying capacity concept. The proposed methodology proved to be quite useful, even if there are some limits, such as the fact the EF method doesn’t allow for accounting multifunctionality of ecosystems (e.g. carbon storage and wood provision from forests).

5. References

Sustainability assessment of forest biomass supply chain at local scale: carrying capacity of the system for energy valorisation

Salvatore Martire¹, Alessandro Grassini¹, Valentina Castellani¹ and Serenella Sala¹,²

¹DISAT, Department of the Environment and Landscape Sciences, Piazza della Scienza 1, 20126 Milano, Italy
²European Commission - Joint Research Centre Institute for Environment and Sustainability, Sustainability Assessment Unit, Via Enrico Fermi 2749; T.P. 270; I-21027 Ispra (VA), Italy
E-mail contact: salvatore.martire@unimib.it

1. Introduction

The multifunctionality of forest has been remarked constantly in European policies. Forests can ensure several ecosystem services: providing raw material for goods, regulating local and global climate, buffering weather events, regulating the hydrological cycles, protecting watersheds and their vegetation [1]. The valorisation of forest biomass is recognized as a new frontier of economically sustainable and environmentally friendly processes, however it is not possible to assume a positive comprehensive balance in term of sustainability of products based only on the fact that they are bio-based [2].

Evaluation of the trade-off between the benefits coming from forest resources’ use and the conservation of forest ecosystems is needed. Considering the use of biomass for energy purpose, on one hand the use of wood resources should be based on an evaluation of the “carrying capacity” of the forest ecosystem and site-specific characteristics (e.g. the local accessibility of raw material and the distance from the processing plant to the delivery point); on the other hand, the role of biomass valorisation has to be assessed considering the socio economic benefit or drawbacks due to the further development of the supply chain. E.g, positive effect related to an increase employment in less developed mountain areas and to a direct relation between population and territory needs to be quantified. In the context of a site–specific sustainability assessment of a wood energy supply chain, the research focuses on development of an expeditious methodology to obtain georeferred quantity of biomass at local scale for mountain forest areas, in order to facilitate energy planning that considers the local system carrying capacity and the potential of substitution of fossil fuels.

2. Methodology

Proposed methodology for a comprehensive sustainability assessment was presented in [2]. The methodology developed for the site-specific assessment of the biomass availability, with respect to carrying capacity, is summarized in Figure 1. It consists of quantification and mapping (using Geographic Information System) of forest biomass that considers local features (e.g. abundance, spatial distribution and type of species) as reported in local territorial plans and it applies Life Cycle Assessment for supporting the overall environmental assessment.

Biomass value calculated has been converted from volume to mass, considering species features and water content. The result is compared with current utilization of wood, and waste products from forestry processing are estimated, in order to quantify the mass available for energy valorisation. Then, the Energy potential is
estimated, from biomass quantity and from wood features, principally the lower calorific value and water content for each species. Finally, the potential of substitution of fossil fuels is calculated, knowing energy potential from available biomass for energy use.

3. Results

The methodology is applied to two mountain areas, Comunità Montana Lario Intelvese (CMLI) and Comunità Montana Triangolo Lariano (CMTL), in Northern Italy (Como Province). Results are summarised in Table 1. Humidity content considered was 20% and 40% (threshold values of the fuel in the case of forest chips boilers). Current utilization is estimated through the elaboration of Forest Activity Statements: 62% for CMLI and 66% for CMTL. Considering data from [3] and [4], combustible fraction adopted to estimate potential available biomass for energy use is 80%.

<table>
<thead>
<tr>
<th>Local authority area</th>
<th>Potential available biomass (t/y)</th>
<th>Current Utilization of wood* (t/y)</th>
<th>Potential available biomass for energy use* (t/y)</th>
<th>Energy potential* (GJ)</th>
<th>Replacement of fossil fuels* (tep)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CMLI</td>
<td>25,277 ± 3,636</td>
<td>15,672</td>
<td>7,684</td>
<td>89,486</td>
<td>2,138</td>
</tr>
<tr>
<td>CMTL</td>
<td>31,110 ± 4,475</td>
<td>20,533</td>
<td>8,462</td>
<td>98,395</td>
<td>2,351</td>
</tr>
</tbody>
</table>

Table 1 - Results (*medium values)

Policy of the Province of Como identifies small biomass plants (power below 1 MW thermal) as optimal solution in order to use the resource in energetic valorisation. CMLI area has already a thermal power plant forest chips for district heating. Considering the consumption of such facilities and the biomass availability calculated, it is estimated that for each study area can be provided for 20-30 similar plants. The location of these facilities should be based not only on the demand for energy, but also on the spatial distribution of biomass and accessibility of forests, considering the different types of roads and paths for transport and storage of firewood. LCA was used to assess the overall environmental impact.

Considering the study conducted by Joint Research Centre (JRC) with CATI (Computer Assisted Telephone Interviewing) methodology and related to the use of wood for domestic heating in Lombardy Region, the value of wood used is 92,290 t for the Province of Como, comparable with results of elaboration of Forest Activity Statements, approximately 80,900 t. Considering that values from Forest Activity Statements could be overestimated, that not all the wood is used for the combustion and that consumption data of the study refer only to domestic heating, it is reasonable to assume that a portion of the wood used for domestic heating comes from outside the province of Como. On the basis of available data, this amount is about 30,000 t. This value is partially bridgeable with full use of the resource in the two local authority areas.

4. Conclusions

The proposed methodology evaluates the possibility for forests to provide the supply of raw material for energy production among ecosystem services. In addition, this assessment aims to integrate considerations to protect the other ecosystem services. Moreover, the methodology is useful for a preliminary assessment of the possibility to considering woody biomass in energy planning at local level. Finally, spatial distribution, quantity and accessibility of wood resources should be compared with the energy demand in order to identify the best location and characteristics of the plants for energy production and to minimize the transport within the supply chain.

5. References


Acknowledgement - The authors thank the Province of Como for funding the activities (Project GPM - Grande Progetto di Montagna).
Is the European honeybee (*Apis mellifera mellifera*) a good representative for other pollinator species?

Ivo Roessink¹, Jozef van der Steen², Muo Kasina³, Mary Gikungu ⁴ and Roberta Nocelli⁵

¹Alterra, Wageningen, The Netherlands  
²Plant Research International, Wageningen, The Netherlands  
³Kenya Agriculture Research Institute, Nairobi, Kenya  
⁴National Museums of Kenya, Nairobi, Kenya  
⁵Federal University of Sao Carlos, Araras, Brazil  
E-mail contact: ivo.roessink@wur.nl

1. Introduction

Pollinators are important components of biodiversity and provide a key ecosystem service through pollination (Klein et al., 2007). Honeybees, mainly *Apis mellifera*, are the most economically valuable pollinators for crop monocultures worldwide (Wantanabe, 1994), however, for several high-value crops, e.g., coffee, *Apis* pollination is less effective than pollination by local wild pollinator species (Klein et al., 2003). The value of pollination for agriculture in the European Union alone is estimated at €14.2 billion per year (Gallai et al., 2008) and globally the economic value of pollination amounts to €153 billion, representing 9.5% of the world agricultural production of human food.

Worldwide an increase of high-value crop farming and an accompanying increased dependency on pollination services occurs. For instance, data from Brazil indicate that total cropping area has grown with 70% and as a consequence pesticide use increased by 700%. The current pollinator risk assessment is based on the European honeybee (*Apis mellifera mellifera*) and it is not clear if this is representative for other pollinator species.

In a first attempt to test if *Apis mellifera mellifera* is a good representative for other pollinators a first-tier contact LD₅₀ test using dimethoate was performed with several pollinator species originating from The Netherlands, Brazil, and Kenya, respectively. Thus acquired LD₅₀ data was used to construct an Species Sensitivity Distribution curve ranking the different species by their response to direct contact with the toxicant.

2. Materials and methods

In the three participating countries all tests were performed using the same disposable cages, pipettes, and type of sugar solution. The test compound was the organophosphate dimethoate since this is the toxic standard in the OECD/EPPO guideline. Test lasted at least 48 hours and the 24 hour LD₅₀ value was used for the sensitivity comparison.

Test animals differed per country (see Table 1) and during the test were incubated at relevant hive temperatures.

<table>
<thead>
<tr>
<th>The Netherlands</th>
<th>Brazil</th>
<th>Kenya</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Apis mellifera mellifera</em></td>
<td>European honeybee</td>
<td><em>Apis</em> sp. (africanized)</td>
</tr>
<tr>
<td><em>Bombus terrestris</em></td>
<td>Bumble bee</td>
<td><em>Scaptotrigona postica</em></td>
</tr>
</tbody>
</table>

*Table 1: Tested pollinators per country*
3. Results and discussion

Control mortalities were always below 20% and the $X^2$ of the calculated LD$_{50}$ values always approximated 1, indicating that the observed data did fit the model used and consequently that calculated values were considered reliable. Figure 1 shows the LD$_{50}$ values of the tested pollinators for direct contact with dimethoate. From this figure it becomes clear that *Apis mellifera* is not the most sensitive organism.

Nevertheless, the difference in sensitivity between the European honeybee (*A. m. mellifera*) and the most sensitive species the African honeybee (*A. m. scutellata*) only comprised a factor 3. Striking is, however, that both stingless bee species are more sensitive than the European honeybee, while the bumble bee is considerably less sensitive (a factor 27). In this first test only social bees have been included and lacking are representatives of solitary bees. Tests on some of the latter species and chemicals with different modes of action are planned early 2011 to expand the data set.

4. Conclusions

Although the European honeybee (*Apis mellifera mellifera*) is not the most sensitive pollinator in the test its use in risk assessment is at this point not immediately challenged. This because the results of a risk assessment based on European honeybee data provides the same result as one based on African honeybee data. However, currently this conclusion is only based on dimethoate and more data on solitary bees and compounds with other modes-of-action is needed to fully substantiate this.

5. References


Acknowledgement - The authors thank their fellow participants of the ‘Pesticide Risk for Wild Pollinators’ project Tjeerd Blacquire, Harold van der Valk, Irene Koomen, Marcia Ribeiro, Leticia, Osmar Malaspina, Thaisa Roat, Andrigo Pereira, Stefan Carvalho, Priscilla Cintra-Socolowski, Nieke van Hessel, Pamela Kibyab, Galdys Maina, Chris Odhiambo, and Barbara Herren for their creative input and the BOCI program of the Dutch Ministry of Agriculture for financial support.
1. Introduction

In spite of countless papers invoking the concept, it can be argued that the term *sustainable sediment management* has little value, as sediments are not managed for their own sake, nor, in many cases, are they managed in a truly sustainable manner. Managing sustainability in ecosystems requires the understanding and management of all aspects, both natural and anthropogenic, of the ecological and biophysical environment in a manner that sustains ecosystem services. Sediments, or the soil/sediment continuum are essential, but just part of that system and their management does not stand alone. At best, we can provide frameworks for understanding and managing the role of sediments in sustaining ecosystem services. Until recently, most regional sediment risk prioritizations and site-specific sediment Ecological Risk Assessments (ERAs) have focused primarily on the risk of contaminants in sediments on associated organisms. There is, however, a growing trend to include a consideration of ecosystem services, the benefits that people obtain from ecosystems, within decision frameworks. Expressing ecological processes and resources in terms of the goods and services they provide links our scientific understanding of the environment to socioeconomic factors. The move to link ERA and Natural Resource Damage Assessments (NRDA) to evaluate impacts; the use of tools such as Net Environmental Benefit Analysis (NEBA) and Habitat Equivalency Analysis (HEA) to compare remediation and restoration scenarios and emerging legislation such as the European Environmental Liability Directive provide opportunities to consider ecosystem services alongside more traditional decision drivers. However, although sediments figure extensively in the Millennium Ecosystem Assessment, contaminated sediment was not the dominant concern. Rather, land and water use and management practices on the landscape scale profoundly affect sediment quality and fate. Habitat change and loss, due to changes in sediment inputs, whether reductions (resulting in the loss of beaches, storm protection, nutrient inputs, etc.) or increases (resulting in lake, reservoir and wetland infilling, coral reef smothering, etc); eutrophication and reductions in nutrient inputs, and disturbance due to development and fishing practices were major drivers, with significant consequences for biodiversity and the provision and resilience of ecosystem functions and services. Thus, whilst an evaluation of contaminated sediment impact on ecosystem services may consider the impacts of habitat or substrate quality alone, sustainable management requires an evaluation of the interacting positive and negative roles of sediment in both the use and sustainability of a broad range of ecosystem services at the landscape scale. This paper will describe an effort to provide a language and conceptual framework in support of that goal.

2. Sediment Status and Ecosystem Services

For the most part, soil and sediment themselves do not provide ecosystem services. Rather, soil and sediment status provides a range of functions essential to the viability and sustainability of a variety of ecosystem services. Sediment (or soil) status, defined here as a combination of the attributes quality, quantity, transport and location, is controlled by landscape and watershed biophysical conditions. These attributes are affected by natural and intrinsic conditions and by anthropogenic management of the landscape to optimize preferred ecosystem services. A range of biotic and abiotic endpoints have sediment status requirements; the extent to which sediment status meets those needs affects the role of sediment (positive or negative) in terms of that endpoint, and in terms of how sediment effects ecosystem services provided by or represented by that endpoint (see Figures 1 and 2).

3. Conclusions

ERA concepts have been adapted to identify the pathways of impact by which sediments link the utilization of ecosystem services on land affects downstream aquatic ecosystem services; these approaches will be described. The development of sediment ecological risk assessment (SEcoRA) approaches will allow for a better understanding of the interacting positive and negative roles of sediment in the maintenance of ecosystems and the socioeconomic functioning of rivers, considering various dynamic aspects of the interactions between sediment status and various endpoints in a spatially explicit manner. Ultimately, however, ecosystem management decisions will require that stakeholders and policy makers make
(hopefully) well-informed decisions about the tradeoffs implicit in various decisions. In terms of sediments
SEcoRA may provide a basis for the evaluation of the consequences of choices made at the landscape
scale to utilize ecosystem services on the viability and sustainability of downstream aquatic ecosystem
services. The results of these analyses could then feed into ecosystem service-based cost/benefit analyses
and decision tools. However, although sediments or the soil/sediment continuum are essential, they are just
part of the ecosystem and their management does not stand alone. These are complex problems which
require the understanding and management of all aspects, both natural and anthropogenic, of the ecological
and biophysical environment in a manner that sustains ecosystem services. Successfully addressing these
issues requires that we are innovative, integrative, adaptive and collaborative – approaches must continue to
evolve if we are to sustainably manage the impacts our choices have on ecosystems.

![Figure 1: Conceptual diagram illustrating the processes on the landscape scale that drive soil/sediment attributes. These attributes must all be considered in order to evaluate the role of sediments in terms of various co-located endpoints and services. From [2].](image1)

![Figure 2: Illustration of landscape/aquatic interactions via the soil/sediment continuum. Landscape management to optimize preferred ecosystem services have a range of impacts on soil/sediment status. The dynamic nature of sediments in the hydrologic system means that landscape management affects the viability and sustainability of aquatic ecosystem services at the watershed scale. Sediment ecological risk assessment (SEcoRA) should characterize these interactions in a spatially explicit manner to help stakeholders make more informed decisions about the utilization and preservation of ecosystem services at various interacting scales. From [2].](image2)

4. References

Modelling the impact of endocrine disruptions on aquatic ecosystems: an experimental lake study

Ludiwine Clouzot¹, Chris Metcalfe², Karen Kidd³ and Peter A. Vanrolleghem¹

¹modelEAU, Université Laval, 1065, avenue de la Médecine, Québec G1V 0A6, QC, Canada
²Trent university, 1600 West Bank Drive, Peterborough K9J 7B8, ON, Canada
³Canadian Rivers Institute, University of New Brunswick, Saint John, E2L 4L5, NB, Canada
E-mail contact: ludiwine.clouzot.1@ulaval.ca

1. Introduction

Endocrine disruptions were first observed in 1994 in caged trout exposed to sewage effluents [1]. Nowadays, the scientific community has highlighted endocrine disruptions in different trophic levels, eg., invertebrates, amphibians, fish, reptiles, birds and mamals. Many laboratory experiments have been performed to identify endocrine disrupting chemicals (EDCs) and their consequences on aquatic and terrestrial species. Then, a wide range of molecules (hormones, pesticides, phthalates, alkylphenols, etc.) originating from sewage effluents have been identified as EDCs. Despite growing concern towards EDCs, impact on wild populations and consequences on ecosystem services remain unknown. Single-species tests are preferred over experiments in enclosed ecosystems for the experimental ease of work and the higher reproducibility. However, environmental risk assessment (ERA) is evolving from analysis of single-species tests to population modelling. Indeed, modelling is a promising tool to help better understand and predict EDC impacts on ecosystems.

Ecosystem modelling is not used as a general tool in ERA because of the need for extensive calibration for a specific ecosystem. However, the goal of ERA and ecosystem-based management (EBM) is to maintain ecosystem functions and services. Thus, ecosystem modelling is the logical next step in predicting impact of chemicals on aquatic and terrestrial ecosystems. This study aims to develop a simplified ecosystem model that can be used as a tool to predict EDC impacts on aquatic ecosystems. The model development is more focused on predicting ecological effects of EDCs rather than their impact on population dynamics.

2. Experimental approaches

2.1. Development of a simplified ecosystem model

A simplified ecosystem model was previously developed to predict the effects of chemicals on lentic ecosystems [2]. This dynamic model succeeded in predicting ecological effects of chemicals by considering direct effects but also ecological interactions (feeding and competition relationships). This model consisted of (i) a food web model (ii) toxic effect sub-models and (iii) a model for nutrient and detritus cycling. An object oriented framework for ecosystem modelling was developed in the software package WEST with equations based on the AQUATOX model (USEPA, 2002). This model base is extended for EDC effect modelling. Reproductive endpoints had to be added in the model to incorporate the endocrine disruptions that are commonly measured (breeding, vitellogenin, histopathology, etc.).

Field data are used to help in developing and validating the model. This study is based on published data of the EC1 project of the Canadian Water Network (CWN) which aims to assess impacts on aquatic organisms of emerging contaminants in wastewater discharges (relevant to Canadian conditions). This CWN project is based on an experimental lake in northwestern Ontario and three river systems across Canada (Grand, North Saskatchewan, Saint Lawrence). A multi-year whole-ecosystem study is performed at the experimental lake with exposure of well-defined fish and lower-trophic-level populations to environmentally-relevant concentrations of the synthetic hormone 17α-ethinylestradiol (EE2) [3]. EE2 was chosen because it is one of the most potent EDCs and its environmental concentration is known to impact endocrine system and reproductive functions of aquatic organisms. Data from the experimental lake were used to start the development of the ecosystem model because only one EDC is applied to a lentic system located in an undisturbed watershed, which are simplified conditions compared to river systems exposed to multiple stressors.

2.2. Experimental lake

The experimental lake contains naturally reproducing populations of fish, benthic invertebrates, zooplankton and algae (Figure 1). The study started in 1999 with baseline data collected until 2000 on aquatic
populations in the experimental lake and references lakes. Between 2001 and 2003, EE2 was added continuously in the experimental lake. Since 2004, EE2 addition was stopped to measure ecosystem stability and recovery after stressor removal. During all the study, fish have been collected to examine reproductive behavior, vitellogenin, sex steroids, thyroid hormones, vitamin stores, gonadal and kidney tissues, gonad and liver size. Besides, population-level parameters have been monitored such as fertilization success, sex ratios, abundance, age-to-maturity, size distributions and growth rates.

**Figure 1: Food web of the experimental lake.**

Juveniles and adults of *Fathead minnow* were used to examine population size and structure in the experimental lake and in reference lakes from the beginning of the study (1999) to the beginning of the recovery period (2005) (Figure 2). In contrast with the reference lake, the *Fathead minnow* population in the experimental lake collapsed after the second year of EE2 addition. This reproductive failure was maintained after the EE2 addition was stopped, although few small individuals indicate some reproduction was occurring.

**Figure 2: Length frequency distribution of Fathead minnow captured during the fall of 1999-2005 in a reference lake (A) and the experimental lake. Mean SE daily trap-net data for adults and juveniles for the fall catches are shown in the panels. [4]**

3. Conclusions

A model is developed to predict ecological consequences of EDCs. Results from the experimental lake were very useful to develop the simplified ecosystem model of aquatic populations and reproductive endpoints were added in line with the endocrine disruptions measured in the lake study.

4. References

Response of red fox populations to rodent field controls with bromadiolone: a 6 year study on regional scale

Marion Jacquot 1, Michaël Cœurdassier 1, Geoffroy Couval 2, Régis Renaude 3, Denis Truchetet 4, Francis Raoul 1 and Patrick Giraudoux 1

1. Chrono-environnement, UMR UFC/CNRS 6249 USC INRA, Université de Franche-Comté, Place Leclerc, F-25030 BESANCON cedex.
2. FREDON Franche-Comté, 12 rue de Franche-Comté, Espace Valentin Est, F-25048 BESANCON cedex.
3. Fédération Départementale Des Chasseurs Du Doubs, rue Chatelard, F-25360 GONSANS.
4. DRAAF/SRAL de Franche-Comté, 191 rue de Belfort, F-25043 BESANCON cedex.
E-mail contact: marion.jacquot@univ-fcomte.fr

1. Introduction

Anticoagulants rodenticides are mostly used to control rodent populations [1]. Treatments can occur on large areas and subsequently cause severe secondary poisoning of rodent predators. In France bromadiolone is the only pesticide authorized to control the cyclic fossorial form of the water vole Arvicola terrestris sherman in grasslands. In 90’s, hundreds of predators (mainly common buzzard, red fox, and red kite) died after large-scale treatments with wheat bait (60,000 ha) in the Doubs department [2]. Since 2001, legislation imposes to control voles under a lower population density limit than before and farmers are encouraged to treat as early as possible before or in the early stage of the population growth phase. The present study aims at verifying whether A.terrestris controls with bromadiolone have a long term depleting effect on fox populations and whether the changes in treatment practices decrease undesirable side effects on fox populations.

2. Materials and methods

Treatment intensity was quantified as the quantity of baits in tons (50 ppm bromadiolone) used each year on each commune (administrative division of some squares kilometers). Data on treatment intensities were recorded by the Fédération Régionale de Défense contre les Organismes Nuisibles (FREDON). Red fox populations were monitored by the Fédération des Chasseurs du Doubs (game association) by night spotlight counts. Counts were performed along 472 transects of 1-2 km distributed on the whole area of the Doubs department. For each transect a Kilometric Abundanc Index (KAI) was calculated as the number of foxes recorded in average on 1 km transect. Then values were interpolated on the centroid of each commune by ordinary kriging.

Fox counts were carried out in spring (march/april) and small mammal controls in autumn. Therefore, fox densities per commune for year n (2004 to 2009) were analysed as the response to treatments of year n-1 (2003 to 2008), and to treatments year n-1 and n-2, the latter to investigate longer delays of secondary poisoning on fox populations. Those variable combinations were included in linear models which were compared using Akaike information criterion corrected for sample size (AICc).

Finally we compared quantities of baits (tons) used per hectare treated per commune between years to characterize the evolution of treatment practices (Kruskai-Wallis test).

3. Results

The model with the lowest AICc selected to explain kriged fox densities a year n includes bromadiolone treatments of the years n-1 and n-2. Intensive treatments led to KAI decrease. Figure 1 shows that treatment impact was important in 2004, fox counts being lower in the areas where treatments were carried out in 2003. Visual examination of maps (data not shown) shows that KAI generally stayed extremely low in large areas until 2005 and partially recovered the following years. The same areas were treated again from 2006 to 2008 during the next vole outbreak and bait quantities per hectare were decreased by 2 at the minimum. Those treatments were not followed by a decrease of fox KAI.
4. Discussion

Usually, the impact of bromadiolone treatments on wildlife is known by circumstantial collection of corpses by people (farmers, hunters, ecologists, etc.) in the field routed to veterinary laboratories by the SAGIR (a network of the ONCFS, the National Game Office) but no systematic research is organized [3]. For instance, the death of 128 foxes due to massive bromadiolone treatments in 1997-1998 was reported by the SAGIR [4,5]. This does not permit to evaluate the real impact of treatment on populations. In a more standard way, Raoul et al. [6] monitored fox populations using index methods from 1989 to 2000 on an 100 km² area. After the autumn 1997 treatment, they reported a strong decrease of the fox index, and that it stayed extremely low until the end of the study.

The present work, to our knowledge, is the first one to address the issue of the impact of bromadiolone treatments on natural populations of foxes on a very large scale (about 5,000 km²), using a dense network of roadside counts on the whole area. It has also permitted to evaluate the resilience of populations to treatment. It clearly shows that the effect of massive treatments at high vole population density, as those carried out in 2003, have a negative impact on fox populations for at least two years. However the SAGIR network reports the finding of only 2 fox bodies poisoned with bromadiolone in the Doubs in 2003, which corresponds to the average number of poisoned foxes found per year over the period 2003-2008 [7].

Our approach shows an additional example of how monitoring wildlife population on the long term using index method may provide valuable informations about adverse effects of pesticide treatment. It also shows that early treatments (carried out at low density of vole population) are likely to have a lesser impact on fox populations.

5. References


Acknowledgement - We thank Olivier Mastain and Betty Plaquin for providing us data on wildlife poisoning in the Doubs department for the period 2003-2009.
1. Introduction

In-situ sediment treatment with a strong sorbent is a new remediation technique that relies on the repartitioning of hydrophobic contaminants and lowering contaminant availability to water and biota. Thus far, little attention has been devoted to the potential of sorbent amendments to restore a pollution-impacted benthic community. Efforts to assess the response of macroinvertebrates to sediment contaminants often lack of a dominant stressor, which limits assessment of ecosystem benefits of remedial strategies [1]. Contaminants in sediment that cause chronic toxicity can simplify the community structure by reducing the abundance of sensitive species [2]. Adverse effects are related to exposure, which is a function of the species’ interactions with the contaminated environment. Recently, the SETAC research community was advised to give trait-based analysis for risk assessment more attention [3]. However, the forecast of remediation success after in-situ treatment to recover the benthic community structure to reference conditions is challenging because data from full-scale, in-situ sorbent amendments does not exist to date. Here, a modeling framework allows linking functional feeding traits with exposure and it further permits comparing the expected remedial success to reference conditions.

2. Materials and methods

2.1 Benthic community.

The present study hypothesizes that pollution-induced changes of the benthic community can be observed at the U.S. EPA Superfund site at Hunters Point (HP), California, relative to 30 reference sites with similar physical habitats in the Central San Francisco Bay. Analysis based on number of species, total abundance, the Shannon-Wiener Diversity index, and dominant species did not reveal differences. Thus, the benthic samples were analyzed based on biological traits of functional ecology. Functional groups were defined for (1) feeding traits, (2) reproductive traits, and (3) position in and protection from the sediment based on differences in exposure to the contaminated environment.

2.2 Sediment characteristics.

The study evaluated the dominant stressor(s). All sites show similar TOC (1.3±0.4%) and fraction of fines (79±20%). Information about contamination levels in sediment (top 5 cm) for all sites were compared including total PCBs, heavy molecular weight polyaromatic hydrocarbons (HPAHs), light molecular weight PAHs (LPHAs), total dichloro-diphenyl-trichloroethanes (DDTs), dieldrin, copper, lead, arsenic, mercury, and nickel.

2.3 Ecosystem recovery scenarios.

A biodynamic model was employed to estimate PCB availability and tissue concentrations for organisms with different feeding traits and under different PCB exposure conditions.

\[
\frac{dC_{org}}{dt} = \text{C}_{\text{sed}} \cdot \text{IR} \cdot \text{AE}_{\text{sed}} + \text{C}_w \cdot k_w - C_{org, t} \cdot (k_e + k_g)
\]

With \( C_{org} \) the PCB concentration in the organism (µg/g dry tissue); \( C_{sed} \) the PCB concentration in the sediment (µg/g dry wt); \( IR \) the ingestion rate (g particles/g dry wt·d); \( AE_{sed} \) the assimilation efficiency of PCBs from sediment [-]; \( k_w \) the aqueous uptake rate constant (L/g·d); \( C_w \) the aqueous PCB concentration (µg/L); \( k_e \) the rate constant of loss (1/d); and \( k_g \) the growth rate constant (1/d).
Feeding strategies considered: the filter and surface-deposit feeding clam *Macoma balthica*, the deposit feeding polychaete *Neanthes arenaceodentata*, and the filter feeding mussel *Mytilus edulis*. Exposure conditions considered: (1) Hunters Point (HP), (2) the reference sites (3) the Effective Range Low, ERL, for PCBs, (4) the Effective Range Median, ERM, for PCBs, (5) the PCB cleanup goal for HP, (6) a site in Oakland Harbor, and (7) the expected remedial response to an in-situ activated carbon amendment. The values for \( C_{\text{sed}} \) of the scenarios considered range over 2 orders of magnitude. The model was used to approximate the reduction of PCB availability (represented by PCB assimilation efficiency, \( AE_{\text{sed}} \)) required to achieve tissue concentrations at HP comparable to the different exposure scenarios.

3. Results & discussion

Benthic community analysis shows that HP is deprived of deposit feeders, subsurface carnivores, egg laying species, and species with no/weak protective barrier in comparison to reference sites (example in Fig. 1). Even though abundance is similar, the HP site shows lower species diversity within these functional groups than the intertidal reference sites. Sediment chemistry showed that PCBS are the major risk drivers at HP (1570 ppb) and that the reference sites contain much lower levels of PCB contamination (9 ppb). The biodynamic model shows how the feeding modes of organisms are linked to differences in exposure. The deposit feeder *N. arenaceodentata* accumulates 20-times more PCBS in its lipids than the facultative deposit feeder *M. balthica* and up to 180-times more than the filter feeder *M. edulis* accumulates though the aqueous phase (Fig. 2). A comparison of exposure scenarios suggests that PCB tissue concentrations at HP are two orders of magnitude higher than at the reference sites. The model predicts that a full-scale sediment amendment with activated carbon can reduce PCB uptake at HP by up to 85 to 90% under favorable field and treatment conditions, which corresponds to exposure conditions suggested by the sediment quality guidelines and the cleanup goal for HP (Fig. 2).

4. Conclusions

The comparison of benthic communities based on functional ecology proved to be a sensitive approach to indentify pollution-induced changes at Hunters Point. Species with functional traits that resemble high exposure show lower abundances and species richness. In the absence of long-term monitoring of the response of a benthic community after a full-scale, in-situ sorbent amendment of contaminated sediment, we show how a modeling framework allows simulating bioaccumulation for organisms with different feeding traits and evaluate the expected remedial response of a sorbent amendment relative to reference conditions. The predicted remedial response suggests that ecosystem recovery is expected to progress to satisfying levels that would comply with the sediment quality guidelines and the cleanup goal for HP. The present study demonstrates how the remedial success of an untested sorbent amendment, which lowers the PCB availability, can be compared to reference conditions and traditional cleanup goals that are commonly based on total sediment concentrations. Such comparative analysis improves the understanding of the remedial possibilities and regulatory acceptance for sediment remediation in general, and for sorbent amendments specifically.

5. References


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Recovery potential of diatomic biofilms after industrial contamination (Cd, Zn) : field and experimental studies

Adeline Arini1,2, Agnès Feurtet-Mazel1, Michel Coste2, François Delmas2.

1Université Bordeaux 1, CNRS, UMR 5805 EPOC, Equipe GEMA, Place Peyneau, 33120 Arcachon, France
2Cemagref, UR Réseaux, Epuration et Qualité des Eaux, 50 av. de Verdun, 33612 Cestas, France.
E-mail contact: a.arini@epoc.u-bordeaux1.fr

1. Introduction

The Gironde fluvio-estuarine system (France) has been subjected to polymetallic pollution (Cadmium and Zinc) for over a century, coming from an industrial site specialized in zinc ore treatment. Since 2007 a major remediation phase has been initiated in the industrial site to improve the chemical and ecological state of the natural environment.

In this remedial context, and in reference to the initial state of contamination determined by previous studies (National program Ecodyn) [1], this study forms part of a multidisciplinary project ANR (RE-Syst). It was conducted on periphytic diatoms biofilms, dominant primary producers in freshwaters and it aims to combine in situ and laboratory studies to assess first biological modifications on biofilms in decontamination conditions in order to better understand the effects of the remediation procedure carried out on the industrial site.

2. Materials and methods

The colonization of biofilms was conducted during 24 days on a site undergoing industrial discharges and in parallel on a metal-free site located upstream from the factory, considered as control.

After 24 days of colonization, biofilms from the polluted site were separated into several batches. A first was left exposed in the contaminated site. A second was translocated to the metal-free site to undergo a decontamination under natural conditions. A third batch was brought in the laboratory, kept into artificial streams under controlled conditions for 56 days, and also divided into 2 lots. A first was kept under contamination conditions with metallic concentrations similar as those found in situ. A second was maintained under decontamination conditions, into metal-free medium, to highlight the recovery potential of biofilms. Finally, biofilms from the metal-free site were also brought in the laboratory as control biofilms.

![Experimental diagram of biofilm translocations.](image)

Different tests were then performed: analyses of metal bioaccumulation, taxonomic investigations, biovolumes and teratological forms measurements, diatom densities numerations, completed by physicochemical measurements in water.
3. Results and discussion

3.1. Recovery potential under natural conditions

Contaminated biofilms translocated to the metal-free site showed a very fast decontamination potential. Indeed, metallic concentrations bioaccumulated into biofilm from the polluted site were not significantly different from the control biofilms after only 24 days of decontamination.

Also, after 24 days, only a few species, like Eolimna minima, considered as metal-resistant, were still found into biofilms in decontamination, but in very low densities in comparison with their densities just before the transplantation or with densities numerated into biofilms maintained in the contaminated site. However, most of species were similar to those found into control biofilms.

3.2. Recovery potential under controlled conditions

Metal concentrations into biofilms showed a sharp decrease but both Cd and Zn stay significatively higher than concentrations analyzed into control biofilms after 56 days of decontamination.

![Figure 1: Kinetics of Cd(A) and Zn (B) concentrations into biofilms under laboratory conditions (n=3). Kruskal-Wallis test (p<0.05) : a : significatively different between control and decontaminated biofilms, b : significatively different between control and contaminated biofilms.](image)

Otherwise, taxonomic inventories did not highlight a complete recovery of diatom communities. Indeed, at the end of the experiment, although we could observe the reappearance of sensitive species, metal-resistant species, initially present into contaminated biofilms, persisted with high abundances into biofilm under decontamination conditions.

4. Conclusions

Results from field study showed that a short period of 24 days, after translocation, was necessary to reach biofilm recovery. Those results are promising for the recovery potential of impacted hydrosystems. However, it is important to consider that those biofilms underwent a brutal stop of contamination and also a brutal change of environmental variables when they were translocated from the contaminated to the metal-free site, which strongly influence diatoms assemblages. Indeed, by comparing results from field and experimental studies, it appears that under laboratory conditions, time needed for the disappearance of metal-resistant species in favor of sensitive species is delayed compared to field experiments. This suggests, under field conditions, important species immigration and emigration from the river which prevail over multiplication rates of pre-established species [2]. The laboratory experiment proved that without this process, the restructuration is longer (more than 56 days). This demonstrates the interest of laboratory experiment under controlled conditions. Complementary experiments would be necessary to assess the real importance of diatom import or export into biofilm and their effects on the evolution and then recovery potential of diatom community structures.

5. References

Acknowledgement - This work was supported by the Agence Nationale de la Recherche (ANR) through its programme CES (Contamination Ecosystèmes Santé) under the reference ANR 08-CES-014 and developed in the RE-SYST project. The authors would like to thank Henri Bouillard, Véronique Duflo, from the Gema team for their technical assistance in the field, and Muriel Bonnet and Maryse Boudigues from the Cemagref laboratory for the water sample analyses.
Establishing environmental risk based management for industrial operations in (sub-) Arctic marine areas. Linking early warning signs to whole organism effects from individual to population levels.

Baussant, T¹, Jonsson H¹(1), Ingvarsdóttir A¹, Bechmann RK¹, Taban IC¹, Skadheim A¹(1), Pampanin DM¹, Bamber S¹, Ravagnan E¹(1), Hjermann DØ², Christie OJ³, Pinturier L⁴, Bracco L⁵, Buffagni M⁶, Sanni S¹

¹ International Research Institute of Stavanger (IRIS) - Biomiljø, Mekjarvik 12, N-4070 Randaberg, Norway
² Centre for Ecological and Evolutionary Synthesis (CEES), Dep. of Biology, University of Oslo, Norway
³ InfoStrat, N-4319, Sandnes, Norway
⁴ TOTAL E&P NORGE AS, N-4001, Stavanger, Norway
⁵ Eni Norge AS, N-4313, Sandnes, Norway
⁶ Eni E&P Division, 20097, San Donato, Milano, Italy
E-mail contact: steinar.sanni@iris.no

1. Introduction

Population and community responses to multiple stressors can be assessed by combining fate and effect modelling with information from whole organisms and individual internal processes. This evaluation is followed by population dynamics modelling. Herein we present such an approach, based on combination of experimental studies, field monitoring and model tools.

Two subsequent projects (BioSea I & II; 2001-10), focused on establishment of environmental monitoring-, risk-, and model tools for oil based discharges to the marine environment. Crude oil consists of a large number of chemical constituents representing a combination of different stressors. The following achievements were made, linking sub-individual biomarker signals to whole organism effects at individual and population levels to aid environmental management.

− Critical harmful (fitness) effect concentrations of dispersed oil were determined based on parameters expressing survival and growth of vulnerable life stages.
− Biomarker threshold values were determined where fitness effects of individual organisms were observed.
− A model approach was developed to assess adverse effects on population level for northern shrimp.

2. Environmental risk - critical levels for whole organisms

In environmental risk assessment, risk can be defined in relation to probability of adverse biological effects. Adverse effects signify when pollutant load in the environment exceeds levels that imply damage to important parts of the ecosystems (Norwegian Ministry of Environment, White paper 58, in Norwegian, 1997). The BioSea Project assumption that such critical levels can be defined by the concentrations where effects are seen/not seen in larval stages of important species is in accordance with guidelines by e.g. [1, 2].

Critical levels of oil exposure were determined for two key species from the Barents Sea, one fish species (halibut) and one invertebrate species (northern shrimp). The fitness effect of larval stages exposed to dispersed oil at the lowest concentration was selected to provide conservative critical levels. No effects were observed in northern shrimp at 0,12 ± 0,01 µg/l PAH, while effects were observed at 0,26 µg/l PAH. No effects were observed in fish at 2,4 ± 0,3 µg/l PAH, while effects were observed at 5,5 µg/l PAH.

3. Monitoring - thresholds for biomarker signals

Biomarker threshold levels were defined as responses measured at critical levels for the whole organisms. Where no threshold data could be obtained from the experimental work, an ‘elevated background level’ was defined by a 90 percentile value from data and used as biomarker threshold value. These principles are the same as used by ICES/OSPAR for developing assessment criteria for biological effects of contaminants monitoring [3]. Threshold levels of key biomarker monitoring are shown in Table 1.

Biomarker threshold levels at different oil concentrations can also be correlated to risk assessment, using species specific critical oil concentrations as effect limit values in the risk model. When the model predicts that a critical level is exceeded, it implies that the biomarker threshold levels are also exceeded. The link is based on statistics and there are loopholes in the mechanical understanding of causal relationships [4].
Table 1: Threshold levels in selected biomarker methods for monitoring of oil based discharges.

<table>
<thead>
<tr>
<th>Background / Threshold</th>
<th>‘Green’ Biomarker range</th>
<th>‘Amber’ Biomarker range</th>
<th>‘Red’ Biomarker range</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>safe level</td>
<td>uncertain range</td>
<td>action level</td>
</tr>
<tr>
<td>source</td>
<td>NOEC-fitness - corresponding level (or 90 percentile field background)</td>
<td>between tested NOEC and LOEC</td>
<td>LOEC-fitness - corresponding level</td>
</tr>
<tr>
<td>Fish species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atlantic Cod</td>
<td>&lt;10</td>
<td>10-23</td>
<td>&gt;23</td>
</tr>
<tr>
<td>Halibut</td>
<td>&lt;15</td>
<td>15-19</td>
<td>&gt;19</td>
</tr>
<tr>
<td>Long rough dab</td>
<td>&lt;10</td>
<td>10-12</td>
<td>&gt;12</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atlantic Cod</td>
<td>&lt;35</td>
<td>35-85</td>
<td>&gt;85</td>
</tr>
<tr>
<td>Halibut</td>
<td>&lt;55</td>
<td>55-60</td>
<td>&gt;60</td>
</tr>
<tr>
<td>Long rough dab</td>
<td>&lt;100</td>
<td>100-110</td>
<td>&gt;110</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atlantic Cod</td>
<td>&lt;1,8</td>
<td>1.6-4.7</td>
<td>&gt;6.7</td>
</tr>
<tr>
<td>Halibut</td>
<td>&lt;0.7</td>
<td>0.7-5.8</td>
<td>&gt;5.8</td>
</tr>
<tr>
<td>Long rough dab</td>
<td>&lt;0.5</td>
<td>0.5-4.0</td>
<td>&gt;4.0</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Atlantic Cod</td>
<td>&lt;0.17</td>
<td>0.17-0.29</td>
<td>&gt;0.29</td>
</tr>
<tr>
<td>Halibut</td>
<td>&lt;40</td>
<td>40-75</td>
<td>&gt;75</td>
</tr>
<tr>
<td>Long rough dab</td>
<td>&lt;160</td>
<td>160-315</td>
<td>&gt;315</td>
</tr>
<tr>
<td>Invertebrate species</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pandalus borealis</td>
<td>&gt;70</td>
<td>70-90</td>
<td>&lt;90</td>
</tr>
<tr>
<td>Mytilus edulis</td>
<td>&gt;70</td>
<td>70-90</td>
<td>&lt;90</td>
</tr>
<tr>
<td>Chlamys islandica</td>
<td>&gt;70</td>
<td>70-90</td>
<td>&lt;90</td>
</tr>
</tbody>
</table>

Nevertheless, establishment of such links can be a useful step to establish tools to administer integrated assessment of different levels of biological organisation. Function of biomarkers and their threshold levels in the assessment system is that they indicate a deviation from normal and predicted conditions. Exceeded biomarker thresholds can show occurrence of metabolic or toxic reactions and the type of contamination, all useful information for determination of follow-up actions.

4. Ecological evaluation – population and community modelling

Linking biomarker threshold levels to adverse environmental effects at population or community levels requires other assessment tools. Relating more complex processes, from low level biomarker responses and effects on whole organisms, to environmental effect at population and community levels requires further research. Models focusing on population dynamics can be developed to assess effects in populations given individual fitness impairment such as the model for different fish species in the Arctic Barents Sea region [5].

A population model for northern shrimp in the Barents Sea was developed within the BioSea II project. Population dynamics were simulated using closed life cycle population model incorporating both reproduction and survival. It could be run as a simulation with given external variables such as climate and impact of fishing. Effects of harvesting, predation, and climate were determined using abundance estimates for shrimps and statistical analyses (General Additive Model; GAMs). Effects of harvesting and predation were strong, while temperature affected recruitment. The resulting model then simulated abundance of the population in the Barents Sea in the period 1982-2007.

The model was used to predict effects from reduction in recruitment due to added mortality (oil exposure). Analysis of sensitivity was performed to determine uncertainty in parameter values. The analysis indicated that with 5 % increase in mortality above normal levels in the recruitment phase, there was a loss of 2-3% of the adult population with temporary population decrease affecting the population for 2 years. This indicates that dose:response data for fitness can be used as basis to simulate approximate population effects for the shrimp. This type of population models can be combined into simple community models, if data on relevant species are available. The assessment system established based on the BioSea II results consist of a discharge model, providing predictions of physical/chemical fate, with the capability to estimate effects on organisms and biomarker responses. The predictions can be further evaluated by population and simple community models. The control monitoring in the field is based on biomarkers showing when threshold levels associated with fitness effects have been exceeded. These biomarkers are standard biomarkers also recommended for monitoring other contaminants [3]. This provides a potential to relate and obtain synergies with other monitoring and assessment programs.

5. References

Fate of organophosphorus flame retardants -
Determination of their metabolites in human urine

Thorsten Reemtsma\textsuperscript{1, 2}, Jana Lingott \textsuperscript{1} and Stefanie Roegler\textsuperscript{1}

\textsuperscript{1}Technical University of Berlin, Department of Water Quality Control, Sekr KF 4, Strasse des 17 Juni 135, 10623 Berlin, Germany
\textsuperscript{2}Helmholtz Center for Environmental Research (UFZ), Permoserstr. 15, 04318 Leipzig, Germany.
E-mail contact: thorsten.reemtsma@ufz.de

1. Introduction

Trialkylphosphates (TAP) are a group of flame retardants that is used increasingly, also as substitute for brominated flame retardants. Besides that TAP are used as pesticides, plasticizers and as additive in many industrial and consumer products. Owing to their widespread use TAP are also widely distributed in the environment \cite{1}. Due to the presence in consumer products humans are exposed to TAP, and TAP have been found in biomonitoring studies, in human breast milk as well as in urine.

Depending on their alcohol moiety TAP are more or less stable and hydrolysis may occur. These hydrolysis products, dialkyl phosphates (DAP) and monoalkyl phosphates (MAP), are far less thoroughly investigated. This was partly due to the lack of analytical methods and of reference compounds required for method development and quantitation \cite{2}.

However, restricting our view to the parent TAP provides an incomplete picture of the occurrence of organophosphorus compounds, in the environment \cite{1} as well as in humans.

This study aimed at:

- adopting a previous method developed for wastewater \cite{3} to human urine and to extend the number of analytes included
- applying the method exemplarily to human urine, to test whether DAP and MAP are of relevance in the human body and whether they contribute to the exposure of humans to organophosphorus compounds.

2. Materials and methods

A method was developed for the detection 14 metabolites of TAP (DAP and MAP) in human urine \cite{4}. This method is based on liquid-chromatography-mass spectrometry (LC-MS/MS) using ion-pair chromatography \cite{3}.

The following analytes were included: dimethylthiophosphate (DMTP), diethylphosphate (DEP), diethylthiophosphat (DETP), mono-2-chloropropyl phosphate (MCPP), mono-n-butylphosphate (MnBP), diethyldithiophosphate (DEDTP), monophenylphosphate (MPhP), monobutoxyethylphosphate (MBEP), di-iso-butylphosphate (DiBP), di-n-butylphosphate (DnBP), diphenylphosphate (DPhP), monoethylhexyl-phosphate (MEHP), dibutoxyethylphosphate (DBEP) and diethylhexylphosphate (DEHP).

For six of the analytes reference material was synthesized in this study because it is not commercially available.

3. Results and discussion

3.1. Analytical Method

The method was easy to handle and resulted in a median LOQ of 0.6 – 11 $\mu$g/L. For some analytes matrix effects were strong and variable. While standard addition could compensate for this, a method involving an improved clean-up may result in more uniform analyte response. For some of the MAP, namely MPhP, MBEP and MCPP lower detection limits are desirable for further investigations. Beyond these 14 analytes other MAP and DAP could be included into the method.
3.2. Application to human urine

Highest concentrations in human urine (µg/L range) were found for MnBP, DEP, DPhP. The concentrations in the different samples were highly variable, with a median of the total concentration of all analytes of 20 µg/L and a 95 percentile of 250 µg/L. Five MAP and two DAP were detected for the first time in human urine.

These first results generated with the new LC-MS/MS method suggest that the body burden for organophosphorus compounds may be much higher than visible from the sole analysis of the TAP.

It needs to be clarified whether the DAP and MAP determined in human urine had been formed from TAP in the human body after uptake or were transformed before uptake and incorporated as DAP and MAP, already.

4. Conclusions

The inclusion of TAP metabolites in future monitoring studies, in the environment as well as in biomonitoring, should improve our knowledge on the fate of organophosphorus flame retardants.

In environmental studies this would improve our understanding of the environmental fate of TAP. It would also help to assess which route of exposure is most relevant for humans. In biomonitoring the inclusion of TAP metabolites will provide a more comprehensive picture of the exposure of humans to organophosphorus compounds. This is a prerequisite for an adequate risk assessment.

5. References


Brominated flame retardants and Dechlorane Plus in air and seawater from the German Bight, North Sea

Axel Möller1, Zhiyong Xie1, Renate Sturm1, and Ralf Ebinghaus1

1Helmholtz-Zentrum Geesthacht, Centre for Materials and Coastal Research, Institute of Coastal Research, Department for Environmental Chemistry, Max-Planck-Strasse 1, 21502 Geesthacht, Germany
E-mail contact: axel.moeller@hzg.de

1. Introduction

Brominated flame retardants (BFRs) have been used for more than 50 years to reduce the flammability of various industrial and commercial products like plastics, textiles and furniture. Polybrominated diphenyl ethers (PBDEs), which are currently the most well studied BFRs, are known to be toxic, bioaccumulative, persistent, are ubiquitous in the environment and undergo atmospheric long-range transport [1; 2]. As a result of their excessive use and long-range transport potential, they have been found worldwide in various environmental compartments with an increasing temporal trend, even in remote areas like the Arctic [3]. Therefore, the usage of the technical PBDE mixtures was banned in the European Union and the Penta- and OctaBDE mixtures were recently included in Annex A of the Stockholm Convention on Persistent Organic Pollutants (POPs).

This leads to an industrial shift towards “new”, non-regulated non-PBDE BFRs such as 1,2-bis(2,4,6-tribromophenoxy)ethane (BTBPE) which has been introduced as replacement for the OctaBDE mixture. Dechlorane Plus (DP), a highly chlorinated flame retardant, has been introduced as replacement for the toxic Mirex in the 1960s and was suggested to replace the banned BDE-209 in the EU [4]. Data on alternative BFRs and DP in the environment, especially in the marine environment, are rare and recent publications mainly focused on their occurrence in biotic samples and, concerning DP, on source-near samples. Here we present the occurrence, distribution and air-seawater exchange of PBDEs, several non-PBDE BFRs and DP in air and surface seawater from the North Sea, Germany.

2. Materials and methods

Sampling in the German Bight of the North Sea took place during three sampling cruises with the German Research Vessel RV Heincke in March, Mai and September 2009 (Figure 1).

One day air samples (~350 m³) were taken at the upper deck using a high-volume air sampler equipped with a glass fiber filter (GF/F) for airborne particles and a PUF/XAD-2 column for the gaseous phase. Water samples (100-200 L) were simultaneously taken via the ship’s intake system using glass fiber filters (GF/C) combined with PUF/PAD-2 packed glass columns.

Extraction and cleanup was done in a clean-lab class 10000. Samples were spiked with 13C6-hexabromobenzene (13C6-HBB), 13C12-BDE-77, 13C12-BDE-138, 13C12-BDE-209 and 13C10-synDP as surrogate standards and extracted for 16h in a Soxhlet apparatus using dichloromethane. The extracts were evaporated to 1-2 mL and further cleaned on a 2.5 g silica column (10 % water deactivated). BFRs and DPs were eluted with 20 mL hexane, evaporated to the final volume of 30 µL and 10 ng 13C-hexachlorobenzene was added as injection standard. The samples were analyzed for 10 PBDE congeners (BDE-28, BDE-47, BDE-66, BDE-85, BDE-99, BDE-100, BDE-153, BDE-154, BDE-183 and BDE-209), several non-PBDE BFRs (e.g., HBB, 2,3-dibromopropyl-2,4,6-tribromophenyl ether (DPTE), BTBPE and pentabromotoluene (PBT)) and for two DP stereoisomers (syn- and antiDP). Analysis was performed by a gas chromatography-mass spectrometry system (6890 GC/5975 MSD) in electron capture negative chemical ionization mode (ECNI).
3. Results and discussion

3.1. PBDEs

The $\sum_{10}$PBDE concentrations ranged from <1 to 15 pg m$^{-3}$ and from <1 to 10 pg L$^{-1}$ in air and seawater, respectively. Thereby, the PBDE congener pattern was dominated by the PentaBDE congeners (BDE-47, -99 and -100) and by BDE-209. The highest peak concentrations were observed for BDE-209 what might be a result of the later banishment of BDE-209 compared to Penta- and OctaBDE and the ongoing usage of BDE-209 in the U.S. and China and, possibly, import of products containing BDE-209 to Europe.

The atmospheric samples were highly influenced by the air mass origin while no significant seasonal trends were observed. The highest atmospheric concentrations were found at stations containing continental air masses (e.g., from Germany, the Netherlands) while lower concentrations were observed for oceanic air masses and air masses originating from the Scandinavian countries.

3.2. Non-PBDE BFRs and Dechlorane Plus

Among the non-PBDE BFRs, HBB and DPTE dominated with concentrations similar to those of the PentaBDE congeners. Besides HBB and DPTE, other non-PBDE BFRs such as PBT were detected in comparably low concentrations.

Dechlorane Plus was detected in all samples from <1 to 23 pg m$^{-3}$ in air and <1 to 10 pg L$^{-1}$ in seawater. Thereby, peak concentrations were detected at the same stations as observed for BDE-209. This indicates the replacement of BDE-209 by DP in industrial applications. The fraction of the syn-isomer ($f_{\text{syn}} = [\text{synDP}]/([\text{synDP}]+[\text{antiDP}])$) in “continental” air samples was close to the technical mixture ($f_{\text{syn}} = 0.32$ [5]) while “oceanic” samples showed an enrichment of the syn-isomer caused by degradation of the anti-isomer during atmospheric transport as proved in our recent study [6].

4. Conclusions

This study shows the presence of several non-PBDE BFRs, especially HBB and DPTE, and Dechlorane Plus in similar concentrations as the banned PBDEs. Germany, representative of Western Europe, is a source of BFRs and DP in the marine environment while they are emitted into seawater via air-sea gas exchange, dry deposition and riverine fluxes.

5. References


Acknowledgement - The authors thank the captain and the crew of RV Heincke and Armando Caba (HZG) for sampling
1. Introduction

Rapid and increasing development of antibiotic resistant bacteria (AR) is eroding the huge benefits of antibiotics in the treatment of disease. Although this problem partially results from inappropriate antibiotic use in medicine and agriculture, there is growing evidence that untreated wastes, which contain organic and heavy metal pollutants also play a role in environmental AR development. One pollution setting where elevated AR might be apparent is downstream of historic abandoned zinc (Zn) and lead (Pb) mines that are scattered across countryside in Northern England. Such sites are of interest because Zn and Pb are often the only pollutants at a given site; therefore, such locations are well suited for the assessing specific effects of heavy metal pollution on AR development. As background, strong evidence exists that shows heavy metals in polluted water and sediments can cause AR due to the co-development of AR that parallels metal resistance in exposed organisms.

This study compared AR using both culturing and genetic methods to quantify AR at various locations in the River Tyne watershed in Northern England. The setting is ideal for assessing the effects of metal pollution on AR because one arm of the river is heavily impacted by mining (the South Tyne) and has very high sediment Zn and Pb levels, whereas the other arm (the North Tyne) has similar mineralogy, but no historic mining. Therefore, by comparing AR in the North and South Tyne using culturing and genetic methods, we can assess the effect of Zn and Pb on AR and also compare culturing versus genetic signals for detecting AR in metal-impacted settings.

2. Materials and methods

Water column and sediment samples were collected using previous methods at five locations in the Tyne watershed, including three impacted sites on the South Tyne and two unimpacted sites on the North Tyne (see Figure 1). All culturing was performed using serial dilution methods on R2A agar media amended with 0, 1000, and 2000 ug/mL of Zn, Pb, ampicillin and tetracycline in different combinations. Resistance strains were defined as colony forming units on media with elevated levels of each antibiotic agent. Resistant colony numbers were quantified by plate counting. Cross-and multi-resistance was assessed by sub-culturing previously resistant colonies on different media and also by combining different metals and antibiotics within the same media. All gene quantification was performed using qPCR and probe-primers sets previously reported, and focused on antibiotic resistance genes (ARG) for β-lactam and tetracycline antibiotics.

3. Results and discussion

3.1. Ambient sediment metal levels

Table 1 summarises mean Zn and Pb levels in composite sediment samples collected at the five sampling sites. The Nent, South Tyne and West Allen are in the South Tyne catchment (i.e., the mining area), whereas Wark’s burn and North Tyne in the northern...
catchment. Relative levels exemplify the huge impact of historic mining of metal levels in the area, which dominated the region for over 200 years; although active mining has not occurred since the 1970s.

3.2. Resistant bacteria and resistance genes in the sediments

Figure 2 presents percentages of resistant cultured colonies to 1000 μg/ml ampicillin (relative to unamended media) at the sites shown in Table 1. Percent resistant colonies were almost 100 times greater at mining exposed sites relative to pristine sites, and significantly correlated with Zn and Pb levels in the sediments (P < 0.05). Interestingly, the relative numbers of ampicillin resistant colonies were lower on plates with Zn in the plate media, suggesting the mode of resistant differed among cultured isolates.

Despite strong correlations between metal levels and resistant colonies, no quantified ARG associated with β-lactam and tetracycline resistance correlated with observed metal levels, which suggests that observed in cultivable organisms was conferred by other mechanisms, possibly uptake-efflux restriction(2). These results show that solely relying on ARG for tracking resistance can provide very misleading results. This is especially important at these sites because 87% of ampicillin resistant colonies also were multi-resistant to all other metals and antibiotics, which confirms that non-specific resistant mechanisms dominate resistance in most of the isolates.

4. Conclusions

Elevated levels of antibiotic resistant colonies were found at the impacted sites by mining activity; however, no equivalent patterns were observed for resistance genes. This suggests that studies that solely rely on ARG for detecting apparent resistance can provide misleading results, depending upon dominant resistance mechanisms, and we suggest that all studies on environmental antibiotic resistance include culturing methods to both detect environmental strains not covered by defined resistance mechanisms and associated genes.

5. References

Effects of veterinary medicines introduced via manure into soil on transferable antibiotic resistance in soil bacterial communities

Kornelia Smalla1, Holger Heuer1, Chu Thi Than Binh1, Christoph Kopmann1, Ute Zimmerling1, Ellen Krögerrecllenfort1, and Eva Top2

1Julius Kühn-Institut - Federal Research Centre for Cultivated Plants (JKI), Institute for Epidemiology and Pathogen Diagnostics, Braunschweig, Germany
2Department of Biological Sciences, University of Idaho, 258 Life Sciences South Moscow ID 83844-3051
E-mail contact: Kornelia.smalla@jki.bund.de

1. Introduction

Due to the use of veterinary antibiotics in animal husbandry, considerable amounts of antibiotics are introduced to soil with spread manure. Manure can also contain a high load of bacteria carrying transferable antibiotic resistances. It is supposed that selective pressure by antibiotics contributes to changes in the structure and function of the microbial communities by selecting resistant populations, and adaptation of sensitive populations through the acquisition of resistance genes [5]. Antibiotics entering the environments can be regarded as ecological factors shaping microbial communities and fostering their evolution [1]. The approach of capturing transferable plasmids (conjugal or mobilizable) into a selectable recipient strain offers the chance to study the genetic structure and transferability of plasmids independent from the cultivability of the original hosts. Transferable multi-antibiotic resistance plasmids could be easily captured in E. coli from 15 out of 16 field-scale manures and were detectable by qPCR in total community DNA from manure slurries indicating that manure is another important reservoir of transferable antibiotic resistances [2,3]. Here we report on the isolation and characterization of plasmids that belong to the previously proposed novel group of LowG+C plasmids [4] and IncP-1ε group in various independent micro- and mesocosm experiments that were performed to study the effect of SDZ entering the soil via manure on soil bacterial communities. Three of the plasmids were completely sequenced. In addition, a real-time PCR system was established to provide quantitative data on the abundance of populations carrying IncP-1ε plasmids in manure slurries, in agricultural and none-agricultural soils.

2. Materials and methods

Capture of plasmids from soil bacteria in a plasmid-free rifampicin resistant E. coli recipient was done as previously described [2]. Shortly, soil was shaken overhead with glass beads for two hours in 1:10 diluted Tryptic Soy Broth (BD Diagnostic Systems, Heidelberg, Germany), and mixed with E. coli cells. Coarse particles were sedimented, cells from supernatants were pelleted and transferred to a membrane filter on Plate Count Agar (Merck, Darmstadt, Germany). After overnight incubation at 28°C, the suspended mating mixtures were spread plated on Mueller-Hinton agar NCCLS (Merck) supplemented with SDZ and rifampicin to select for transconjugants that captured a sulfonamide resistance plasmid.

Southern blot restriction analysis was done as previously described [2].

Sequencing of shotgun libraries from the plasmids, sequence assembly and gap closure by primer walking were performed by the U.S. Department of Energy Joint Genome Institute (Walnut Creek, CA). Putative open reading frames in the complete nucleotide sequences were compared by Blastx and Blastn searches to GenBank sequences.

3. Results and discussion

3.1. Exogenously isolated plasmids

Conjugative plasmids conferring sulfonamide resistance were captured from manure or soil bacteria by exogenous plasmid isolation in E. coli. In two microcosm and a mesocosm experiment with manured soil, a total of 204 captured plasmids were analysed. The majority of plasmids captured belonged to the recently discovered group of LowG+C plasmids and 15% to the IncP-1ε subgroup. Restriction profiles and the plasmid encoded resistances showed that a high diversity of antibiotic resistance plasmids was captured from soil and rhizosphere samples. Although potential transconjugants were only selected based on the
captured SDZ resistance, the plasmids captured in *E. coli* typically conferred combinations of antibiotic resistances such as: oxytetracycline, sulfamerazin; chloramphenicol, oxytetracycline, sulfamerazin, streptomycin; ampicillin, oxytetracycline, sulfamerazin, streptomycin; chloramphenicol, oxytetracycline, sulfamerazin, streptomycin, trimethoprim. The resistances reflect the frequent use of these antibiotics in animal husbandry. All of the IncP-1ε plasmids hybridized with the sulfadiazine resistance gene *sul1* and the integrase gene *int1* of class 1 integrons.

### 3.2 Sequencing of IncP-1ε plasmids

The backbone of all four plasmids was nearly identical to that of pKJK5, the first published complete sequence of the IncP-1ε plasmids. It comprised genetic modules for replication, partitioning and regulation, mating pair formation and conjugal transfer, and a region with genes of unknown function. The plasmids differ in the gene cassettes which were captured into the attachment site of the integron. They harbour either *aadA* (pHH3414), *aadA* and two copies of *catB* (pHH128), or were devoid of any gene cassette (pHH3408). The *sul1* gene in the 3’-CS of the integrons conferred sulfonamide resistance.

### 4. Conclusions

Spreading manure on agricultural soils was shown to promote spreading of transferrable antibiotic resistances and residual veterinary medicines in agricultural soils. Our data indicate that LowG+C and IncP-1ε plasmids play an important role in the dissemination of multiple antibiotic resistance in agroecosystems.

### 5. References


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Does fertilization with sewage sludge promote antibiotic resistance in bacteria isolated from food crops?

Edward Topp¹, Andrew Scott¹, Lyne Sabourin¹ and Peter Duenk²

¹Agriculture and Agri-Food Canada, London ON Canada N5V 4T3
²Department of Biology, University of Western Ontario, London ON N6A 5B7
E-mail contact: ed.topp@agr.gc.ca

1. Introduction

The recycling of municipal sewage sludge onto agricultural land as a valued source of crop nutrients is a common farming practice in many jurisdictions, including the province of Ontario Canada. Prior to application, the sewage sludge may be subjected to various treatments that influence the microbial and chemical composition including aerobic digestion, anaerobic digestion, composting, heating, or addition of alkali. In the absence of rigorous treatment, sewage sludge can contain a variety of antibiotic residues, as well as an abundance of bacteria that are resistant to various antibiotics [1,2]. What impact these chemical or microbial contaminants might have in the agro-environment, particularly if they promote antibiotic resistance, has received little attention [3].

The purpose of the present study was to evaluate the abundance and the characteristics of bacteria recovered from crops grown in land fertilized with sewage sludge, compared to land that is not. A field experiment was undertaken according to normal farming practices permitted in Ontario Canada. Control and sludge-treated plots were cropped to tomatoes, potatoes, carrots and sweet corn (maize). At harvest, bacteria obtained from the crops were plated onto medium containing a variety of antibiotics at breakpoint concentration to establish the frequency of resistance to each antibiotic within the culturable bacterial populations. A subset of the recovered bacteria were evaluated for resistance to all tested antibiotics to establish patterns of multiple resistance.

2. Materials and methods

Field work. The experiment was started at the experimental field station of the University of Western Ontario in May 2009. Dewatered, anaerobically-digested sewage sludge obtained from a municipality of 425 thousand inhabitants was manually uniformly applied at a rate of 8000 dry kg/ha to a plot 72 m² and incorporated to a depth of 12 cm. A second control plot of the same dimensions was managed in the same way except for the addition of biosolids. The two plots were each subdivided into randomly distributed triplicate blocks that were cropped in triplicate with tomatoes, potatoes, sweet corn or carrots. Crops were planted in May 2010, a year after the application of biosolids, and harvested in August 2010.

Laboratory work. After harvest, bacteria were washed off the surface of the crops by mechanical agitation in buffer. Suspensions were plated onto Iso-Sensitest agar medium (Oxoid Canada, Ottawa, ON) without antibiotics, or supplemented with a range of antibiotics at clinical breakpoint concentrations (Table 1). Colonies that grew in the presence of the antibiotics were counted as ‘antibiotic resistant’. Bacteria obtained from tomatoes, carrots and corn were chosen for further study because these crops can often be eaten raw. A selection of isolates that grew in the presence of cefoxitin [2nd generation cephalosporin], cefotaxime [3rd generation cephalosporin] or augmentin [amoxicillin + clavulinate] were evaluated for resistance to the complete panel of antibiotics.

Isolate collections were prepared in a microtiter plate format to aid in screening for multiple antibiotic resistance. Isolates were picked from primary isolation plates containing either cefoxitin, cefotaxime or augmentin and purified by streaking onto Iso-Sensitest agar containing the same breakpoint concentration of antibiotic that the isolate was primarly isolated on. Plates were incubated at 30°C for 48 hours, where upon the isolates were picked into microtiter plates containing sterile Mueller-Hinton broth (Difco, Thermo-Fisher Scientific, Mississauga, ON) containing the same breakpoint concentration of antibiotic as the isolate was originally isolated from. Microtiter plates were sealed with PCR film and incubated for 48 hours at 30°C. Sterile 30% (v/v) glycerol was added to each well of the microtiter plates to a final concentration of 15% (v/v), and the plates were sealed with fresh PCR film and archived at -80°C until required for screening.

MAR screening was carried out on crop and biosolid isolates using three different concentrations of each antibiotic in the panel (1/2 breakpoint, breakpoint and 2X breakpoint). Isolates were prepared for screening by replicating the archived plates into microtiter plates containing sterile Mueller-Hinton broth and incubating the replicated plates at 30°C for 48 hours. Plates were then replicated into microtiter plates containing sterile...
0.02% (v/v) Tween 20 using a slot-pin replicator (V&P Scientific, San Diego, CA). The Tween 20 replication plates were used as the master inoculation plate for screening. Isolates were screened by inoculation onto Mueller-Hinton agar containing the specified concentration of antibiotic using a slot-pin replicator. Plates were incubated at 30°C for 48 hours prior to scoring results. Isolates were scored for growth according to the following criteria: 0 – No growth; 1 – Growth; 2 – Faint Growth; and 3 – Spotty Growth. Results were recorded in MS Excel 2007.

3. Results and discussion

Suspensions of bacteria obtained from each crop were plated onto media containing 64mg/L of amikacin, 32/16mg/L augmentin (amoxicillin/clavulanic acid), 32mg/L ampicillin, 128mg/L bacitracin, 64mg/L cefotaxime, 32mg/L cefoxitin, 8mg/L ceftriaxone, 32mg/L chloramphenicol, 4mg/L ciprofloxacin, 8mg/L erythromycin, 16mg/L gentamycin, 64mg/L kanamycin, 32mg/L lincomycin, 8mg/L linezolid, 32mg/L nalidixic acid, 128mg/L nitrofurantoin, 16mg/L norfloxacine, 16mg/L penicillin, 64mg/L streptomycin, 512mg/L sulfamethoxazole, 16mg/L tetracycline, 16mg/L trimethoprim, 4/76mg/L co-trimoxazole (trimethoprim/sulfamethoxazole), 32mg/L tylosin, 32mg/L vancomycin, 64mg/L chlorotetracycline, 512mg/L sulfamethazine. There was no effect of treatment [± sewage sludge] on the fraction of cultivable bacteria that grew in the presence of any of the antibiotics.

Collections of tomato and carrot bacteria that were resistant to cefoxitin, cefotaxime or augmentin on primary isolation were often resistant to other antibiotics. In most cases isolates were resistant to multiple antibiotics. Preliminary analysis suggests that bacteria resistant to multiple antibiotics (eg. >10) were more frequently detected on tomatoes and sweet corn grown in soil fertilized with sewage sludge than in soil without sewage sludge.

4. Conclusions

This study is to our knowledge the first evaluating in detail the impact of sewage sludge fertilization on the abundance and characteristics of antibiotic-resistant bacteria found on food crops. Our preliminary results suggest that this practice may not increase the frequency of resistance to individual antibiotics in food crop bacteria, but may in some cases increase the abundance of bacteria that are resistant to multiple antibiotics. If confirmed, this would certainly be an undesirable consequence of this agricultural practice.

5. References


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Assessing the environmental hazard of antibiotic resistance
Considerations from a regulatory view

Jens Schönfeld, Anette Küster, Bettina Rechenberg

Federal Environment Agency (UBA), Division Chemical and Biological Safety, Section– Environmental Risk Assessment of Pharmaceuticals
Wörlitzer Platz 1, D-06844 Dessau-Roßlau, Germany
E-mail contact: jens.schoenfeld@uba.de

1. Introduction
The development of antibiotic resistance in microorganisms has been identified as a major global public health problem (WHO 2001 [1], UNEP 2005 [2]). The importance of the topic has been addressed in the current guidance for the authorization of veterinary (CVM/P/VICH/644/2001 [3]) and human pharmaceuticals (CPMP/EWP/520/96 [4]). In contrast, the potential adverse impact of the development and spreading of antibiotic resistances on the environment is not reflected in the current guidance for the environmental risk assessment for human as well as veterinary pharmaceuticals [5, 6]. The adverse effects of antibiotic resistance in the environment might hereby comprise two aspects. On the one hand, the efficacy of human and veterinary antibiotics might be affected by increasing the probability of environmental exposure of human pathogens to resistant microorganisms, thus being a public health concern in the end. On the other hand, the environment might be affected directly through antibiotic resistances threatening its integrity or single populations of organisms (plants, animals, microorganisms). In this study, conclusions are drawn from a regulatory point of view in order to evaluate the need to include antibiotic resistance in the environmental risk assessment of pharmaceuticals. The work was performed first, on the basis of the recent scientific literature and second, on consumption data of antibiotics given for Germany.

2. Materials and methods
In a first step, the recent scientific literature on the development and/or spread of antibiotic resistance was screened for the occurrence of adverse effects of antibiotic resistance in the environment. The aim was to give a comprehensive overview of the full range of observable effects and to identify the key parameters for the selection and dissemination of antibiotic resistance. For each individual antibiotic or class of antibiotic, respectively, the type of resistance developed (specific, unspecific) and the potential for horizontal transfer of the resistance genes was recorded. In a next step, a comparison and evaluation of methods for the determination of threshold concentrations of antibiotics at which resistance is likely to be induced was conducted using available literature studies. Finally, derived thresholds from the literature were linked to consumption data for Germany to assess the probability that these concentrations might be reached in environmental compartments.

3. Results and discussion
Internationally harmonised guidelines for the assessment of resistance studies are presently not available. The lack of standardisation in resistance determination studies and their evaluation renders it difficult to compare results from different environmental compartments.

3.1. Identification of key parameters for the selection and dissemination of antibiotic resistance.
The incidence of environmental concentrations of antibiotics at sub-therapeutic levels was identified as an important driver of the development of antibiotic resistance [5]. Resistance development generally occurs already at the Minimum Effect Concentration (MEC) at which growth rate of bacteria is affected. MECs are about tenfold below the Minimum Inhibitory Concentrations (MICs) [6]. The occurrence of hormesis effects in a resistance study, i.e. contrasting activities at subinhibitory and inhibitory concentrations, gives a good indication for sub-therapeutic effects [5]. The actual measurable concentrations of antibiotics are certainly directly correlated with the respective consumption data (see 3.3), the second important driver of selection of antibiotics (Fig. 1). The spread of antibiotics in the environment is mainly governed by physical forces, like those generated by wind and flowing water, and biological forces (Fig. 1). The biological forces can be differentiated into forces which arise from human activity or the activity of animals and the transfer of mobile genetic elements, which harbour antibiotic resistance, across bacterial species borders.
Furthermore, another frequently observed adverse effect of antibiotic resistance in the environment was a shift in bacterial community structure.

**Figure 1: Key factors for the selection and dissemination of antibiotic resistance.**

### 3.2. Comparison and evaluation of methods for the determination of resistance-inducing threshold concentrations of antibiotics.

The comparison of methods revealed that the determination of a Minimum Effect Concentration (MEC) for antibiotics allows setting a threshold concentration above which the development of resistance is likely to occur. The principle of the determination method has a potential to be transferred into a standardised guideline.

### 3.3. Surveillance of antibiotics consumption data.

In most of the cases the consumption data show that MEC thresholds would be exceeded in the environmental compartments.

### 4. Conclusions

From the recent scientific literature there are indications for adverse effects of the spread of antibiotaical resistance in the environment. The literature analyses support the requirement of including antibiotic resistance in the environmental risk assessment of pharmaceuticals. Determination of a Minimum Effect Concentration (MEC) for antibiotics is regarded as an appropriate method for setting a threshold concentration above which the development of resistance is likely to occur.

### 5. References


Human exposure to PBDEs in Europe and North America

Natalie von Goetz¹, David Trudel¹, Martin Scheringer¹ and Konrad Hungerbühler¹

¹Swiss Federal Institute of Technology (ETH) Zurich, Wolfgang-Pauli-Str. 10, 8093 Zurich, Switzerland

E-mail contact: natalie.von.goetz@chem.ethz.ch

1. Introduction

Polybrominated diphenylethers (PBDEs) are flame retardants used to reduce the ignition and spread of fire in a broad range of products such as computer screens, mattresses, or upholstery in transport vehicles. Because of their toxicological properties, lower-brominated PBDEs (penta and octa mixtures) have been banned in Europe and voluntarily phased out in North America. Deca-BDE is still in use in most states of the US, but will be phased out by the end of 2013. In Europe deca-BDE is no longer used in electronics and electrical applications.

The aim of this study is to show with a consistent methodology whether the different legal settings, the different use patterns of PBDEs in North America and Europe, as well as the differences in behaviour (e.g. eating habits) lead to different dose levels of PBDEs taken in and up by humans.

2. Materials and methods

The exposure was modelled probabilistically for five regions (North America, United Kingdom, Northern Europe, Central Europe, and Southern Europe), seven consumer groups (infants, toddlers, children, female and male teenagers, female and male adults), the eight environmentally most relevant PBDE congeners (BDE-28, 47, 99, 100, 153, 154, 183, and 209), and eight pathways (oral intake via food, dust, soil, and organic films, dermal uptake via dust, soil, and organic films, and inhalation uptake via air). Based on the total doses, elimination half-lives for adults were calculated for each congener by using a one-compartment pharmacokinetic model [1].

3. Results and discussion

The median doses are in the range of 1.8-13.0 ng/kg bw/day for the sum of all investigated congeners (sum PBDE). The 95th percentiles of the dose distributions are in the range of 11-480 ng/kg bw/day and highest for infants in the region North America (see Table 1). The dominating congeners are BDE-209, BDE-47 and BDE-99 for all regions and all consumer groups (not shown).

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Table 1: The 95th percentiles of the dose distributions for sum PBDE in different regions per consumer group

In general, Americans experience higher doses of PBDEs than Europeans. This is most pronounced for the individuals allocated in the higher percentiles of the dose distribution. Consumption of food and inadvertent ingestion of dust, as well as dermal contact to dust contribute most to the exposure to PBDEs (see Figure 1). In most cases, especially in Northern, Central and Southern Europe, food is the dominant pathway for median dose estimates, whereas in the higher dose percentiles the contribution of dust becomes more important and in about half of the cases even dominant. Infants experience the highest doses, followed by
toddlers and children, and teenagers and adults (hockey-stick like dose pattern), with about three to six times the dose of adults.

Figure 1: Contribution of different sources to sum PBDE in Central Europe and North America per consumer group (pathways oral soil, dermal soil and dermal film insignificant).

The hockey-stick like dose pattern is also visible in biomonitoring data, which supports our model results. The elimination half-lives calculated on the basis of the dose distributions are lower for the PBDEs of lower molecular weight than determined in comparable studies [1], but on the same order of magnitude. For Deca-BDE we calculated about the same half-life as determined earlier for workers [2].

Most likely, the reason for Americans to experience higher doses of PBDEs is that more consumer products (e.g. mattresses) are treated with flame retardants in North America compared to Europe, resulting in higher PBDE concentrations in dust. In all regions, oral uptake of food and dust and dermal uptake of dust are the most important pathways due to the persistent and bioaccumulative nature of PBDEs and their application in products that are used mainly indoors (as for example mattresses, sofas, cushions, blankets). Younger consumers take up higher doses mainly due to their higher ingestion of food and dust when normalized to their body weight.

4. Conclusions

Using a consistent methodology we show that Americans take up higher doses of PBDEs than Europeans. We quantify the contributions of food and dust as important sources of exposure and show that infants take up the highest doses. There are, however, large uncertainties associated with the oral dust intake rates and dermal uptake rates for PBDEs that are propagated into the dose estimates.

5. References


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From Sources to Urban Fate: a Contrast of PCBS, PBDES, PAHs AND Synthetic Musks

Diamond ML\textsuperscript{1,2}, Helm PA\textsuperscript{3}, Csiszar SA\textsuperscript{2}, Melymuk L\textsuperscript{2}, Robson M\textsuperscript{1}, Giang A\textsuperscript{2}, Backus S\textsuperscript{4}, Bradley L\textsuperscript{4}, Gilbert B\textsuperscript{3}, Daggupaty S\textsuperscript{5} and Jantunen LM\textsuperscript{5}

\textsuperscript{1}Department of Geography, University of Toronto, Toronto, Canada, M5S 3G3; \\
\textsuperscript{2}Dept of Chemical Engineering & Applied Chemistry, University of Toronto, Toronto, Canada, M5S 3E5; \\
\textsuperscript{3}Environmental Monitoring & Reporting Branch, Ontario Ministry of Environment, Toronto Canada, M9P 3V6; \\
\textsuperscript{4}Ontario Water Quality Monitoring, Environment Canada, Burlington, Canada, L7R 4A6; \\
\textsuperscript{5}Air Quality Research, Environment Canada, Toronto, Canada, M3H 5T4

E-mail contact: miriam.diamond@utoronto.ca

1. Introduction
Diamond and Harrad\textsuperscript{1} proposed a conceptual model for human exposure to chemicals contained within a stock or inventory of materials and products (Fig 1). For chemicals use in everyday commodities, this inventory is held mainly indoors, which is the first environment into which the chemicals migrate. From indoors chemicals migrate outdoors into the urban environment. Persistent chemicals will be exported from the urban area mainly by atmospheric movement and a smaller, but significant fraction will find its way to urban soils and surface waters. Very persistent chemicals will be transferred through terrestrial and aquatic food webs from which the chemical may return to the urban area in foods that we eat.

We quantified and contrasted the emissions, movement and inventory of 4 compounds (2 compounds for the inventory) in Canada’s largest urban area, Toronto, with a population of 2.5 million. Through material flow analysis, measurements and modelling, we examined PCBs (e.g., in interior and exterior building sealants), PBDEs (in the pre-2008 electrical and electronic products, textiles, etc.), polycyclic musks or PCMs (synthetic fragrances in “down the drain” consumer products), and PAH (combustion by-products and pavement coatings).

2. Materials and methods

Spatial inventories of PCBs in-use, in-storage and in exterior sealants\textsuperscript{2,3} and PBDEs in computers, carpets and vehicles were compiled for the City of Toronto.

Measurements of each compound class were obtained for air, precipitation, soil (not discussed here), tributaries and waste water treatment final effluent. Passive air samples were collected using “dome”-type polyurethane foam (PUF) passive samplers every four months from 19 sites, on east-west and north-south transects centered on the downtown core of the city. Hi-vol samples were taken downtown only, the same location at which precipitation was collected. Surface water samples were collected on a monthly basis from 11 sites across the GTA using an automated ISCO 6712 peristaltic pump. Samples were extracted via PFE using a Dionex ASE 300 and then cleaned up using a combination of silica and Florisil SPE. PAH samples and PCM samples were analysed by GC-LRMS and PCB and PBDE samples by GC-HRMS. Surface water samples were analyzed at the Ontario Ministry of the Environment, Toronto, Canada, precipitation samples at the National Laboratory for Environmental Testing, Environment Canada in Burlington, Canada and air...
samples at the University of Toronto, Toronto, Canada. For all sample types a rigorous system of field blanks and reference materials analysis was followed to ensure the accuracy and precision of the samples.

Spatially explicit modelling was used to estimate total emissions and fate in the urban area of PCBs and PBDEs using a combined boundary layer atmospheric transport and multimedia fugacity model (BLFM-MUM) developed at the University of Toronto in conjunction with Environment Canada.

3. Results and discussion

Indoor air concentrations of PCBs, PBDEs and PCMs all exceeded those outdoors illustrating the importance of the indoor environment for exposure and as an intermediary environment in the fate of these compounds. The magnitude of outdoor air concentrations of PCBs and PBDEs corresponded to their respective spatial inventories (Figure 2a,b). PCB, PBDE and PCM air concentrations were also related to population and building density.

Modelling results showed that prevailing winds advected 85-98% of emissions of all compounds from the urban area providing an opportunity for persistent PCBs and PBDEs to enter the terrestrial food web. Measurements corroborated that a fraction of PCB, PBDE and PAH emissions entered urban soils and surface waters where the later, we postulate, originate from wash off of atmospherically deposited surface films on impervious surfaces and entrained soil during storm events. In contrast, the fate of more volatile PCMs is controlled by waste water treatment plant discharges and atmospheric processes (Fig 3).

4. Conclusions

PBDEs are released from indoors to the outdoor environment, from which air and surface waters deliver these emissions to the surrounding environment. PBDEs also originate from treated waste water effluent. The fate of PCBs, which have the largest emissions in the downtown area where the greatest inventory was located, is mostly controlled by atmospheric movement. PAH emissions are dominated by transfer to surface waters whereas “down-the-drain” PCMs are mostly emitted to the environment via treated waste water treatment plant discharges. These results connect chemicals in consumer and building materials and products and their environmental pathways, all of which contribute to ecosystem and human exposure.

5. References


Acknowledgement - Funding was provided by Great Lakes Air Deposition Program, NSERC of Canada, Environment Canada, Ontario Ministry of the Environment and the City of Toronto, Canada.
1. Introduction

Traditional plastic were developed to be enduring and resistant to all forms of degradation. Their versatility allows them to be used in many applications, but disposal of plastic contributes to the growth in municipal wastes [1]. The use of biodegradable polymers in specific applications and sectors (i.e. compostable waste bags, carrier bags, single-use tableware, mulch films) can be an alternative to the disposal in landfill, can reduce the cost of waste management and the accumulation in the environment.

A biodegradable polymer is completely converted by microorganisms (under aerobic condition) to carbon dioxide, water and new biomass, as described by the following equation:

\[ \text{C}_{\text{polymer}} + \text{O}_2 \rightarrow \text{CO}_2 + \text{H}_2\text{O} + \text{C}_{\text{biomass}} + \text{C}_{\text{residues}} \]

The most acceptable disposal method for biodegradable polymers is composting, but products used in agriculture (in particular mulch films) are applied to soil and at the end of their life they can be left there. American Society for Testing and Materials [2] and International Standard Organization [3] published standard methods for determining aerobic biodegradation in soil of plastic materials by measuring the amount of carbon dioxide evolved in a closed or a ventilated respirometer respectively. However, CO\(_2\) is only one part of the carbon balance. The determination of biomass development, of polymer sub-products and their fate in soil is very important in order to obtain a complete description of the process. Most commercial biodegradable plastics are made with biodegradable polyesters. The aim of the work was to evaluate mineralization in soil of a model polyester compared with cellulose and polycaprolactone (PCL), a biodegradable polyester which has, for example, an important biomedical role [4].

2. Materials and methods

The experimental polyester was obtained by condensation of butanediol and sebacic acid. PCL and cellulose were used as reference materials. A sandy agricultural soil, collected in Arborio (Italy), was used for the test.

The test was carried out according to ASTM 5988-96 [2]. Powdered polymers (2 g) were added to 500 g of a soil-compost-salts mixture and put in hermetically closed jars. Blank jars, without test substances, were also prepared. Each jar contained a beaker filled with 0.5 M KOH (40 ml), which was regularly titrated with 0.25 M HCl in order to measure the CO\(_2\) production within the jar. The measurement was carried out every 3-4 days in the first two weeks and every 7-10 days thereafter. The amount of CO\(_2\) produced in the blank jars (mg CO\(_2\) blank) was subtracted to the amount of CO\(_2\) measured in the test jars (mg CO\(_2\) molecule), and than converted to mineralization percentage, according to the equation:

\[
\text{mineralization} = \frac{(\text{mg CO}_2\text{ molecule} - \text{mg CO}_2\text{ blank}) / \text{sample weight}}{\% \text{C} \times 3.6667 \times 0.01}
\]

where the constant 3.6667 is the ratio between CO\(_2\) molecular weight and C atomic weight.

The mineralization of the tested substances was monitored for about five months.

3. Results and discussion

Figure 1 shows the mineralization curves of the tested substances. Each point represents the values obtained from experimental data in the jars prepared for the tested polymers. The reproducibility of results is very good: measures in similar jars are, in practice, the same. PCL and cellulose mineralization have a very short lag phase (3-4 days) and in the first 25 days the mineralization is the same. Then PCL mineralization is higher then cellulose one. After about four months of incubation both PCL and cellulose reach a not well defined plateau phase and final mineralization mean values are about 60 % and 51 % respectively.

New polyester mineralization is different from the cellulose or PCL ones. The experimental curves show a lag phase around 15 days after which mineralization starts and proceeds regularly until the end of the test. The plateau phase is not completely reached, and mineralization value is about 53 %.
The new polyester mineralization percentage obtained by respirometric test justifies only the half of the carbon contained in the molecule. To completely describe the biodegradation, it is important to determine the biomass production and quantify the polymer eventually remained in soil or its sub-products.

The overall biodegradation process of a polyester can be described by two consecutive reactions. In fact, it is first hydrolyzed by extracellular enzymes in its monomeric units and then metabolized by microorganisms. After about five months of incubation in soil, PCL and the new polyester were mineralized more than cellulose (used as reference material).

As regards the model polyester, at the end of the experiment, only half of its carbon evolved into CO₂. Monomers used for its synthesis, butanediol and sebamic acid, have been shown to be fully biodegradable. As a matter of fact they are mineralized within 40 days (about 50% and 52% respectively)[5]. Results obtained by the application of a mathematical model (based on the experimental data of CO₂ production and on the chemical-physical monomers properties [6]) show that about 50% of butanediol and sebacic acid, when added to the soil, were converted into biomass, so that their biodegradation was assumed to be complete. Therefore, it can be reasonably supposed that no carbon linked to material residues can be found in soil at the end of the test. Moreover, polymer mineralization, normalised to the reference substance, can be considered 100%.

4. Conclusions

Respirometric tests for determining mineralization in soil of polymers are very accurate and important to describe the principal products of the biodegradation process in soil (CO₂).

The results of this study indicate that the experimental polyesters were fully biodegraded. This conclusion can be reached because (1) the mineralisation is similar to cellulose, considered as a fully biodegradable polymer (2) the monomers of the polyester have been shown to be fully biodegradable. To complete this study it is necessary to complete the carbon balance and to develop a simple and reliable method to determine the biomass production.

5. Reference


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Toxic metals derived from plastic litter on a beach

Etsuko Nakashima¹, Atsuhiko Isobe¹, Shin’ichiro Kako¹, Shinya Magome², Noriko Deki¹, Takaaki Itai¹ and Shin Takahashi¹

¹ Center for Marine Environmental Studies (CMES), Ehime University
2-5 Bunkyo-cho, Matsuyama, Ehime, 790-8577, Japan
² Sanyo Techno Marine, Inc.
1-3-17, Nihonbashi Horidome-cho, Chu-ku, Tokyo, 103-0012, Japan
E-mail contact: nshima@mepl1.cmes.ehime-u.ac.jp

1. Introduction

The marine pollution by beach litter has been recognized as a serious trans-border environmental issue due to their flotation and transportation over a long distance [1]. Hence, the protection of the marine environment from such pollution must involve international cooperation with neighboring countries. On the other hand, marine litter poses a great threat to marine wildlife because of the ingestion of plastics by animals and entanglement in drift nets [2]. However, only few reports on toxic metals derived from marine litter are available [3]. In polymers, toxic metals are widely used as plasticizers, catalysts, stabilizing additives, and pigments [3-6]. These metals may leach out and transfer from the plastic to animals in the environment when plastic are degraded or digested. Hence, the present study attempts:

- To establish a reliable method to measure the total litter mass over a beach by aerial photography using a digital camera attached to a balloon.
- To estimate the mass of toxic metals in marine plastic litter over a beach using the total litter mass measured with the balloon.

2. Materials and methods

2.1 Measurement of total litter on Ookushi beach

A beach survey was carried out on October 22, 2009 on the Ookushi beach in Goto Islands, Nagasaki, Japan. The beach litter-covered area over a beach was measured by aerial photography using a digital camera attached to a balloon. In order to estimate the total litter mass (kg) over the beach by multiplying the total litter-covered area (m²) obtained by aerial photography, measurements of the beach litter mass per unit area (kg/m²) were carried out. We randomly chose ten square boxes each with an area of 4 m² (2 m × 2 m) over which beach litter covered completely.

2.2 Sampling and Classification of beach litter material

Besides the in-situ beach survey mentioned above, we collected litter samples randomly from each square box (4 m²) on the beach to investigate the materials in it, especially the types of plastics and polymers. To measure the mass of each material, all collected litter samples carried to our laboratory were classified into specific categories. Special attention was given to differentiate plastic litter into their polymer types (e.g. polypropylene, polyethylene, etc.) using a near-infrared spectrometer (Plascan-SH, OPT Research Inc. Tokyo, Japan) and a Fourier transform infrared spectrophotometer (FT-IR, ALPHA, Bruker Optics) because different metals are present in different quantities in plastic polymer types [3-6].

2.3 Chemical analysis

To estimate litter-derived toxic metals, we used a handheld X-ray fluorescence analyzer (XRF: Innov-X, a-6500). The toxic metals which are regulated by EU regulation on packaging and packaging waste such as lead (Pb) and total chromium (Cr). Accuracy of these analyses was examined using standard reference materials, EC680k and EC681k (European Reference Materials). Uncertainty were determined by 10 measurements using reference materials. Quantitation limit (10σ) were determined by 10 measurements of reference materials and virgin polyethylene pellets (Grand Polymer Co. Ltd. Japan).
3. Results and discussion

3.1. Total mass of beach litter on the Ookushi beach
The total area covered by beach litter is found as 123.5 m² on the entire beach. The average of litter mass per unit area within the ten boxes on the beach is 5.8 kg/m². Multiplying the total litter-covered area (123.5 m²) by the averaged 5.8 kg/m² yields the best estimate of the total litter mass of 716 ± 259 kg by a t-test with a 95% confidence limit.

3.2. Composition and total mass of each polymer type
Plastics prevail among various materials on the ten boxes of the Ookushi beach, the mass of plastics accounts for 74% (i.e., 530 ± 201 kg). Furthermore, smaller density plastics such as polyethylene (PE), polypropylene (PP) are predominant than heavier materials (not shown). Therefore, we now focus on the estimation of toxic metals in PE plastic litter on the Ookushi beach as follows. The estimated PE plastic mass over the beach is 234 ± 96 kg. Pb and total Cr are measured by analyzing 432 pieces of PE plastic samples. The results of other polymers will be shown in our presentation.

3.3. Estimation of toxic metals derived from PE plastic litter
The concentrations of Pb and total Cr range from less than the quantification limits to 10,000 mg/kg. Pb and total Cr contained in PE plastic litter occasionally exceed 100 mg/kg, that is the EU regulation on packaging and packaging wastes. Although concentrations of Pb and total Cr vary largely, we compute the average and standard deviation of Pb and total Cr as follows: The concentrations of Pb and total Cr derived from PE plastic litter over Ookushi beach are estimated at 44.7 ± 13.7 mg/kg and 13.7 ± 5.5 mg/kg, respectively. Therefore, it is possible to estimate the total mass (g) of Pb and total Cr carried by PE litter by multiplying the concentration (mg/kg) with the estimated PE plastic mass over the beach (234 ± 96 kg): Pb and total Cr mass over the beach are calculated as 10.4 ± 5.2 g and 3.2 ± 1.9 g, respectively. These toxic metals in PE plastics are often used in pigments such as lead chromate [6] and potentially leach out in to the beach environment during degradation of plastics.

4. Conclusions
A reliable method to estimate mass of toxic metals included in plastic litter over a beach is established in the present study. Toxic metals from marine plastics potentially leach out in the beach environment during degradation. Our future research will be focused on their bioaccessibility/bioavailability to the resident organisms.

5. References

Acknowledgement - This research was partly supported by grants from the Environment Research and Technology Development Fund (B-1007) of the Ministry of the Environment and the Global COE (Center of Excellence) Program of the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Japan.
Understanding leaching from plastics into the environment: Lessons learned from 30 years of Food Contact Plastics use

Jane Muncke

1. Introduction

Since the start of plastics mass production around 60 years ago the prevalence of environmental plastics debris has increased, and the extent of this contamination is gradually becoming apparent now [1, 2]. Recent sampling shows that plastics constitute the majority of marine debris [3]. Due to its material properties plastic is not readily degraded, though mineralization can occur under certain conditions [4]. Weathering slowly leads to mechanical break-down, resulting in small plastic fragments being distributed worldwide [5]. This observed widespread distribution of plastic fragments implies chemical interactions with the biotic and abiotic environment [6]. While the adsorption of persistent environmental organic pollutants (POPs) to plastic particles is being studied [7], the release and migration of chemicals from the plastic into the environment is far less well characterized [8].

The chemistry of plastics is complex. Plastics are made from monomers, small organic molecules that are chemically linked to each other by polymerization to form the polymer. Polymers can be made up of ten thousand monomers. To obtain the finished plastic material properties the polymer is compounded with different additives and pigments. Subsequently the plastic is then shaped into the finished article. During these process steps reaction products are generated, for example when polymerization is terminated early and oligomers, small molecules made up of 2 to 10 monomers, are produced [9].

This paper reviews the extensive scientific information on plastic composition and leaching available from food contact material (FCM) research. A good understanding of plastics’ qualitative and quantitative characteristics will be imperative to address environmental pollution and understand toxicity of plastic leachate.

2. Food Contact Material Literature

For environmental research the rich body of literature concerning migration from food contact material (FCM) plastics into food and food simulants can be a very useful source of information. Since the introduction of plastic FCM around 3 decades ago the understanding of chemical diffusion into foods has been a major concern and challenge for regulators and industry [10]. The authorization of monomers or additives for use in FCM requires pre-market testing of diffusion behaviour and toxicity properties [11].

To assess chemical leaching, migration from the FCM into so called food simulants is assessed. Food simulants are distilled water, 3% acetic acid, 10% ethanol, 50% ethanol, and olive oil. For partitioning of FCM substances into the food simulants data are abundant for all commonly used plastic types. For additives, the authorization application can include calculated migration data, based on commonly used models [12].

In addition, other compounds than monomers and additives can migrate from plastics, too. Plastics contain so called non-intentionally added substances (NIAS), side products from polymerization and compounding. Such NIAS are for instance oligomers. For example, styrene and polyethylene terephthalate (PET) (plastics #6 and #1) contain cyclic trimers [13, 14] that have been found to leach into food simulant or food [15, 16]. Other NIAS migrating from PET bottles are formaldehyde and acetaldehyde, reaction products formed during the blow moulding process [17].

3. Conclusions

Even if produced under controlled lab conditions not all reaction and break-down products of a plastic can be identified [9]. Plastics essentially are mixtures of known and unknown compounds. The well-studied plastics used for FCM, containing known starting materials and additives, are an appropriate starting point for understanding plastics chemistry and leaching behaviour. Furthermore, plastics used to package foods and beverages constitute between 10 to 20% of all plastics currently manufactured. As single-use items the highly abundant types of plastic packaging are thought to constitute a significant fraction of marine plastics debris [18]. A closer look at their chemical composition is useful for assessing and characterizing the nature of plastics chemical pollution.
4. References


Comparison of cartilage and bone malformations in the head of zebrafish embryos after exposure with dithiocarbamates and hydrazides

Ruben Strecker¹, Thomas Braunbeck¹

¹ Aquatic Ecology and Toxicology Section, Department of Zoology, University of Heidelberg, Im Neuenheimer Feld 230; D-69120 Heidelberg, Germany

E-mail contact: Ruben.Strecker@zoo.uni-heidelberg.de

1. Introduction

In search of alternatives to animal experiments, the zebrafish embryo test (ZFET) has received increasing attention as a refinement or even a replacement for the acute fish toxicity test [1]. Using the ZFET, not only acute mortality can be investigated but also specific sublethal malformations can easily be analysed. Dithiocarbamates such as disulfiram (DSF) are well-known teratogens causing wave-like deformation of the notochord and cartilage malformation in fish embryos [2,3,4]. Although different in chemical class, molecular weight and log $K_{ow}$, hydrazides, especially acetic hydrazide (ACH) – a degradation product of isoniazide – generate similar morphological effects. Similar results have also been found for benzhydrazide, formic acid hydrazide and isoniazide itself. The cranium of adult zebrafish consists of 74 ossifications needing at least 70 days for completion [5]. Two types of bones are present in higher teleosts: dermal bones, developing directly within connective tissue and cartilage bones. Visible dermal bones in the head of 6 days old zebrafish larvae are the parahenoid, the opercles, the cleithrum and more or less visible the occipitals, branchiostegal rays, maxilla and entopterygoids. The fifth ceratobranchial is the only cartilage bone at this developmental stage. The ossified front of the notochord is defined as a perichordal bone. Effects of both chemical classes on the development of each cartilage and bone in the cranium during zebrafish embryogenesis – with main focus on hydrazides – were investigated in order to identify the most susceptible skeletal elements.

2. Materials and methods

Alizarin red S (Sigma Aldrich, Deisenhofen) and alcian blue 8 GS (Serva, Heidelberg) dyes were used for staining. 6-Day old larvae were killed with an overdose of benzocaine and stained as described by the protocol of Walker and Kimmel [6] with slight modifications. Briefly, embryos were fixed and rocked for 2 h in 4 % paraformaldehyde, stained overnight using acid free double stain solution with 120 mM MgCl₂, dehydrated with ethanol, bleached with 3 % H₂O₂ and 1 % KOH for 25 minutes and stored in 50 % glycerol and 0.1 % KOH at 4°C. After staining, all embryos were examined using microscopes with adequate imaging hard- and software (Stemi 2000-C with Canon Power Shot G7 and Olympus CKX41 with C-5069 Wide Zoom Camera and Analysis 5.0 Software). Quantification of cartilage and bone malformations was assessed in a semi-quantitative approach: all cartilage elements classified as “1” were normally developed, cartilages classified as “2” showed little malformations, “3” represented strong and “4” very strong malformations or no longer detectable skeletal elements. For ossification, the approach was identical. Fig. 1 A shows a 6 days old control zebrafish embryo.

3. Results and discussion

Concentrations of 20 µg/L DSF caused no malformations in cartilages of the neurocranium, only little effects on the pharyngeal skeleton and strong alterations on bones. DSF induced strong cartilage malformations after exposure to ≥ 80 µg/L, whereas acetic hydrazide caused cartilage alterations from ≥ 1.5 g/L (Fig. 1 B). In the two lowest DSF concentrations (20 and 80 µg/L), the notochord showed in about 75 % stronger ossification than in controls. Wave-like deformation of the notochords (Fig. 1 C) occurred after exposure to DSF at all tested concentration, whereas at the two lowest concentrations of acetic hydrazide (0.375 and 0.75 g/L) mainly breaks of the notochord (Fig. 1 D) were observed. Concentrations of acetic hydrazide higher than 1.5 g/L resulted in undulated notochords similar in appearance to those seen with DSF. Van Boxtel et al. [2] found that DTCs inhibit lysyl oxidase activity, a group of cuproenzymes, and a knockdown of lox genes sensitizes zebrafish embryos to dithiocarbamate exposure. The mode of action of acid hydrazides to cartilages, bones and the notochord seems to be similar to DSF. It was shown in early biochemical studies...
[7, 8] that various hydrazides inhibit the copper-containing enzyme lysyl oxidase, causing increased solubility of collagen. Lysyl oxidase is needed for cross-linking of collagen and elastin.

![Figure 1](image)

**Fig. 1: Cartilage and notochord malformations caused by exposure to acetic hydrazide. Both undulated notochords and breaks of the notochords in lower concentrations are characteristic to acetic hydrazide exposure. (A) Negative control, (B) malformed pharyngeal cartilage, (C) undulated notochord, (D) break of the notochord with subsequent accumulation of notochord materials.**

4. Conclusions

Both substance classes cause notochord and cartilage malformations similar in their morphological appearance. Cartilages of the neurocranium, such as the ethmoid plate, proved to be more stable than cartilages of the pharyngeal skeleton such as, e.g., Meckel’s cartilage. Hence, ossifications are much more susceptible to the test compounds than cartilage, with bone mass reduction being the most prominent alteration.

5. References


Further development of a gene expression fish embryo test as a potential alternative to the fish early life stage test

Mirco Weil1, Lisa Vorberg1, Janine Rooch1, Nils Klüver2, Stefan Scholz2, Lixin Yang3, Jessica Legradi3, Uwe Straehle3, Frank Sacher4, Karen Duis1

1 ECT Oekotoxikologie GmbH, Boettgerstr. 2-14, D-65439 Floersheim am Main, Germany
2 Department of Bioanalytical Ecotoxicology, Helmholtz Centre for Environmental Research – UFZ, Permoserstraße 15, D-04318 Leipzig, Germany
3 Institute of Toxicology and Genetics, Karlsruhe Institute of Technology, P.O. Box 3640, D-76021 Karlsruhe, Germany
4 DVGW-Technologiezentrum Wasser (TZW), Karlsruher Strasse 84, D-76139 Karlsruhe, Germany

E-mail contact: m.weil@ect.de

1. Introduction
While the fish embryo toxicity test with zebrafish has been developed as a replacement for the acute fish toxicity test [1, 2], there are to date no alternatives to chronic fish toxicity tests, such as the fish early life stage test [3]. In an earlier project, the zebrafish embryo test was extended by an additional endpoint, the analysis of differential expression of marker genes, genes that are sensitive to toxicants [4, 5]. Using a reverse transcription polymerase chain reaction (RT-PCR) based protocol, the effects of 14 substances on the differential expression of seven marker genes were evaluated. For most tested substances, lowest observed effect concentrations (LOECs) derived with the gene expression Danio rerio embryo test (Gene-DarT) agreed reasonably well with LOECs of fish early life stage tests. However, for some substances, larger differences were observed indicating the need to improve the Gene-DarT.

The objectives of the current project are to further develop the Gene-DarT by (1) identifying additional marker genes, (2) optimising the test protocol to allow an effective expression analysis of the larger set of marker genes and (3) using this protocol for testing a range of substances in order to identify the application range and limitations of the test.

2. Materials and methods
A list of 10 model substances and 30 test substances was compiled covering different modes of action, excess toxicities and acute-to-chronic ratios, and a wide range of toxicities in the fish early life stage test.

2.1. Identification of marker genes
Marker genes were identified by microarray analyses following exposure of zebrafish embryos to the ten model substances. The 10 most up-regulated and the 10 most down-regulated genes for each model substance were chosen for verification with quantitative real time PCR (qRT-PCR). A hierarchical cluster analysis of the gene responses (microarray data) was performed for the qRT-PCR validated genes that exhibited robust expression patterns (i.e. a differential expression similar to the microarray data in all replicates). One gene of each cluster was selected for inclusion in the set of marker genes.

2.2. Optimization of the test protocol and substance testing
A multiplex RT-PCR method was developed for expression analysis of the larger set of marker genes. This protocol has been used for testing of approx. 20 test substances. Zebrafish embryos were exposed from 1 to 49 hours post fertilisation to at least five test substance concentrations. Range finding tests were performed under static conditions without replication, definitive tests were performed using a flow-through system and four replicates per concentration and control. Tests solutions from the lowest, middle and highest test concentration and the control were sampled for chemical analysis. Total RNA was extracted by phenol-chloroform extraction, mRNA was transcribed into cDNA, and marker gene sequences were amplified by multiplex PCR. Subsequently, PCR products were analysed using capillary gel electrophoresis.
3. Results and discussion

3.1. Identification of marker genes

In the microarray experiments, sensitive genes were identified for all ten model substances. Using qRT-PCR to verify the expression of 176 of the identified genes, 84 genes were confirmed. Fifty-seven of these genes showed a very robust expression pattern. The hierarchical cluster analysis of these 57 genes resulted in 19 clusters (Fig. 1). One representative gene of each cluster was selected for the Gene-DarT protocol.

Fig. 1: Hierarchical clustering analysis of gene responses (microarray data) in zebrafish embryos exposed to the 10 model substances. The analysis was performed for the qRT-PCR validated genes with very robust expression patterns. The objective was to identify genes with similar expression patterns. The data are means of all biological replicates (n=3).

3.2. Effects of the test substances on differential expression of the marker genes

Range finding tests were performed with 19 test substances. Preliminary results indicate that cyp1a, fabp11, hmox-1, si:ch211 and f7i are the most sensitive marker genes. In the selected concentration range, none of the marker genes was influenced by all test substances. Definitive tests were performed with three test substances and effects on the expression of the following genes were detected: fabp11 (diuron), cyp1a (propanil), zgc:77906, si:ch211, zgc:110712 (2-chloroethanol) and fads2 (2-chloroethanol and diuron). Differential gene expression was observed at substance concentrations that deviate by a factor of not more than ±6 from the effect concentrations determined in fish early life stage tests.

Currently, the effects of further substances on differential gene expression are evaluated in definitive tests. Based on the results, the suitability of the marker genes and the correlation of the results of the Gene-DarT with fish early life stage toxicity data will be evaluated. First conclusions on the application range and the limitations of the Gene-DarT will be drawn.

4. References


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**Danio rerio embryos as a convenient animal alternative model for neurotoxicity assessment**

Elke Muth-Köhne, Vera Delov, Arne Wichmann, Viktoria Schiller, Christoph Schäfers, Martina Fenske

1Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Aachen, Germany
2Institute for Molecular Biotechnology (Biology VII), RWTH Aachen University, Germany
3Institute for Environmental Research (Biology V), RWTH Aachen University, Germany
4Fraunhofer Institute for Molecular Biology and Applied Ecology IME, Schmallenberg, Germany

E-mail contact: elke.muth-koehne@molbiotech.rwth-aachen.de

1. **Introduction**

The developing nervous system is far more sensitive to injury caused by neurotoxic substances than is the adult one. This conflicts with the growing number of chemicals released into the environment deemed to be neuroactive. Despite the relevance of neurotoxic effects in the hazard evaluation of chemicals, testing for neurotoxicity is currently only based on expensive, time-consuming and ethically disputed mammalian experiments, which are also unsuitable for large scale screening applications. Moreover, the present testing approaches endorsed by the OECD or the US-EPA, focus on the assessment of the human health risks only, while the environmental relevance of the neurotoxic compounds remains disregarded.

In ecotoxicology, the embryo toxicity test with zebrafish (Danio rerio; zFET; [1]) is used to determine acute and developmental toxicity of chemicals and is currently being reviewed and validated at the OECD level as an alternative to the fish acute toxicity test [2]. The zebrafish is already a well-established model in developmental and neurobiology, and there is good evidence suggesting that the embryos qualify as promising animal alternative for neurotoxicity testing. Our studies indicate that the examination of spinal motor neuron defects could significantly improve the sensitivity of the zFET for neurotoxins. Two distinguishable types of spinal motor neurons form the spinal cord in the developing embryo, the early-developing primary motor neurons (PMNs) and later-born secondary motor neurons (SMNs). We test the hypothesis if PMN and SMN development could be an informative additional assessment parameter for the zFET to determine the developmental neurotoxic potential of environmental chemicals.

2. **Materials and methods**

The pesticides thiocyclam and cartap (both nAChR blockers), as well as the pharmaceutical disulfiram (aldehyde dehydrogenase blocker) were selected due to their specific phenotype in the FET [3] and as described in the literature [4,5]. After 48 hours, embryos were dechorionated, fixed, and stained with the primary antibodies znp1 (PMNs) and zn8 (SMNs, both [6]). The severity of the motor neuron defects were classified according to Tab. 1 [7,8]. EC values derived from the standard zFET were compared to the EC values determined by the neurotoxicity zFET. Ethanol was used as a positive control for neurotoxic effects [8,9].

<table>
<thead>
<tr>
<th>Motor axon defect</th>
<th>Classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Truncated at the horizontal myoseptum</td>
<td>Severe</td>
</tr>
<tr>
<td>Truncated at the horizontal myoseptum + excessively branched</td>
<td>Severe</td>
</tr>
<tr>
<td>Excessively branched but not truncated</td>
<td>Moderate</td>
</tr>
<tr>
<td>Innervates neighboring myotom</td>
<td>Moderate</td>
</tr>
<tr>
<td>Ectopic branches or ventral roots but overall normal axon morphology</td>
<td>Mild</td>
</tr>
<tr>
<td>Defasciculated axons</td>
<td>Mild</td>
</tr>
<tr>
<td>Axons lacking stereotyped morphology but no excessive branches</td>
<td>Mild</td>
</tr>
</tbody>
</table>

*Table 1: Classification of motor axon defects observed in zebrafish embryos.*

3. **Results and discussion**

3.1. **Thiocyclam treatment severely impairs motor axon growth**

Initial experiments revealed a decreased motor activity of thiocyclam-treated embryos after 24 h. Additional effects were a concentration-dependent occurrence (concentration ranging between 0.08 and 0.187 mg/l; compare Fig. 1A) wavy notochord and a faint pigmentation, which could be related to defects in
neurodevelopment. The same concentrations were used to determine defects in PMN and SMN development. With increasing concentration, motor axons become visibly disorganised and excessively branched, thereby innervating neighbouring myotoms (Fig. 1A). Treated embryos exhibit decreasing numbers of unaffected MNs, while MNs with mild and moderate effects prevail (Fig. 1B; here exemplarily shown for PMNs). Concentration-effect curves determined for thiocyclam with the standard zFET and for the neurotoxicity zFET revealed that the neurotoxicity assessment leads to a shift in the effective concentration (Fig. 1C).

3.2. Cartap and disulfiram effects resemble thiocyclam-mediated effects

Neurotoxicity was also determined for cartap and disulfiram. Preliminary results revealed defects of PMN and SMN development resembling the defects found for thiocyclam. Concentration-effect curves for the neurotoxicity zFET were in accordance with the effect curves of the normal zFET 48 hpf. These results indicate that a similar phenotype leads to comparable defects in neural development.

4. Conclusions

Neuronal damage can be detected in a fast and convenient manner using the zebrafish embryo test zFET. Concentration-effect relationships for adverse neurotoxic effects can be assessed by immunostainings and are applicable to determine the developmental neurotoxic potential of diverse substances. The EC values for neurotoxicity are in good accordance with the standard zFET values, and the method could be considered as an alternative approach to mammalian animal experiments, hitherto still the method of choice for neurotoxicity assessment.

5. References

Transgenic Fluorescent Zebrafish- a promising tool to refine the zebrafish embryo toxicity test zFET

Vera Delov1,2, Elke Muth-Köhne1, Arne Wichmann1,3, Viktoria Schiller1,2, Martina Fenske1, Christoph Schäfers4

1 Fraunhofer Institute for Molecular Biology and Applied Ecology, Aachen, Germany
2 Institute for Molecular Biotechnology (Bio VII), RWTH University, Aachen, Germany
3 Institute for Environmental Research (Bio V), RWTH University, Aachen, Germany
4 Fraunhofer Institute for Molecular Biology and Applied Ecology, Schmallenberg, Germany
E-mail contact: vera.delov@rwth-aachen.de

1. Introduction

The fish embryo toxicity test zFET is highly attractive as an animal alternative testing approach in ecotoxicology. To date, the application of the zFET has focused on acute toxicity assessment, but there is plenty of evidence for its high potential to also evaluate specific and even chronic toxicity like teratogenicity or endocrine disruption. This, however, requires a refined endpoint evaluation using detailed morphological and submorphological analysis methods, which are so far neither established nor standardised. For screening, the zFET procedures will also need adjustments to high throughput applications. The use of gene expression markers can be instrumented for a better understanding of adverse effect mechanisms. They can also help to increase the sensitivity and specificity of the zFET in comparison to the solely on macroscopic morphological endpoints based original test assessment.

This project seeks to test the suitability of a transgenic zebrafish line for the zFET to test for chemical toxicity on vasculogenesis. The transgenic line Tg(fli1:EGFP)y1 expresses enhanced green fluorescent protein (EGFP) in the vasculature under the control of the fli1 promoter [1]. Fli1 belongs to the ETS transcription family and is crucial for the development of the vascular system. Thus, fli1 driven EGFP-expression may serve as a relevant marker for the detection of vascular defects.

2. Materials and methods

Zebrafish embryos of the Tg(fli1:EGFP)y1 line and the wild type strain were exposed to triclosan (0.5 – 0.8 mg/L), genistein (2 – 4 mg/L), fenazaquin (0.03 – 0.27 mg/L) and cartap (0.2 – 1.3 mg/L) for 48 h. The treatment and assessment was carried out according to DIN 38415-6 and OECD Draft TG 2006 [2,3]. Lethal and sublethal effects were determined and the EC50-values were calculated.

Furthermore, the intersegmental vessels (ISVs) of the treated fluorescent fish embryos were measured using fluorescent imaging and the free software ImageJ (Figure 1). To differentiate the obtained effects, the muscle fibers of the embryos were immunobiochemically counterstained.

![Figure 1: Measurement of the intersegmental vessels (ISVs) of 48 hpf Danio rerio embryos using ImageJ. DLAV = dorsal lateral veine, ISV = intersegmental vessel.](image)

3. Results and discussion

3.1. Morphological test endpoints via bright field assessment

Morphologically observable effects comprised malformation of heart, a reduced blood circulation and edema. Reduced blood circulation occurred at concentrations of 0.8 mg/L triclosan, 2.38 mg/L genistein and 0.07 mg/L fenazaquin. For genistein, fenazaquin and cartap, somite and tail malformations were additinally
recorded. Cartap-treated embryos showed also dose-dependent notochord malformations and a lack of pigmentation at concentrations exceeding 0.5 mg/L.

3.2. Fluorescent assessment reveals vasotoxicity

Using the fluorescent $Tg(fli1:EGFP)^{y1}$ line, a concentration-dependent increase of vascular developmental defects could be demonstrated for triclosan, genistein and fenazaquin. At the time of abstract submission, the impact of cartap on the blood vessels of zebrafish embryos was not confirmed.

![Figure 2: A) Relative ISV-length (%) after 24 h and 48 h exposure with triclosan. B) Dose response curve of triclosan (48 h treatment).](image)

Figure 2 A shows that the relative ISV-length decreases with increasing substance concentration. Significant differences of the ISV-length between the treated and the control zebrafish already occurred after 24 h of exposure. These early defects or retardation of the intersegmental vessel-growth were undetectable in bright field microscopy. Furthermore, the fluorescent assessment revealed effects on the vasculature at lower exposure concentration than the bright field assessment (see Figure 2 B). Thus, EGFP-expression driven by the fli1 promoter leads to an earlier and more sensitive detection of vasotoxicity than in wild type zebrafish embryos. Additionally, immunolabelling offers the possibility to specify the results and to differentiate between primary and secondary effects caused by vasotoxicants.

4. Conclusions

A defect of the development of the blood circulatory and vascular system of fish embryos is an important toxicological endpoint in the zFET, which should be considered as lethal. However, the earliest time point for the detection of such adverse effects in wild type embryos is 36 h post fertilization (hpf) of the eggs. Fli1 is one of the first ETS transcription factors, and the onset of Fli-expression in the developing endothelial vessels is as early as 14 hpf. Hence, adverse effects on vasculogenesis and angiogenesis can be detected earlier or even at all in the $Tg(fli1:EGFP)^{y1}$ embryos by using a fluorescent signal-based assessment method. The results demonstrate that the transgenic zebrafish line $Tg(fli1:EGFP)^{y1}$ is a promising tool to refine the zFET for vasotoxic substances.

5. References


Acknowledgement - The authors thank the Federal Institute for Risk Assessment bfr-ZEBET for the financial support of this work.
Combined toxicity of estrogen receptor agonists and antagonists in a fish *in vitro* assay

Karina Petersen\(^1,2\) and Knut Erik Tollefsen\(^1,3\)

\(^1\)Norwegian Institute for Water Research, Oslo, Norway  
\(^2\)University of Oslo, Oslo, Norway  
\(^3\)University of life sciences, Ås, Norway  
E-mail contact: kpe@niva.no

1. Introduction

Primary hepatocytes from fish such as rainbow trout (*Oncorhynchus mykiss*) represent a well-characterised high-throughput screening tool for single chemicals and mixtures [1, 2]. These native cells provide assessment of multiple mode of action (MoA) and has successfully characterised the potency of both estrogen receptor (ER) agonists and ER antagonists *in vitro* through use of the estrogenic biomarker vitellogenin (Vtg) [2, 3]. Fish in the environment are rarely exposed to just one chemical at the time and the growing concern about the combined effect of mixtures of chemicals has resulted in development of prediction models for the combined effect of mixtures. Two of these prediction models, the concentration addition (CA) and the independent action (IA) prediction models are widely applied for direct endpoints like toxicity to algae, *in vivo* induction of Vtg and *in vitro* activation of the ER [4, 5, 6]. Because the CA prediction model seems to be able to predict the combined effect of mixtures in various experimental models, the screening of combined effect of chemical mixtures *in vitro* could be a useful tool to identify chemical mixtures of interest and perform a preliminary screening of potential interactions between chemicals in the mixture. In the last years, several studies have shown that CA can accurately predict the combined effect of mixtures of ER agonists both *in vivo* and *in vitro* [6, 7]. The present work assess the CA and IA prediction models’s ability to predict the combined effect of mixtures of ER agonists and antagonists in a primary culture of rainbow trout hepatocytes.

2. Materials and methods

Primary cultures of rainbow trout hepatocytes were exposed to individual ER agonists to determine the activation of ER-mediated production of Vtg. The inhibition of Vtg production was determined by exposing hepatocytes to ER antagonists in combination with a fixed concentration of 17β-estradiol (E2, 0.63nM). After 96h of exposure, the cell growth media were pipetted off and both cells and the cell growth media was stored at -80°C for subsequent Vtg (ER agonists and antagonists) and CYP1A (ER antagonists) analysis by capture ELISA. The CYP1A-mediated EROD activity were additionally measured for the ER antagonists. Results for the ER agonists were expressed as percentage of a positive control exposed to 30nM E2 and results for the ER antagonists were expressed as percentage of the Vtg production induced by the fixed E2 concentration (0.63nM) alone. Cytotoxicity was determined in parallel by the combined use of Alamar blue and 5-carboxyfluorescein diacetate acetoxymethyl ester (CFDA-AM). The individual concentration-response curves of Vtg production and inhibition were used to calculate the composition of mixtures according to the CA prediction model. The observed effects of the mixtures were compared to the CA and IA model predictions to identify interactions in the mixtures.

3. Results and discussion

3.1. Combined effect of mixtures of ER agonists

Although nine of the tested compounds showed a concentration dependent increase in the production of Vtg, the individual concentration-response curves for the different compounds differed in potency and efficacy (results not shown). Mixtures of seven (bisphenol A, o,p’-DDT, 4-t-octylphenol, 17β-estradiol, estril, estrone and diethylstilbestrol) and nine (bisphenol A, o,p’-DDT, 4-t-octylphenol, dibenzothiophene, musk ketone, 17β-estradiol, estril, estrone and diethylstilbestrol) ER agonists were designed according to the CA prediction model and tested in the primary rainbow trout hepatocyte bioassay. Both mixtures followed the prediction models at the low to medium relative mixture concentrations (fig. 1). Deviations from the prediction models were observed at the higher relative mixture concentrations suggesting antagonistic effects in the mixtures. None of the mixtures showed prominent cytotoxicity.
3.2. Combined effect of mixtures of ER antagonists

All tested ER antagonists; benzo(a)pyrene (BAP), benzo(k)fluoranthene (BKF), benzo(b)fluoranthene (BBF), benzo(a)antracene (BAA), indeno[1,2,3-cd]pyrene (IP), PCB126, PCB77, TCDD, β-naphtoflavone (BNF), ZM189.154 and hydroxy-tamoxiphene (OH-TAM), showed a concentration dependent inhibition of the E2-induced production of Vtg. Most compounds were able to decrease the E2-induced Vtg production from 100 to about or below 20%, and a few compounds completely inhibited the E2-induced production of Vtg at the highest tested concentrations. Several mixture toxicity scenarios are currently under investigation; 1- five PAH’s (BAP, BKF, BBF, BAA and IP), 2 - two PCB’s and TCDD, 3 - ZM and OH-TAM, and 4 - all tested compounds.

4. Conclusions

The rainbow trout primary hepatocytes have proven to be a fast and reliable method for assessment of estrogenic and anti-estrogenic properties of compounds and highlight the potential for use in high throughput screening of chemicals, either singly or in multi-compound mixtures. Except for the deviations from the prediction models at the higher relative mixture concentrations of ER agonists, our results are in agreement with other in vitro and in vivo studies where CA has shown to accurately predict the combined effect of ER agonists [5, 6]. Our results show that the use of in vitro native cells such as primary rainbow trout hepatocytes in combination with mixture toxicity prediction models may become useful tools in assessing the combined effects of ER agonists and ongoing studies with ER antagonists will determine whether the prediction models are able to successfully predict more complex MoA such as that caused by ER antagonists.

5. References


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Alternatives to in vivo tests to detect endocrine disrupting chemicals (EDCs) in fish and amphibians

S. Scholz1, P. Renner1, L.S. Ortego2, S. Belanger3, F. Busquet4, R. Davi5, B. Demeneix6, J. Denny7, M. Léonard8, M. McMaster9, D. Villeneuve7 and M. Embry10

1Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany
2Bayer Crop Science, Research Triangle Park, NC, USA
3Procter and Gamble, Cincinnati, OH, USA
4European Commission, Joint Research Centre, IHCP, ECVAM, Ispra, Italy
5ExxonMobil Biomedical Sciences, Inc., Amandale, NJ, USA
6Musee Nationale d’Histoire naturelle, Paris, France
7US Environmental Protection Agency, Mid-Continent Ecology Division, Duluth, MN, USA
8L’Oreal, Aulnay Sous Bois, France
9Environment Canada, Burlington, ON, Canada
10ILSI Health and Environmental Sciences Institute, Washington, DC, USA

E-mail contact: stefan.scholz@ufz.de

1. Introduction

A wide variety of both developmental and reproductive disorders observed in wildlife species have clearly been linked to the exposure to environmental contaminants which act as endocrine disrupting chemicals (EDCs). Such chemicals are of high environmental relevance, since many essential physiological processes which impact on the individual’s health, such as growth and development, stress response, and ultimately reproduction and population development are controlled by hormones.

The importance of identifying potential endocrine disruption for environmental risk assessment has also been recognised by regulatory bodies and is expressed in recent legislations, for instance, the registration of chemicals [1]. Further, REACH also demands that tests on vertebrate animals are refined, reduced or replaced (3Rs) whenever possible. This is also illustrated in the recently published European Directive 2010/63/EU on the protection of laboratory animals [2]. For medicinal products for human use in Europe, biological effect evaluation of potential EDCs has to be addressed for environmental risk assessment irrespective of the expected quantity released into the environment [3].

In 2009, the US EPA began implementation of the Endocrine Disruptor Screening Program which includes Tier 1 screening assays in fish and frog species which are closely aligned with the OECD test guideline series 229 and 231. However, these assays use a large number of animals and are quite long in duration relative to an ideal screening assay. As the Tier 1 assays screen a large number of chemicals for possible endocrine activity and prioritize them for testing, shorter-term and alternative to animal tests would be advantageous.

2. Materials and methods

Peer-reviewed literature as well as publicly available government documents from 1995 to the present were screened to identify key literature relating to alternatives for the detection / testing of EDCs in fish and amphibians. Specifically, the screen was focussed on estrogen, androgen, and thyroid hormone pathways and the following assay systems: cell lines (piscine/amphibian cell lines or mammalian cell line if a reporter construct of piscine/amphibian origin was used), yeast screening assay (if a reporter construct of piscine/amphibian origin was used), primary cells (fish and amphibians), ligand binding assays (if homogenates or receptors from fish or amphibians were used), and amphibian or fish embryos. The data were compiled with details on the assay systems and effect levels. In order to facilitate data evaluation a graphical representation based on a dot plot and correlation analyses were used. For graphical representation and comparative analysis, the match of certain quality criteria was required such as availability of NOEC and LOEC or EC50 levels, similar molecular endpoints etc…

3. Results and discussion

The literature review covered about 180 different compounds with data on alternative assay systems extracted from more than 70 publications and reports. The majority of the studies utilised molecular
endpoints related to the mode of action, such as induction of estrogen/androgen sensitive target genes, reporter genes controlled by hormone responsive elements.

The greatest amount of data was identified for assays screening potential (anti)estrogenic compounds in fish. For this mode of action the large number of available data also allowed a qualitative evaluation of sensitivity. (1) Despite the fact that data originated from different laboratories and that different protocols have been used a relatively high intra-assay concordance was observed. (2) Transgenic reporter systems appear to be on average about an order of magnitude more sensitive than other alternative assays. (3) Correlation was limited by the number of in vivo data. However, in case that in vivo and alternative assay data were available, the latter exhibited a similar sensitivity (Fig. 1).

**Figure 1: Alternatives to fish tests for detecting (anti)estrogenic effects.** The figure represents an example with a close-up for selected compounds. Similar representations were compiled for androgen and thyroid hormone disrupting compounds and for alternatives to amphibian tests. Dots represent lowest observed effect concentrations or EC/IC₅₀ data.

4. Conclusions

Alternative assays can provide similar sensitivity as in vivo assays if the endocrine disruption is based on receptor binding. For other modes of action, such as interference with steroid synthesis, the applicability of these alternative assays is limited. However, such compounds have been tested with very low frequency and hence, it is difficult to provide a quantitative statement. Each assay system exhibits advantages and limitations. However, transgenic reporter assays appear to be promising, due to their sensitivity and simplicity of measurement. Fish or amphibian embryos may also represent a promising test system. They represent a more complex system which is – in terms of toxicokinetics - closer to the in vivo situation. In combination with reporter genes they also provide a high sensitivity. However, in contrast to other assays, only few data are available. Validating these alternative test methods would help proving the relevance and the reproducibility of these assays. In summary, alternative assays with cells, embryos etc. could be established as alternatives to testing of animals at least as part of a tiered screening approach.

5. References


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Exploring the potential of the ZFET beyond acute toxicity

Martina Fenske¹, Elke Muth-Köhne¹, Viktoria Schiller¹², Arne Wichmann¹³, Vera Delov¹², Catherine Turner⁴, Ralf Kriehuber⁵ and Christoph Schäfers¹

¹Fraunhofer Institute for Molecular Biology and Applied Ecology IME, 52074 Aachen and 57392 Schmallenberg, Germany
²RWTH Aachen University, Institute of Molecular Biotechnology (BioVIII), 52074 Aachen, Germany
³RWTH Aachen University, Institute for Environmental Research (BioV), 52074 Aachen, Germany
⁴University of Liverpool, Centre for Genomic Research, Liverpool, L69 7ZB, UK
⁵Forschungszentrum Jülich GmbH, Department of Safety and Radiation Protection, 52425 Juelich, Germany

E-mail contact: martina.fenske@ime.fraunhofer.de

1. Introduction

Animal testing demand estimates for REACH list long-term (chronic) fish toxicity among the top five of toxicity endpoint needs [1], together with another chronic test, the 2-generation developmental toxicity test (OECD 416), coming first. The pressure to find regulatory accepted alternative approaches for chronic toxicity assessments in fish is therefore high, but is also opposed by the fundamental requirement for any alternative approach to be good enough to not increase the uncertainty in hazard and risk assessment.

In terms of fish toxicity test alternatives, the fish embryo toxicity test FET can certainly be considered the most promising approach, and in the context of the OECD Test Guideline Program, the validation of a protocol for the FET with zebrafish (zfET) to replace the fish acute test is already underway. Meanwhile, the applicability of the FET to also evaluate specific non-acute toxicity and teratogenicity or to predict chronic adverse outcome pathways, is widely acknowledged [2,3]. A prerequisite to take full advantage of the potential of the FET, however, is to agree on common standard protocols for specific applications and to address sources of uncertainty of the test procedures.

In our studies, we address basic FET related methodological issues like the selection of test vessels or the test duration, to facilitate standardisation and prevalidation of the assay, but also seek to refine the FET through the integration of additional toxicological endpoints. One focus is on transcriptomics as an endpoint and the evaluation of its value to inform on underlying mechanism of toxicity.

2. Materials and methods

Fish embryo tests with zebrafish were conducted either following the DIN 38 415-6 and OECD Draft (2006) test guidelines or following a prolonged test protocol, which includes post-hatch stages until 120 hpf. Data of several studies will be presented, covering different aspects of the FET of the above mentioned objectives.

- Exposure of newly fertilised embryos for 120h in either 24-well or 96-well plates to several insecticides (e.g., abamectin and tebufenpyrad), with the oxygen content monitored continually. The aim was to investigate the differences between the two well-plate types and the influence of the oxygen saturation.
- Exposure of newly fertilised embryos for 48h to the solvent dimethylformamide (DMF) or 4-tert-pentylphenol (TPP) or in combination; RNA was extracted from the embryos and analysed on a custom oligonucleotide microarray to investigate the influence of organic solvent use on the toxicity of a test compound and on the transcriptome.
- Exposure of newly fertilised embryos for 48h to the phytoestrogen genistein; extraction of RNA and analysis on Agilent Zebrafish Oligo Microarray chips and by quantitative RT-PCR. The aim of the study was to investigate how genistein affects gene expression and whether the estrogenic mode of action can be discerned from the transcriptome response.

3. Results and discussion

3.1. Influence of the multiwell plate type on the FET and the role of oxygen saturation

Oxygen can become a limiting factor in the extended FET (efET) when 96-well plates and static conditions are used (Figure1). Significant differences in the toxicity and the morphological phenotype were found between different plate types for some of the insecticides tested, but this issue was resolved when the test solution was renewed every 24h. Overall, the efET proved more sensitive than the FET for insecticides, given all assessable sub-lethal effects are considered for the effect level determination.
3.2. The potential confounding influence of solvent use on FET-derived toxicity

<table>
<thead>
<tr>
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<th>MTE</th>
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<th>MTE cut-off</th>
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<td>Sig. probes</td>
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<td>0</td>
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<tr>
<td>TPP (100 µL L⁻¹)</td>
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<td>0.411</td>
<td>0</td>
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Table 1: Number of differentially expressed probes at different FDR (false discovery rate) cut-off values for all DMF and TPP treatments compared to the ISO control treatment. MTE: Minimum Total Error—the FDR at which the percentage of the whole data set that have been erroneously identified is minimal.

Exposure to DMF and TPP alone and in combination, can modulate the gene expression of thousands of genes (Table 1), including many belonging to gene regulatory groups involved in important biological and metabolic processes. The numbers of genes displaying regulation by TPP were also concentration sensitive when compared to the ISO or the DMF control treatments, and a larger response was apparent in the low TPP concentration mixture than in the high TPP concentration in contrast to the single compounds.

Some genes indicating oestrogenic activity were found at the higher concentration to be differentially expressed by TPP, but only with small fold changes and therefore not unequivocally confirming the presumed weak oestrogenicity of TPP.

3.3. Transcriptomics to identify endocrine disruption MOA in the FET

Genistein enhanced the expression of mainly estrogenic genes and there was a good coherence between the genes responding in the embryos and in adult zebrafish [4]. We could also demonstrate that similar pathways were affected in medaka embryos than in zebrafish.

4. Conclusions

The zebrafish embryo toxicity test ZFET holds numerous opportunities as an alternative approach, which can provide a more highly refined view of toxic effects, from which meaningful patterns of response can be discerned and related to functional deficits, and from which more reliable prognostic indicators of toxicological effect can be extracted. In the context of regulatory chronic toxicity assessments, however, the ZFET will require a thorough validation and standardisation of procedures to minimise the level of uncertainty of in particular, integrated molecular omics-based methodologies.

References

Evaluation of the OECD 210 Fish Early Life Stage Chronic Toxicity Test - Setting the Target for Future Animal Alternative Efforts

James T. Oris\textsuperscript{1}, Scott E. Belanger\textsuperscript{2}, and A. John Bailer\textsuperscript{3}

\textsuperscript{1}Department of Zoology, Miami University, Oxford, OH 45056 USA
\textsuperscript{2}Product Safety and Regulatory Affairs, The Procter & Gamble Co., Cincinnati, OH 45253 USA
\textsuperscript{3}Department of Statistics, Miami University, Oxford, OH 45056 USA
E-mail contact: orisjt@muohio.edu

1. Introduction

Several promising animal alternatives approaches now exist for acute fish toxicity and bioaccumulation. In the context of international chemical management programs a larger need now looms to address chronic fish toxicity alternatives. Many obstacles need to be overcome to make chronic ecotoxicity alternatives a reality; however, a fundamental tenet of any approach should be that the alternative should not increase uncertainty in hazard and risk assessment. Therefore, understanding the present level of uncertainty in identification of hazards based on traditional assays sets the bar for future alternative developments.

An important consideration of animal welfare in toxicity testing is the recognition that statistical significance does not equal biological significance. Tests should be optimized to use the minimum number of organisms that can provide a statistical determination of a biologically significant effect. If a test is over-sized, too many organisms are being used. If a test is under-sized and biologically significant effects cannot be detected, the test will be inconclusive and animals wasted. In our opinion, neither situation is desirable or ethical.

The chronic fish toxicity assay most commonly used to establish chronic effects is the OECD 210, Fish Early Life Stage Test. The test guideline (TG) for OECD 210 was proposed in 1988 and adopted in 1992. The TG appears to be based on consensus, and provides general guidance on test design, methodology, and statistical analysis. In the OECD 210, early life stages of fish are exposed to a range of concentrations of a dissolved test substance, with the intent of estimating chronic lethal and sub-lethal effects [1]. Four freshwater and 1 saltwater fish species are recommended for testing, but a wide variety of other, well-documented freshwater and saltwater species have been used as test subjects. Tests are initiated with a minimum of 60 newly fertilized eggs per treatment concentration divided equally among a minimum of 2 replicate chambers. Typically five concentrations and appropriate controls (dilution water, dilution water plus dissolution solvent) are recommended. Test duration depends on species, and lasts until control fish are free-feeding (duration range = 28 – 60 days). Endpoints measured include hatching success, post-hatch survival, length, weight, and developmental abnormalities. No specific length measurement (e.g., standard v. total) is recommended, and dry-weight is the recommended measure for weight. Involvement of a statistician is recommended in the design and analysis of the experiment and much flexibility is allowed; however, analysis of variance techniques are suggested as a minimum level of data analysis. The No Observable Effect Concentration (NOEC) and Lowest Observable Effect Concentration (LOEC) are typically reported for each measured endpoint.

The OECD 210 TG has been in use as adopted for nearly 20 years. It has served an important role in the assessment of chemical safety and there have been hundreds, if not thousands of tests performed. However, to our knowledge there has been no systematic analysis of the test design or statistical analysis since the TG was adopted. In an effort to reduce uncertainty in hazard estimates of chemicals and the ongoing desire to reduce the use of animals in toxicity tests, we conducted an analysis of data compiled from OECD 210 tests conducted by industry labs. The focus of this analysis was the statistical characteristics of control treatments, with the goal of providing recommendations on optimizing the design of the OECD 210 to minimize animal usage needed to detect biologically significant outcomes and minimize the number of inconclusive experimental outcomes.

2. Materials and methods

We collated a database (>100 studies and compounds from 15 different laboratories) to probe the data characteristics of the OECD 210. Studies were constrained to fathead minnow (72%), rainbow trout (12%) and zebrafish (16%), which form the majority of studies conducted that were available. Studies were summarized with respect to experimental design, water quality, quantifying chemical exposure, and measured test endpoints (hatchability, post-hatch survival, wet and dry weight, length, developmental
abnormalities). Information was collected at the level of individual replicates with the goal of determining the statistical power of the reviewed studies to detect biologically meaningful effects. Binomial endpoints (hatching success and post-hatch survival) were examined for indications of over-dispersion (extra-binomial variability) [2] in an effort to determine whether individual fish or test chamber could be used as a replicate. Analysis of the ability to detect statistical differences in endpoints between control and treatment was conducted for each test using appropriate power analysis techniques [3]. Power to detect differences based on each species recommended control performance minimums relative to actual performance for binomial endpoints were summarized across all tests. Power to detect differences for other endpoints (length, wet-weight, dry-weight) based on each species measured control performance were summarized across all tests. Sensitivity of each endpoint for each species was compared to provide insight on optimal experimental design and analysis.

3. Results and discussion

Tests for overdispersion in tests indicated that extra binomial variation was frequently present, indicating that the unit of replication should be test chamber and not individual fish. Thus, in order to increase the sensitivity of the OECD 210 test, it is recommended to maximize the number of test chambers rather than the number of fish per chamber. For example, 4 chambers with 15 fish will be more powerful than 2 chambers with 30 fish each. All fish performed above recommended minimal levels of hatching and post-hatch survival. Because control performance in binomial endpoints can affect the ability to detect differences, it is recommended that minimum performance standards for control hatching and post-hatch survival be based on broadly attainable levels for each individual species. Fathead minnow and rainbow trout could generally achieve 80% and zebrafish could generally achieve 90% levels of both hatching and post-hatch survival. Power analysis indicated that most endpoints could detect a 20% change relative to controls when using at least 4 replicate chambers. Zebrafish tended to be the least variable in all endpoints and thus were able to detect smaller differences in endpoints compared to other species. Wet weight was 3x more sensitive for zebrafish and 1.5x more sensitive for zebrafish and fathead minnows compared to dry weight, even though the current TG recommends dry weight as a preferable measure of growth. The most sensitive endpoint was length for all species, followed generally by wet weight, dry weight, hatching success, and post-hatch survival.

4. Conclusions

Results of the analysis indicated that improvements in the sensitivity of the test could be made by maximizing the number of replicate chambers per treatment concentration rather than by maximizing the number of organisms per chamber, by increasing the acceptable level of control hatching success and larval survival compared to current levels, by using wet weight measurements rather than dry weight, and by focusing test effort on species that demonstrate less variability in outcome measures. From these analyses we provide evidence to support the level of uncertainty and power to expect from traditional OECD 210 studies as a target for future animal alternative methods for chronic toxicity testing in fish.

5. References


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The OECD fish testing framework project: Summary of workshop recommendations

J. Wheeler1, G. Ankley2, M. Embry3, A. Gourmelon4, T. Iguchi5, G. Maack6, P. Matthiessen7, L. Musset4, L. Touart8, C. Willett9

1Syngenta Ltd, Bracknell, UK
2US EPA, Duluth, MN, USA
3ILSI HESI, Washington, DC, USA
4OECD, Paris, France
5NIBB, Okazaki, Japan
6UBA, Dessau, Germany
7Consultant, UK
8USEPA, Washington, DC, USA
9PETA, Norfolk, VA, USA

Disclaimer: The opinions expressed and the arguments employed herein are those of the authors and do not necessarily reflect the official views of the OECD or of the governments of its member countries

E-mail contact: james.wheeler@syngenta.com

1. Introduction

An integrated Fish Testing Framework project was initiated in mid-2009 as OECD Project 2.30 with the United States as the lead country. The objectives of the project were to review the regulatory needs and data requirements for fish testing and review the currency of existing OECD Test Guidelines. In addition, the project aimed to support animal welfare concerns by identifying unnecessary test methods, minimizing the number of in vivo fish tests, and ensuring the optimal use of data derived from in vivo studies. A September 2010 workshop with participation from over 40 experts was organized with the goal of producing a Fish Testing Framework guidance document that provides a detailed discussion of issues, relevant endpoints, and considerations for a harmonized testing framework for fish.

2. Materials and methods

Over 40 participants from OECD members countries and stakeholder groups participated in the workshop, where various chapters drafted by a project steering team in preparation for the meeting were discussed and revised. In addition to detailed reviews of the individual OECD fish test guidelines, topic areas discussed included general test methods, regulatory needs and data requirements for fish testing, statistical considerations, animal welfare considerations and alternative approaches to testing. General guidance on possible strategies for approaching hazard testing with fish was developed, illustrating broad principles which can then be adapted for specific circumstances and types of chemicals. Numerous preliminary conclusions and recommendations were developed by the participants as a result of the individual chapter discussions.

3. Results and discussion

3.1. Regulatory needs and data requirements for fish testing

Fish tests are the subject of several OECD test guidelines (TGs) and were originally developed at the request of OECD member countries to suit a regulatory need and ensure the Mutual Acceptance of data. However, as many of the test guidelines were published several decades ago, it is now worth reconsidering their applicability to current regulatory requirements and to possible future developments. In addition, more recent guidelines were developed at a time of increased scientific and technical knowledge of fish testing in general leading to a disparity amongst guidelines. A review of the fish testing requirements of a range of regulations in several OECD jurisdictions covering various types of chemicals (pesticides, biocides, industrial chemicals, pharmaceuticals) was performed and discussed.

3.2. Statistical considerations

The statistical methods used to analyze results of regulatory ecotoxicology studies must be consistent with regulatory frameworks, must be statistically robust, and maximize efficiency in terms of animal use, time and cost. Some of the key topic areas discussed included definitions of replicates, biological versus statistical
significance, calculation of NOEC, LOEC values, and ECx values, alternate study designs, replicates, use of solvent/carrier controls, and power analysis.

3.3. General test considerations

In addition to the statistical considerations noted above, various general test considerations that are applicable to a wide variety of the OECD fish TGs were addressed. These topics included concentration setting, preparation of test solutions, acclimation and culture maintenance, selection of test species, chemical analysis, water and diet quality criteria, and test acceptance criteria.

3.4. Animal welfare considerations and alternative approaches to testing

Background on and current approaches to replacing, reducing and refining (the “3Rs”) the use of animals in testing including social and legal impetus for reducing reliance on animal testing as well as general approaches to the 3Rs were highlighted. During discussion specific examples of 3R approaches from ecological or fish testing were evaluated for their applicability to existing fish TGs and/or integration into a fish testing framework.

3.5. Detailed review of Test Guidelines

All existing OECD Test Guidelines and proposed test guidelines were reviewed by the workshop participants. Specific information that was evaluated includes deliverables (data/information), prerequisites, strengths and limitations, statistical considerations, terminology, concentration setting, quality assurance, animal minimization, delivery or solvent use, and species effectiveness. In addition, general aspects related to clarity or interpretations of TGs were also highlighted.

3.6. Possible fish testing strategies

A generic approach for fish testing was presented to reflect the latest scientific advances in order to meet risk assessment needs and reduce vertebrate testing. This generic approach provides some general guidance on possible strategies for approaching hazard testing with fish, recognizing that no single approach will be appropriate for all scenarios.

4. Conclusions

Preliminary conclusions and recommendations by the workshop participants focused largely on revisions to existing TGs and/or the need for expert group discussions, workshops, or reviews to address critical issues.

Existing TGs that warrant revision and updating include the 210 (fish early-life stage test) and the 203 (fish acute test); several other TGs (212, 215) may require updating upon completion of the Fish Embryo Test (FET; current a draft TG) validation. TG204 (Fish, Prolonged Toxicity Test: 14-day Study) was recommended for deletion.

Several issues may necessitate expert working groups, workshops, or review papers to sufficiently address the topics before any TG revisions can be made. These include: evaluation of solvent effects (including statistical analysis); choice of test concentrations for fish endocrine screening assays (TG229 and 230); test acceptance and validity criteria; water and nutrition quality; review and analysis of existing fish data to further inform TG229 development; development of high-quality fish chronic data sets; review of practical applications of mode of action and pathways that can help avoid unnecessary testing including consideration of additional biomarkers in some assays; assessment of the appropriateness of the various recommended or optional fish species in TGs; and harmonization of definitions across various TGs (e.g., life stages, acute, chronic, spawning status, etc.).

Acknowledgement - The authors thank all of the workshop participants for their active input and critical evaluation.
Comparing prioritisation schemes for environmental risk assessment of human pharmaceuticals

Vendela Roos¹, Jerker Fick², Lina Gunnarsson³, DG Joakim Larsson³ and Christina Rudén¹

¹Division of Philosophy, Royal Institute of Technology (KTH), SE-100 44 Stockholm, Sweden
²Department of Chemistry, Umeå University, SE-901 87 Umeå, Sweden
³Department of Neuroscience and Physiology, the Sahlgrenska Academy at the University of Gothenburg, SE-405 30 Göteborg, Sweden
E-mail contact: vendela.asp@abe.kth.se

1. Introduction

The presence of pharmaceuticals in the aquatic environment, and the concerns that these pharmaceuticals might negatively affect aquatic organisms, has gained increasing attention over the last years. Active pharmaceutical ingredients (APIs) are excreted in the urine and/or faeces of users, and end up in our waterways as a result of poor removal in sewage treatment plants. Pharmaceuticals may also be released into the aquatic environment from pharmaceutical production facilities. The environmental fate of pharmaceuticals, particularly their effects on aquatic organisms, are largely unknown, and it is therefore important to prioritise pharmaceutical substances for ecotoxicity testing and environmental monitoring.

An ideal prioritisation procedure is systematic, transparent, based on relevant data and applicable to every API on the market. Several prioritisation and ranking schemes have been proposed, however the usefulness of many models is limited due to lack of appropriate data. The present study aims to compare and discuss ranking and prioritisation schemes based on different types of data, with particular focus on the prioritisation process developed within the Swedish MistraPharma research programme.

2. Methodology

We have systematically applied the fish plasma prioritisation model, originally proposed by Huggett et al (1), to approximately 800 APIs on the Swedish market covered by the current European legislation, i.e. excluding vitamins, electrolytes, amino acids, peptides, proteins, carbohydrates, lipids, vaccines and herbal medicinal products. In addition, antibiotic and antiviral substances were excluded since they do not primarily target human proteins.

The fish plasma model (Equations 1 and 2) estimates fish plasma concentrations of pharmaceuticals based on PEC and lipophilicity (logP) values and compares them to human therapeutic plasma levels, thereby creating a concentration ratio (CR). The CR is thought to reflect the likelihood for pharmacological effects in fish, assuming that orthologs to the human drug targets are functionally conserved in fish.

\[
CR = \frac{H_{TPC}}{F_{SSPC}}
\]

Equation 1: CR=concentration ratio, \( H_{TPC} \)=human therapeutic plasma concentration, \( F_{SSPC} \)=fish steady state plasma concentration.

\[
F_{SSPC} = 10^{((\log P \times 0.73) - 0.88)} \times PEC
\]

Equation 2: \( F_{SSPC} \)=fish steady state plasma concentration, \( \log P \)=octanol:water partition coefficient, \( PEC \)=predicted environmental concentration.

We have also compiled chemical, sales, environmental effect, and pharmacological data for approximately 600 APIs currently on the Swedish market. Parameters include amounts of defined daily doses (DDD) sold per year, predicted environmental concentrations (PEC), predicted no effect concentrations (PNEC), data on...
persistency, bioaccumulation and toxicity (PBT), and quantitative structure-activity relationship (QSAR)- derived chronic effect data. All data were collected from publicly available sources. APIs were ranked using one or several parameters at a time and relative rank positions were calculated in order to compensate for differences in data coverage between parameters. The results of the different ranking methods will be compared.

3. Results and discussion

3.1. Data availability and coverage

The conditions for conducting a study such as the present are probably better in Sweden than anywhere else in the world. For example, the Swedish pharmaceutical industry participates in a voluntary initiative to publish otherwise confidential environmental effect data online (2). That said, while sales statistics and logP values are available for all 600 APIs in our study, PNEC values are only available for one-third of the substances. PBT values, which provide a rough chemical/biological classification, are available for approximately two-thirds of the APIs. These and additional aspects of data availability and coverage are discussed in the presentation.

3.2. Criteria selection and combinations

A useful prioritisation scheme would be based on data that cover as many pharmaceutical substances as possible. Several previously proposed prioritisation schemes include environmental effect measures. While obviously an attractive thought, such approaches generate circular arguments whereby pharmaceuticals would only be selected for further testing if they were already identified as potentially hazardous to the environment.

Another approach is to make use of the abundant pharmacological data that is generated during the pharmaceutical development process, particularly with respect to mode-of-action. The suitability and feasibility of different criteria selection approaches are further discussed in the presentation.

4. Conclusions

Pharmaceuticals may be ranked and prioritised for further risk assessment according to any chemical, sales, and/or effect criteria or combinations thereof. The fish plasma model, proposed by Huggett et al (1) and applied by the MistraPharma research programme, is based on a combination of different types of data (sales statistics, chemical properties, and pharmacological data) that are available for almost all pharmaceutical substances. The model thus takes into account both the estimated internal exposure at the drug target(s) and an estimate of potency (based on read-across from human potencies). This makes it a theoretically attractive concept for predicting risks for pharmacological effects in fish, whether adverse or not.

We would like to stress, however, that any prioritisation scheme should be evaluated empirically for accuracy prior to implementation. Existing empirical data illustrate some of the current difficulties in theoretically predicting bioconcentration for certain drugs. Still, a comparison of predicted or measured fish plasma levels and human therapeutic levels correctly identifies ethinylestradiol and levonorgestrel as pharmaceuticals of high environmental risks. This adds confidence to the overall concept of the fish plasma model.

5. References


Developmental *Environmental Assessment Regulations for Pharmaceuticals: Guided By The Science*

A. Graham M. Rattray¹, R. Gordon Stringer¹ and Andrew Beck²

¹Environmental Impact Initiative, Health Canada, Ottawa ON Canada
²Environmental Assessment Unit, Health Canada, Ottawa ON
E-mail contact: graham.rattray@hc-gc.sc.ca

1. Introduction

While timely access to pharmaceuticals and health care products is of utmost concern to the government of Canada, there is growing recognition and concern that substances in these types of products are being found in the environment in concentrations that may pose a risk. The *Canadian Environmental Protection Act* (CEPA) 1999 requires that all new substances for use in Canada be evaluated for their potential risks to the Canadian environment and human health. Currently substances in products regulated by the *Food and Drugs Act* (F&DA) (including therapeutic drug ingredients) are legally obliged to undergo an environmental assessment. However the current environmental assessment regulations were developed primarily for industrial chemicals and are not appropriate for assessing the potential environmental risk of the types of substances and release scenarios associated with drug products. As such, Health Canada, in consultation with representatives from industry, non-governmental organizations and consumer groups, initiated a project to develop *Environmental Assessment Regulations* (EARs) with specific information requirements for these types of substances. A framework has been developed which seeks to align with the drug development process while leveraging a testing strategy that considers the potential fate and exposure profile of the drug substance to direct the type of ecotoxicological testing to be required.

The starting premise in the development of a regulatory framework for drug substances is that the regulatory requirements must be science-based and in proportion to potential for risk and allow for the continually expanding knowledgebase of the behaviour of active ingredients in the environment to refine requirements for environmental assessments. Action limits and data requirements should be commensurate with the characteristics of the compound and its expected release into and fate in the environment. Fate/exposure directed toxicity testing ensures that all compartments of the environment are considered and will be protected, allowing the science to establish testing requirements, rather than being driven by prescriptive measures. This talk will explore some of these concepts.

2. Materials and methods

As this talk will focus on the results of scientific analyses and literature reviews conducted for the purpose of regulation development, the methodology is not in the form of laboratory research.

3. Results and discussion

As this talk will focus on the results of scientific analyses and literature reviews conducted for the purpose of regulation development, the methodology is not in the form of laboratory research.

4. Conclusions

The scientific basis for proposed regulatory frameworks to assess the environmental impacts of new substances in pharmaceuticals and personal care products are to be presented. The emphasis will be on the science of conducting an initial fate and exposure assessment to target the appropriate compartment of concern and concommitant toxicity testing.

5. References
Multi-biomarker approach to assess sub-lethal effects induced by a mixture of three common non-steroidal anti-inflammatory drugs (NSAIDs) on the zebra mussel (*Dreissena polymorpha*)

Marco Parolini¹, Andrea Binelli¹, Alfredo Provini¹

¹ Department of Biology, University of Milan, Via Celoria 26, 20133 Milan, Italy
E-mail contact: marco.parolini@unimi.it

1. Introduction

The non-steroidal anti-inflammatory drugs (NSAIDs) are the sixth most sold drugs worldwide with an estimated annual production of several kilotons [1] so that they are commonly revealed in aquatic ecosystems. Considering their distribution, the acute toxicity of the most common NSAIDs (among which acetylsalicylic acid, diclofenac, ibuprofen) was tested on non-target organisms belonging to different levels of the biological organization [2,3], even if few studies were aimed to investigate their chronic effects, which are much more probable. Moreover, these investigations have only evaluated the potential toxicity of single molecules [4,5], excluding the major environmental problem due to drug mixtures.

This work deals with a fundamental ecotoxicological topic based on the chronic toxicity evaluation of a drug mixture on non-target organisms. In detail, our research was aimed to the investigation of sub-lethal effects induced by three concentrations of a mixture of three common NSAIDs, namely diclofenac, ibuprofen and paracetamol, on a reference freshwater biological model, the zebra mussel (*Dreissena polymorpha*). Our goal was reached by the application of a suite of eight different biomarkers in order to highlight cytogenotoxic effects, as well as the unbalance of the oxidative status of treated-specimens. The single cell gel electrophoresis (SCGE) assay, the DNA Diffusion assay, the micronucleus test (MN test) and Neutral Red Retention Assay (NRRA) were applied on mussel haemocytes as cytogenotoxic biomarkers. In addition, the activity of catalase (CAT), superoxide dismutase (SOD), glutathione peroxidase (GPx) and the phase II detoxifying enzyme glutathione S-transferase (GST) was measured in the cytosolic fraction extracted from a pool of entire bivalves in order to reveal a possible oxidative status unbalance induced by the drug mixture.

2. Materials and methods

Several zebra mussel specimens having similar shell length (20 mm) were collected in the pristine site of Lake of Lugano. Mussels were purified by eventual xenobiotics under laboratory conditions and exposed to three concentrations of the NSAIDs mixture. Mother solutions (10 mg L⁻¹) for each NSAID were prepared in bi-distilled water. The lowest tested concentration (Low) was prepared by adding the exact volume of each mother solution directly in exposure aquaria (10 L) in order to reach levels reflecting the median of the current concentrations measured in surface water worldwide [6]. The intermediate one (Mid) corresponded to the median of levels found in the outlet of waste water treatment plants [6], while the highest one (High) reflected the median of predicted environmental concentration (PEC) calculated for each drug [7]. 96 h in vivo exposures were conducted in semi-static conditions, the whole water volume was changed daily and the exact volume of each chemical was added up to the selected concentration. Specimens were fed daily 2 h before water change and contamination. Several bivalves (n=33) were collected each day to measure cytogenotoxic biomarkers in haemocytes, while the whole soft tissue of other 20 specimens was frozen in liquid nitrogen and stored at –80 °C until the enzymatic activity was measured.

3. Results and discussion

As showed in figure 1, each administered concentration of NSAIDs mixture was able to induce a significant \((p<0.05)\) increase of primary genetic damage, already after 24 h of exposure. In addition, DNA fragmentation seems to prelude fixed genetic injuries, as highlighted by the significant \((p<0.05)\) induction of both apoptotic and micronucleated cells. In addition, it is interesting to note the unexpected reduction of DNA damage found at the end of exposure at each concentration. The marked destabilization of the lysosome membranes (NRRA) showed that exposure to this mixture was able to cause a noteworthy increase of cellular stress in treated-bivalves, above all at the highest concentration, probably due to the rise of oxidative stress, pointed out by the moderate unbalance of oxidative status of zebra mussel specimens revealed at each concentration, which seems to be the main responsible of the onset of genetic damage. Considering the high potential toxicity of NSAIDs mixture on *D. polymorpha* further in-depth analyses should be necessary in order to investigate the involved mechanism of action and enlarge the knowledge on this fundamental ecotoxicological topic.
4. Conclusions

✓ NSAIDs mixture is much more dangerous than single compounds towards D. polymorpha;
✓ considering genotoxicity end-points, mixture exert its toxicity according to the concentration addition of each its single component;
✓ in-depth analyses by applying more introspective techniques (e.g. “omic techniques”) should be absolutely necessary to enlarge the knowledge on the mixture toxicity towards non-target organisms and to obtain more exhaustive information about their mechanism of action.

5. References

Bioaccumulation and molecular effects of the contraceptive hormone levonorgestrel in the non-target organism *Dreissena polymorpha*

Valeska Contardo-Jara¹, Claudia Lorenz¹, Stephan Pflugmacher², Gunnar Nützmann¹, Werner Kloas¹, Claudia Wiegand³

¹Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 301, 12587 Berlin, Germany
²Technische Universität Berlin, Institute of Ecology, Franklinstraße 29, 10587 Berlin, Germany
³University of Southern Denmark, Institute of Biology, Campusvej 55, 5230 Odense M, Denmark

E-mail contact: contardo@igb-berlin.de

1. Introduction

There is increasing concern about the adverse effects of contraceptive steroids found in wastewaters worldwide. Bio-active estrogens are the most used pharmaceuticals in developed countries. It has been reported that they are adversely affecting vertebrates, mostly by “feminization” of males. Contraceptive hormones have been found not only in influents and effluents of municipal sewage treatment plants, but also in surface waters in the ng L⁻¹ range. In particular, levonorgestrel (LNG), a synthetic steroid used as a contraceptive, as well as a post-coital contraception modality (“the morning after pill”), has been detected in the effluent of STP (~18 ng L⁻¹), in surface waters (~7 ng L⁻¹), in ground water (~11 ng L⁻¹) and in sediment (~2 ng g⁻¹) (de Alda et al., 2002; Vulliet et al., 2008).

For fish exposed to LNG no no-observed-effect concentration (NOEC) were established as an inhibition of reproduction occurred even at concentrations below 1 ng L⁻¹ (Zeilinger et al., 2009). Exposure to higher concentrations provoked the masculinization of females. Fick et al. (2010) furthermore evidenced a reduction of fish fertility at concentrations below 1 ng L⁻¹. Surprisingly, bioconcentration was so strong that amounts of LNG in blood plasma of fish even exceeded the human therapeutic plasma level.

The effects of LNG on invertebrates, in particular on mussels are as yet unexplored. The species *Dreissena polymorpha* was depicted as a model organism to study adverse effects of LNG on mussels. The aims of this study were (1) to explore to which extent LNG bioaccumulates in *D. polymorpha*, thus the bioconcentration factor (BCF) was assessed and (2) to examine if LNG has a direct effect on cell functions of biotransformation, elimination, prevention from oxidative stress and protein damage. Time and concentration kinetics of aryl hydrocarbon receptor (AH-R), pi class glutathione S-transferase (piGST), P-glycoprotein (P-gp), superoxide dismutase (SOD), catalase (CAT), metallothionein (MT) and heat shock protein 70 (hsp70) were studied at the molecular level.

2. Materials and methods

Mussels were exposed (3 tanks of 20 mussels for each exposure concentration and the solvent control) for seven days in the flow-through system to increasing concentrations of LNG: 0, 0.312, 3.12 and 6.24 µg L⁻¹, which corresponds to 0, 10⁻⁹, 10⁻⁸ and 2x10⁻⁸ mol L⁻¹, respectively. Each tank contained 7 L exposure medium and a water exchange rate of approximately seven tank volumes per day. Constancy of the applied concentrations was therewith guaranteed, which was further monitored daily throughout the entire duration of the experiment by LC-MS/MS. Ethanol (0.0005%) was used as the solvent control. Gill and digestive gland tissue of ten mussels per treatment (n=10) were sampled after 1, 4 and 7 days of exposure. Real-time PCR assays for elongation factor 1-alpha (EF1-α), piGST, AH-R, P-gp, SOD, CAT, MT and hsp70 were run in a Stratagene Mx3005p qPCR cycler to study changes in mRNA levels. Whole tissue from six mussels (n=6) per test concentration was sampled at each time point for analysis of LNG tissue content, which was determined after dichloromethane/methanol extraction by LC-MS/MS. The BCF was calculated by dividing the LNG tissue concentration by the mean of the actual measured exposure medium concentration.

3. Results and discussion

3.1. Bioaccumulation of LNG

Within four days in the lowest applied exposure concentration of LNG (0.312 µg L⁻¹), it was 95-fold bioconcentrated, whereas mussels exposed to the higher concentrations (3.12 and 6.24 µg L⁻¹) displayed much lower BCFs (30 and 56, respectively). After one week, amounts of LNG in mussels exposed to the two
lower concentrations were increased compared to the four days exposure. In turn, only for the highest concentration a decrease of the BCF within one week could be observed, which may probably hint on enhanced regulatory processes, as e.g. metabolisation and excretion. In this treatment group mRNA up-regulations of the membrane bound P-gp responsible for unspecific elimination were strongest compared to the other treatment groups in the digestive gland after four and in gills after seven days exposure, explaining the observed pattern of bioconcentration.

Huggett et al. (2003) calculated the potential for chronic receptor mediated responses in fish and predicted a BCF of 46 for LNG in fish plasma, based on lipophilicity, human pharmacology and toxicology studies. In turn, a 14-days study of fish exposed to effluents yielded to BCFs of LNG up to 260-fold higher than predicted (Fick et al., 2010). This was explained by special sex-steroid binding globulins located in fish gills working as a trap for steroids in the surrounding water. In our study the BCF in D. polymorpha was much lower compared to the study by Fick et al. (2010), however, still higher than predicted by Huggett et al. (2003). It is unknown if mussels exhibit the mentioned sex-steroid binding globulins in their gills being therewith presumably prevented from such strong active up-take of LNG from the water.

3.2. Effects on gene expression

After only one day we found an immediate up-regulation of piGST in both examined tissues in all treatment groups, indicating phase II biotransformation processes. LC-MS/MS measurement of tissue samples of exposed mussels revealed the presence of a LNG cysteine conjugate and a LNG glucoronic acid conjugate in exposed mussels. Also enhanced ROS generation could have been the reason for piGST elevation in our study, as also SOD and MT were significantly up-regulated after only one day in the digestive gland.

Surprisingly, in gills AH-R was immediately down-regulated after one day exposure. mRNA levels persisted decreased during the whole experiment. In invertebrate species AH-R plays an important physiological role in cell functions including cell growth, death, and migration (Hahn et al., 2009). This would mean that due to the permanent down-regulation of the AH-R fundamental cell functions are disturbed, having a negative influence on the organism.

After exposure for four days responses were most pronounced in the digestive gland with up-regulated P-gp, as well as increased levels of SOD and MT mRNA in mussels exposed to the highest concentration, suggesting enhanced elimination processes and ongoing oxidative stress. As the MXR mechanism mediated by the P-gp is ATP dependent, the augmented energy requirement may impair growth and reproduction in exposed organisms, contributing to the negative effects of LNG in the mussel.

Finally, after one week exposure to LNG enhanced elimination processes were indicated for mussels exposed to the highest concentration by the up-regulation of P-gp in gills. An enhanced requirement for protein repair, transport or protective processes was evidenced by hsp70 induction in gills.

4. Conclusions

This study proves that LNG can accumulate in mussels especially at low concentrations to a higher degree than predicted based on its lipophilicity. Our study provides insight how cell processes such as biotransformation, elimination and prevention from oxidative stress can be influenced in mussels by exposure to the contraceptive LNG.

5. References

Do nonprescription pain relievers have endocrine disrupting potential in zebrafish (*Danio rerio*)?

Jane Ebsen Morthorst¹, Birgit Friis Lund¹, Henrik Holbech¹, Poul Bjerregaard¹, Andrea Lister² and Glen Van Der Kraak²

¹Institute of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense M, Denmark
²Department of Integrative Biology, University of Guelph, Ontario N1G 2W1, Canada
E-mail contact: jamor@biology.sdu.dk

1. Introduction

Nonprescription pain relievers such as ibuprofen (ipren, ibumetin), acetylsalicylic acid (aspirin) and paracetamol (panodil, pinex, pamol) exert their effect by inhibiting enzymes involved in prostaglandin synthesis. Prostaglandins are signalling molecules involved in induction of pain, inflammation and fever but they are also involved in various housekeeping functions like platelet aggregation and renal blood flow. Nonprescription pain relievers (mild analgesics) used to treat pain, fever and inflammation and therefore found in most homes. Recently, the Association of Danish Pharmacies estimated an average annual consumption of 175 pills of mild analgesics per Dane. Due to high consumption of mild analgesics in many industrialised countries several analgesics have been detected in waste water and surface water [1,2]. In mammalian studies inhibition of prostaglandin synthesis have been found to affect steroid levels and sexual behaviour [3,4]. Recently, also maternal consumption of paracetamol during pregnancy has been correlated with increased risk of cryptorchidism in newborn boys, and administration of paracetamol to pregnant dams reduced the anogenital distance of male rats [5].

In a set of studies we investigated the effects of prostaglandin inhibitors on sexual differentiation and reproductive parameters in zebrafish, which is a commonly used test organism in endocrine research. Zebrafish vitellogenin levels and sex ratio have been demonstrated to be sensitive endpoints after exposure to endocrine disrupters with different mechanisms of action [6,7]. The primary investigated endpoints were: sex ratio, prostaglandin E2 (PGE2) levels, 17β-estradiol (E2) levels, 11-keto testosterone (11-KT) levels, vitellogenin levels and expression of genes involved in steroid and prostaglandin synthesis.

2. Materials and methods

Paracetamol and acetylsalicylic acid were tested int the Fish Sexual Development Tests (FSDT) at nominal concentrations ranging from 0.25-25 mg/L and 0.32-10 mg/L, respectively. Zebrafish were exposed in a flow-through test system from 0-60 days post hatch (dph) and the actual concentrations were 0 (control), 0.22, 2.26 and 29.55 mg/L (paracetamol) and 0 (control), 0.2, 0.5, 1.7 and 8.2 mg/L (acetylsalicylic acid). The investigated endpoints were vitellogenin levels and sex ratio based on histological examination of the gonads. In addition, two experiments with adult zebrafish were performed. In the first experiment fish were exposed to paracetamol (1.9 mg/L) or acetylsalicylic acid (3.8 mg/L) in a flow-through test system for 14 days and levels of PGE2, 11-KT and E2 were determined by EIA and compared to the control groups. In the second experiment zebrafish were exposed to ibuprofen concentrations from 20-500 µg/L in a semi-static test system during a seven day period. The actual concentrations were: 0 (control), 21, 201 and 506 µg/L. Male and female levels of PGE2, E2 and 11-KT were determined by EIA and ovarian and testicular expression levels of genes involved in steroid or prostaglandin synthesis were investigated by q-PCR. Additionally, vitellogenin levels, egg production and female gonadosomatic index (GSI) were also investigated.

The actual water concentrations in each experiment were determined by LC-MS-MS.

3. Results and discussion

Exposure to paracetamol or acetylsalicylic acid during development and sexual differentiation did not affect sex ratio or vitellogenin levels of sexually mature zebrafish.

Short term adult exposure to ibuprofen reduced PGE2 levels in a dose response relationship but E2 and 11-KT levels remained unchanged (Fig. 1) and the same tendencies were observed after acetylsalicylic acid exposure. Expression of genes involved in prostaglandin and steroid synthesis remained unchanged after exposure to ibuprofen and female GSI and egg production was not significantly different from the control. A reduction in male vitellogenin was observed at the two highest ibuprofen concentrations.
4. Conclusions

Sex ratio and vitellogenin levels in sexually mature zebrafish remained unchanged after exposure to acetylsalicylic acid and paracetamol during sexual differentiation. Both acetylsalicylic acid and ibuprofen reduced PGE2 levels in male and female zebrafish after short term adult exposure but steroid levels remained unchanged. Ibuprofen did not affect other reproductive endpoints except for vitellogenin levels in males. In conclusion, commonly used reproductive parameters in zebrafish do not seem to be sensitive towards prostaglandin inhibitors.

5. References

Effects of the antidepressant venlafaxine (Effexor®), on fish brain chemistry and predation behavior

Joseph H. Bisesi1,2, Stephen J. Klaine1,3

1Clemson University Institute of Environmental Toxicology (CU-ENTOX), 509 Westinghouse Rd, Pendleton, SC 29670, USA
2Graduate Program in Environmental Toxicology, 509 Westinghouse Rd, Pendleton, SC 29670, USA
3Department of Biological Sciences, Clemson University, 132 Long Hall, Clemson, SC 29634, USA
E-mail contact: Bisesi@clemson.edu

1. Introduction

Antidepressants found in wastewater effluent and receiving streams [1] have been shown to be relatively non-toxic using traditional fish aquatic toxicity testing [2]. But the psychototropic mode of action of these compounds warrants examination of the behavioural effects these chemicals may have on aquatic organisms. Previous results indicate the antidepressant fluoxetine (Prozac®), causes decreased brain serotonin levels in fish, resulting in a decreased ability to capture prey [3].

The antidepressant venlafaxine (Effexor®) has been found at low µg/L concentrations in wastewater effluent [4]. It has a slightly different mode of action than fluoxetine, in that it is designed to alter brain serotonin as well as norepinephrine. The objective of this study was to determine the effects of venlafaxine on fish brain chemistry and predation behaviour.

2. Materials and methods

A predator-prey bioassay previously designed in our lab, using hybrid striped bass (Morone chrysops x Morone saxatilis) as the exposed predator species and fathead minnows (Pimephales promelas) as prey, was used to address our objectives. Hybrid striped bass (bass) were exposed to venlafaxine for a period of 6 days, followed by a 6 day depuration period to examine recovery. Concentrations tested ranged from 50 – 500 µg/L. Feeding events took place on days 0, 3, 6, 9, and 12. During each feeding event bass were given 4 minnows and the time to capture each minnow was quantified, with a maximum of 25 minutes to consume all minnows. Time to capture each minnow was compared among treatments to determine behavioural effects.

In addition to behavioral endpoints, 5 bass were removed after each feeding event, euthanized and brains removed for monoamine analysis. Brain chemistry was compared to the behavioural results to determine if any correlation existed between brain chemistry changes resulting from fluoxetine exposure, and quantified behavioral effects.

3. Results and Discussion

Bass exposed to 250 and 500 µg/L venlafaxine showed significant increases in time to capture prey 1 by day 6 and prey 2 and 3 by day 3. The 50 µg/L treatment showed a significant increase in time to capture prey 3 by day 6. All treatments were able to recover their ability to capture prey 1 by day 12. Time to capture prey 2 and 3 did not appear to recover for the 250 and 500 µg/L treatments, though this could not be shown statistically due to low replication by day 12 (figure 1).

The results indicate that at the lowest exposure concentration, 50 µg/L, the bass are still able to efficiently capture their first two prey, but no longer seem interested in the remaining prey. We hypothesize that at low concentrations, venlafaxine may act as an appetite suppressant, while at higher concentrations it may also affect motor function. The results also showed that after a recovery period, bass from all treatments are able to recover their ability to capture their first prey. But trends in time to capture prey 2 and 3 lead us to believe that the two highest treatments are unable to recover after a 6 day depuration period. We hypothesize that
even after a depuration period, residual monoamine effects in the brain may be acting as an appetite suppressant. Forthcoming brain monoamine data should shed some light on these hypotheses.

Figure 1. Time to capture prey 1, 2, and 3 for hybrid striped bass exposed to venlafaxine. Error bars = ±1 Standard Error.

4. Conclusions

Venlafaxine causes significant decreases in a basses ability to capture prey. At low concentrations there seems to be an effect appetite only, whereas higher concentrations may affect both appetite and motor function. After a depuration period bass are able to recover their ability to capture a first prey but residual chemical effects may still supress appetite. We hope that future brain monoamine data will provide insight into the biochemical mechanisms of the behavioural effects we have seen.

5. References


Effects of two pharmaceutical drugs on the microbial community of a river water ecosystem

Barra Caracciolo A, Grenni P, Ademollo N, Patrolecco L

Water Research Institute – National Research Council, Via Salaria km 29,300 00015 Monterotondo, Rome, Italy
E-mail contact: barracaracciolo@irsa.cnr.it

1. Introduction

The presence of pharmaceuticals as widespread ecosystem contaminants has increased attention among researchers, lawmakers, regulators, and the public in the last years. After administration, they are not, or only partially, metabolized and can be excreted with their metabolites in urine. They continuously reach the environment, and for this reason they have been constant detected in the aquatic environment, at concentrations ranging from µg/L in the effluents of wastewater treatment plants to ng/L in surface waters. Pharmaceuticals are specifically designed to be biologically active, but there is limited understanding of their ecological effects on non-target organisms in the environment, included microorganisms [1, 2].

Microorganisms have a key role in the cycles of elements and in ecosystem energy flows but, thanks to their adaptability and metabolic potentiality, they are able to degrade many xenobiotic molecules. Consequently they are essential in the overall processes that contribute to the quality state of natural ecosystems. For these reasons it is essential to evaluate both the potential detrimental effects of pharmaceuticals on structure and functioning of microbial communities and if the latter show homeostatic capabilities versus these contaminants, degrading them.

The aim of this work was to evaluate the effects of two river ecosystem contaminants, naproxen (anti-inflammatory) and gemfibrozil (lipid regulator), on the structure and functioning of an autochthonous microbial community. For this purpose, water samples collected from river Tiber (Rome, Italy), were used for setting up different microcosms (presence/absence of the microbial community) and treated with naproxen or gemfibrozil or both pharmaceuticals at a concentration of 100 µg/l. The degradations of the two pharmaceuticals were assessed and the bacterial community structure and functioning were investigated. At different times, bacterial abundance (DAPI counts), cell viability (live/dead method) and phylogenetic composition (by fluorescence in situ hybridization) were assessed and compared to those of microbiological control (no-treated water samples).

2. Materials and methods

Water samples were collected from River Tiber (Southern Rome, downstream Magliana WWTP) using sterile bottles. A previous monitoring survey highlighted gemfibrozil and naproxen as contaminants (65 ng/l and 200 ng/l respectively) of this ecosystem.

The experimental set-up consisted of closed sterile flasks (100 ml capacity). Some microcosms were filled with 50mL of river water and treated with the pharmaceutical (gemfibrozil or naproxen or both drugs, Figure 1) to give a final concentration of 100 µg/l. In order to evaluate the role of microorganisms in the degradation additional microcosms (sterile) were set up with previously sterilized water (120°C, 20 min) and treated with pharmaceutical, as previously described. Finally some microcosms filled with just 50mL of river water were used as microbiological controls, to assess the effects of the pharmaceuticals on the bacterial community. Microcosms were incubated at 20°C on an orbital shaker (125 rpm) in the dark. At selected times two replicate microcosms were collected (distruptive samples) for each conditions (treated, sterile, control) and sub-samples used for chemical (HPLC UV fluorescence) or microbiological analysis. All operations were conducted under sterile conditions. pH and oxygen concentration were measured at each sampling time.

Figure 1: Chemical structure of Gemfibrozil (on the left) and Naproxen (on the right).
Pharmaceuticals were detected by HPLC-UV-fluorescence. Microbiological analysis were performed by epifluorescence microscopy techniques, such as direct count of bacterial abundance (DAPI count), viability (Live/Dead, cell viability assay), and Fluorescence In Situ Hybridization (FISH) [3, 4].

3. Results and discussion

The degradation vs time of gemfibrozil and naproxen are shown in Fig 2. Both pharmaceuticals, although at different times, were biodegraded as it can be seen in the microbiologically active water (MAW) condition. In fact in the sterile conditions any significant compound decreasing was observed.

Figure 2 Degradation of Gemfibrozil (on the left) and Naproxen (on the right) in presence of the river microbial community (MAW) and in river water sterilized.

The DT$_{50}$ of naproxen was about 27 days in MAW and in correspondence of this time the bacterial number increased compared to control (no-treated) water samples. At the same time, the results of fluorescence in situ hybridization showed an increase of Bacteria groups such as Alpha, Beta and Gamma-Proteobacteria in presence of the naproxen drug.

The DT$_{50}$ of gemfibrozil was more than 70 days in MAW and the end of the experiment (150 days) about 15% of the initial concentration was still found. An increase in bacterial number was observed in correspondence of DT$_{50}$ and an increase in Gamma-Proteobacteria presence was observed.

The DT$_{50}$ of naproxen was more than 30 days in co-presence of gemfibrozil and the lag phase before the degradation started was greater.

4. Conclusions

The overall results show an important role of the natural microbial community in the naproxen and gemfibrozil degradation and it was associated with the increase of some specific bacterial groups, suggesting the importance of microbial ecology studies in the evaluation of pharmaceutical environmental fate.

5. References


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Drugs of abuse as new aquatic contaminants: cocaine effects on zebra mussel (*Dreissena polymorpha*)

Alessandra Pedriali¹, Andrea Binelli¹, Marco Parolini¹, Consuelo Riva¹, Alfredo Provini¹.

¹University of Milan, Department of Biology, Ecology Section, via Celoria 26, 20133, Milan Italy. E-mail contact: alessandra.pedriali@unimi.it

1. Introduction

Illicit drugs are common contaminants of the aquatic environments whose contamination appears to be widespread, with consumers being the major source. Even if environmental concentrations are relatively low or moderate, risks for the aquatic biocoenosis and consequently for human health cannot be excluded since some of these substances have proved pharmacological activities in mammalian systems, and their presence as complex mixtures in surface waters may lead to unforeseen pharmacological interactions causing toxic effects to aquatic organisms. Biological effects from drug residues can in fact occur even at low environmental concentrations [1].

Cocaine is one of the most common illicitly used drugs in the world and it exhibits profound CNS and cardiovascular toxicity [2]. This drug was chosen for this study since a considerable amount of data are available for this substance concerning consumption and concentrations in waste and surface waters, which allow us to validate the proposed model. Present study is the first work to evaluate the cocaine cyto-genotoxic effects on aquatic non-target organisms, such as the freshwater bivalve *Dreissena polymorpha*. These preliminary tests are part of a broader project that tries the application of a multidisciplinary approach based on the integration of various modern methods such as the analysis of biomarkers and proteomic approach, that will allow us to determine both the different contamination footprint due to this new environmental contaminant and the effects of physiological/functional mold at the proteome level.

2. Materials and methods

We chose three different cocaine concentrations very similar to those found in environment, in order to give information useful in the real world: 40 ng/L, 220 ng/L e 10000 ng/L. We exposed *in vivo* several hundred *D. polymorpha* specimens to the three different concentrations. A control aquarium without contaminant was been also predisposed. Exposure lasted five days (96 h): every 24 h a sufficient number of specimens was been sampled and the battery of biomarkers was been immediately applied. Exposure assays were conducted in semi-static conditions. We tested three different biomarkers of genotoxicity: Comet test, that shows the increase of DNA fragmentation (reversible damage), Micronucleus test and Apoptosis test, which allow the determination of fixed and irreversible DNA damages. We also evaluated the Neutral Red Retention Assay, a classical biomarker of cellular stress.

3. Results and discussion

Results of exposures (Fig. 1) showed only a slight increase of DNA fragmentation with a significant rise of primary damage only at t=96 h at the intermediate (p<0.05) and highest (p<0.01) tested doses, although we noticed significant overall time/effect (F=3.4, p <0.05) and dose/effect (F=14:37, p<0.01) relationships. In agreement with data obtained by the Comet assay, we observed a significant increase of micronucleated cells, compared to the corresponding control, only at t=72 h (p<0.05) and t=96 h (p<0.01) at the highest concentration. By contrast, Apoptosis test showed that cocaine was able to induce a significant increase of apoptotic cell frequency in an overall dose-dependent (F=98.34, p<0.01) and time-dependent (F=10:45, p<0.01) manner and significant differences (p<0.01) in the apoptotic frequency were noticed after already 24 hours of exposure at the intermediate and high dose. Finally, data obtained by the NRRA showed a significant destabilization of lysosomal membranes at the two highest doses tested with a significant increase of cellular stress in bivalves from t=72 h for the intermediate dose and from t=48 h for the highest one.

Cocaine has been shown to be able to induce apoptosis in different mammalian lines cell [3] and pathological studies have implicated oxidative damage in the mechanism of cocaine-induced cell injury [4]. Actually, we reported a significant increase of apoptotic cells at the same time to a depletion of the neutral red retention time from lysosomal membranes. On the other hand, the destabilization of lysosomal membranes seems to be ascribed mainly to the action of reactive oxygen species (ROS) produced during exposure to xenobiotics [5].
4. Conclusions
All the biomarkers have marked a significant cocaine effect at the end of exposure at the highest dose. This can lead to the hypothesis that, with the increasing of the exposure time, a significant increase in both the primary and irreversible DNA damage would be detected, and also a general increase in cell stress rate. Our preliminary results suggest then a probable indirect mechanism of action of cocaine, through the increasing oxidative stress that can lead to the programmed cell death by the activation of the apoptotic pathway or by the interaction with the electron transport chain. Against this background, we will carry out further in-depth studies. We will verify the possible induction of oxidative stress through the analysis of the activity of three antioxidant phase I enzymes (catalase, superoxide dismutase and glutathione peroxidase), as well as the phase II detoxifying enzyme glutathione S-transferase.

5. References
1. Introduction

Presently, human nutrition has become more than ever a major issue, focusing the attention of scientists and stakeholders regarding food safety. The fast increase in animal farming to meet the needs of the continuously growing world population carries with it additional and important concerns as is the use of veterinary medicinal drugs (VMD). Pharmaceutical use is a well described practice to promote animal health, whether for prophylactic or therapeutic reasons, which dictated the need to implement regulations to assure a reduction of hazards to consumers (defined as "a biological, chemical or physical agent in, or condition of, food with the potential to cause an adverse health effect" according to Regulation (EC) No. 178/2002 [1]). In the light of these regulations some pharmaceuticals have been banned from use in animal food production since the risks to human health were too high and include VMD such as furaltadone and chloramphenicol. Despite this, there are still reports of their use as they are highly effective and inexpensive.

More recently, the awareness that the surrounding natural ecosystems can potentially be contaminated by pharmaceuticals has risen and the extent of their effects in non-target organisms is already under the scope of researchers. However, little attention has been given so far to primary producers such as macroalgae, which are in the bottom of the trophic webs. The present study tested the effects of two antibiotic agents, furaltadone (FTD) and chloramphenicol (CAP), in the growth of Ulva lactuca.

2. Materials and methods

An experimental design was established with macroalgae subjected to prophylactic (25 µg/ml) and therapeutic (50 µg/ml) concentrations of FTD. Macroalgae were collected in the Mondego estuary (Portugal) and after acclimation for 2 weeks were placed in 250 ml glass containers with natural filtered seawater (30 psu) and placed in an orbital shaker in a room with constant temperature (20°C) and photoperiod (14 light:10 dark), for 120 hours [2]. A control group was established for the duration of the trial. At the beginning and end of each time point, macroalgal disks were photographed and analysed to determine variations in growth. The same experimental setup was repeated with CAP.
3. Results and discussion

Results showed differences in macroalgal growth when submitted to prophylactic and therapeutic concentrations of FTD in the water, statistically different from the control group and between treatments. The therapeutic concentration led to algal death after 48 hours whereas the prophylactic treatment was able to attain growth, however lower than the control group.

As for the CAP trial, macroalgae behaved very differently. Growth was statistically different from the control group and between treatments, with the therapeutic concentration presenting an accentuated growth in comparison to the other groups.

The two antibiotics tested represent two distinct classes of pharmaceuticals with different chemical properties which act in contrasting ways on the growth of *U. lactuca*. The fact that the macroalgae reacted distinctly to the two drugs indicates different sensibilities to their presence.

![Figure 3: Growth measured as variation in disk area (cm²) for prophylactic concentration.](image1)

![Figure 4: Growth measured as variation in disk area (cm²) for therapeutic concentration.](image2)

4. Conclusions

The exposure of macroalgae to pharmaceuticals in the environment will have significant effects on growth that will depend on the characteristics and also on the concentrations of the chemical present. Moreover the ability of the macroalgae to tolerate the amount that is taken up from the water is fundamental to maintain growth and prevent decayment which will have a greater impact on the ecosystem. Also, the eventual presence of mechanisms of detoxification will dictate the extent to which pharmaceuticals can enter the food chain via primary producers.

5. References


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**In vivo experiments for the evaluation of different biomarkers and alterations in proteins expression profile of Triclosan in Zebra mussel (D. polymorpha)**

Riva Consuelo¹, Binelli Andrea¹, Marco Parolini¹, Rusconi Francesco², Colombo Graziano², Pedrali Alessandra¹, Zippel Renata², Provini Alfredo¹.

¹University of Milan, Department of Biology, Ecology Section, via Celoria 26, 20133, Milan Italy.
²University of Milan, Department of Biomolecular Sciences and Biotechnology, via Celoria 26, 20133, Milan Italy.

E-mail contact: consuelo.riva@unimi.it

1. Introduction

Among the emerging class of environmental pollutants of PPCPs (Pharmaceuticals and Personal Care Products), one of the most widely used groups is that of antibacterial agents. While these products at low concentrations are probably not pharmaceutically active in humans, they may still be potential pollutants in aquatic environments [1]. Triclosan (TCS, 2,4,4'-trichloro-2'-hydroxydiphenyl ether) is one of the main known antibacterial agents and its increasing environmental levels is causing growing concern about its presence in freshwaters. In aquatic organisms, TCS has been assessed for its acute toxicity by traditional in vivo ecotoxicological tests according to established guidelines, using biological models from different trophic levels, such as algae, crustaceans and fish [2]. However, very few data are available on other sub-lethal effects (genotoxicity, cytotoxicity, oxidative stress, alteration of protein expression profile), which can also demonstrate the possible mechanism of action of TCS.

The aim of this study was to assess the effects of TCS and to investigate its possible mechanism of action in the freshwater bivalve zebra mussel (*Dreissena polymorpha*). For this purpose we used a battery of biomarkers of genotoxicity and cytotoxicity in haemocytes of *D. polymorpha*. We used the single cell gel electrophoresis (SCGE) assay, the micronucleus test (MN test) and the measure of the apoptotic frequency (Halo assay) to measure the genotoxic potential of TCS; lysosomal membrane stability was measured by the neutral red retention assay (NRRA) to identify cellular stress alterations. We also evaluated the activity of enzymes related both to oxidative stress (superoxide dismutase -SOD-, catalase -CAT- and glutathione peroxidase -GPX-) and phase II metabolism (glutathione S-transferase -GST-) on the cytosolic fraction of the whole body mussels. Finally, we applied a proteomic technique in order to identify changes in protein expression profiles in the mussel gills.

To the best of our knowledge these data represent the first comprehensive study regarding the investigation of the effect and the possible mechanism of action of TCS in this sentinel-organism that links the pelagic and benthic compartments.

2. Materials and methods

Experiments were performed for 96 h in semi-static conditions with daily changes of the entire volume of water and the addition of TCS up to the selected concentrations (1 nM, 2 nM and 3 nM). Another two aquaria were prepared with only tap water (control) and dimethyl sulfoxide (DMSO) as solvent control. Four pools of mussels were collected each day and immediately analyzed for the SCGE assay, MN test, apoptosis frequencies and NRRA. Another pool of mussels was dissected each day, frozen in liquid nitrogen and stored at -80 °C for the determination of the activity of SOD, CAT, GPx and GST. Finally, at the end of the exposure, the gills of eight animals (four male and four female) were removed, frozen in liquid nitrogen and stored at -80 °C until analysis. A comparative study of the protein profiles generated from analytical two-dimensional electrophoresis gels was performed and changes in protein expression were analysed by computer analysis. The differentially expressed proteins were identified by peptide mass fingerprint or MS/MS analysis with a MALDI spectrometer (Ultraflex TOF/TOF, Bruker, Germany).
3. Results and discussion

We observed significant increases in all of the genotoxic biomarkers examined as early as 24 h after initial exposure, as well as a clear destabilization of lysosomal membranes after 48 h (Fig.1 a,b,c,d). In addition, the genotoxicity of TCS in Zebra mussel haemocytes has previously been demonstrated by our research group with an in vitro experiment, while no statistical differences between exposed and untreated haemocytes were noticed in the NRRA in vitro test [3]. On the other hand, TCS seems only slightly affect the antioxidant defense mechanisms in *D. polymorpha*, especially at the highest concentration tested (Fig. 1 e,f,g,h).

The results that we obtained from our both in vivo an in vitro experiments allow us to suggest that TCS may act as a DNA adduct or a DNA intercalant to directly exert genotoxic effects. In fact, TCS has been reported to act as a precursor of the 2,8-dicholodibenzo-p-dioxin [4], which is a well known DNA-intercalating agent.

The role of the identified protein involved in this hypothetical mechanism of action will be discussed.

![Figure 1: Temporal trend (h) of the means (± SEM) of LDRs (a), apoptosis frequency (b), micronuclei frequency (c) and neutral red retention times (d), calculated for Zebra mussel haemocytes for controls, solvent controls and treated samples with TCS. Temporal trend of the means (± SEM) of CAT (e), SOD (f), GPx (g) and GSH (h) activity evaluated in Zebra mussel specimens from controls, solvent controls and treated samples with TCS. Significant values (two-way ANOVA, Bonferroni post-hoc test, *p<0.05, **p<0.01) refer to the comparison between treated samples and controls at the same time.](image)

4. Conclusions

Our results indicate that the mechanism of action of TCS in *D. polymorpha* seems mainly connected to a direct effect on DNA instead of an increase of the oxydative stress produced by the Reactive Oxygenen Species (ROS). Other, in-depth studies, such as the role played by TCS metabolites, will be necessary to fully understand the effects of TCS on this sentinel organism. Moreover our data demonstrate the potential genotoxicity of environmental levels of TCS in zebra mussel specimens and point out a possible danger for the entire aquatic community.

5. References


Triclosan persistence through wastewater treatment plants and its potential effects on fluvial systems

Marta Ricart1,2, Helena Guasch2, Damià Barceló1,3, Chloé Bonnineau2, Frits Gillissen4, Marínel-la Farré3, Josep Ferrer5, Miquel Lürling4, Soizic Morin6, Lorenzo Proia2, Anna M. Romani2, Lluís Sala7, Sergi Sabater1,2

1Catalan Institute for Water Research, Emili Grahit 101, 17003 Girona, Spain
2Institute of Aquatic Ecology, University of Girona, Campus Montilivi, 17071 Girona, Spain
3Department of Environmental Chemistry, IIQAB-CSIC, Jordi Girona, 18-26, 08034 Barcelona, Spain.
4Aquatic Ecology & Water Quality Management Group, Wageningen University, PO Box 47, 6700 AA, Wageningen, The Netherlands
5Empresa Mixta d’Aigües de la Costa Brava, S.A., Plaça Josep Pla 4, 3r, 1ª, 17001 Girona, Spain
6Cemagref Lyon, 3 bis quai Chauveau, CP 220, 69336 Lyon, Cedex 09, Lyon, France
7Consorci de la Costa Brava, Plaça Josep Pla 4, 3r, 1ª, 17001 Girona, Spain

E-mail contact: mricart@icra.cat

1. Introduction

Though WWTP (Wastewater treatment plants) function as partial barriers to pollutants, they are not specifically designed for the effective removal of organic compounds such as personal care products. Thus, WWTP effluents can potentially alter the water quality of fluvial ecosystems when some of these chemical compounds reach the aquatic environment as a result of an incomplete removal during wastewater treatment processes. The so-called emerging compounds are thought to be potential threats to environmental ecosystems. Among them, triclosan is a commonly used bactericide that survives several degradation steps in WWTP and is present in effluents from WWTP and in the receiving river systems [1].

Triclosan toxicity in fluvial biofilms was assessed in the first part of the study. The objective of this first part was to mimic the effects of triclosan in WWTP-dominated rivers by examining the effects of triclosan at the biofilm community level, including effects on its target (bacteria) and non-target (algae) organisms.

Since in fluvial systems organisms are exposed to a multitude of toxicologically and structurally different chemical compounds [2], the second part of the study evaluated the toxicity of binary mixtures of toxicants, one of them being triclosan. The main objective of this second part was to evaluate the toxicity of chemicals from different classes: triclosan, diuron (herbicide) and propranolol (pharmaceutical product) when applied singly and in binary mixtures as well, and to test the applicability of the concepts concentration addition (CA) and independent action (IA) to predict the toxicity of the mixtures investigated.

2. Materials and methods

A set of experimental channels was used to examine the short-term effects of triclosan (from 0.05 to 500 µg/L) on biofilm algae and bacteria. After 48 hours of exposure, a set of endpoints was used to assess its toxicity. Algal-related endpoints included photosynthetic efficiency, non-photochemical quenching and diatom cell viability. The toxicity on bacteria was analysed with the bacterial abundance, distinguishing between live and dead cells.

In the second part of the study, the toxicity of both the single compounds and their binary mixtures was evaluated on the green algae Scenedesmus obliquus. A set of dose response assays were performed in the same way for both individual toxicants (Diuron (D), Propranolol (P) and Triclosan (T)) and mixtures (Mix 1: D+T and Mix 2: P+T). After 48 hours of toxicant exposure, a set of endpoints was used to assess toxicity, including photosynthetic efficiency, chlorophyll-a concentration and biovolume concentration. Then, observed toxicity was compared with the toxicity predictions obtained with the models CA and IA.

3. Results and discussion

Environmentally relevant triclosan concentrations affected both algal and bacterial components of the biofilm, though toxicity was higher in bacteria than in algae. Triclosan caused a clear increase of bacterial mortality (EC10 = 0.6µg/L) (Fig.1). In this case, bacterial toxicity was attributed to the triclosan mode of action, which inhibits the bacterial fatty acid synthesis [3]. Although less pronounced, effects on the autotrophic component of biofilms were also observed. The photosynthetic efficiency of algae was inhibited (EC10 = 3.4µg/L), as well as the non-photochemical quenching mechanisms (EC10 = 1.3µg/L). Diatom cell viability was also affected.
with increasing concentrations of triclosan, showing an increase in diatom mortality ($EC_{10}=3.7\mu g/L$). Though the photosynthetic parameters were the most sensitive to triclosan exposure, all the algal-related endpoints were affected, being progressively inhibited with increasing concentrations of triclosan, what would suggest a direct effect of the bactericide. However, the lack of information on the mode of action of triclosan on algae prevents from concluding that direct effects of triclosan on algae were detected.

The combination effect of diuron and triclosan showed an important reduction of all the endpoints with increasing doses of the mixture. The IA model has been found to be able to predict the toxicity of dissimilarly acting mixtures of chemicals [4], which was confirmed with the results of this study. The mixture composed by propranolol and triclosan caused an inhibition of all the measured endpoints. The observed toxicity of these toxicants applied together was stronger than predicted by the models, indicating a synergistic effect between the two compounds (Fig. 2). This synergism might be attributed to the potential interactions among stressors; the presence of one compound might enhance the presence of the other. That is the rationale behind synergisms, which have serious implications for risk assessment since they result in a higher toxic effect than predicted by models. Although rare, synergisms have been mainly reported for mixtures of similarly acting chemicals [5]. The results of this study demonstrate that dissimilarly acting chemicals can exhibit synergistic influence beyond their individual influences.

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4. Conclusions

Triclosan is present at low concentrations in fluvial systems. However, the capacity of triclosan to survive through WWTP processes, the toxicity detected on the co-occurring non-target components (algae) of the biofilm community and the increase in toxicity when mixed with other substances demonstrates that triclosan may be toxic to fluvial communities. The results obtained highlight the need of studies covering both single exposures and mixtures and their application to different levels of biological organization (populations and communities) in order to better assess the environmental risk of emerging pollutants.

5. References


In situ feeding assay with *Gammarus fossarum*: move forward to an ecologically relevant biomonitoring of water chemical quality.

R. Coulaud¹, O. Geffard¹, H. Quéau¹, J. Garric¹, S. Charles², A. Chaumot¹

¹ Cemagref, UR MALY, Laboratoire d’écotoxicologie, Lyon, France.
² Université de Lyon, F-69000, Lyon ; Université Lyon 1 ; CNRS, UMR5558, Laboratoire de Biométrie et Biologie Evolutive, F-69622, Villeurbanne, France
E-mail contact: romain.coulaud@cemagref.fr; arnaud.chaumot@cemagref.fr

1. Introduction

In aquatic ecosystems, organisms are constantly exposed to different levels of physical and chemical stressors. To estimate and predict their biological effects, the need of relevant tools has considerably increased in the last decades, notably in the regulatory framework for the diagnosis of ecological impacts of chemicals. Biological responses, like feeding activity, locomotive behaviour or reproduction, have been studied using several methods and showed a high sensitivity through the different taxonomic groups (invertebrates, fish, amphibians). As shown by many laboratory studies since 1990s, a large range of stressors can inhibit gammarids feeding rate. Feeding inhibition also constitutes in most case one of the first traits observed in responses to environmental pollutions. Studying the effects of pollutants on the feeding behaviour is of ecological concern; as it can be related to life history traits like growth, survival or fertility thus allowing assessing the effects of toxicants on higher biological organization levels, such as population [1, 2].

However, the feeding rate can be affected by many biotic and abiotic factors, which can limit the relevance of the observed responses for water quality assessment. To make the feeding rate more robust as a specific indicator of water toxicity, this variability must be characterized and taken into account in the interpretation of measured feeding activities. Furthermore, a mechanistic modelling approach is proposed as a perspective to perform this extrapolation between biological scales. [3].

2. Experimental approaches

2.1. Definition of a reference level of feeding activity integrating environmental factors

In a first part, we illustrate how taking into account of the influence of biotic and abiotic factors (body size, temperature, water hardness) on feeding activity allows one to improve the interpretation of in situ feeding rate assays for the evaluation of water quality. For this, we performed a three-steps approach: (i) we characterized the influence of these confounding factors in laboratory conditions, (ii) we validated the robustness of feeding activity reference values; these latter were established through in situ caging experiments with transplanted standard organisms in reference streams with contrasted abiotic profiles at different seasons (iii) finally, by considering in situ caging in contaminated streams (in the Lot watershed and the Amous watershed), we underlined the importance of taking into account the influence of such factors for a better toxicological bio-monitoring of freshwater ecosystems (Figure 1).

2.2. Link between feeding inhibition and potential impacts on population dynamics

In a second part, we show how feeding activity can be related to life history traits such as fecundity and we propose a modelling methodology to link impact on feeding activity to potential effects on population dynamics. For this purpose, we developed an environmentally realistic Leslie population model which considered size-structured populations. This model takes into account the influence of environmental conditions (phenology) on population dynamics and allowed one to mechanistically link individual-level demographic parameters to the dynamics of native populations. Ultimately, we illustrate how couple fitness related endpoints measured in bioassays procedures to population modelling reveals useful for population level assessment of water quality.
Figure 1: Case study of the Lot watershed and the Amous watershed A) before normalisation (FRinsitu, mm³.d⁻¹.organism⁻¹) of gammarids and B) after normalisation: feeding index (FI = FR in situ−FR predicted with control of abiotic facors. a = false-positive feeding inhibition due to water temperature, b = false-negative feeding inhibition due to water temperature.

3. Conclusion

- Our results underline how considering environmental conditions in the definition of reference values of biological activities used as endpoints for toxicity testing, highly improve the in situ approach for site-specific studies and extended to large scale and long term biomonitoring programs.

- Our results show how couple fitness related endpoints measured in bioassays procedures to population modelling allows one to propose indicators for an ecologically relevant assessment of water quality or chemical compound toxicity.

4. Research needs

The links between feeding activity and life history traits represent a perspective in order to propose more ecologically relevant endpoints for the assessment of water quality, notably to propose assessment at the population level. Nevertheless, these links need to be improve and further research would be proceeded in order to gain insights into these relationships. Furthermore, previous works on the development of fitness-related molecular biomarkers in *G. fossarum* [4] could be integrate into the proposed multi-level toxicity assessment scheme.

5. References

Effects at a daily resolution of imidacloprid on the individual feeding activity of *Gammarus pulex* (L.)

Annika Agatz\textsuperscript{1,2}, Roman Ashauer\textsuperscript{3}, Colin Brown\textsuperscript{1}

\textsuperscript{1}University of York, Heslington, York, YO10 5DD, United Kingdom
\textsuperscript{2}The Food and Environment Research Agency, Sand Hutton, York, YO41 1LZ, United Kingdom
\textsuperscript{3}Eawag-Aquatic Research, Überlandstrasse 133, Dübendorf, Switzerland

E-mail contact: annika.agatz@fera.gsi.gov.uk

1. Introduction

Ecosystems are usually exposed to low concentrations of toxicants. Environmentally-relevant concentrations of toxicants rarely cause mortality. Whilst observing behavioural changes of organisms evoked by relatively short, low concentration stress in ex-situ studies is a useful tool.

Methods to detect the effect are required. Depending on the underlying question, existing methods have to be modified or new methods developed.

Intraspecific variability is an important factor that influences experimental results, particularly when studying sub-lethal effects. Differences between individuals in the endpoint of interest can be equal or even greater than the effect caused by the toxicant, making it necessary to reduce the variability by, for example, focusing on a sub-group of the test species.

A method to measure the individual feeding activity of *Gammarus pulex* on a daily basis was developed, taking into account the intraspecific variability caused by food source and infection with acanthocephalan parasites (experiment 1) and body mass (experiment 2). The method was used to observe the influence of the neonicotinoid insecticide imidacloprid on feeding activity in a chronic exposure test.

2. Materials and methods

All semi-static experiments were undertaken using artificial pond water (APW) as medium. The medium was changed every 2\textsuperscript{nd} or 3\textsuperscript{rd} day and the food source was horse chestnut leaf discs (Ø 1.6 cm) inoculated with *Cladosporium* sp. The constant environmental conditions were 13 ± 1°C, a light dark rhythm of 12:12h and a pH between 7.5 and 8.1.

In the first experiment, gammarids in a group of five organisms per replicate (250 ml APW) with and without infection with acanthocephalan parasites were fed with one of three different types of leaf discs. The food sources differed according to presence/absence and duration of inoculation with decomposing fungi.

In the second experiment, the feeding activity of 75 organisms with a body mass between 0.48 and 14.6 mg (dw, dry weight) was observed at a daily resolution for a total period of nine days. The organisms were kept individually in 90 ml medium.

The experiment measuring the influence of imidacloprid was divided into an initial (2d), exposure (4d) and recovery phase (5d). Organisms with a body mass bigger than 4.6 mg (dw) were exposed to five different concentrations of the pesticide. The highest concentration corresponded approximately to the lethal concentration for 20% of the organisms for the same exposure duration [1].

3. Results and discussion

3.1. The influence of food source and parasite infection to the feeding activity

Inoculation of leaf material with a fungi led to a 36.7% higher feeding rate compared to the rate with non-inoculated food. A reduction of the feeding rate of 18.0% was observed when a parasite infection was present. When the food was not inoculated and organisms were infected with a parasite, the feeding rate was reduced by 69.0%. Furthermore, it could be observed that the feeding rate measured at a daily resolution in a group of five organisms was not stable.

In the present study, the impacts of parasite infection as well as the influence of food quality separately were significant over a measurement period of four days. The results are comparable to the literature [2]. Parasite infection and lower food quality together yield an even stronger reduction of the feeding rate of *Gammarus pulex* in relation to uninfected and optimally-fed organisms.
3.2. The influence of body mass to the feeding activity

Smaller organisms (dw <5 mg) had a three times higher feeding rate than bigger organisms. Fluctuations in the individual feeding rates were lower when observing organisms bigger than 5 mg (dw). A 30% reduction of the feeding rate was measured over an observation time of 9 days.

In terms of size dependence of the feeding rate, the results of this experiment agreed with those in the literature [3; 4]. Furthermore it could be demonstrated that daily measurement of feeding activity on an individual level was feasible, but there were some unidentified factors which reduced the feeding rate over time. Possible influences could be the isolation of the organisms, feeding with only one food source and the lack of water flow.

3.3. The influence of imidacloprid to the feeding activity

A time and concentration dependence of the feeding activity could be observed. The 50% effect concentrations for exposure with imidacloprid decreased over time from 25 to 3.8 µg/L (Table 1). Gammarids were able to “recover” from an inhibition following exposure for five days at concentrations lower than 30 µg/L. Recovery in this case is defined as an increase of the feeding rate compared to the exposure phase. For organisms exposed to concentrations lower than 30 µg/L the recovery evoked a significantly higher feeding rate compared to the control. This led to very low no observed effect concentrations while recovery was taken into account (Table 1). At higher concentrations no recovery was observed. Focus on the median feeding rate over the whole experimental duration concentrations up to 9 µg/L led in a overall higher feeding rate compared to the control.

<table>
<thead>
<tr>
<th>EC50 (µg/L)</th>
<th>95% Fiducial limit</th>
<th>NOEC (µg/L) exposure phase</th>
<th>NOEC (µg/L) recovery phase</th>
</tr>
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<tbody>
<tr>
<td>24h</td>
<td>25.02</td>
<td>10.48 – 151.66</td>
<td>30</td>
</tr>
<tr>
<td>48h</td>
<td>11.35</td>
<td>4.96 – 33.61</td>
<td>30</td>
</tr>
<tr>
<td>72h</td>
<td>10.69</td>
<td>4.11 – 35.33</td>
<td>2.7</td>
</tr>
<tr>
<td>96h</td>
<td>3.83</td>
<td>1.16 – 8.45</td>
<td>2.7</td>
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Table 1: 50% effect concentrations (EC50), 95% Fiducial limit and no observed effect concentrations (NOEC) for the endpoint individual feeding rate (mg/mg*day) for different time points within and after exposure of Gammarus pulex with imidacloprid.

The observed effect concentration for an exposure duration of four days was 20 to 380 times lower than the lethal concentration of 170-450 µg/L [1]. Furthermore, the effect concentrations were within the range of measured [5] and estimated [6] environmental concentrations. However, the negative effect at low concentrations turned into a positive one when the recovery phase was taken into account.

4. Conclusions

Intra-specific variability makes it difficult to study sub-lethal effects on an individual level and experimental design should minimise variability wherever possible. It is known that lethal and sub-lethal impacts can still be seen after the toxicant is removed from the exposure medium. In this experiment, a secondary effect (higher feeding rate in previously impacted individuals) also persisted beyond the time when the toxicant was eliminated from the water phase. Whilst effects on feeding rate were observed at very low concentrations of imidacloprid, these effects were cancelled out at intermediate concentrations by higher feeding rates during the recovery phase. It is essential that experimental duration should be sufficient to study such compensatory effects.

5. References


Acknowledgement - The authors thank the Marie Curie Initial Training Network, 7th Framework Programme “CREAM”: Mechanistic Effect Models for Ecological Risk Assessment of Chemicals. www.cream-itn.eu
Non-guideline studies refine and improve the aquatic risk assessment of forest insecticides imidacloprid and neem

David Kreutzweiser and Dean Thompson

1 Canadian Forest Service, Natural Resources Canada, 1219 Queen St. East, Sault Ste Marie, Ontario P6A 2E5, Canada
E-mail contact: dave.kreutzweiser@nrcan.gc.ca

1. Introduction
Most pesticide risk assessment frameworks include a problem formulation phase in which relevant measurement and assessment endpoints are identified. Functional attributes or measurement endpoints are not typically used in risk assessments under the PCPA in Canada [1] or FIFRA in the USA [2] because it is generally accepted that protection of structural endpoints will protect ecosystem function. However, when specific functional attributes can be identified and are known or suspected to be at risk from a pesticide, they could be included in the data submission for a risk assessment. We provide two examples from non-guideline mesocosm studies in which we evaluated the risk of synthetic (imidacloprid) and natural (neem) insecticides in the context of forest insect pest control to aquatic ecosystems, by combining known ecological, use pattern, and exposure scenario information to direct an adaptive, not tiered, sequence of studies.

2. Materials and methods
Two examples are provided. In the first, aqueous concentrations of neem (azadirachtin) were tested in outdoor forest mesocosms [3] to simulate aerial applications of neem for foliar insect control. Initial mesocosm studies focused on standard higher-tier structural endpoints (population levels, community structure). Based on results from those experiments in which neem was selectively toxic to adult copepods, and because adult copepods were known to have a relatively-long life cycle (1 year) and to contribute a major component of zooplankton biomass, subsequent mesocosm experiments were conducted focusing on community respiration (oxygen uptake), whole system metabolism (dissolved oxygen concentrations and conductivity as functional indicators of ecosystem stress), and food web structure and functional guilds.

In the second example, trunk-injected systemic applications of imidacloprid and neem for wood-boring insect control in riparian (shoreline) trees were found to retain measureable foliar concentrations at autumn leaf-fall and potentially posed risk of harm to decomposer organisms and processes in leaf litter. Laboratory mesocosm experiments were conducted to assess that risk of harm to decomposition processes [4]. In this case, measurement endpoints were survival, feeding, and avoidance rates of obligate leaf-shredding aquatic insects.

3. Results and discussion

3.1. Aqueous concentrations in outdoor mesocosms to simulate aerial applications of neem
Initial mesocosm results indicated delayed (probably growth-regulating effects) but significant reductions in adult copepod populations at realistic concentrations, with the dominant calanoid copepod virtually eliminated by 42 days post-treatment. This was accompanied by short-term reciprocal increases in cladocerans. Subsequent mesocosm results indicated that population declines among adult copepods persisted into the following year, and the resultant shifts in zooplankton community structure were sufficient to induce measureable reductions in community respiration (Figure 1A), whole system metabolism, and food web stability (Figure 1B).

3.2. Foliar concentrations in laboratory mesocosms from systemic tree applications of imidacloprid and neem
Leaves collected at senescence from systemically-treated trees to control wood-boring insect pests contained measureable concentrations of imidacloprid and neem (azadirachtin), although foliar concentrations in leaves from imidacloprid-treated trees were at least 10-fold higher than in leaves from
neem-treated trees. At foliar concentrations resulting from operational field trials with imidacloprid, leaf litter breakdown was significantly reduced by sub-lethal feeding inhibition effects on key leaf-shredding aquatic insects (Table 1) and impaired microbial decomposition activity. Adverse effects on leaf-shredding insects were shown to result from sub-lethal behavioural effects, not avoidance. No such reductions resulted from exposure to leaves from neem-treated trees, even at intentional over-dose (Table 1).

Figure 1. A, average (± SE) community respiration (O₂ uptake mg L⁻¹ h⁻¹), and B, food web directed connectance in mesocosms treated with neem (expressed as active ingredient azadirachtin)

<table>
<thead>
<tr>
<th>Mass loss</th>
<th>A</th>
<th>B</th>
<th>C</th>
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<tr>
<td></td>
<td>17%*</td>
<td>146%</td>
<td>98%</td>
</tr>
</tbody>
</table>

Table 1: Average mass loss of leaf material (as % of control mass loss) in mesocosms with realistic foliar imidacloprid (A) realistic neem (B) and 25X realistic neem (C) concentrations. * indicates significantly different from controls (p<0.05)

4. Conclusions

Here we show how hypotheses generated by combining known ecological, use pattern, and exposure scenario information can be used to direct adaptive, non-guideline studies for assessing risk to aquatic communities. Simulated aerial applications of neem resulted in measurable structural changes in zooplankton communities. But the change was unique (selective toxicity to key taxonomic group) and prompted directed community-level testing to address specific hypotheses, i.e., that reductions in calanoid copepods would invoke community and system-level functional disruptions. This would not have been predicted based on the relatively-low sensitivity of cladocerans. Systemic applications of imidacloprid and neem to trees for a specific insect pest presented a unique route of non-target exposure and risk of adverse effects on leaf litter decomposition (a critical ecosystem function in forest soils and water bodies). In that case, the protection goal was maintaining leaf litter decomposition, the community at risk was decomposer invertebrates feeding on leaves from insecticide-treated trees, and the selection of test species was directed to a specific functional group because of the unique route of exposure to decomposer organisms identified in the risk hypotheses. We suggest non-guideline studies like those illustrated here can, and should, provide ecologically-relevant ancillary data for regulatory guideline data submissions to increase environmental realism, reduce uncertainties, and improve overall risk assessments for pesticides.

5. References


Acknowledgement – Excellent technical assistance in these studies was provided by S Capell, D Chartrand, K Good, S Grimball, J Harnden, E Muto, M Roberts, B Wujtajszek, and Korrie Young. Partially funded by Enhanced Pest Management Method S&T Program, Natural Resources Canada, and the Ontario Ministry of Natural Resources.
Effects and Environmental consequences of U exposure on the fish *Danio rerio*

Simon O¹, Floc’h E¹, Geffroy B¹ and Gilbin R¹

¹Laboratoire de Radioécologie et d’Ecotoxicologie, Institut de Radioprotection et de Sûreté Nucléaire, Bât 183, BP 3, 13115 Saint-Paul-Lez-Durance Cedex, France
E-mail contact: olivier.simon@irsn.fr

1. Introduction

Exposure to metals can cause a number of abnormalities in adult (reproductive capacity) and egg/larvae (survival rate) fish. These endpoints may have serious implications for normal population dynamics and community structures. The OECD guidelines currently propose that the early life stages (ELS) of fish be used as an experimental model to assess toxic effects from contaminants. Moreover, appropriate laboratory tests could quantify the reproductive success (fertilization, fecundity, egg and larval mortality) together with modelling approaches for assessing the potential for population consequences. Hence, the objectives were to measure to impact on live-history traits of fish (food, acclimatizing modalities, age of exposure) in order to develop ecotoxicological tests more accurate for the environmental risk assessment.

Uranium’s environmental prominence is currently increasing because of new mining and milling activities to support the resurging commercial nuclear power industry. Such anthropogenic activities can increase the environmental concentrations of U and cause the metal to accumulate in biota. In Canadian lake, very high concentration of U in water (200-3000 µg.L⁻¹) are detected as compared to control lake (5.2 µg.L⁻¹). The health-based guideline value of Uranium in drinking water is 20 µg.L⁻¹. The main direct exposures of DU (depleted Uranium; 20 to 250 µg.L⁻¹) was performed on adults and eggs *Danio rerio*.

The aims were (1) to identify the U effects on eggs after 96h of exposure (2) to confirm the results in another egg species *Oryzias latipes* and *Onchorynchus mykiis*, (3) identify the U effects on adults reproduction after a short exposure time (20d) and after 200d (from eggs to mature adults).

2. Materials and methods

Adult fish, purchased from Aquasylva, Pertuis, France were acclimated to tap water and/or artificial water. Experiments of U exposure was performed in artificial water at pH 6.5. Its composition resulted in a compromise between the conditions necessary for healthy and the optimal U bioavailability). Groups of genitors were placed separately in small spawning aquarium (3 L) to keep the fish from eating the newly spawned eggs. Embryos were obtained from spawning genitors (3 groups of 12 eggs per condition). Mature adults were exposed during 20 days. The obtained eggs were then exposed during 200 days to study the U effects on the whole life cycle. Effects on reproduction capacity were performed from 10 groups (sex ratio : 2♂:1♀) per condition. We focused only on the first spawn. Accumulation levels in organs was also measured but not presented in the extended abstract.

The Table 1 summarized the exposure modalities (species, state of the biological model, age of exposure, exposure duration U concentration in water) and effects.

<table>
<thead>
<tr>
<th>Species</th>
<th>Biological stage</th>
<th>Age of exposure</th>
<th>Exposure duration</th>
<th>U concentration in water (µg.L⁻¹)</th>
<th>Endpoints</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Danio rerio</em></td>
<td>Eggs</td>
<td>3 hpf</td>
<td>96 hpf</td>
<td>0, 30, 100, 300, 1000</td>
<td>Viability, Hatching, Malformation, embryo development</td>
</tr>
<tr>
<td><em>Oryzias latipes</em></td>
<td>Eggs</td>
<td>24 hpf</td>
<td>600 hpf</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Onchorynchus mykiis</em></td>
<td>Eggs</td>
<td>336 hpf</td>
<td>240 hpf</td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Danio rerio</em></td>
<td>adult</td>
<td>Mature</td>
<td>20d</td>
<td>0, 20, 250</td>
<td>Fecundity, Viability</td>
</tr>
<tr>
<td></td>
<td>adult</td>
<td>Eggs</td>
<td>200 d</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Table 1. Exposure modalities (species, state of the biological model, age of exposure (hpf : hours post fertilisation, d: days), exposure duration U concentration in water) and effects*
3. Results and discussion

No mortality in eggs and no disruption of the main embryo development stages were observed after direct 96h-U exposure. However, early life stage are significantly affected by the U exposure; effects of U on hatching success starting at the concentration of 300 $\mu$g.L$^{-1}$ causing a delay in hatching time (Figure 1). Metals (cadmium, copper, nickel, lead) were known to affect the hatching kinetic.

Moreover, this biological effect at the egg stage varied as a function of acclimatization modalities of parents. Eggs (D. rerio, O. latipes, O. mykiss) obtained from genitors without acclimatization in artificial water showed a significant delay in hatching time only at the concentration of 1000 $\mu$g.L$^{-1}$ in D. rerio, O. mykiss and O. Latipes. Moreover, U exposure at lower concentrations caused hatching to occur earlier than in control. Results indicated that the sensibility of egg response was influenced by the water quality of adults acclimatization period.

Effects on the adults reproduction efficiency after the first spawning and egg viability were given in the Table 2. Uranium exposure reduced the total number of spawned eggs. For the higher concentration, 20 days of exposure reduced (x5) although no eggs was spawned after the 200 days of exposure condition. For the 20 $\mu$g.L$^{-1}$ exposure, the number of eggs was reduced by 3-2.5. Moreover, the viability of eggs was reduced after 200d. In the case of whole life cycle exposure, sex ratio, fecundity, viability and the ability to spawn were widely modified by U exposure. A population model included viability, fecundity and ability to spawn endpoint could be used to evaluate the U effects. Dynamic of population was directly altered by uranium exposure.

<table>
<thead>
<tr>
<th>Female mass (g)</th>
<th>Control 200d</th>
<th>20 $\mu$g/L 200d</th>
<th>250$\mu$g/L 200d</th>
<th>Control 20d</th>
<th>20$\mu$g/L 20d</th>
<th>250$\mu$g/L 20d</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.4730 ± 0.11</td>
<td>0.4348 ± 0.11</td>
<td>0.5286 ± 0.12</td>
<td>0.4502 ± 0.17*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex ratio (F:M)</td>
<td>1:1.7</td>
<td>1:1.1</td>
<td>1:8.6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ability to reproduce (%)</td>
<td>100</td>
<td>70</td>
<td>0</td>
<td>90</td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>Total number of eggs</td>
<td>2764</td>
<td>836</td>
<td>0</td>
<td>1330</td>
<td>553</td>
<td>246</td>
</tr>
<tr>
<td>Viability at 24 hpf</td>
<td>79 ± 18</td>
<td>32 ± 27</td>
<td>75 ± 13</td>
<td>88 ± 21</td>
<td>75 ± 14</td>
<td></td>
</tr>
<tr>
<td>Viability at 96 hpf</td>
<td>75 ± 18</td>
<td>21 ± 24</td>
<td>70 ± 18</td>
<td>88 ± 15</td>
<td>75 ± 11</td>
<td></td>
</tr>
</tbody>
</table>

Table 2. Effect of U on adult reproduction efficiency and egg after the first spawn. 10 groups(1:2) were performed per condition. The ability to reproduce indicated the percentage of groups of mature fish per condition which spawned. * measured before the beginning of the exposure.

The U sensibility of adult response was influenced by the age of the beginning of the exposure modalities.

4. Conclusions

Bioassays based on the early life stages gave sensitive information of U exposure. Hatching success was a good marker for U concentration upper 250 $\mu$g.L$^{-1}$. Experiments based on adult stage showed significant U effect of the total number of eggs and on the ability to spawn from the 250 $\mu$gU.L$^{-1}$ concentration. Finally, the whole life cycle experiment shown a drastic reduction of eggs spawned and a weak viability from the 20 $\mu$gU.L$^{-1}$ concentration. Thus, U exposure could affect the dynamic of population and led to significant consequences on the structure and the function of the ecosystem at low concentration. As expected, the most representative test (long term of exposure/whole live cycle) will provide better information on the environmental risk of U exposure.
1. Introduction

KEYBIOEFFECTS was a Marie Curie Research Training Network (RTN) aiming to gain a deeper understanding of the anthropogenic toxic compounds released in European water bodies. In this presentation, the main research findings will be summarized and a few examples presented in detail to illustrate our research output and open questions.

Fig. 1 Scheme showing the main elements required to address cause-effect relationships of pollution in fluvial ecosystems. Environmental risk assessment is commonly based on Priority Pollutants (PPs) data and toxicity results obtained from standardized toxicity tests (solid lines). Keybioeffects research focused on several knowledge gaps (dashed lines): identification, toxicity, fate and monitoring of emerging compounds and transformation products; bioavailability, trophic transfer, direct and indirect effects of chemicals on natural populations and communities and the evaluation of the environmental effects of chemical pollution in several European river ecosystems.
2. Results and discussion

Several researchers focused their studies on environmental risk assessment of pollutants. Sophisticated modelling approaches were developed using environmental data provided by water agencies (Catalonia, NE Spain and Adour-Garonne watershed, SE France). Based on available data, it was possible to map areas with higher potential impact due to pesticides, heavy metals or surfactants [1, 2]. In other investigations, the simultaneous study of chemical and biological data allowed to link chemical pollution with population or community changes. In the river Llobregat (Catalonia), biodiversity losses were associated with herbicide (triazines) pollution, and this finding was supported by results obtained in microcosms under controlled exposure conditions [3]. In another investigation [4], fish (Squalius cephalus) morphology (life traits) was significantly affected by the presence of toxic pesticides investigated along a pollution gradient in the Adour-Garonne river basin. Omic technologies such as transcriptomics and metabolomics were developed by other researchers [5, 6]. These new approaches are expected to contribute to better address the multi-faceted effects of chemicals on biota as well as the interaction with other chemical, physical and biological stressors in future environmental studies.

The influence of chemical pressure on river organisms was confirmed at a smaller scale by means of active bio-monitoring: “in situ” translocations experiments. This approach allowed demonstrating the impact of metal pollution (Zn) and the herbicide prometryn on fluvial communities (biofilms) under real conditions. Other investigations performed in microcosms under controlled exposure conditions were used to develop or validate a set of biomarkers of toxic exposure using fluvial biofilm communities [6, 7].

Furthermore, KEYBIOEFFECTS provided tools to overcome existing gaps in the identification of key toxicants using Effect-Directed Analysis and computer tools [8, 9, 10, 11], identification of transformation products [12], and in the quantification of the influence of environmental conditions on toxicant bioavailability, retention and trophic transfer [13, 14].

3. Conclusions

Overall, the results obtained demonstrate that chemical pollution, including priority and emerging compounds as well as non-identified toxic compounds, is posing a risk to the ecological integrity of European rivers. Furthermore, sampling strategies, experimental approaches, analytical and bio-analytical methods applied or developed within the network are recommended as innovative and sensitive tools to better address the environmental risk of pollutants and identify the causes of ecological deterioration as the European Water Framework Directive requires.

4. References


Acknowledgement - EU project: KEYBIOEFFECTS (MRTN-CT-2006-035695).
Effects of ingestion of fungicide- and insecticide-coated seeds on red-legged partridge (*Alectoris rufa*) health

Manuel E. Ortiz-Santaliestra¹, Ana López-Antia¹, François Mougeot² and Rafael Mateo¹

¹Instituto de Investigación en recursos Cinegéticos CSIC-UCLM-JCCM. Ronda de Toledo s/n., 13071 Ciudad Real (Spain)
²Estación Experimental de Zonas Áridas CSIC. Cañada de San Urbano s/n 04120 Almería (Spain)
E-mail contact: ManuelE.Ortiz@uclm.es

1. Introduction
Pesticide application is suspected to be a major cause of population decline in farmland birds. Direct intoxication by ingestion of pesticides incorporated into plants or invertebrates is a major threat for these birds. Cereal seeds are usually coated with fungicides or insecticides before sowing. These seeds constitute a main part of the diet of species such as the red-legged partridge (*Alectoris rufa*) during sowing seasons in autumn and late winter¹. Although the most toxic chemicals are being legally restricted, some of the currently used fungicides and insecticides can cause adverse health effects when ingested by birds thus threatening individual fitness and population viability. The aim of this study was to investigate the effects of coated seed ingestion on red-legged partridge physiology and general fitness. We tested three currently used chemicals, an insecticide (imidacloprid) and two fungicides (difenoconazole and thiram).

2. Materials and methods
Each experiment consisted in two groups of six pairs of partridges each. One of the groups was given wheat seeds treated with the recommended dose for seed coating, whereas the other group was given seeds treated with a concentration twice the recommended one to evaluate the risk of pesticide abuse by farmers. A third group of six couples was fed with untreated wheat seeds and used as control.

Treated seeds were administered for a 10-day period, after which all animals were transferred to an untreated diet and monitored for an additional 12 days. Animals were weighed, ventilation rates were measured and blood samples were collected at the end of the exposure and monitoring periods. Additionally, we took pictures of the beak and eye ring to estimate the colour intensity of both areas and the percent of pigmentation of the eye ring. Blood samples were used for measuring hematocrit; testosterone and estradiol levels were quantified in plasma samples using ELISA commercial kits; vitamin (retinol, tocopherol, retinol palmitate) and carotenoid (lutein, zeaxanthin) profiles in plasma were obtained by HPLC as described by Rodriguez-Estival et al.[²]; oxidative stress parameters were quantified according to the protocols detailed in Mateo et al.[³], and included lipid peroxidation (measured by TBARs), antioxidant enzymes (glutathione peroxidase and superoxide dismutase) and the ratio between reduced and oxidized glutathione.

We tested the efficiency of both cellular and humoral immune responses. The former was analysed using the phytohemagglutinin (PHA) test[¹] whereas the latter was tested in vitro by means of a hemaglutination tests that quantifies the amount of specific antibodies produced after challenging with sheep red blood cells (SRBCs).[⁵]

3. Results and discussion
Partridge survival was affected by the highest concentrations of imidacloprid and thiram (58% and 42% mortality, respectively). Interestingly, lethal effects of the fungicide were observed mostly in males (60% vs 29% in females). Lower concentrations of both pesticides induced significant weight loss, consistent with the fact that symptoms of anorexia were found during necropsies of dead animals. However, survivors were able to recover once the exposure to coated seeds ceased (Fig. 1).

Sublethal concentrations of imidacloprid and thiram also induced oxidative stress, as deduced by the increase in the activity of antioxidant enzymes. Superoxide dismutase was significantly stimulated by thiram when compared to controls (F₂,₂₈=7.539; p=0.002), whereas imidacloprid induced the activity of glutathione peroxidase (F₂,₂₂=6.651; p=0.006).

Immunocompetence was affected by the exposure to imidacloprid. Animals exposed to the highest insecticide concentration showed an impaired cellular immune response as revealed by the PHA test (F₂,₂₅=4.809; p=0.017). When we analysed both sexes separately, the influence of imidacloprid on cellular immune response was only observed for males. The humoral response was not affected by the pesticides.
Finally, some animals fed with coated seeds suffer alteration of their normal pattern of ornamentation. Beak redness was reduced by the lowest imidacloprid concentration; however, when we introduced the sex as a cofactor and in spite of the apparently big differences among treatments for both males and females, the differences were only statistically significant in females (Fig. 2). The two fungicides at their highest concentrations significantly reduced the percentage of eye ring pigmentation (thiram: $F_{2,32}=6.158; p=0.005$. difenoconazole: $F_{2,32}=4.245; p=0.023$).

4. Conclusions

• Adverse health effects were caused by the insecticide treatments on partridges, and appeared more severe than those provoked by the fungicides treatments
• Imidacloprid and thiram reduced body condition by inducing anorexia. At lower, environmentally realistic, doses, animals were able to recover quickly when fed again with an untreated diet. Artificially-increased concentrations caused increased mortality after only 10 days of exposure
• The two pesticides induced oxidative damage at environmental concentrations
• Some of the observed responses, like thiram-related mortality or imidacloprid effects on beak redness, were dependent on sex, which could ultimately affect the reproduction process by altering breeding investment, mate choice or sex ratio
• Imidacloprid appears as an immunosuppressive toxicant, affecting the cellular immune response

5. References


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Ecosystem Exposure and Adverse Human Health Effects of POPs: A Data Analysis Approach by a Discrete Mathematical Method

Kristina Voigt¹, Rainer Brüggemann², Karl-Werner Schramm³

¹ Helmholtz Zentrum Muenchen - German Research Center for Environmental Health (GmbH), Institute of Biomathematics and Biometry, Neuherberg, Germany
² Leibniz-Institute of Fresh Water Ecology and Inland Fisheries, Berlin, Germany
³ Helmholtz Zentrum Muenchen - German Research Center for Environmental Health (GmbH), Institute of Ecological Chemistry, Neuherberg, Germany

E-mail contact: kvoigt@helmholtz-muenchen.de, brg_home@web.de, schramm@helmholtz-muenchen.de

1. Introduction

It has been evident for decades that environmental chemicals pose an enormous risk to the environment as well as to humans. The effects of environmental contaminants on health are a major concern because exposure is associated with the alteration of human sex ratio, as well as with a number of diseases, including cancer, diabetes, congenital malformations and infertility, etc. It is clear that there is increasing pressure to intensify the research and to more efficiently evaluate the data on persistent and bioaccumulative chemicals in the environment as well as in human bodies.

Our research focuses on data analyses concerning environmental chemicals in order to gain information out of the data and draw conclusions concerning the prevention of those chemicals. Furthermore, we investigate if and how the physical-chemical properties logPow and BCF are associated with the contamination of human breast milk samples in 32 selected POPs.

2. Materials and methods

2.1. Data analysis method: Hasse diagram technique and PyHasse software

The data analysis method is based on the theory partially ordered sets. The theory of partial order is a discipline of Discrete Mathematics and one may consider partial order as an example of mathematics without numerical arithmetic. The graphical representation of partial orders is laid down in so-called Hasse diagrams. The software package applied is named PyHasse, written by the second author is still under development. A good overview on the basic ideas of partial order as well as their application can be found in [1] and [2].

In complex data sets it is often necessary to compare different sets of criteria (attributes). In the similarity analysis we intend to calculate the similarity of different posets (partially ordered sets). This similarity analysis is an important feature of PyHasse. We distinguish among the following similarity relations: Isotone, antitone, indifferent, weak isotone and equivalence relations. For further reading see Voigt et al [3].

2.2. Data set of 32 POPs

In our data-analysis approach we investigated data sets of breast milk samples of women in Denmark and Finland which contained measurable levels of 32 Persistent Organic Pollutants (POPs). Out of the 65 samples (complete data matrix) in each country we selected two different subsets according to healthy boys and boys with cryptorchidism, a malformation of the testis.

The data have already been evaluated by classical statistical methods [4] as well as by the Hasse diagram technique [3]. In this study we want to compare the logPow/BCF data of 32 POPs with the occurrence of these chemicals in breast milk samples of Finnish and Danish women.

3. Results and discussion

In Figure 1 the data matrix of 32 POPs (objects) and 2 criteria (logPow, BCF) is set up in a Hasse diagram.
Figure 1: Hasse Diagram 32 Chemicals evaluated by Pow and BCF.

The chemical B155 (22'44'66'-BB) which is equivalent to the chemical B153 (22'44'55'-BB), both brominated compounds are the maximal objects. This means that their two factor BCF and logPow together are the highest in comparison to all other chemicals with which they are connected in the downward direction. In this diagram means they are comparable to all other chemicals. The minimal objects are the chemical CHCE (cis-Heptachloroepoxide) which is equivalent to PHCE (+ Enantiomer cis-Heptachloroepoxide) and END1 (Endosulfan-1). These chemicals have the lowest logPow/BCF values.

In the next evaluation step we want to know whether and how the above shown 32x2 Hasse diagram is similar to the evaluation of the cryptorchidism/healthy data sets. The similarity sequence follows DKh>DKc>FLc>FLh. Note: Only the isotone fraction was considered as a similarity measure. This means that in the healthy boys’ data of Denmark the greatest similarity between logPow/BCF is encountered.

4. Conclusions

In the Hasse diagram technique the ordering of the chemicals, their different positions in the Hasse diagrams and the quantification using partial order theoretical method are, however, new and important amendments in the findings of chemicals influencing the state of health of human beings. The comparisons (similarity analysis) of data sets of Danish and Finnish breast milk samples (healthy, cryptorchidism) with logPow/BCF of the 32 chemicals reveals similar structures of the diagrams. It shows that the physical chemical properties logPow and BCF are good indicators for the ranking of the used POPs.

It has been demonstrated that ranking methods like the Hasse diagram technique and further features found in the PyHasse software package are a helpful tool for evaluating the ecosystem exposure and its impact on human health.

5. References

Modulation of immune parameters by chemical environmental pressures in wild populations of European bullhead, *Cottus sp.*, from Vesle basin (Champagne, France).

Anne Bado-Nilles¹,², Sabrina Jolly², Alain Geffard¹, Nadou Cadic⁴, Béatrice Gaignaire³, Jean-Marc Porcher², Wilfried Sanchez² & Stéphane Betoulle¹

¹Université Reims Champagne Ardenne, UFR Sciences Exactes et Naturelles, Unité de Recherche Vignes et Vins de Champagne - Stress Environnement (URVVC-SE) EA 2069, Laboratoire Ecologie – Ecotoxicologie, Moulin de la Housse BP1039, 51687 Reims.


³Institut de Radioprotection et de Sureté Nucléaire (IRSN), Laboratoire de radioécologie et écotoxicologie, Centre de Cadarache, Bât 186, B.P. 3, 13115 Saint-Paul lez Durance, France.

⁴Office National d’Etude des Milieux Aquatiques (ONEMA), Délégation Nord-Est, 57000 Marly, France.

E-mail contact: Anne.Bado-Nilles@ineris.fr

1. Introduction

Champagne is one of the French regions where pesticide uses are particularly high either for agriculture or viticulture. A large number of these chemical substances present in the aquatic environment are able to disturb homeostasis and physiological adaptations of organisms by modulating one or more biological functions. The natural immune functions, which are particularly important in ectotherm vertebrate as fish, are potential target of numerous xenobiotics. In fact, many cellular responses as phagocytosis, cellular mortality and cellular subpopulations distributions are used as immunotoxicity biomarkers in fish. In the present study, these three immunological biomarkers were developed to evaluate immune status of European bullhead, *Cottus sp.*, a sedentary fish, in the presence of various agricultural pressures in the Vesle River system.

2. Materials and methods

Five different sites on the Vesle basin were selected due to their various environmental anthropogenic pressures. All the chosen sites were in possession of wild populations of European bullheads (Figure 1). These sites were located on two rivers, the Vesle (Bouy, Prunay and Muizon) and the Ardre (Serzy and Courtagnon). Situated in a forested sector, Courtagnon was used as a reference site with no direct environmental inputs of chemicals. Serzy station was localized within an intensive viticulture area. Bouy site was highly influenced by intensive cereal farming. Prunay station was impacted by agricultural and viticultural pressures. Finally, Muizon site was chosen due to their position in the downstream of Reims City (Champagne, France).

![Figure 1: Localization of sampling sites on Vesle river system, with A = localization of sites in France and B = localization of the selected sampling sites in Vesle basin.](image)

During spring, summer and autumn 2010, 20 adult European bullheads were caught by electrofishing in each selected sites. Spleens were interesting due to sampling facilities and to their immune function. After spleen tissue disruption, leucocytes were isolated using density gradient centrifugation and cell suspensions were adjusted to 10⁶ cells.mL⁻¹. Leucocyte subpopulation compositions, cell mortality and phagocytosis activity were analysed with a Cyan™ ADP flow cytometer connected to hypercyt®intellicyt (Beckmann Coulter).
3. Results and discussion

3.1. Morphological and seasonal variations

Fish biometric characteristics (length and weight) seem to have no impact on selected immune parameters. The seasonal variation induced modifications of selected immune biomarkers without discrepancy between studied sites. In summer, fish had normal leucocyte population distributions with roughly 80% of lymphocytes and 20% of granulocytes-monocytes. In spring and autumn, lymphocyte proportions were reduced whereas those of granulocytes-monocytes increased. For cellular mortalities, a peak was detected in spring and the bottom value in summer. Phagocytosis activity was significantly higher in fish caught in autumn (Figure 2).

![Figure 2: Seasonal variations of immune-related biomarkers for European bullhead. Values correspond to mean of all sites (n = 100) ± ET. For each biomarker, the same letter indicated no statistical difference (p ≤ 0.05).](image)

3.2. Impact of various environmental pressures

Independently of seasonal variations, environmental characteristics of each site seem to disturb selected immune markers (Figure 3). The alteration of splenic leucocyte distributions in Bouy and Prunay sites compared to other stations were rather identical between seasons. The highest cell mortalities were observed in sites situated in areas with intensive agriculture or viticulture (Bouy, Prunay and Serzy). Moreover, phagocytosis activities were significantly reduced in fish caught in the three same sites, excepted in summer for Prunay and in autumn for Bouy. We can notice here that bullhead sampling in autumn at Bouy station suffered from numerous visible pathologies.

![Figure 3: Biomarker responses for European bullhead captured at the different sampling sites and season with A = lymphocytes (%), B = granulocytes-monocytes (%), C = leucocyte mortality (%) and D = phagocytosis (%). Values correspond to mean (n = 20) ± ET. For each season, sites annotated with different letters are statistically significant (p ≤ 0.05).](image)

4. Conclusions

These first results may indicate possible immunotoxicological impacts on bullhead from Vesle basin, of seasonal variations and agri-viticultural practices, with more impact of cereal farming influence than of wine-growing area. Hence, further ambitious studies with multi-annual development, have to complete the present data in order to improve the knowledge of pesticides induced immunomodulations. Moreover, data are needed to better characterize the effects of biotic and abiotic confounding factors on these biomarker base levels and to define their natural variability ranges for assessment of wild fish health.

Acknowledgements - We thank “Office National de l’Eau et des Milieux Aquatiques” for the technical assistance in fish sampling. We also acknowledge the financial support of the Post-Grenelle Programm 190 (DEVIL programme) of the French Ministry for Environment.
Multi-endpoint assessment of the effects of chlorpyrifos ingestion in a lacertid lizard (Podarcis bocagei)

M.J. Amaral1,2, R.C. Bicho1, M.A. Carretero2, J.C. Sanchez-Hernandez3, R. Valente1, A.M.R. Faustino4, A.M.V.M. Soares1 and R.M. Mann1,5

1CESAM & Departamento de Biologia, Universidade de Aveiro, 3810-193 Aveiro, Portugal
2CIBIO, Centro de Investigação em Biodiversidade e Recursos Genéticos, Campus Agrário de Vairão, 4485-661 Vairão, Portugal
3Facultad de Ciencias del Medio Ambiente, Universidad de Castilla-La Mancha, Avda. Carlos III, E-45071 Toledo, España
4Departamento de Patologia e Imunologia Molecular, ICBAS, Universidade do Porto, 4050-123 Porto, Portugal
5Hydrobiology, Auchenflower, Queensland 4066, Australia

E-mail contact: mjamaral@ua.pt

1. Introduction

Lizards are among the least studied groups in ecotoxicology, and despite a recent increase in the number of studies, there is still a lack of knowledge regarding their response to environmental contamination [1]. In Europe, lacertid lizards have been identified as potential model species for reptile ecotoxicology [2]. Chlorpyrifos (CPF) is one of the main organophosphorus insecticides used in agricultural areas and has been reported as moderately toxic and a cholinesterase inhibitor. The main goal of our project was to use a multi-endpoint approach to assess the effects of environmentally relevant doses of chlorpyrifos in the lacertid Podarcis bocagei.

2. Materials and methods

Adult male P. bocagei (n=36) were captured in a non-contaminated site and exposed under controlled conditions to a commercial formulation of chlorpyrifos (CI CLONE 48 EC - 480 g/L, SAPEC AGRO). Individuals were assigned to three treatments: control (dH2O), CPF1 (96 ng cpf/meal) and CPF2 (960 ng cpf/meal). The CPF was injected into live mealworms, which were provided as meals every second day over a 20-day period.

After 20 days feeding, 18 individuals (6 from each treatment) were fasted and subjected to individual performance tests: sprint speed in a horizontal surface, time of latency to attack prey (TLA) and prey manipulation time (MAN) were estimated for each individual.

The remaining lizards were sacrificed and dissected for biochemical assays, histopathological analysis of liver and testes and hemoparasite prevalence/intensity. The activities of antioxidant defense and detoxifying enzymes, glutathione S-transferase (GST), glutathione reductase (GR), glutathione peroxidase (GPx) and ratio of reduced and oxidized glutathione (GSH/GSSG) were measured in the liver, intestine, testis and brain of P. bocagei. Acetylcholinesterase (AChE) and carboxylesterase (CbE) inactivation or inhibition was measured in brain, liver, intestine and testis respectively. Histological changes in liver were detected microscopically and evaluated qualitatively and quantitatively. Spermatogenesis was assessed by measuring seminiferous tubule diameters and size of spermatogenic phases. Blood smears were scanned for hemoparasites and parasite prevalence was estimated as the percentage of infected lizards. Parasite intensity was estimated as the percentage of infected erythrocytes in 2000 cells.

3. Results and discussion

3.1. Individual Endpoints

No mortality or clinical symptoms of poisoning were observed during the test. There were no significant differences regarding running speed between treatments. In the predatory behavior experiment one of the 18 lizards did not swallow the prey in the allowed time although all...
individuals attacked the tenebrio (from one to four times). There was a trend for animals exposed to chlorpyrifos to take more time to subdue and swallow the prey. There were no statistical differences in the proportion of lizards with hemoparasites.

### 3.2. Biochemistry

The activity of the oxidative stress enzymes showed a marked tissue variation, with intestine having lower enzyme activities than liver, testis or brain. However, no differences in activities of the three enzymes (GST, GR, GPx), or in GSH/GSSG ratios were observed regarding treatment. Exposition to chlorpyrifos did not produce any variation in these enzymes regarding the control group in any of the tissues. Esterase activity was significantly inhibited by chlorpyrifos (Figure 3a,b). CbEs had a higher sensitivity to the insecticide than AChE. CbE activity was dependent of tissue and substrate used. 4-NPV had a higher hydrolytic activity.

![Figure 3a,b – Carboxylesterase activity (CbE - 4-NPV substrate) and acetylcholinesterase (AChE) in different tissues of *P. bocagei* exposed to sub-lethal concentrations of chlorpyrifos for a period of 20 days. Bars represent means ± SE (n=6). Statistical differences *p<0.05, ***p<0.001.](image1)

### 3.3. Histopathology

In general, no major histopathological changes were observed in livers of treated animals, although CPF2 exposed individuals presented higher levels of congestion.

Seminiferous tubule diameters did not differ (Figure 4) and all spermatogenic stages were present in control and treated individuals. A tendency toward larger lumen was observed in treated individuals. No differences were detected for the other phases (Figure 5).

![Figure 4 and 5 - Seminiferous tubule diameters and size of spermatogenesis phases of *P. bocagei* exposed to sub-lethal concentrations of chlorpyrifos for a period of 20 days. Bars represent means ± SE (n=6).](image2)

### 4. Conclusions

When exposed to environmentally relevant doses of chlorpyrifos *P. bocagei* displayed carboxylesterase and acetylcholinesterase inhibition in all tissues examined. These biomarkers of exposure were accompanied by behavioural symptoms of neurotoxicosis, including reduced capacity to respond to prey cues. Although not significant, histopathological changes also indicate negative effects of this compound. The implication of these studies is that environmental exposure to chlorpyrifos may affect individual fitness.

### 5. References


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Effects of Roundup® on the protein expression patterns in the liver and brain of metamorphosing common frog *Rana temporaria*

Gillardin Virginie¹, Silvestre Frédéric¹, Oriol Annick¹, Dieu Marc², Raes Martine² and Kestemont Patrick²

¹ Unit of Research in Organisms Biology (URBO), Faculty of Namur (FUNDP), rue de Bruxelles n°61, 5000 Namur, Belgium.
² Unit of Research in Cellular Biology (URBC), Faculty of Namur (FUNDP), rue de Bruxelles n°61, 5000 Namur

E-mail contact: vgillard@fundp.ac.be

1. Introduction

The worldwide decline of amphibian populations, reported in a large variety of habitats, is often linked to man-driven causes such as destruction, disturbance, and fragmentation of the habitat (Hayes et al., 2002; Prugh et al., 2008). Ultraviolet radiations, introduction of predators or competitors, and acid rain have also been suggested as potential causes. Finally, the environmental presence of chemical compounds such as pesticides has been considered as a possible factor contributing to the reported decline (Sparling et al., 2001).

One of the widely used herbicides is glyphosate which is, among other things, formulated as Roundup® (Monsanto, Saint-Louis, MO) (Ortiz-Santaliestra et al., 2010). Literature has already stated the worldwide use of glyphosate. In Europe, on the basis of six European countries, the application of glyphosate in croplands has been estimated to 1.68 millions of ha which corresponds to about 2.5 millions of kg (Dewar, 2009). The massive employ of the chemical may thus implicates some run-off or diffusion into water supplies. In this context, Giesy et al. (2000) reported glyphosate concentrations in surface water up to 1.7 mg (a.e.)/L.

Up to now, little information is known about the toxicological effects of Roundup® on amphibians. Moreover, the results are mainly related to general toxicological aspects such as the survey, growth, or appearance of malformations, and nothing is known about the mechanisms by which Roundup® may express its toxicity. Since the understanding of the mechanisms by which this herbicide may interact with the normal gene expression is of special interest, we evaluated the potential effects of this relevant environmental pollutant on the protein expression in the brain and liver of metamorphosing *Rana temporaria*.

2. Materials and methods

In an experimental point of view, tadpoles at stage 28 (Gosner index) were exposed to either 1mg or 10µg a.e./L of Roundup® until they reached metamorphosis at stage 44 (Gosner index). For the proteomic analysis, a 2D-DIGE minimal labeling approach coupled to nano flow liquid chromatography tandem mass spectrometry (nano-LC-MS/MS) was applied to detect and identify proteins differentially expressed in the different organs (liver and Brain) and Roundup® conditions.

3. Results and discussion

For the first part of the experiment, when considering the effects of Roundup® on the liver, results showed that 9 spots from the 10µg a.e./L Roundup® condition, and 15 spots from the 1 mg a.e./L Roundup® condition displayed a significant increase or decrease (p<0.05) of abundance compared to the courant water control. In total, 10 proteins were identified. Among these proteins, 3 were commonly identified between both experimental conditions.

The results suggest that Roundup® may impair, in the liver, mechanisms such as the energetical metabolism (glycerol-3-phosphate dehydrogenase, transaldolase), cellular cycle regulation (60S acidic ribosomal protein P0), hepatic proteolysis (monomeric alpha-macroglobulin), mitochondrial function (carbamoyl-phosphate synthase), and hepatic haem biosynthesis (coproporphyrinogen oxidase).

In the second part of the experiment, when considering the effects of Roundup® on the brain, results showed that 30 spots from the 10µg a.e./L Roundup® condition, and 25 spots from the 1 mg a.e./L Roundup® condition displayed a significant increase or decrease (p<0.05) of abundance compared to the
courant water control. In total, 35 proteins were clearly identified. Among other things, functions such as calcium signalling (Annexin A5), neuronal differentiation (Dihydropyrimidase-like3, Dihydropyrimidinase-related protein 5), cellular cycle regulation (Membrane protein-palmitoylated, ndrg2 protein,…), cytoskeleton (Microtubule-associated protein RP/EB family member 1, Alpha-tropomyosin, Putative actin-capping protein Z beta subunit variant 1, vinculin,…), ions transport (ATPase, H+ transporting, lysosomal 70kDa, V1 subunit A 5), and proteins splicing (Splicing factor, arginine/serine-rich 9) were targeted by Roundup®.

4. Conclusions

In conclusion, the present study is the first to highlight impacts of environmental relevant concentrations of Roundup® on the proteome of metamorphosing amphibians. In the liver, it has been shown that the Roundup® toxicity could be related to interactions with well-known mechanisms such as the energetical metabolism, hepatic proteolysis and mitochondrial function. The comparative analysis of protein data sets in the brain enabled to think that biological functions such as cellular signalling, neuronal differentiation and ion transport are also targeted by Roundup®. These data demonstrate that environmentally relevant exposure to Roundup® can deeply modify amphibian proteome and argue that these changes have to be taken into account while estimating the toxicological hazard of wild amphibian populations exposed to those chemicals.

5. References


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The role of metabolization processes in micropollutants bioaccumulation by adult green frogs: comparison of benzo[a]pyrene and fipronil toxicokinetics

Worms IAM¹, Reynaud S¹, Veyrenc S¹, Portier J¹ and Raveton M¹

¹Equipe P3E, Laboratoire d’ECologie Alpine, Université de Grenoble, BP 53, 2233 rue de la piscine, 38041 Grenoble Cedex 9
E-mail contact: wormsisabelle@rocketmail.com

1. Introduction

The implication of micropollutants (MP) in the global amphibian biodiversity decline is nowadays well accepted. However, there is a paucity of data concerning the biophysico-chemical interactions of MP with adult frogs.

Benzo[a]pyrene (BaP), is classified as a priority pollutant on the basis of its known or suspected carcinogenicity especially to fish [1;2]. Effects described in mammals and aquatic animals for fipronil concern endocrine disruption and developmental toxicity [3;4].

Indeed, the aim of this study was to compare the toxicokinetics of benzo[a]pyrene (BaP) and fipronil (fip), two MP deferring by their origin (combustion vs agriculture) and by their chemical properties (log $K_{ow,BaP}=6$ vs log $K_{ow,fip}=2.8$).

2. Materials and methods

Green adults frogs, Pelophylax kl. Esculentus were exposed in glass carboys containing 500mL tap water spiked with benzo[a]pyrene or fipronil to give a concentration of 10 ppb. For the determination of bioaccumulation factors and metabolization study, $^{14}$C-labelled BaP or fip were added in trace amount in the exposure media. After dissection and organic extraction of organs, tissues and whole frog, radioactivity counting as well as thin layer chromatography analysis (TLC) were performed using final extracts made of ethanol according to [5].

3. Results and discussion

3.1. Uptake and bioaccumulation of BaP and fip

Despite the three order magnitude difference in $K_{ow}$ between BaP and fip, similar short term uptake rates, determined on the first day of exposure, were measured. Moreover, frog BAFs measured for fipronil were allways 2-3 times higher than obtained for BaP (data not shown). These results contradict with well accepted idea for which an increase in $K_{ow}$ will lead to higher absorption rate as well as accumulation [6;7].

Results obtained for BaP distribution within frog organs (figure 1A) were highly heterogeneous with higher BAFs found for gall bladder (GB), intestine and kidneys, organes involved in enterohepatic cycle, although other organs, including liver, and tissues were around or below a value of 10.
For fipronil, the distribution was more homogeneous and BAFs found for GB, that is ten-time less than found for BaP, and fat bodies were the same order of magnitude (figure 1B). Based on these results, the general depuration process involving the enterohepatic system, seems more efficient for BaP than for fip and should be the result of the metabolism efficiency of liver detoxification enzymes.

### 3.2. Metabolization

In its main accumulation site that is gall bladder, BaP is found solely as highly polar products even after one day of exposure. This indicates that the liver has the capability to metabolized rapidly benzo[a]pyrene by the mean of oxidation and then conjugation, and this assumption is comforted by the induction we measured in frog liver for both P450 and GST activities after one and two days of exposure.

Although the presence of highly polar products were also found as fipronil metabolites in gall bladder, the main one was identified to be sulfone-fipronil, which has the tendency to accumulate over time in liver, and most of the organs and tissues involved in frog reproduction. The involvement of cytochrome P450 in the oxidation of fipronil into sulfone-fipronil have been reported already reported for mamals and fishes. But according to the ECOD test be used here for activity measurement, a depression in oxidation activities in liver was obtained over the 6 days of exposure to fipronil. In addition, only a slight increase in GST activity was recorded after 4 days of exposure. In the case of fip, it seems that both oxidation and conjugation enzymes were less efficient than for BaP, and the cause of increased-BAF obtained for fipronil.

### 4. Conclusions

Our results show that metabolization of benzo[a]pyrene by adult green frog follows the general process of the enterohepatic systems relates to the induction of several detoxification enzymes (e.g. P450 and GSTs). This general process seems to be limited by the conjugation step of sulfone-fipronil indeed, in all the organs and tissues (despite involved in depuration process) increased-bioaccumulation factors for fipronil, minimum 10-times higher than for BaP, were found. Considering the high level of sulfone-fipronil in organs and tissues involved in female reproduction, it could be anticipated that together with parental molecule long term effects on the global population might occurred, by decreasing the success of fecundation and development of embryos.

### 5. References


Acknowledgement – Many thanks are given to Pr Anne Maitre and the team of TIMC, for usefull discussions on benzo[a]pyrene metabolism.
1. Introduction

The dawn of a new paradigm in energy supply – biofuels – points to the continued expansion of industrial agriculture in the near future. Land for biofuel crop cultivation should expand at least 2 to 3.5 times until 2030 [1] and, considering that no less than 88% of all suitable agricultural land on Earth is located in Central and South America, and in sub-Saharan Africa [2], continents that currently cultivate only 20 and 24% of their total suitable land area, it is reasonable to assume that most of this biofuel crop expansion will take place in the tropics. While expected to result in significant economic gains for tropical countries, this expansion has to be carefully planned and monitored as industrial agriculture is one of the most environmentally harmful human activities, being directly involved in native habitat destruction and in the contamination of water resources through the employment of fertilizers and pesticides.

Brazil provides an ideal scenario for investigating the relationships between land use change due to biofuel crop expansion, environmental contamination, and biodiversity loss. From one side, the country is the world’s largest producer of sugarcane and second largest producer of soybean, which are important sources of ethanol and biodiesel respectively. Brazil also recently became the world’s largest consumer of pesticides, and a recent review demonstrated that 225 and 457 pesticide formulations containing 62 and 133 active ingredients, many of which hazardous to biodiversity, are registered for application in sugarcane and soybean fields in the country [3]. From another side, Brazil harbors an estimated 13% of the world’s biodiversity with no less than 900 amphibian species [4].

This study proposes to test the hypothesis that the expansion of the most important biofuel crops in Brazil - sugarcane and soybean – is associated with significant changes in amphibian community composition, diversity and structure, and that these changes are consistent with agrochemical contamination in the environment. We focused on amphibians because of their importance for the functioning of freshwater ecosystems, because of their conservation status under the threat of population declines, and because of their value as model systems in ecology and ecotoxicology for their excellent tractability in laboratory, mesocosm and field experiments - which are complementary components of our research program.

2. Materials and methods

This study is based on field surveys in replicated water bodies distributed across a gradient of environmental degradation, i.e., that comprised of native habitats, pastures and plantations. Although native habitats offer the appropriate reference sites for assessing the impacts of agricultural expansion overall, a comparison between pastures and plantations offers stronger insights in the assessment of eventual impacts of agrochemicals, as both are structurally degraded but plantations are, in addition, subject to agrochemical contamination.

We compared the correlates of land use on amphibian communities across gradients in environmental degradation in two locations: one representing a landscape dominated by soybean (Fazenda Tanguro and surroundings, headwaters of the Xingu River in Mato Grosso, Southern Amazon; gradient represented by transitional cerrado-Amazonian rainforest, pastures and soybean fields) and one dominated by sugarcane (Estação Ecológica do Jataí and surroundings, State of São Paulo; gradient represented by cerrado forest, cerrado grasslands, pastures and sugarcane fields).

We conducted field surveys for amphibian larvae employing pipe sampling with sampling effort scaled to pond surface area. In sugarcane landscapes, in addition, pipe sampling was complemented by dipnetting for retrieval of rare species, and calling surveys for improving identification to species level. Larval sampling was preceded by habitat characterization and analyses of basic water quality parameters. We also collected water, sediment and tadpoles for analyses of residues of metals of ecotoxicological relevance, and pesticides. Sampling surveys were conducted twice in a breeding season, i.e., early and mid-rainy season.
November 2008 and April 2009 for soybean, November 2009 and April 2010 for sugarcane. Number of ponds sampled equaled 36 (soybean) and 31 (sugarcane).

3. Results and discussion

Overall, we found a strong signal of land use on amphibian communities both in sugarcane and soybean landscapes. As for sugarcane landscapes, amphibian community composition across the gradient appeared to be nested, with amphibian species in plantations being a subset of those found in pastures, and those in pastures being a subset of those found in cerrado forests. Nevertheless, some species appeared to be favored by conversion to plantation; *Leptodactylus fuscus*, for example, found in 6 of 10 ponds in cerrado forest, and in 1 of 10 ponds in pastures, was found in 11 of 12 ponds in sugarcane fields, where it was also very abundant. As for soybean landscapes, amphibian community composition in soybean fields was a subset of that found in pastures. However, amphibians were more frequent, dense, abundant and diverse (at least at the genus level) in pastures than in plantations or forests. This result, at first surprising, could result from the difficulty in sampling rare species (comparatively more frequent in diverse habitats), from an increase in species with direct development or non-aquatic larvae in the forest, or from the original patchiness of forested and open areas in the region, i.e., forest clearing could actually have benefited common grassland species.

Overall, a loss of species as one goes from pastures to plantations is consistent with an effect for habitat contamination, and we witnessed die-offs in amphibian larvae of several species in ponds in sugarcane fields that could be consistent with the timing of application of pesticides. Likewise, in soybean fields strong community level effects appeared to occur after pesticide applications. We are currently attempting to associate more closely the timing of pesticide applications and biodiversity sampling. We are also conducting analyses on residue levels in field collected tadpoles to understand to what degree amphibian species are subject to agrochemical contamination and how do these residue levels correspond to those accumulated by conspecifics exposed in the laboratory and mesocosms to commonly used pesticides.

4. Conclusions

- Land use in agroindustrial landscapes had strong effects in amphibian community composition, structure, and diversity.
- An impoverishment of species as one moves from pasture to plantations in both soybean and sugarcane-dominated landscapes is consistent with an effect for agrochemical contamination in influencing community composition and structure. Die-offs and impoverished amphibian communities in ponds in plantations following pesticide applications are also consistent with a hypothesis of relevance of agrochemicals as factors influencing amphibian distributions. However, more observational and experimental studies have to be conducted to establish a link between environmental contamination and species distributions and therefore community structure.

5. References


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Atrazine and Glyphosate in the Environment and its Implications for Amphibians

Christine A. Bishop¹ Tana V. McDaniel² Sara L. Ashpole³, Shane R. de Solla²

¹Environment Canada, 5421 Robertson Road, Delta, British Columbia Canada V4K3N2
²Environment Canada, Box 5050, Burlington Ontario Canada L74 4A6
³Dept. Of Planning, University of Waterloo, 200 University Avenue West
Waterloo, Ontario Canada N2L 3G1
Email contact: cab.bishop@ec.gc.ca

1. Introduction

Herbicides are the top selling pesticides on a global basis. Glyphosate and atrazine are the highest selling herbicides today. Although atrazine has been banned in the European Union, it is still used in north and south America, and Asia. Glyphosate is used globally. While they are not highly persistent, herbicides persist long enough that amphibians, and to a lesser extent reptiles, can be exposed as eggs, juveniles and adults to intermittent yet chronic concentrations of herbicides throughout their lifetime.

2. Materials and methods

Our review of the literature found that, initially registered by Ciba-Geigy in 1958, the triazine herbicide atrazine which inhibits photosynthesis swiftly replaced 2,4-D as the dominant herbicide for use on field corn since it offered selective weed control and reduced damage to crops. In Asia, atrazine use is on the rise. In China, in 2002, use was estimated at 2,273 tonnes per year and is expected to continue increasing (Ren et al. 2002).

Monsanto Corp. (St. Louis, MO USA) began to commercially produce glyphosate in 1974. Its mode of action is to disrupt amino acid production in plants. But this chemical did not easily penetrate the waxy cuticle of plants, so a surfactant was typically added or it was recommended by the manufacturer to add a surfactant to dissolve glyphosate on the plant surface and enter the plant. The most common surfactant used with glyphosate a derivative of animals fat: polyethoxylated tallowamine (POEA). Because this combination was toxic to many types of broadleafed plants, it was only after the introduction of glyphosate-resistant genes into crop plants that the use of glyphosate really took off. Roundup-Ready® crops include corn, soybeans, cotton, canola and sugar beets. The weeds would die but the crops were untouched by the herbicide. Glyphosate is now the highest selling herbicide in the world (Baylis 2000). Monsanto is largest producer, but other companies produce glyphosate-based herbicides as well.

3. Results and discussion

The effects of atrazine on amphibians in particular have received wide attention in recent years (Hayes et al. 2002; Hecker et al. 2004, Kiesecker 2002, Rohr et al. 2008 a, b) to the extent that the use of atrazine has been reviewed within the USA based solely on its potential to affect gonadal development in amphibians (Steeger et al. 2007). The ecosystems and food webs inhabited by herpetofauna may also be altered by atrazine (Rohr et al. 2008a,b). There is a growing realization that the impacts of herbicides directly on amphibians and reptiles and indirectly on ecosystems may have bottom up effects on the health and survival of amphibians. Immunosuppression, particularly when herpetofauna are exposed to herbicides in combination with other pesticides and/or certain water chemistry profiles may lead to disease or increased exposure to parasitism (Christin et al. 2003, 2004). Our field studies in Canada, indicate that even hatching of amphibian eggs may be affected by atrazine exposure combined with other pesticides but this can be species specific (Bishop et al. 2010). In the south Okanagan valley, an arid but key agricultural and viniculture of Canada, is a classic location where high biodiversity and human development meet. The intensive use of pesticides combined with loss of habitat and degradation of habitat due to other factors (irrigation, introduced fish and amphibians) led our team to counter by creating new habitats for amphibians with conservation covenants in the hope that this will preserve the many species at risk that depend on water in this desert region.

Glyphosate combined with its surfactants can be highly toxic to amphibians. As a result of this toxicity, its use was restricted from overspray on water courses and wetlands in Australia. It is still used extensively with aerial application in Canada in forestry operations in order to kill broadleaf plants that compete with conifers.
desirable for wood and paper production. As with atrazine, studies in mesocosms also indicate glyphosate has potential community level effects that can affect amphibians (Relyea 2003, 2004a, b).

4. Conclusions

Until recently, these compounds were registered and their toxicities to organisms have been extensively reviewed without due regard for the unique sensitivities of amphibians and potential bottom up effects of herbicides on wetland ecosystems.

5. References


Acknowledgement- Pesticide Science Fund.
1. Introduction

Molluscs are raising attention as ecotoxical test organisms due to their high diversity and importance in aquatic ecosystems. The ovoviviparous prosobranch gastropod *Potamopyrgus antipodarum* (freshwater mudsnail) responds very sensitively to xenobiotics and has therefore been proposed as OECD standard test organism [1]. Endocrine disrupting chemicals influence the reproduction of *P. antipodarum* which can be assessed by embryo numbers in the broodpouch. Estrogenic substances cause an increase in embryo numbers, androgenic a decrease. However, the knowledge about the endocrine system of *P. antipodarum* is rather limited. We investigate in this study if *P. antipodarum* possesses an estrogen receptor (ER) and if this receptor is differentially expressed under (xeno-)hormone exposures.

2. Materials and methods

2.1. Isolation of *Potamopyrgus*-estrogen receptor

To identify the *Potamopyrgus*-ER whole tissue of 25 snails was pooled, RNA extracted and cDNA synthesised. PCRs were conducted with primers derived from the gastropod *Nucella lapillus* ER (GenBank: EF591073). The amplified fragment was cloned and sequenced. The *Potamopyrgus*-ER sequence was elongated with primers designed from the ER-fragment. The deduced amino acid sequence was compared with sequences from GenBank on the National Center for Biotechnology Information (NCBI) server (http://www.ncbi.nlm.nih.gov).

2.2. (Xeno-)hormone exposure and quantitative real-time PCRs

To quantify the ER-response to (xeno-)hormones 20 individuals of *P. antipodarum* were exposed each to 17α-ethinylestradiol (25, 50, 100 ng/L), bisphenol A (40 µg/L) and 17α-methyltestosterone (300 ng/L) for 1, 7, 28, and 56 days. Water and solvent (dimethyl sulfoxide) controls were included. After exposure total RNA was isolated. TaqMan™ probe and primers for the identified ER were established and quantitative Real-Time PCRs were performed.

3. Results and discussion

3.1. Identification of *Potamopyrgus*-estrogen receptor

*Potamopyrgus antipodarum* possesses an ER. The DNA-binding domain (fig. 1) has an amino acid identity of 92% compared to the ER of *Nucella lapillus* (84% to human ERα) and 83% in the ligand-binding domain (38% to human ERα).
3.2. Differential expression of Potamopyrgus-estrogen receptor

The Potamopyrgus-ER is transcriptionally regulated as demonstrated by quantitative Real-Time PCRs of (xeno-) hormone exposed snails. Exemplarily shown are the results after 56 days of exposure to ethinylestradiol (EE₂). The ER-expression-level in control (NC – only water) and solvent control (SC – dimethyl sulfoxide) is similar to snails exposed to 25 ng EE₂/L (fig. 2a). But an exposure to 50 ng EE₂/L results in an earlier exponential amplification (fig. 2a) and hence in a lower cycle threshold (fig. 2b) implying an up-regulation of the ER.

4. Conclusions

Potamopyrgus antipodarum possesses an ER-like transcript which is regulated under exposure of environmentally relevant (xeno-)hormone concentrations. Due to the sensitivity (in-/decreased embryo numbers and transcriptional regulation of ER) P. antipodarum is a promising test organism.

5. References


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Quantification of vitellogenin by mass spectrometry (LC-MS/MS) in the freshwater amphipod, *Gammarus fossarum*: 
a potential endocrine disruption biomarker

Guillaume Jubeaux¹, Romain Simon², Fabien Audouard-Combe¹, Hervé Quéau¹, Arnaud Salvador², Arnaud Chaumot¹, Jeanne Garric¹, Olivier Geffard¹.

¹ Cemagref, Unité de Recherche MALY, Laboratoire d’écotoxicologie, Lyon, France.  
² Université de Lyon1, UMR 5180, Sciences Analytiques, Villeurbanne, France. 
E-mail contact : guillaume.jubeaux@cemagref.fr

1. Introduction

Vitellogenins (Vg) are the precursor of the egg-yolk proteins, vitellins, which provide energy reserves for embryonic development in oviparous organisms. Induction of Vg in males or juveniles is a well known effect of xenoestrogenic contaminants in fish, and has been extensively used as biomarker both in laboratory and field studies [1]. Some methods have been proposed for the quantification of Vg in fish and other vertebrate species. Among them, immunological assays (ELISA kits) have been developed for a variety of species. Despite their obvious ecological importance, relatively few works have been carried out on invertebrates and consequently few tools are available to diagnose an endocrine disruptor exposure, although there is growing evidence that crustaceans, such as amphipods, may be susceptible to these compounds. At present, only indirect methods, such as the organic alkali-labile phosphate (APL) assay, have been proposed and applied in invertebrates species. From an analytical point of view, efforts should also be directed toward developing more specific methods to measure Vg levels in invertebrates. In this aim, absolute quantification of proteins using mass spectrometry (LC-MS/MS) has thus naturally emerged as an alternative approach.

In this context, the aim of this study was to develop and propose a new methodology allowing a specific measurement of the Vg in an ecologically and ecotoxicology relevant species, the freshwater amphipod, *Gammarus fossarum*. For this end, we followed an approach in three steps, i) we developed a quantitative SRM (Single Reaction Monitoring) assay for the detection of proteotypic Vg peptide, ii) the specificity of the prototypic peptide as indicator of the functional Vg was validated, by assessing its natural variability during the reproductive cycle of female organisms and iii) the use of this new specific endocrine disruption biomarker was evaluated, by studying the modulation of the proteotypic peptide in relation to an exposure to known endocrine disruptors in Crustaceans.

2. Experimental approaches

2.1. Development of Vg analysis by mass spectrometry

The aim of development step is to detect by mass spectrometry peptides deduced from amino acid sequence (197 aa ; Genbank number accession : GU985184). After tryptic digestion of a sample of vitellogenic oocytes, we have selected eight peptides showing best analytic intensities. To verify analyte specificity, we have measured them in male gonad and in previtellogenic and vitellogenic oocytes discared from mature females. Thereafter, possibility of individual measurement has been tested and confirmed. ILIPGVGK has shown the best intensity and specificity and has been selected for development of an absolute quantification method. Vg is quantified by adding valine labelled peptide (ILIPGV*GK) in sample. Linearity of quantification has been checked.

2.2. Validation of Vg function

The second step aims to check natural variability of the Vg peptide ILIPGVGK during oocyte maturation (when vitellogenesis takes place) and embryo development (when accumulated vitellins were metabolized) [2] (Fig.1). Our results show : i) a great correlation between oocyte growth (µm²) and Vg content, and ii) a strong decrease of Vg content in relation to embryonic development. These results validate the use of ILIPGVGK peptide as a reliable method for Vg quantification.
2.3. Modulation of Vg

In this part, we will show experiments which have been set up to evaluate the potential use of this assay as a specific biomarker of endocrine disruptor exposure. For this, female organisms have been exposed to two compounds known to interact on endocrine regulation of crustacean, the 20-hydroxyecdysone and the methyl-farnesoate (Fig.2). Our results show that Vg synthesis was modulated by these two compounds, whereas no effects on the moulting cycle was observed. Consequently this tool can be proposed as a reliable method to evaluate impact of endocrine disruptors in this species.

3. Conclusion & Research needs

► A rapid and specific method for Vg measurement in *G. fossarum* is now available [3].
► This method permits to highlight Vg modulation after exposure to endocrine disruptors.
► Consequently, it can be proposed as a specific biomarker of endocrine disruption.
► In perspectives, we will study the pertinence and the possibility to use this tool in males.
► We will give toxicological relevance of this measurement by highlighting the link between Vg modulation and offspring impairments.

4. References

H295R cells as a steroidogenic model: A broader picture using simultaneous chemical analysis of 7 key steroid hormones exposed to 3 endocrine disruptors

Frederik Knud Nielsen1, Cecilie Hurup Hansen1, Jennifer Anna Fey1, Martin Hansen1, Naja Wessel Jacobsen1, Erland Björklund1 and Bjarne Styrishave1

1Copenhagen University, Faculty of Pharmaceutical Sciences, Universitetsparken 2, DK-2100 Copenhagen, Denmark. E-mail contact: fkn@farma.ku.dk

1. Introduction

Much effort has focused on substances interfering with hormone function, and more recently research on substances that interfere with the natural production of hormones has developed. Important work in this field is the validation of the H295R cell line as a steroidogenic model, used for detecting effects on the adrenocortical production of steroid hormones [1]. Focus has been directed mainly on the production of estradiol, testosterone, progesterone, and expression of enzymes involved in the steroidogenic pathway, however expression of enzymes does not always reflect hormone production [1,2]. Determining the simultaneous production of several hormones throughout the pathway will contribute to a better understanding of effects on the steroidogenesis. Other steroid hormones of endocrinological importance produced in the steroidogenic pathway in H295R cell line are pregnenolone, progesterone, dehydroepiandrosterone, androstenedione, estrone, aldosterone, corticosterone and cortisol.

Hormone measurement in the low ppb concentration range are often indirect methods such as RIA and ELISA using commercially available assays [3]. Even though such methods are sensitive they also have several disadvantages. ELISA kits provide only information on steroids one at a time, and in some progesterone assays cross-reactivity is substantial, amounting to 61% for pregnenolone [1]. Gazdar et al [4] showed that in the H295 cell line some of these steroid hormones were detectable with GC and reported that pregnenolone was quantifiable while progesterone was not detected. Measuring progesterone in such cell lines, using an ELISA assay with 61% cross-reactivity, may therefore lead to substantial errors clearly demonstrating that new methods for steroid measurements are in demand.

The GC-MS-MS method applied in the present study offers the analysis of several steroids in the low ppb range with high-selectivity and quantification of individual steroid simultaneously in individual samples. In the present study prochloraz, ketoconazole and genistein were tested, as previous studies have given inputs to understanding where in the steroidogenic pathway they interfere. However, by applying simultaneous chemical analysis of 7 key steroid hormones in the pathway, a better understanding of the rate limiting steps can be achieved.

2. Materials and methods

The H295R steroid hormone synthesis assay was performed (with minor modifications) and validated as described in the draft OECD H295R assay guideline (http://www.oecd.org/dataoecd/56/11/44285292.pdf). In short, the cells were grown in 24 well plates (COSTAR, Bucks, UK). 3×10⁵ cells in 1 ml supplemented medium were plated into each well, and the cells were allowed to settle for 24 hours. The medium was changed after 24 hours and the test compounds were added. Each compound was tested in 7 concentration levels on three plates and the experiment was repeated on two different days. On each test plate a solvent control (SC) was included, either methanol or DMSO. Maximum solvent concentration was 1% known not to affect the cells. After 72 hours of incubation, the medium was carefully removed, adjusted to pH 3 with diluted phosphorous acid and stored at -20°C for later analysis of steroid hormones on the GC-MS/MS.

All chemical analysis of steroid hormones was done according to a simplified version of the method described by Hansen et al [5]. In short, 3 pooled wells (3000 µl) of cell medium was added 20ng deuterated internal standards and subsequently concentrated on 500 mg Bond Elut C18 solid phase extraction cartridges (Varian Inc., CA). Steroids were eluted with 5 ml acetone and 20 ng of AE2 was added as a derivitization standard. The liquids were evaporated to dryness, and the residues derivatized with TMS for one hour at 60°C. The liquid was then evaporated and the residues dissolved in 200 µl heptane containing 20 ng of MeE1 as instrument standard and analyzed using GC-MS-MS. The analytical system consisted of a Varian 3800 gas chromatograph (Varian Inc, CA) with a large-volume programmable-temperature-vaporizer (PTV) injector coupled to a Varian 1200 triple-quadrupole mass spectrometry system (Varian Inc, CA).
3. Results and discussion

Although all 3 test compounds also lead to decreases in testosterone and 17β-estradiol as previously described in the literature, effects are observed on all other analyzed steroids. These data also demonstrate that the steroidogenic pathway is disturbed at different stages by the 3 test compounds (Table 1).

![Table 1](https://example.com/table1.png)

Table 1: Arrows indicate increasing or decreasing hormone concentrations in media from H295R cells exposed to prochloraz, ketoconazole and genistein for 72 hours. Increases are also indicated by bold text and shading. Corresponding EC50 values have been stated where 4 parameter log logistic regressions could be fitted, otherwise N.A is stated. Italics are used where no trend for increase or reduction could be identified. Absolute concentrations of hormones in solvent control media from H295R cells after 72 hours is shown. Asterisk(*) indicates measurements between LOQ & LOD.

Exposure to prochloraz resulted in significant dose dependent increases in hormone concentrations upstream of 17-alpha hydroxylase and 17,20 lyase. Downstream dose dependent reductions were observed for DHEA, androstenedione and estrone, while no clear trend was visible for testosterone and 17β-estradiol.

Exposure to ketoconazole resulted in significant dose-dependent decrease in all measured hormones, except progesterone, downstream of cholesterol side-chain cleavage enzyme, indicating that this enzyme or earlier processes are affected by ketoconazole.

Exposure to Genistein resulted in significant dose-dependent increases in hormone levels upstream of the 3-Beta hydroxysteroid dehydrogenase. Downstream dose-dependent reductions of androstenedione and estrone were observed while tendencies of reduced levels of testosterone and 17β-estradiol were present. No trend was visible for progesterone.

Progesterone levels in solvent controls was a factor of 22 lower than pregnenolone, demonstrating that using ELISA kits to measure progesterone with 61% cross reactivity to pregnenolone will lead to serious overestimations of progesterone levels. Progesterone levels have been reported in the 7-14 ng/ml range [1,2] using ELISA kits but the present data (table 1) suggest that the actual levels in the H295R cell line are considerably lower. Estrone levels were a factor of 7 higher than 17β-estradiol which also indicates risk of overestimating estradiol levels using ELISA kits with cross reactivity to estrone.

4. Conclusions

Applying the analytical method used in this study to measure hormone production in the H295R cell line offers significant advantages compared to immunoassays.

- Accurate simultaneous hormone baseline measurements of seven key hormones (pregnenolone, progesterone, dehydroepiandrosterone, androstenedione, estrone, testosterone and 17β-estradiol)
- No cross reactivity
- Differentiation of where the steroidogenic pathway is affected by test compounds

5. References

Effect Directed Analysis of estrogenic effects in sediments of the river Elbe

Sebastian Schmitt, Georg Reifferscheid, Evelyn Claus, Michael Schlüsener, Sebastian Buchinger

Federal Institute of Hydrology, Am Mainzer Tor 1, D-56068 Koblenz / Germany
E-mail contact: buchinger@bafg.de

1. Introduction

Endocrine disrupting chemicals (EDC) are listed in the ANNEX VIII of the WFD as compounds that are concerned to have reasonable effects on human health and wildlife. The high ecological relevance of estrogenic potentials was evidentiary demonstrated for example by Kidd et al. 2007 (1). It was concluded that concentrations of estrogens and their mimics observed in freshwaters can impact the sustainability of wild fish populations.

Many attempts have been made to identify contaminants that are responsible for observed estrogen effects in a number of environmental water and sediment samples (2,3). The estrogenic effect is often higher than expected based on the assumption of concentration addition which is unsatisfying as it may be possible that substances adding up to the observed effect stay undetected and unconsidered (3,4).

In order to further identify compounds that might contribute to the overall estrogenic potential of sediments in the river Elbe an organic sediment extract was characterized by effect directed analysis. Furthermore, the estrogenic potential of mixtures of the structurally identified compounds was analyzed in order to assess the mixture toxicity of the substances that are present in the environmental sample.

2. Materials and methods

Sediment samples were extracted with an accelerated solvent extractor (ASE 100) and separated via liquid chromatography using a preparative column into five fractions with increasing polarity. The total extract and all fractions were tested for estrogenicity with the yeast estrogen screen (YES) (5). Positive fractions were analyzed by GC/MS and LC/MS. Liquid chromatography was performed on a Synergi RP-MAX column and a SecurityGuard / Phenomenex, Torrance, USA. An Orbitrap mass analyser was used at a resolution of R=100.000.

3. Results and discussion

Only the most polar fraction (fraction 5) of the analyzed sediment extract showed an estrogenic potential. The fraction 5 was analyzed by GC and LC/MS. Structurally identified compounds were quantified and the estrogenic activity of the pure compounds determined by the YES (Section 3.1). The assessment of mixture toxicity is shown in section 3.2.

3.1. Identified sediment associated (xeno)estrogens

The Figure 1 shows the dose-response relationships of all estrogen-active substances that were structurally identified in the sediment fraction together with the respective sediment concentrations. Beside the natural estrogens estradiol and estrone the xenoestrogen nonylphenol (as a technical mixture) was found in higher concentrations (2.2 mg/kg). These three compounds contribute most to the overall estrogenic potential of the sample. Interestingly, the antiseptic chlorophene (o-benzyl-p-chlorophenol) showed an estrogenic potential in the YES with EC50 values of 0.8 mg/L. No estrogenic activity is predicted for o-benzyl-p-chlorophenol by QSAR-methods (Danish-QSAR). Chlorophene was also reported to have no estrogenic potential in the ER-CALUX assay (6). Chlorophene is a widely used antiseptic in hospitals and personal care products as well as in household formulations. It is most likely that this compound is realeased in the environment by sewage treatment plants due to its only partial removal.
3.2. Mixture toxicity

For the estrogenic compounds concentration additivity could be shown as to be expected because of the same mode of action. Compounds with no estrogenic potential as pure substances were included in these experiments in order to determine if they could modulate estrogenic effects. It was found that the estrogenic potential of the technical nonylphenol increases with the concentration of tocopherol in the sample (Fig. 2).

4. Conclusions

Nonylphenols are the main anthropogen contaminants, but natural estrogens like estradiol and estron are accountable for the effect as well. The disinfectant chlorophene shows a xenooestrogenic potential in the YES that is comparable to those of some nonylphenoles. The analysis of estrogenic potentials of mixtures comprising of both, xenostrogens and non-active substances indicate a modulation of the estrogenic effect by compounds that do not stimulate the estrogen receptor by themselves. The non-active compound tocopherol enhanced in a concentration dependent matter the estrogenic potential of the technical mixture of nonylphenols. This finding indicates the importance of mixture effects and underlines the need for bio-assays for the integrative analysis of environmental samples.

5. References

Long-term effects of a binary mixture of perfluorooctane sulfonate (PFOS) and bisphenol A (BPA) in zebrafish

Susanne Keiter¹, Harald Färber², Henrik Holbech³, Dirk Skutlarek², Magnus Engwall⁴ and Thomas Braunbeck¹

¹Department of Zoology, Heidelberg University, 69120 Heidelberg, Germany
²Institute for Hygiene and Public Health, University of Bonn, 53105 Bonn, Germany
³Institute of Biology, University of Southern Denmark, 5230 Odense M, Denmark
⁴Man-Technology-Environment Research Center (MTM), Academy of Science and Technology, Örebro University, 701 82 Örebro, Sweden

Email contact: jernbro@zoo.uni-heidelberg.de

1. Introduction

The frequent use of perfluorinated chemicals (PFCs) in industrial applications and domestic products has led on a global basis to a continuous detection of PFCs in a wide range of environmental matrices including aquatic systems. Perfluorooctane sulfonate (PFOS) is the most commonly detected PFC in biotic and abiotic samples. To date, the understanding of the potential effects of PFOS towards biological systems has reached substantial progress. However, the majority of studies have focused on acute effects, leaving long-term effects largely unexplored. Given the persistent properties of PFOS and other PFCs, chronic effects following low-dose exposure is a topic of great relevance for both human and environmental health. As PFOS has been reported to possess membrane-altering properties [1, 2] a long-term assessment in combination with other pollutants should be a promising strategy to shed more light on the complex toxicology of PFOS. Since PFOS has been shown to act as an endocrine disruptor in fish [3] a combined investigation with another endocrine disrupting chemical (EDC) would represent an approach where specific endpoints such as sex steroid levels could be measured and compared, thus providing a more direct hint of any interactive effects.

In the present study we investigated the effects of waterborne PFOS both alone and in a binary mixture with the known EDC bisphenol A (BPA) over two full generations of the zebrafish (Danio rerio).

2. Materials and methods

The long-term study was designed to allow a simultaneous evaluation of PFOS, BPA and a binary mixture of them at exposure concentrations covering ecologically relevant concentrations. A flow-through system was applied, and endpoints such as survival, growth, reproductive success, vitellogenin (VTG) and histological alterations in thyroid, liver and gonads were examined. Data were collected at 90 and 180 days post-fertilization (dpf) and were compared in between the treatments as well as in between the two generations.
3. Results and discussion

Considering the global distribution of PFOS in wildlife and humans, the interactive effects of PFOS with other compounds could represent a cause for potential environmental and human health risks. In a previous study [2], we could show that PFOS increased the genotoxic potential of cyclophosphamide (CPP) *in vitro*, thus supporting the theory of a synergistic potential of PFOS when present in a chemical mixture. Our results in this *in vivo* study indicate no synergistic effects of PFOS when combined with BPA. PFOS (300 µg/L) was found to reduce growth in F1 generation and to cause 100 % mortality of the F1 generation offspring within 7 dpf. PFOS (300 µg/L) was further found to induce lipid accumulation in liver of F1 generation fish. A parallel finding in PFOS (300 µg/L) exposed fish was the occurrence of granuloma, presumably as a result of bacterial infection. Identical granuloma structures were detected in lower PFOS concentrations in F2 generation fish, indicating a suppressed immune system over generations. PFOS has previously been reported to assert immunotoxicological effects [4] thus supporting the findings in this study. An interesting observation was the depressed plasma VTG concentrations with PFOS exposure in both generations. Similar observations were made in a previous study [3] where higher concentrations of PFOS were shown to decrease sex steroid concentrations. It was speculated whether this phenomenon could be attributed to a PFOS-reduced serum cholesterol level. An increased β-oxidation of fatty acids by PFOS [5] could possibly serve as an alternative explanation.

4. Conclusions

- BPA exposure induced elevated plasma VTG levels whereas the opposite was true for PFOS.
- PFOS did not increase the endocrine disrupting potential of BPA, when present in a binary mixture.
- PFOS (300 µg/L) caused an accumulation of lipid droplets in the liver of males.
- The occurrence of granulomas in lower PFOS concentrations in F2 generation fish compared to F1 generation fish implies a suppressed immune system over generations.
- No reproductive effects were found.

5. References


Contamination of river ecosystem by compounds with specific modes of action assessed in biotic and abiotic matrices using in vitro bioassays

Veronika Jálová1, Tomáš Ocelka2, Roman Grabin2, Jana Jurčíková2, Jarmila Halířová3, Jana Blahová4, Zdeňka Svobodová4, John P. Giesy5, Klára Hilscherová1, Luděk Bláha1

1Research Centre for Toxic Compounds in the Environment (RECETOX), Masaryk University, Kamenice 126/3, CZ625 00 Brno, Czech Republic
2Institute of Public Health Ostrava, National Reference Laboratory for POPs, Ostrava, Czech Republic
3Czech Hydrometeorological Institute, Brno, Czech Republic
4Dept. of Veterinary Public Health and Toxicology, University of Veterinary and Pharmaceutical Sciences, Brno, Czech Republic
5Dept. of Biomedical Veterinary Sciences and Toxicology Centre, University of Saskatchewan, Saskatoon, Saskatchewan, Canada
E-mail contact: jalova@recetox.muni.cz

1. Introduction

Wide range of synthetic and natural compounds present in the environment can cause various adverse effects in organisms, e.g. modulating of endocrine system, reproduction, developmental processes, immune system, behavior and/or central nervous system [1, 2]. City agglomerations represent a source of contamination by chemicals of different origin and action. Major sources of endocrine-disruptive compounds in aquatic environment include municipal and industrial waste waters. Despite generally efficient treatment processes in wastewater treatment plants, some compounds still can enter aquatic environment and pose a risk for wildlife (e.g. feminization of fish associated with exposure to effluents from WWTPs).

Monitoring studies dealing with assessment of contamination by endocrine-disruptive compounds in the aquatic environment are often focused on abiotic matrices, mainly sediments, surface and waste waters. However, presence of environmental contaminants in abiotic compartments does not necessarily indicate uptake and potential effects on organisms. In this context, use of biological samples (e.g. fish tissue) for biotests should provide more accurate information about contamination and its reflection in organisms.

The aim of a two year long study was the complex assessment of contamination by compounds with specific modes of action in river ecosystem and evaluation of impacts of city agglomeration with large municipal wastewater treatment plant. Specific activities, namely dioxin-like, anti/estrogenic, anti/androgenic, and also cytotoxic effects have been determined in abiotic and biotic matrices using battery of in vitro bioassays.

2. Materials and methods

The studied area of the second largest city in the Czech Republic (400,000 inhabitants) with variety of industrial activities and large WWTP is spread in the basin of two rivers. The WWTP belongs to the most modern WWTPs in the Czech Republic and provides mechanical, biological and chemical treatment of municipal and industrial waste waters from the city of Brno and wide surroundings.

Contamination of aquatic environment by endocrine-disruptive compounds has been assessed in three types of matrices: surface and waste waters sampled by passive samplers, sediments and fish bile. Sediments were collected from selected locations in spring and autumn seasons (for two years; 4 sampling campaigns). River water and waste waters from influent and effluent of the WWTP were sampled using two types of passive samplers – semipermeable membrane devices (SPMD) for hydrophobic compounds and polar organic chemical integrative samplers (POCIS) for polar pesticides and pharmaceuticals. In addition to abiotic samples and for better characterization of direct effects of aquatic organisms, the chub (Leuciscus cephalus L.) was selected as the most suitable indicator species. Organic extracts of sediments, passive samplers (SPMD, POCIS) and fish bile have been tested by battery of in vitro bioassays with recombinant yeast and mammalian cell lines. Four reporter gene bioassays have been used to measure receptor-mediated activities of organic extracts of abiotic and biological samples. Total steroid/dioxin-like activity was assessed in stably transfected cell lines containing a steroid/dioxin-like responsive element linked to a luciferase reporter gene. Hormonal effects were examined either singly or in co-exposure with competing endogenous ligand (17β-estradiol, dihydrotestosterone). Dioxin-like activity elicited via aryl hydrocarbon receptor (AhR) was determined with H4IIE-luc (rat hepatoma cell line) bioassay, the estrogen receptor (ER)-
mediated activity in MVLN cells (human breast carcinoma) [3]. Anti/androgenicity and glucocorticoid activity was assessed in a bioassay with MDA-kb2 cells (human breast carcinoma) [4]. Biotest with Saccharomyces cerevisiae yeast strain served for assessment of cytotoxicity [5]. Relative potency estimates (BIOTEQ, EEQ, AEQ), antiestrogenic, antiandrogenic and cytotoxic effects have been calculated according to Villeneuve et al. [6] and Novak et al. [3].

3. Results and discussion

Cytotoxic effects were detected after exposure to all types of samples. Cytotoxicity in sediments increased in both rivers after flowing through the city and also downstream of the WWTP. However, there was no evidence in passive samplers extracts that the city impacted river water, resp. no increase in cytotoxicity. Results of bioassays with passive samplers extracts demonstrated very efficient removal of cytotoxic compounds during the waste water treatment in the WWTP.

Dioxin-like (AhR-mediated) activity was detected in sediments and SPMD extracts from most sampling sites. The POCIS exerted dioxin-like activity mostly in the samples from WWTP with higher activity in effluents than in influents. This can be caused by lower removal efficiency of contaminants, release of particle-bound pollutants during WW treatment or incomplete sampling of waste water.

Estrogenic and androgenic activities were observed only in POCIS samples from the WWTP and in fish bile extracts. Results showed significant decrease of estrogenicity and androgenicity after treatment of waste waters. Antiestrogenic and antiandrogenic activities were detected in all abiotic matrices from most sampling sites.

Despite the seasonal variability there was a pattern in sediment samples with the lowest effects in upstream locations and increased activity downstream of the city for cytotoxicity and dioxin-like activity. Passive samplers showed mostly considerable variability with no apparent trends.

4. Conclusions

The results of in vitro bioassays documented presence of cytotoxic, dioxin-like, antiestrogenic and antiandrogenic compounds in all abiotic matrices. Fish bile samples elicited mainly cytotoxic, estrogenic, androgenic and low dioxin-like activities. Passive samplers extracts showed efficient elimination of compounds with cytotoxic, estrogenic and androgenic activities by treatment processes in the WWTP. The biotest results indicate that both hydrophobic and hydrophilic compounds contributed to the observed biological activities. Bioassays enabled complex assessment of contamination of abiotic and biological matrices in river ecosystem by endocrine-disruptive compounds and possible risks for aquatic organisms.

References


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A novel type of endocrine disrupting effect: Octylphenol and 17β-oestradiol cause malformations in eelpout embryos

Poul Bjerregaard, Randi V. Nielsen, Nanna Brande-Lavridsen, Bodil Korsgaard
Institute of Biology, University of Southern Denmark, Campusvej 55, DK-5230 Odense, Denmark
E-mail contact: poul@biology.sdu.dk

1. Introduction
In recent years, the eelpout has been used as a monitoring organism in the environment by several Baltic countries because the viviparous life history of this species allows detection of malformations among the offspring, contrary to the situation in oviparous fish species where seriously malformed fish larvae would have a lower probability of being detected in the environment.

Monitoring programmes (i.e. in Denmark and Germany) have revealed increasing frequencies of malformations among eelpout embryos during recent years [1,2]. Malformations can be induced in oviparous fish species by exposure to chemicals in the laboratory and the implicit assumption in the eelpout monitoring programmes is that the observed increase in malformations might be caused by changing environmental conditions – including exposure to chemicals. In a recent review of Danish data on malformations, potential sources of discharge of chemicals, oxygen depletion, etc., it was concluded that increased frequencies of malformations were associated with high anthropogenic input to the coastal areas, but no specific chemical or groups of chemicals could be pinpointed as causal agents [3].

Although exposure to chemicals is suspected as causal agents, the published scientific literature shows no experimental evidence linking malformations to exposure to chemicals. This lack of experimental evidence is probably caused by the challenges involved in the investigation of teratogenic effects in the eelpout. Female eelpout undergo vitellogenesis and oogenesis during spring and summer and the mating normally takes place during late August – early September. The fertilised eggs stay in the ovary and they hatch there after approximately one month’s larval development. Unpublished observations from our laboratory indicate that the period in which the eelpout larvae are vulnerable to the teratogenic effects of chemicals is fairly short, the critical period being days or maybe one to two weeks after fertilisation. To be able to reveal teratogenic effects, it is therefore crucial that the pregnant eelpout females are caught in the environment and brought to the laboratory for exposure as soon as possible after the fertilisation. On the other hand, the females cannot be caught until there is certainty that the vast majority of the females have been fertilised; we do not know if it possible to induce mating in captivity.

Malformations upon exposure of pregnant eelpout to octylphenol and estradiol have been observed - but not published - previously in experiments with other purposes [4] and the aim of the present investigation was to clarify and record the malformation upon exposure to these two chemicals.

2. Materials and methods
Eelpout with fertilised eggs in the ovaries were caught in seines by local fishermen in the coastal waters south of Funen, around the island of Birkholm primo September. The fish were transported to the university’s Marine Biological Station in Kerteminde and maintained in large tanks supplied with running seawater until September 13, where exposure began.

Two groups of 10 female eelpout were exposed to nominal concentrations of 500 ng l⁻¹ E2 or 100 µg l⁻¹ OP in a flow through system from September 13. Seawater and stock solutions were supplied by peristaltic pumps. The chemicals were dissolved in isopropanol; a control group received vehicle alone. The actual exposure concentrations were determined by LC-MS-MS. The experiment was terminated October 12, after 29 days' exposure. The various types of abnormal development among the embryos (early death, malformations of eyes and spinal cord) were evaluated and the frequencies were recorded for each experimental group. Plasma vitellogenin concentrations and calcium concentrations in the ovarian fluid was determined (in individuals in which ovarian fluid could be obtained). Length and weight of the embryos were determined and the weight of the ovarian sac was determined.
3. Results and discussion

In the control group, 77.3% of the embryos developed normally, while only 0.9% and 8.8% did so in the groups exposed to E2 and OP, respectively (Fig. 1). Early death (with or without malformations) and eye and/or spinal cord malformations were observed (Table 1).

<table>
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<tr>
<th></th>
<th>Control</th>
<th>E2</th>
<th>OP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Early death</td>
<td>8.2</td>
<td>70.2</td>
<td>6.5</td>
</tr>
<tr>
<td>Eye deformation</td>
<td>4.5</td>
<td>14.2</td>
<td>6.7</td>
</tr>
<tr>
<td>Spinal malformation</td>
<td>15.2</td>
<td>15.4</td>
<td>82.7</td>
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Table 1: Abundance of various types of abnormal development as percentage of total embryos. Embryos showing more than one type of effect count in 2 or 3 categories.

Early death dominated in the group exposed to E2 and spinal cord deformations dominated in the group exposed to octylphenol (Table 1).

4. Conclusions

Exposure of pregnant eelpout females to E2 and octylphenol at concentrations in the upper range of concentrations found in the environment results in abundant malformations among the embryos. This is a novel type of endocrine disrupting effect, and obviously, we want to establish dose-response relationships and no-effect-levels in further investigations. Likewise, we want to elucidate the mechanisms underlying this effect.

5. References

Progress of the Japanese program on endocrine disruption: from ExTEND2005 to EXTEND2010

Kunihiko Yamazaki, Masato Homma and Teruyoshi Hayamizu

Ministry of the Environment, Japan, 1-2-2 Kasumigaseki, Chiyoda-ku, Tokyo 100-8975 Japan
E-mail contact: KUNIHIKO_YAMAZAKI@env.go.jp

1. From SPEED’98 to EXTEND2010

In 1998 Environment Agency established the “Strategic Programs on Environmental Endocrine Disruptors: SPEED’98” to respond to growing public concern to and scientific uncertainty of endocrine disruption. It was succeeded by another program of the Ministry of the Environment (MOE) named as “ExTEND2005” and relevant research and development were conducted under this framework.

MOE reviewed its previous activities conducted under the ExTEND2005 and published a new program titled “Further Actions on Endocrine Disrupting Effects of Chemical Substances: EXTEND 2010” in July this year. MOE is to proceed with various initiatives on the endocrine disrupting effects of chemical substances under this new framework..

2. Principles of the EXTEND2010

EXTEND2010 inherited the framework from the preceding ExTEND2005 with appropriate improvements. This new program emphasizes that the establishment of assessment methodologies and its implementation should be accelerated with a view to conduct risk management. Priority is put on the effects to the environment, the same as the preceding ExTEND2005, and human health risks which may be caused by chemical substances in the environment should also be addressed. International cooperation to establish test methods and assessment methodologies should be promoted.

3. Tasks under the EXTEND2010

EXTEND2010 is composed by the following seven tasks.
1) Promotion of biological research on wildlife and fundamental research
2) Development of test methods and establishment of an assessment framework
3) Environmental monitoring and exposure assessment
4) Evaluation of actions and effects
5) Risk assessment and risk management
6) Promotion of information sharing
7) Enhancement of international cooperation

4. Progress of the assessment of chemicals on endocrine disrupting effects in Japan

As well as promoting research on fundamental science and wildlife observation, test protocols of fish, amphibian and invertebrates have been developed through bilateral or multilateral collaborations. Reliability evaluation of existing knowledge that might be relevant to endocrine disruption is being conducted to select possible candidate chemicals subject to testing to assess their endocrine disrupting effects to aquatic organisms. Framework for assessing endocrine disrupting effects to organisms in the environment is being developed and in vitro tests are conducted for some of the candidate chemicals on a trial basis.

Progress and updated situation of the assessment of chemicals in EXTEND2010 will be demonstrated.

5. References

  http://www.env.go.jp/en/chemi/ed/extend2010_full.pdf (Tentative Translation) (in English)

*) “EXTEND” in EXTEND2010 stands for “Extended Tasks on Endocrine Disruption”, while ExTEND2005 was named after “Enhanced Tack on Endocrine Disruption”.
A proteomic approach to the development of potential estuarine biomarkers for metal contamination using the Sydney Rock Oyster (*Saccostrea glomerata*), NSW Australia

Emma Thompson¹, Daisy Taylor¹, Sham Nair¹, Gavin Birch², Ross Coleman³, Paul Haynes⁴, David Raftos¹

1. Department of Biological Sciences, Macquarie University, NSW, Australia
2. School of Geosciences, University of Sydney, NSW, Australia
3. Centre for Research on Ecological Impacts of Coastal Cities, University of Sydney, NSW, Australia
4. Department of Chemistry & Biomolecular Sciences, Macquarie University, NSW, Australia

E-mail contact: emma.thompson@mq.edu.au

1. Introduction

The introduction of waste products into rivers and estuaries in industrial and urbanised areas since the industrial revolution has led to significant increases in chemical contamination. As such, it has become imperative to develop effective monitoring methods to protect biota and the environment. Traditional methods of monitoring, such as sediment toxicity, provide fine scale mapping of contaminants [1] but no information regarding their effects on biota, whilst ecotoxicology can lack sensitivity [2]. In contrast, molecular biomarkers provide cause and effect information by linking the effects of contaminants directly to biota [3]. However, traditional single parameter biomarker analyses can be insensitive, especially at low contaminant levels. Proteomics provides a method for identifying potentially hundreds of novel biomarkers simultaneously, even at extremely low levels of contamination, allowing early detection of environmental damage [4]. Proteomics may be able to differentiate between a variety of contaminants in both transient and long-term exposures. It also provides additional information on the biological function of affected proteins, which is essential to understand the biological consequences of contaminants [5].

Bivalves have been used extensively as sentinel species worldwide due to their capacity to bioaccumulate contaminants and their sensitivity to chemical contaminants [6]. The Sydney Rock oyster (*Saccostrea glomerata*) is endemic to Australian estuarine habitats and is found throughout the East coast and so is biologically relevant for the current study, which aims to identify a suite of potential biomarkers for the assessment of metal pollution in Australian estuaries.

2. Materials and methods

Sydney Rock oysters were exposed for four days to three environmentally relevant concentrations (100 µg/l, 50 µg/l and 5 µg/l) of copper, lead and zinc in laboratory-based exposures. Proteins were isolated and purified from extracted haemolymph after metal exposure and then analysed by 2-dimensional electrophoresis (2-DE). 2-DE proteome maps of metal-exposed oysters were then compared to non-exposed control oysters (three 2-DE maps per treatment, each derived by pooling haemolymph from five oysters). A combination of Progenesis spot detection software and manual visualisation followed by statistical analysis identified differentially expressed protein spots for further analysis. Protein characterisation was performed by tandem mass spectrometry (LC-MS/MS) and putative biological functions were assigned using the Global Proteome Machine (GPM) software version 2.1.1 (X!Tandem algorithm). GPM data were searched against a database containing 15,000 protein sequences from bivalve molluscs and the NCBI non-redundant protein sequence database. Identification of differentially expressed proteins by GPM was accepted if the number of peptides was >5, with log(e) <-10 and if the result appeared before any reversed sequences.

3. Results and discussion

3.1 Exposure to 100 µg/l of copper, lead and zinc

On average 161 spots were found per 2-DE proteome map. The spots were distributed predominantly in the pI 4-7 range. The intensities of 21 protein spots differed significantly between controls and metal-exposed oysters (figure 1). Lead exposure induced the most changes in protein concentration yielding a total of 11 differentially expressed spots ten of which increased in concentration. Zinc exposures yielded ten differentially expressed spots, 66% of which decreased in intensity. All of the four differential spots identified
after copper exposure had decreased concentrations relative to controls. Overall a relatively unique pattern of proteins were differentially expressed in response to each type of metal exposure.

Proteins identified by MS analysis of differential protein spots included actin, triosephosphate isomerase, ATP synthase, tropomyosin and tubulin. The proteins had a range of putative biological functions including protein synthesis and stress responses, as shown in figure 2. Cytoskeletal proteins accounted for 25% of the differential proteins, and were associated with all three metal exposures. A further 25% of the annotated proteins were putatively involved in shell calcification and adhesion. These shell associated proteins were primarily linked to zinc exposure.

3.2 Exposure to 50 µg/l and 5 µg/l of copper, lead and zinc

A further set of proteins were identified in response to 50µg/l and 5µg/l metal exposures. Initial analysis of these protein expression profiles suggests some overlap with differential expression patterns identified from 100 µg/l. However, unique sets of proteins were also evident for each metal at each concentration.

4. Conclusions

This study shows that proteomics has the potential to identify novel molecular biomarkers for metal contamination in Australian estuaries. Further work will test the viability of these potential biomarkers in field studies currently being conducted at Lake Macquarie, NSW, Australia. Early data suggest the sensitivity of these biomarkers could be sufficient to provide an early warning system for environmental damage.

5. References


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Searching for stable biomarkers of malachite green exposure in *Pangasianodon hypophthalmus* by a non invasive approach

Marie-Aline Pierrard¹, Patrick Kestemont¹, Edouard Delaive², Marc Dieu², Nguyen Thanh Phuong³, Martine Raes² and Frédéric Silvestre¹

¹Namur University (URBO), 61 rue de Bruxelles, B-5000 Namur, Belgium
²Namur University (URBC), 61 rue de Bruxelles, B-5000 Namur, Belgium
³College of Aquaculture and Fisheries, Cantho University, 3/2 Str., Campus II, Cantho City, Vietnam

E-mail contact: marie-aline.pierrard@fundp.ac.be

1. Introduction

Malachite green (MG), primarily used as a dye, is currently used as an antiseptic to prevent and treat parasites, fungal and bacterial infections in fish. This veterinary drug has been controversial and reported to cause chromosomal fractures, carcinogenesis, mutagenesis, significant alterations in biochemical parameters of blood and especially on immune system in fish and to have genotoxic properties [1]. Despite the fact that MG is banned in fish for human consumption this chemical is still commonly used in some aquaculture because of its low cost, availability and effectiveness. Our concern is more about the rearing of one particular species, the Asian catfish, *Pangasianodon hypophthalmus*, which is one of the most economically important fish worldwide due to some of its features. This fish has a high tolerance to poor water quality and its cheap filets are exported worldwide. However, the health management and excessive use of xenobiotics during its culture raise some questions on human health and risk assessment.

This study aims at identifying signature of protein expression which could work as an early warning signal of MG application even one month after decontamination. The final objective is to apply this biomarker signature to an in situ monitoring in fish farm conditions. Peripheral blood mononuclear cells (PBMCs) were chosen to allow regular sampling in a non invasive way. Also, blood is the perfect exchange medium between rearing water and the organism and PBMC proteome has the advantage to be subject to rapid changes in response to external signals. In ecotoxicology, the use of biomarkers to detect a contamination in the field is often discussed and some authors underline that directly assaying the compound in the rearing water or in the organism is easier, faster and cheaper than developing biomarker systems which are often non specific. However, lots of xenobiotic compounds are not stable in the water and the European legislation is asking for alternatives to animal testing and reduction of animals killed in ecotoxicology [2]. This study was aiming to figure out some molecular effects of MG on fish PBMC by a proteomic approach and to point out a stable biomarker Protein Expression Signature (BPES) to detect the illegal use of malachite green.

2. Material and methods

Experiment was carried out at the College of Aquaculture and Fisheries of the University of Cantho in Vietnam. Asian catfish *P. hypophthalmus* were acclimated at 25 fish per tank for 15 days. The tank is the experimental unit and the experiment was conducted in four replicates. A classic (0.1ppm) dose for therapeutic treatment was applied as used by the Vietnamese fish farmers. MG was put twice with an interval of 72 hours as shown in Fig. 1. Each time of contamination, MG was freshly dissolved in distilled water first, protected from light and put in the corresponding tanks. After the second bath, the continuous renewal of water has been restored until the end of the experiment.

![Figure 1: Experimental design.](image)

Both after the second bath (T1) and after 1 month of decontamination (T2), blood was sampled on 3 fish per tank and PBMC were isolated and finally suspended in DLA buffer for proteomic analysis in Belgium. Two-dimensional differential in gel electrophoresis (2D DIGE) were performed on 24cm, gradient acrylamid 8-13.5%, pH 4-7, IPG strips followed by analysis with DeCyder software. Peptides were analyzed by using nanoflow LC-ESI-MS/MS (Waters) instrument on a CapLC Q-TOF2 mass spectrometer (Waters). Scaffold (version Scaffold-2_06_01, Proteome software Inc., Portland, OR) was used to validate MS/MS based
peptide and protein identifications. At the same time of sampling, we collected blood and muscle from 3 fish per tank for MG residues assay. MG and its principal metabolite leuco-malachite green (LMG) have been extracted from muscle, blood and rearing water to assay their concentration by LC-MS/MS (Agilent 1100, liquid chromatograph, Waldbronn, Germany; API 3000 triple quadrupole, Applied Biosystems/MDS SCIEX, Toronto, Canada).

3. Results and discussion

The water contamination with MG confirmed that the storage of MG is first in the serum [1] and then in the muscle of Pangasius. Also, MG was rapidly converted into its more toxic form, LMG, which has a longer retention time. However, after one month of decontamination MG completely disappeared in both muscle and blood. We still found LMG but at very low concentrations: 10.3 ± 3.8 ng/g muscle and 5.3 ± 1.9 ng/mL blood. Nevertheless, as the zero tolerance of 0.01 mg/kg for the sum of MG and LMG in edible fish has been established [1], concentrations measured in the present study still enable a detection of the illegal use of MG after one month of decontamination, but just at the limit of the ban and only in the muscle.

The number of spots matched in the eight gels was 1195 ± 364 in which 116 showed significant differences in intensity between the treated and the control tanks and which are common for both periods of sampling without effects of sampling time (Anova 2, n=4). Principal component analysis (PCA) shows that the effects of MG on PBMC of Pangasianodon hypophthalmus can explain 78.5% of the variation of protein expression (n = 4, Anova 2, p<0.05). Considering single identification per spot, we identified by LC-MS/MS 26 different proteins which are involved principally in energetic metabolism, protein folding, oxidative stress, cytoskeleton and response to stress. Among the largest expression differences, Proline 4-hydroxylase (P4HB) and Heat Shock 60kD protein 1 were under-expressed 5.84 and 2.83 fold, respectively, at T2 (p<0.05).

Also, MG is known to have a high affinity for DNA [1] and we identified five proteins involved in DNA/RNA binding. The Proliferating cell nuclear antigen (PCNA) regulates DNA replication, modification and repair and was under-expressed 1.91 fold at T2 (p<0.05). As Bose et al. suspected in 2005 [3], MG may be responsible for incomplete replication of DNA and loss of the G2/M checkpoint control leading to the cell cycle arrest. Pre-mRNA-splicing factor (SPF27) involved in mRNA splicing was under-expressed 1.34 times at T2 (p<0.05) supporting the mutagenesis properties of MG [1]. In another way, Heterogeneous nuclear ribonucleoprotein (HnRNP) A/B or D are 1.18 and 1.48 fold over-expressed, respectively, at T2 (p<0.05). HnRNP A2/B1 is a nuclear RNA-binding protein involved in the splicing of mRNA and its subsequent transport from the nucleus to the cytoplasm. HnRNP A2/B1 has been reported to be over-expressed in several human cancers [4].

4. Conclusions

We identified by LC-MS/MS some proteins as potential candidates for a Biomarker Protein Expression Signature (BPES) even one month after exposure of farmed fish to MG, making them stable biomarker candidates of exposure. It suggests that MG has profound effects on various biological processes. This study confirms the necessity to point out a stable multibiomarker to detect the illegal use of malachite green. In the opposite, the assay of MG residues, even in the muscle, seems to be less reliable. These promising results open a new way to the application of fish mononuclear cells to find potential biomarkers of exposure in a non invasive way.

5. References


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The Estuarine Sediment Ecology Array (ESEA): a rapid and comprehensive molecular based approach for environmental monitoring and assessment

Anthony Chariton¹, Leon Court², Matthew Colloff², Matthew Morgan² and Christopher Hardy²

¹CSIRO Land and Water, Lucas Heights, Australia
²CSIRO Ecosystem Sciences, Canberra, Australia
E-mail contact: Anthony.Chariton@csiro.au

1. Introduction

Ecological assessments of estuarine environments are typically restricted to the examination of a small number of macrobenthic taxa (e.g. polychaetes and amphipods) that provide only a narrow view of a system’s true diversity. This is despite strong evidence that many other biotic groups, e.g. meiofauna, may be better indicators of environmental change and condition¹. However, their inclusion is generally considered too difficult and time consuming to be practical. With the exception of computational and statistical advancements, the fundamental approaches used to assess and monitor sedimentary environments has changed little for many decades.

Genetic approaches are seen as the tools of the future in ecological studies². Recent advances in DNA sequencing and microarrays (‘gene chips’), provide a unique opportunity to measure and understand biological complexity at a previously unattainable level. Exploiting these new technologies, we have developed a novel genomic approach for sediment ecological assessment, the Estuarine Sediment Ecology Array (ESEA). ESEA is a custom-designed microarray which contains phylogenetically diagnostic gene probes which can be used to identify the presence of an extensive range of organisms ranging from microorganisms to macrobenthic fauna.

The underlying approach behind ESEA is that DNA is extracted from sediments, the targeted genes are amplified (e.g. 18S rDNA for eukaryotes), and hybridized against the array. When the targeted sequence matches its complementary probes on the array, a fluorescence signal is produced after further downstream processing and the intensity measured. Each probe set is annotated with taxonomic information, enabling rapid identification of the biotic composition of the samples. In this presentation we discuss the basic principals of ecogenomic monitoring, how the array was designed, preliminary results from Sydney Harbour and the potential application of ESEA and other ecogenomic techniques.

2. Materials and methods

ESEA was designed to run on the Affymetrix Genechip platform. Genes used to create the probe sets were derived from two sources: a pyrosequencing study of Sydney Harbour sediments³; and from GenBank, an online gene repository. GenBank sequences were downloaded and then aligned into their respective taxonomic groups, 16S rDNA for prokaryotes and 18S rDNA for eukaryotes. Viable sequences were then cropped to the most variable regions present in most of the sequences for each sequence set. Where possible, two probe sets were created for each sequence from two different variable regions of the gene. As the probes on the Affymetrix arrays have a length of 25 nt and the length of the targeted genes was >200 bp, multiple probes were used to capture the uniqueness of each target sequence. Cross-reaction probe sets were also created for probes common to groups of taxonomically related sequences. This enables the detection of taxa that may not be represented by a unique sequence on the array, albeit at courser taxonomic levels. The design also included a number of measures to ensure the quality and reliability of the data. Briefly, this included 378 Affymetrix control probes; perfect match (PM) and mismatch (MM) sets for as many probes as possible; and multiple probes to reduce false positive and negative signals.

3. Results and discussion

The manufactured product (ESEA) (Figure 1) contains over 250,000 probes designed to encapsulate over 53,000 gene sequences. During the laboratory trial phase, evidence for the array’s viability was demonstrated by the consistent hybridization of varying concentrations of a reference bacterial gene; pronounced differences in the fluorescence between the perfect match (PM) and mismatched (MM) probes;
and the detection of all members of a simple artificially created assemblage, with fluorescence for all spiked taxa increasing with DNA target concentration.

Figure 1: The Estuarine Sediment Ecology Array (ESEA)

Field trials were performed on eukaryotic meiobiota (63-500 micron fraction) assemblages sampled from reference and contaminated locations in Sydney Harbour, Australia. The results of the analysis (Figure 2), revealed marked differences in the composition of biota between the two treatments, with comparisons involving several thousand taxa. The outputs from the ESEA have been calibrated and cross checked using pyrosequenced data derived from the same DNA extracts. Extensive field trials are currently being performed to further validate the ESEA and to examine its potential application across a range of environments.

Figure 2: Summarized ESEA data from the analysis of meiobiota obtained from reference and contaminated sediments. N= the number of probe sets detected with a cut-off of 5.0 (log2).

4. Conclusions

The ESEA and other novel genomic technologies have the potential to redefine the way we examine aquatic ecosystems. Their application now makes it possible comprehensively examine the biological constituents of an environment at a cost similar similar to that of traditional taxa focussed optical based techniques. Furthermore, microarray data can be produced rapidly, enabling scientists to obtain ecological data within the same time frames as analytical techniques such as GCMS.

5. References


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The DanTox-Project – Identification of specific toxicity and molecular modes of action of sediment-bound pollutants in zebrafish

S. Keiter¹, S. Peddinghaus¹, J. Bräunig¹, U. Feiler², C. Hafner³, M. Hammers-Wirtz⁴, N.Y. Ho⁵, B. Kais⁶, J.C. Otte⁵, R. Ottermanns⁴, S. Rastegar⁵, G. Reifferscheid², T. Braunbeck⁶, U. Strähle⁵, H. Hollert¹

¹Institute for Environmental Research, RWTH Aachen University, Worringerweg 1, 52074 Aachen, Germany
²German Federal Institute for Hydrology, BfG, Am Mainzer Tor 1, 56068 Koblenz, Germany
³Hydrotox GmbH, Bötzinger Str. 29, 79111 Freiburg, Germany
⁴Research Institute GAIAC, Worringerweg 1, 52074 Aachen, Germany
⁵Karlsruhe Institute of Technology, Institute of Toxicology and Genetics, P.O. Box 3640, 76021 Karlsruhe, Germany
⁶Aquatic Ecology and Toxicology Group, Department of Zoology, University of Heidelberg, Im Neuenheimer Feld 230, 69120 Heidelberg, Germany

E-mail contact: steffen.keiter@bio5.rwth-aachen.de; henner.hollert@bio5.rwth-aachen.de

1. Introduction

The European Water Framework Directive (EWFD) aims to achieve a good ecological and chemical status in European rivers by the year 2015. In order to fulfill this legal obligation, acquisition of new knowledge from basic research definitely plays a huge role. In particular, since sediments and particulate matters are well known for being sinks and secondary sources for pollutants, applied sediment toxicology is of major relevance in achieving the objectives set by the EWFD [1]. Current bioassays provide insufficient data on the bioavailability of pollutants; thus, sediment contact assays are important tools for the assessment of hazard potentials at the ecosystem level. In addition, existing bioassays for sediment toxicity analyses do not provide sufficient data concerning the mode-of-action of pollutants. Microarrays are a promising tool to obtain more information about the interferences of specific mechanisms in organisms caused by pollutants. In this context, there is a need to develop a vertebrate-based contact assay, which can be used to determine specific ecotoxicological effects caused by contaminated sediments [2, 3]. The aims of the joint research project DanTox are (a) to develop a suitable testing strategy for the assessment of bioavailable toxicants in sediments, (b) to investigate the molecular and cellular mechanisms of sediment toxicity, and (c) to elucidate the causality of biological effects. A major long-term objective will be the development of a targeted cDNA-microarray which will be a useful tool for environmental screening. In the end, this concept will be tested for its suitability for daily use and commercial viability.

2. Materials and methods

Sediment samples were collected from the Rhine River (Altrip and Ehrenbreitstein) and from the Vering Canal in Hamburg (Germany). Freeze-dried sediments were extracted with acetone in a Soxhlet apparatus. Four model chemicals were selected: methylmercury(II)-chloride, chlorpyrifos, Aroclor 1254 and bisphenol A. All chemicals, sediment extracts and freeze-dried sediments will be investigated with mechanism-specific test systems and gene expression analysis. The different methods of the joint research project are categorized into four modules:

Module bioassays: Different biomarkers and endpoints (e.g. Comet assay, EROD assay, fish embryo test, live-imaging of EROD induction, and neurotoxicity) will serve the possibility to get a brought overview of the ecotoxicological hazard potential of native sediments, sediment extracts and selected chemicals using embryos of the zebrafish (Danio rerio).

Module gene expression: In order to get more insight into specific mechanisms of observed effects, the gene expression profile in zebrafish will be investigated after exposure to sediments and the model chemicals using microarrays and qRT-PCR, the latter in order to analyze the transcript abundance of phase I and II metabolism genes (CYP1A1, AHR2, GST and UGT1A1) as well as MT1 and MT2 as markers for metal pollution.

Module data assessment: Data of gene expression and chemical analysis will be statistically analyzed to provide a possibility for a prospective analysis of the chemical contamination of sediments.

Module practical transfer: In order to transfer the newly developed sediment contact tests into routine testing procedures, the test results will be compared with data from selected guideline tests (e.g. algae, Daphnia, and fish test).
3. Results and discussion

3.1. Embryo toxicity

The highest embryotoxic potential was measured for the sample from the Vering Canal after 48 h of exposure (EC50 = 2.6 and 3.6 mg/ml for extract and native sediments, respectively). EC50 values of sediment extracts from Ehrenbreitstein (EC50 = 21.7 mg SEQ/ml) and Altrip (EC50 = 18.1 mg SEQ/ml) suggest that there is a comparable embryotoxic hazard. The latter result was surprising since the sediment from Altrip was chosen as a relative unpolluted reference sampling site.

3.2. EROD induction

The EROD assay measures the induction of the biotransformation phase I enzyme CYP1A fluorometrically and is, therefore, a frequently used biomarker for dioxin-like activity.

**EROD assay:** First results of the EROD assay with zebrafish embryos (48 hpf) showed only a minor EROD induction of 2,3,7,8-tetrachlordibenz-1,4-dioxin (TCDD) compared to results with the cell line RTL-W1, even though several improvements were made to increase EROD induction in fish embryos. Potentially, the natural barrier function of the chorion prevents TCDD from entering and harming the embryo. As a consequence, TCDD might not be a suitable positive control for the EROD assay with *Danio rerio*, if embryos with an intact chorion are used. In contrast to TCDD, the exposure to sediment extracts showed an EROD induction above the basal level of the negative controls. Moreover, clear tendencies towards dose-response relationships could be observed. The sediment extract from Vering Canal showed the highest effect: an 7.7-fold EROD induction over negative controls. Sediments extracts from Ehrenbreitstein and Altrip induced EROD activity by less than 2.3-fold.

**Live-imaging of EROD induction:** The EROD activity in zebrafish embryos can be detected via epifluorescence microscopy and confocal laser scanning microscope [4]. Live-imaging of EROD induction with β-naphtoflavone as a positive control documented CYP1 induction at any time of inspection. Due to their molecular structure, methylmercury(II)chloride and chlorpyrifos showed no EROD induction.

3.3. Gene expression analysis

qRT-PCR revealed clear changes in the transcript abundance of CYP1A GST and UGT1A1 genes for the sediment extracts. Up to 600-fold changes in CYP1A1 could be seen for the extract from Vering Canal. Clear dose-response relationships for CYP1A, GST and UGT1A1 were observed in embryos exposed to extracts from the least contaminated Rhine site, Ehrenbreitstein. No comparable dose-response relationships were observed in embryos exposed to extracts from either the Vering Canal or the second Rhine site, Altrip. This was likely due to already maximal induction of transcript abundance in embryos exposed to the least concentration of both sediment extracts. Transcript abundance of MT1 and MT2 was not significantly altered in embryos exposed to extracts from any of the sites. Overall, dioxin-like pollution seems to be more prevalent than metal pollution in these areas.

4. Conclusions

At the present stage of this project, only results from the fish embryo toxicity test, EROD assay and qRT-PCR analysis are available. First results from the biotest systems indicate that measurement of specific endpoints is a suitable strategy to identify and detect the bioavailable hazard potential of sediments. In addition, a comparison of the results from the EROD assay and qRT-PCR showed similar tendencies for the sediments indicating that the EROD contact assay with zebrafish embryos might be a useful tool for routine testing.

5. References


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The future role of next-generation DNA sequencing and metagenetics in aquatic biological monitoring programs

Erik Pilgrim, Suzanne Jackson, John Martinson, & John Darling

Ecological Exposure Research Division, United States Environmental Protection Agency, Cincinnati, OH, USA
E-mail contact: pilgrim.erik@epa.gov

1. Introduction

The development of current biological monitoring and bioassessment programs was a drastic improvement over previous programs created for monitoring a limited number of specific chemical pollutants. Although these assessment programs are better designed to address the transient and potentially synergistic nature of environmental stressors, the reliance on morphological taxonomic identification of samples has several inherent issues limiting the scope of these programs. Biological monitoring programs suffer from high costs, long completion times, and quality assurance issues from taxonomic disagreements [1]. These programs also rely on animal groups, such as fish and macroinvertebrates, that can be identified by experts, while avoiding microbial, algal, and meiofaunal communities whose members would be difficult or impossible to identify through morphology. These neglected communities likely represent a wealth of information that could be applied to assessing ecological condition. Even for those fauna used in bioassessment, many taxa are not readily identified to species, leading to taxonomic imprecision and a potential loss of information.

Next generation DNA sequencing (NGS) is a revolutionary technology when considered in the context of biological monitoring programs. This technology allows for bulk DNA extraction of virtually all the specimens in a sample, followed by DNA sequencing of genetic loci used for taxonomic identification. NGS generates large amounts of sequence data that can be applied not only to the identification, with finer taxonomic resolution, of those groups currently used in assessment, but to previously intractable groups such as nematodes, diatoms and other algae, protozoans, and other meiofaunal invertebrate phyla.

This presentation will outline the future role that metagenetic data generated by NGS could have in new biological monitoring and assessment programs. The future uses of this metagenetic data would be not only to improve the speed and precision of biological monitoring, but to move programs beyond relatively simple conditions assessments (often ‘good,’ ‘fair,’ or ‘poor’) to the ultimate goal of bioassessment: environmental stressor identification.

2. Program Applications

2.1. Coastal Marine Benthic Assessment

Coastal marine environments receive a variety of terrestrial inputs of stressors. Current monitoring programs predominantly rely on morphological identification of fishes and macroinvertebrates (0.5 or 1.0 mm and larger) and therefore do not include microbiota or meiofauna. Most of these taxa are identifiable to species, but difficult groups remain. Full identification of samples requires considerable time and effort due to the large number of animal phyla present, requiring a wide range of taxonomic expertise.

NGS has several potentially beneficial applications to marine benthic bioassessment. This technique would still provide identification of macroinvertebrates, but would also allow for taxonomic identification of microbial and meiofaunal communities. Several recent studies [2,3] have shown the utility of NGS for uncovering the diversity within intractable marine benthic meiofaunal groups like nematodes, kinorhynchs, and gastrotrichs. These studies outline the revolutionary capability that NGS can have on marine benthic bioassessment in terms of time (weeks to months as opposed to 6-12 months for traditional techniques) and information. Inclusion of microbial and meiofaunal communities, along with macroinvertebrates, will lead to much larger data sets for biological monitoring programs with an eye toward developing programs for stressor identification.

2.2. Stream and River Assessments

Freshwater lotic habitats also receive large and varied inputs of environmental stressors. Like marine programs discussed above, existing monitoring programs utilize morphological identification of fish and macroinvertebrates, although certain aquatic insect groups are often the main focus of ecological condition.
assessments. Typical stream/river assessment programs use a limited number of specimens (often 200-600 specimens per site), as opposed to full enumeration of all specimens, and the identification of many of these larval aquatic insect groups is only to genus level or above. Traditional programs can be costly in time and resources, and discrepancies in taxonomic identification between laboratories further complicates the use of this data for making ecological condition assessments. Data from current monitoring programs is also difficult to compare across regions as different programs collect different data for their ecological condition assessments.

NGS has the potential to address many of the issues facing stream/river bioassessment. This technology would allow for faster identification of virtually all the specimens within a sample. The large and continuously increasing DNA barcode databases would lead to the identification of specimens to species level giving these samples greater taxonomic resolution. As with marine samples, this technique would allow the inclusion of other important groups within the community that are currently not utilized in bioassessment, such as diatoms and other algae, protozoans, and nematodes. The increased taxonomic resolution along with the inclusion of new community biodiversity information will help move biological monitoring toward including environmental stressor identification with ecological condition assessment. Development of NGS as a uniform technique for biological monitoring will also allow critically valuable comparisons of assessments across regions.

3. Conclusions

Next Generation Sequencing represents a revolutionary new technology for application to biological monitoring and assessment programs. This transformative technology will generate considerably more data than currently available for bioassessment with untold future applications. Currently, several research groups from around the world are working to develop consistent, uniform techniques, as well as addressing other issues related to data management, storage, and accessibility. NGS can address many existing problems of current assessment programs including speed of identification, the decline in availability of taxonomic expertise, incomplete taxonomic resolution, and incomparable assessments between regions. This technology is the next logical step toward moving biological monitoring programs beyond condition assessment to stressor identification.

4. Future needs

Current research efforts are focused on dealing with several issues facing the use of metagenetic data for biological monitoring: 1) development of consistent genetic loci for comparison across data sets; 2) continued improved in bioinformatic capabilities for handling large amounts of metagenetic data and for issues related to grouping and identifying taxa; 3) development of an international database for storage and access to previous (and future) data sets; 4) development and implementation of bioassessment protocols based on these metagenetic methods.

5. References

Enchytraeid transcriptome sequencing towards the establishment of a soil ecotoxicogenomics model

Marta Patrícia Castro-Ferreira1,2, Cornelis A. M. Van Gestel1, John Colbourne3, Amadeu Mortágua Velho Maia Soares2, Mónica João Barros Amorim2 and Dick Roelofs1

1Animal Ecology department, Vrije Universiteit Amsterdam, Amsterdam, Netherlands.
2Biology department, University of Aveiro, Aveiro, Portugal.
3Center for Genomics and Bioinformatics, Indiana University, Bloomington, USA.
E-mail contact: marta.ferreira@falw.vu.nl

1. Introduction

Enchytraeids are an ecologically relevant functional group of the soil mesofauna [1, 2] and Enchytraeus crypticus is a potworm frequently used in soil ecotoxicology [1, 3, 4]. We exposed E. crypticus for 21 days to LUFA 2.2 soil spiked with five chemicals with distinct modes of action, in order to assess the effect concentrations (ECx) at physiological endpoints. We aim to study the transcriptome of this enchytraeid, which has no genomic data available, applying ultra-high throughput sequencing technology [5, 6]. In order to obtain most enchytraeid transcriptome information, samples from short-term exposures to distinct ECx, soil moisture, temperature and pH values were pooled together with samples for starvation and developmental stages. The RNA pool was normalized prior to sequencing. Bioinformatic analyses emphasized biological processes associated with the identified transcripts. Subsequently we will assess stress-related processes that may highlight mechanistic information associated with the tested stressors.

2. Materials and methods

Ecotoxicological assays were performed according to standard guidelines [1, 2] using LUFA 2.2 natural soil (Speyer, Germany) and E. crypticus were collected from established laboratory cultures. The test chemicals were carbendazim, cadmium, phenanthrene, 3,5-dichloroaniline and pentachloroaniline. Stock solutions (aqueous for cadmium; acetone-based for organic compounds) were prepared and serially diluted prior to spike the chemicals. Soils spiked with organic compounds were equilibrated for 24 h and opened for acetone evaporation; then were homogeneously mixed and moistened to 50% maximal WHC. Cadmium-spiked and control soils were mixed and homogeneously moistened to 50% maximal WHC and aged for 21 days at 20 ºC. Ten adults with white clitellum and equal size were introduced per 100 mL glass vial containing 30 g soil; then it was supplied with 2 mg oat flakes and closed with perforated aluminum foil. Each treatment comprised five replicates. The exposure lasted 21 days at 20 ºC and 75% air humidity. Food and soil moisture contents were weekly checked and replenished if necessary. At the end all samples were immediately fixated and followed the bengal red staining procedure [1, 4]. The ECx were calculated using the log-logistic model. In equal test conditions, E. crypticus adults were 2-days exposed to ECx of the five chemicals. As represented in Figure 1, short-term exposures to distinct temperatures, pH and soil moisture levels were performed and sampled together with starvation and developmental stages. Total RNA was extracted from ninety independent RNA samples comprising the seven treatment classes listed in Figure 1, using the SV Total RNA Isolation System (Promega). Every RNA sample followed the quality control by Nanodrop measurement and agarose gel electrophoresis. The ninety RNA samples were combined into the RNA pool, which was normalized and submitted to 454 Roche GS FLX Titanium pyrosequencing. The manufacturer assembler Newbler (Overlap-Layout-Consensus algorithm) and the freeware VELVET (de Bruijn graph algorithm) program were used to analyse the 454 output reads. BlastX was used to identify homologous genes in public databases. Blast2GO was applied to link GO terms to BlastX hits. IntreProscan searches were conducted to identify functional domains that were not retrieved from the BlastX analysis. Finally pathway information was retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG).

Figure 1: Enchytraeus crypticus were sampled from the listed treatment classes; RNA was pooled and normalized prior to 454 sequencing; the reads were assembled and Gene Ontology (GO) analyses were performed to link biological process to stressor.
3. Results and discussion

3.1. Estimation of effect concentrations

The EC10 and EC50 results assessed at reproduction are presented in Table 1. In comparison to F. candida [4], E. crypticus showed higher sensitivity to metals and lower susceptibility to organic compounds.

*Table 1: Effect-concentrations (mg a.i./kg d.w. LUFA soil) of the five test chemicals to Enchytraeus crypticus reproduction endpoint, after 21 days of exposure in LUFA 2.2 spiked soil (95% confidence intervals are shown).*

<table>
<thead>
<tr>
<th>Test chemical</th>
<th>EC10</th>
<th>EC50</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbendazim</td>
<td>-</td>
<td>&lt; 1</td>
</tr>
<tr>
<td>Cadmium</td>
<td>14.9 (10.4-19-3)</td>
<td>35.0 (30.4-39.6)</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>35.8 (12.4-59.2)</td>
<td>144 (105-183)</td>
</tr>
<tr>
<td>3,5-Dichloroaniline</td>
<td>93.0 -</td>
<td>101</td>
</tr>
<tr>
<td>Pentachloroaniline</td>
<td>2.53 (0-11.5)</td>
<td>278 (38.2-518)</td>
</tr>
</tbody>
</table>

3.2. RNA pool and 454 sequencing

Ninety high-quality RNA samples were combined into the RNA pool and this was successfully normalized as evenly abundant transcripts were confirmed by q-PCR. The 454 run retrieved approximately one million reads with 4 Mbp mean length.

3.3. Sequence assembly and functional analyses

The distinct assemblies, retrieved from Newbler and VELVET assembler programs, were combined in order to reach more consistent contigs. Subsequently, contigs were functionally annotated using Blast2GO, retrieving information on Gene Ontology (GO) terms, functional protein domains (InterProScan) and enzymatic pathway information (KEGG). We will present the functional annotation of identified transcripts as a crucial step towards understanding the mechanistic basis of toxic effects. For instance, Nota et al. [7] identified biological processes of biotransformation I, II and III in phenanthrene-exposed F. candida; we checked the presence of such biological processes in the output of our analyses.

4. Conclusions

E. crypticus was exposed to five key chemicals in order to empirically assess the physiological endpoints. Comparing to F. candida, the enchytraeid was more susceptible to metals but less to organic compounds. The different sensitivity of E. crypticus emphasizes its relevance as soil ecotoxicological model. This study was based on seven treatment classes (Figure 1) and lead to the identification of important stress-response pathways in the enchytraeid transcriptome. Our data is a crucial starting point for further ecotoxicogenomic studies. To that end we developed a high-density microarray derived from the present transcriptome sequences which will be used to study E. crypticus stress-responses to distinct environmental conditions.

5. References

1. Introduction

Sediments represent secondary sources of contamination for aquatic environments because of their high capacity to sequester and then release a great number of persistent chemicals such as POPs (persistent organic pollutants) and heavy metals. Consequently, sediments represent a threat for benthic organisms in particular for pollutant-sensitive early life stages. This study aims at evaluating metal contamination and biological responses in embryos and larvae of the Japanese Medaka exposed to sediments spiked with environmental concentrations of cadmium (Cd). Medaka embryos were exposed in controlled laboratory conditions during their whole embryonic phase to Cd-spiked sediments. Time-course of metal bioaccumulation as well as gene expression responses and toxic effects were analysed in embryos and newly hatched larvae.

2. Materials and methods

Freeze-dry reference sediment was spiked with concentrated solution of CdCl₂ to obtain three different exposure conditions (Control: 0µg Cd/g d.w., 0.3X: 2µg Cd/g d.w. and 3X: 20µg/g d.w.). Each condition was performed in six replicates (three dedicated to embryonic sampling and three to larval sampling).

One hundred 24hpf (hours post-fertilization)-embryos per replicate were placed in petri dishes containing 17g d.w. sediment and 10ml of ERS (Egg Rearing Solution). Embryos were incubated in direct contact to contaminated sediment up to sampling time (end of organogenesis i.e. 7dpf or hatching) at 26°C, with 12h:12h photoperiod. Embryo viability was checked daily and dead embryos were removed.

Cardiac activity was measured in 5 embryos per replicate at day 6pf and 7pf. At hatching, 15 individuals per replicate were randomly selected for biometric measurement and developmental abnormalities examination.

At each sampling time, three pools of 8 individuals were sampled for rRT-PCR analysis, two pools of 15 individuals for Cd bioaccumulation measurement and two other pools of 15 individuals for MTs content determination. Moreover, sediments and ERS were sampled for Cd analyses.

Cd concentrations in all samples (sediment, ERS, larvae and embryos) were measured by polarized Zeeman atomic absorption spectrophotometry. Total MT proteins were determined by mercury saturation followed by Hg quantification using flameless atomic absorption measurement. After total RNA extraction, retrotranscripted-cDNA gene amplifications were performed with a LightCycler®.

3. Results and discussion

3.1 Tissue accumulation of Cd in embryos and prolarvae

Cd analysis in 7dpf-embryos showed a marked increase of metal bioaccumulation between treatments with Cd contents being 35-fold and 1666-fold higher in comparison to control for 0.3X- and 3X-Cd group. A dose-dependent Cd bioaccumulation was also observed in newly hatched larvae but with Cd body burden significantly lower in comparison to the embryonic stage suggesting a non-negligible adsorption of cadmium on chorion.

3.2 Mortality and developmental effects

Cd treatments affected neither embryos survival nor development time nor hatching success. Surprisingly, 3X-Cd exposure led to a significant increase of 6dpf-embryos heart rate which might be a first metabolic
response to chemical stress, whereas 7dpf-embryos showed bradychardia at the same concentration. Decrease of cardiac activity in fish early life stages exposed to Cd had already been reported [1].

At hatching, the average percentage of abnormal larvae increased from 13% for control group to 65% (p<0.05) and 45% (p=0.07) for 0.3X- and 3X-Cd treatment respectively. Developmental abnormalities within contaminated groups mainly included spine column and cardio-vascular system deformities, in agreement with litterature (Rewiewed in [2]). Interestingly, average total length of prolarvae exposed to 3X-Cd concentration was slightly (3%-increase) but significantly higher compared to control group.

3.4 Biochemical and genetic responses

Total MTs protein contents were not altered by Cd treatments which is in agreement with the unaffected mt expression level in comparison to control group. Table 2 summarizes the gene expression induction factor for twelve selected genes in comparison to related control groups.

<table>
<thead>
<tr>
<th>Table 2: Induction factors of gene expressions observed following Cd-exposure at embryonic and larval stages compared to controls. Only induction factors superior to 2 or inferior to 0.5 were considered.</th>
<th>Embryonic stage</th>
<th>Larval stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.3X-Cd</td>
<td>3X-Cd</td>
</tr>
<tr>
<td><strong>Mitochondrial metabolism</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>coxl</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>nd5</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Metabolisation / detoxication</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mt</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>cyp1a</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Oxydative stress defense</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>sodMn</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>sodCu</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Apoptosis / cell cycle arrest</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>p53</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>bax</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>Oncogenesis / morphogenesis</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>wnt1</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>CNS development</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>emx2</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td><strong>DNA repair mecanisms</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>rad51</td>
<td>/</td>
<td>/</td>
</tr>
<tr>
<td>ogg1</td>
<td>/</td>
<td>/</td>
</tr>
</tbody>
</table>

At the embryonic stage, no effect on gene expression was observed, except a repression of bax and rad51 genes at the highest tested concentration.

On the contrary, p53, bax, rad51 and ogg1 were overexpressed in larvae following exposure to the highest Cd-concentration. These genes may be specifically overexpressed in response to DNA damage induced by Cd exposure. wnt1 expression in larvae was significantly induced at both Cd concentrations. The wnt1 gene product is a cell signalling protein which is involved in embryogenesis and especially in cell differentiation and compartmentalization. wnt1 overexpression have been shown to lead to tumorigenesis [3].

4. Conclusions

This study evidenced significant bioaccumulation and effects of cadmium in early developmental stage of medaka following realistic exposure to environmental concentrations of Cd. The same experimental protocol can be used to monitor effects of various sediment-associated pollutants including heavy metals and POPs. The Medaka embryo-larval assay (MELA) could be used to study mode of action and effects of chemicals and may improve risk assessment of environmentally-persistent chemicals.

5. References


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Effects of habitat and season on food web accumulation of cadmium to the little owl: a modelling study based on field data

Nico van den Brink¹, Aafke Schipper², Sander Wijnhoven³ and Hans Baveco¹

¹Alterra Wageningen UR, P.O. Box 47, NL-6700AA, Wageningen, The Netherlands
²Radboud University Nijmegen, P.O. Box 9010, NL-6500GL Nijmegen, The Netherlands
³Netherlands Institute of Ecology, P.O. Box 140, NL-4400AC Yerseke, The Netherlands

E-mail contact: nico.vandenbrink@wur.nl

1. Introduction

Little owls (Athene noctua) inhabit different habitats, including floodplains, and have a wide range of potential diet items. However, floodplains are contaminated by legacy contaminants, which may pose a toxicological risk to the little owl [1]. It has been shown that accumulation patterns may differ within floodplains, due to spatial variation in habitat characteristics and the occurrence of prey items [2, 3]. In addition to spatial variation, contaminant accumulation to the little owl is expected to vary temporally, because of seasonal changes in diet composition [4]. In this presentation we will discuss seasonal and spatial variation in exposure of little owl to cadmium, a metal known to accumulate in food webs, in relation to both seasonal and spatial shifts in diet composition.

2. Materials and methods

The study was performed in the Woldswaard, a floodplain of the river Neder-Rhine in the Netherlands. Based on soil properties, metal contamination levels, flooding frequency and land use, three habitats were distinguished: floodplain, pasture and orchard. Prey densities were determined per habitat type per month. The diet of the little owl was assumed to consist of earthworms, beetles and small mammals, which generally account for the major part of the diet (based on weight) in North West Europe [5]. Beetle densities were determined on a monthly basis using pit falls. Earthworms were collected by hand-sorting of soil cores, collected in May, June, August and March (2007-2008). Small mammal densities were based on monitoring data from a nearby floodplain area [6]. For months without monitoring data, prey densities were determined by linear interpolation of the densities recorded in the preceding and following months. Monthly prey densities were translated into monthly specific diet compositions by applying diet item specific functional responses [7].

Exposure to cadmium was calculated as the Daily Intake (DI) according to [8]:

\[ DI = \text{DFI} \times \sum (f_i \cdot C_i) \]

where DFI = daily food intake (g), \( f_i \) = weight fraction of prey type \( i \) in little owl’s diet in month \( t \) (dimensionless), and \( C_i \) = cadmium concentration of prey type \( i \) (µg g\(^{-1}\)). Internal cadmium concentrations in invertebrate prey were determined based on empirical regressions (for vegetation and invertebrates) or modelled (for small mammals; according to [8]).

3. Results and discussion

3.1. Seasonality in diet composition

Figure 1A illustrates the modelled seasonal variation in the diet composition for the pasture. Particularly in winter, the diet in this habitat type is dominated by common voles. The contribution of earthworms is highly variable, ranging from 0 to 50% based on weight. In the other two habitat types, similar seasonal variation was shown, with relatively large contributions of earthworms in spring and autumn. Especially in the floodplain, the contribution of earthworms was high in spring (up to 80%).

3.2. Seasonality in DI

The DI in the pasture shows a high seasonal variability (Figure 1B) similar to the diet. The geometric mean of the monthly DIs is below the threshold level indicating absence of risks. However, in the periods February to April and July to October, the DI exceeds the threshold level. These periods are equal to, or even longer than the 12 week exposure period that was used to derived the threshold level [9]. This would indicate risks, in contrast to the results based on the geometric mean. Furthermore, in the first period the breeding season
starts, which may be a more sensitive period for the birds.

![Seasonal variation in diet composition (% based on weight) of the little owl in pasture.](image1)

![Seasonal variation in DI (ug/day) of little owls in pasture.](image2)

**Figure 1. A. Seasonal variation in diet composition (% based on weight) of the little owl in pasture. B Seasonal variation in DI (ug/day) of little owls in pasture. Blue line represents a LOAEL of 148 ug/day, based on a LOAEL for water fowl of 0.8 mg·kg⁻¹·d⁻¹ [9] and a little owl body weight of 185 g.**

DI's differ between habitats, not only in absolute values but also in seasonality (data not shown). The geometric mean of the daily intake is lowest in the orchard, due to the relatively low concentrations in prey and the low fraction of earthworms in the diet (data not shown). However, even in the orchard, the threshold level is reached in the spring period. The highest daily intakes are modelled in the floodplain, reaching up to nearly 400 µg·d⁻¹ in spring. This is related to the comparatively high proportion of earthworms in the diet, caused by the relative low small mammal densities caused by the annual flooding in winter or early spring.

### 4. Conclusions

Modelled exposure of little owls to cadmium showed considerable seasonal and spatial variation. Geometric means of the DI were well below threshold levels, but due to the seasonal variation in diet composition the DI exceeded the threshold values in both spring and fall. This would indicate risks at those moments, which is in contrast with the conclusion based on the geometric mean value. Hence, the assumption of fixed dietary fractions may underestimate risks that may occur seasonally. This presentation illustrates that regular approaches, based on accumulation factors, may not be suitable to include spatial and temporal variation in risk assessment procedures. The approach presented here has the potential for habitat and season specific food web modelling, resulting in a better and more adequate assessments of risks of environmental contaminants to wildlife.

### 5. References


**Acknowledgement** - The authors thank Annemariet van der Hout for the collection of earthworms. AS was funded by the Netherlands Organisation for Scientific Research (LOICZ program); NvdB and HB were funded by the BERISP project (INTERREGIIIB) and INSPECT project (SNOWMAN-network)
Capacities of phospholipid membrane to accumulate neutral organic chemicals

Satoshi Endo¹, Beate Escher², Kai-Uwe Goss¹

¹UFZ Helmholtz Centre for Environmental Research, Permoserstrasse 15, 04318 Leipzig, Germany
²The University of Queensland, National Research Centre for Environmental Toxicology (Entox), 39 Kessels Road, Brisbane, QLD 4108, Australia

E-mail contact: satoshi.endo@ufz.de

1. Introduction

Lipids have been considered as the predominant components for accumulation of organic chemicals in biota. It is a common practice to normalize chemical concentrations in the organism to the total lipid content regardless of the classes of chemicals. Different types of lipid are not considered and the accumulation capacity of “the lipid” is assumed to be identical to the solvent octanol.

Abundant lipids in organisms are storage and membrane lipids. Storage lipids are in the form of triacylglycerides (esters of glycerol and three fatty acids), also called neutral lipids, and predominant in fat tissues. Membrane lipids are the main components of biological membranes and typically contain two (nonpolar) fatty acids and one polar head group. Phospholipids, having phosphate as the head group, are usually predominant in the membrane. Despite the obvious structural differences between the two types of lipid, their differences in accumulation properties have not systematically been addressed.

This study focuses on the equilibrium partition coefficients ($K_{lipw}$) of neutral organic compounds into phospholipid membrane. Artificial liposome membranes are considered as the model phase. Liposomes are lipid-bilayer vesicles typically composed of one or more types of phospholipid and used extensively in membrane studies. It has frequently been shown that the liposome-water partition coefficient ($K_{lipw}$) is a more accurate descriptor than the octanol-water partition coefficient ($K_{ow}$) to estimate chemicals’ membrane affinity and membrane-related processes such as bioconcentration in aquatic organisms, intestinal absorption, and baseline toxicity (narcosis). Contradicting this fact, $K_{ow}$ is still the sole parameter used in most bioaccumulation models, and if $K$ for membrane needs to be modeled, $K_{ow}$ instead of $K_{lipw}$ is typically used as a surrogate. One reason is the far larger availability of experimental data and estimation methods for $K_{ow}$ compared to $K_{lipw}$, although many $K_{lipw}$ values have been measured and reported, too.

Another important aspect of membrane partitioning is that it has been claimed [1, 2] that, for hydrophobic compounds, there is a cutoff log $K_{ow}$ value (or a cutoff molecular size) above which log $K_{lipw}$ does not increase any more or even starts to decrease with log $K_{ow}$ (or molecular size). A recent study [3], however, showed that such a cutoff can largely be explained by experimental artifacts, pointing out that careful and systematic re-evaluation on reported experimental $K_{lipw}$ values are inevitable.

The objectives of this study are to critically evaluate literature $K_{lipw}$ data for neutral compounds and to establish and evaluate methods to estimate $K_{lipw}$. Polyparameter linear free energy relationships (PP-LFERs) are used for both consistency test and $K_{lipw}$ estimation. Based on the calibrated PP-LFER models, relevance of using $K_{ow}$ to derive $K_{lipw}$ and the differences between storage and membrane lipids are discussed.

2. Materials and methods

Experimental $K_{lipw}$ data from more than 30 papers were evaluated. Only liposomes composed of phosphatidycholines (PCs) were considered because of their high abundance in biological membranes and the large data availability. The $K_{lipw}$ values measured below the membrane phase transition temperatures ($T_c$) were not considered, as anomalous changes in $K_{lipw}$ below $T_c$ have been reported. In the end, 377 data for 228 neutral compounds (or neutral species of ionizable compounds) were compiled.

In addition to the literature work, batch experiments were performed to fill gaps in the data set. $K_{lipw}$ for 14 volatile aliphatic compounds were determined by measuring the concentration drop in the headspace caused by the addition of liposome. $K_{lipw}$ for 16 chlorinated hydrophobic compounds were determined by the solid-phase dosing and sampling technique described in ref 4.
3. Results and discussion

There existed a fairly good correlation between log $K_{lipw}$ and log $K_{ow}$. Errors were typically up to ± 1 log units, although there was considerably larger scattering in the region of log $K_{ow} > 6$, due primarily to the too small $K_{lipw}$ values for PCBs from early studies. However, even recent $K_{lipw}$ values measured by polymer-mediated sampling methods exhibited 1–2 log unit differences between PAHs and PCBs of comparable $K_{ow}$.

Two types of PP-LFER [5, 6] were fitted to the data,

$$\log K = c + eE + sS + aA + bB + vV \quad (1)$$

$$\log K = c + lL + sS + aA + bB + vV \quad (2)$$

Both equations fit well to the collected $K_{lipw}$ data, leading to $R^2 = 0.97$ and a standard deviation of 0.3 log units. Again, the early experimental $K_{lipw}$ values for PCBs appear to be too small. In contrast, the recent values for both PAHs and PCBs fit well to the regression equations. It is also suggested that eq 2 is better suited than eq 1 for modeling partitioning of PCBs.

Using the PP-LFER for $K_{lipw}$ derived above, $K$ values representative for membrane lipid-water partitioning were calculated for a number of compounds. Similarly, $K$ values from water to storage lipid were estimated using the PP-LFER for olive oil-water partition coefficients [7], which also has an accuracy of about 0.3 log units. The comparison between the two lipid types suggests, in brief, that $K$ between both lipids differ by only < 1 log unit for low-polarity compounds (e.g., PAHs) and H-bond-acceptor monopolar compounds (e.g., nitrobenzenes). In contrast, bipolar compounds (e.g., phenols) generally favor membrane lipid over storage lipid and the resulting $K$ values can exhibit > 1 log unit differences. Some illustrative examples are shown in the Figure below.

![Figure. PP-LFER-calculated liposome-water partition coefficients vs olive oil-water partition coefficients.](image)

4. Conclusions

PP-LFERs accurately describe the phase properties of liposome and can be used for $K_{ow}$ estimation even for highly hydrophobic compounds. There is no indication of “cut-off” in the recent literature data. Membrane lipid has a much larger capacity than storage lipid for bipolar compounds, e.g., compounds with the -OH group(s). Thus, the “total lipid” may not be a suitable normalizer for these compounds. It should additionally be noted that proteins may also accumulate these compounds, as suggested by our separate study.

5. References


Acknowledgement - The authors thank Stefanie Krämer for providing liposome samples and Simon Spycher for his help in evaluating the literature data.
Integrated testing strategies (ITS) for bioaccumulation: hierarchical scheme of chemistry-driven modules and definition of applicability domains

M. Nendza¹, M. Scheringer², S. Strempel², H. Segner³, A. Lombardo⁴, A. Roncaglioni⁴, E. Benfenati⁴, A. Franco⁵, S. Trapp⁵, M. McLachlan⁶, R. Kühne⁷, R. Rallo⁸, F. Giralt⁸, S. Dimitrov⁹, E. Bleeker¹⁰, T. Vermeire¹⁰

¹ Analytisches Laboratorium, Luhnstedt, Germany
² ETH Zürich, Switzerland
³ University of Bern, Switzerland
⁴ Istituto di Ricerche Farmacologiche "Mario Negri", Milan, Italy
⁵ Technical University of Denmark, Kongens Lyngby, Denmark
⁶ Stockholm University, Sweden
⁷ Helmholtz Centre for Environmental Research – UFZ, Leipzig, Germany
⁸ Universitat Rovira I Virgili, Tarragona, Catalunya
⁹ University "Prof. As. Zlatarov", Bourgas, Bulgaria
¹⁰ National Institute of Public Health and Environment (RIVM), Bilthoven, The Netherlands

E-mail contact: nendza@al-luhnstedt.de

1. Introduction
Assessment of the bioaccumulation potential of chemicals under REACH with integrated testing strategies (ITS) requires multiple tools. Existing data have to be searched and information from chemical structures and physico-chemical properties need to be evaluated prior to considering to conduct in-vivo experiments with vertebrates. The OSIRIS inventory of chemistry-driven and in-silico BCF modules for ITS compiles:

- Sources of existing data
- Computational methods
  - B/nonB classification models
  - QSARs
  - Physiological models
  - Exposure models
  - Read across
- in-vitro tools
- 3R (Refine, Reduce, Replace) modules

2. Materials and methods
The ITS components for bioaccumulation listed in the ECHA Guidance [1,2] have been extended with new knowledge generated in OSIRIS and complemented with feedback from stakeholders on the actual problems in using ITS for chemical registration.

3. Results and discussion

3.1. ITS components for bioaccumulation
The performance of an ITS depends on the efficient combination of modules in a hierarchical manner with consensus-based decision support. A major objective is to be protective, i.e. to minimise the risk of false negative assessments (Figure 1). The weight of evidence contributed by any modelled estimate or experimental result depends on its reliability and relevance and multiple values/studies may contribute to different extents, i.e. different weight factors are allocated to the various building blocks in a Weight of Evidence (WoE) approach.

REACH requires the use of existing data and therefore a literature search must be performed before further processing. If experimental studies of adequate quality, in particular for vertebrates, are available, then no further testing is allowed and the registrant(s) must use them for dossier preparation.

Modelling approaches to BCF use different techniques at different levels of complexity. Classification models frequently use decision trees to discriminate non-B/B/vB chemicals. QSARs for BCF apply mostly log $K_{OW}$.
related physico-chemical properties and theoretical descriptors to predict numerical BCF values for individual compounds. Physiological models aim to predict the dynamic exposure of cells, organisms and foodchains and are a prerequisite for in-vitro → in-vivo extrapolations. Read-across utilises the functional similarity of chemicals to translate information from one analogue to another.

The available in-vitro tools in bioaccumulation assessments either address bioavailability and uptake into organisms (the A in ADME) or metabolism (contribution to ME in ADME). The methods are still exploratory without standard format, and need to be adapted case by case for specific questions and/or compounds.

![Figure 1. Sequential order of BCF modules for ITS.](image)

If an in-vivo bioaccumulation study is unavoidable for the chemical in question (i.e. saving 100% of the experimental animals is not possible), still a reduced test design may be realised, e.g. less concentration levels, reduced exposure duration, less samplings, less animals per concentration level (“refinement” in sensu 3R). At a very early stage of exploration is the idea of multiple endpoints testing, e.g. the combination of chronic NOEC and BCF studies to reduce the total number of animals.

### 3.2. Applicability domains

The relevance and reliability of any alternative tools, be it computational models or in-vitro assays, greatly depend on their applicability domain that shall be defined on the one hand for each individual component of the ITS and on the other hand for the combined modules of the ITS. The latter task cannot be solved in a static manner because for different compounds different sequences of modules may apply and hence different domains may interact.

### 4. Conclusions

The OSIRIS ITS for bioaccumulation (webtool) will be publicly available after further refinement based on stakeholder feedback. Its concepts and modules, as well as validation results, are presented in detail in a dedicated poster corner.

### 5. References


**Acknowledgement** - This work is supported by the EU 6th Framework Integrated Project OSIRIS (contract no. GOCE-ET-2007-037017). http://www.osiris-reach.eu.
Exposure to EDCs Disrupts the Expression of cyp19a Isoforms of the Murray River rainbowfish, Melanotaenia fluviatilis

Admane Holeyappa Shanthanagouda¹, Jawahar G Patil²³ and Dayanthi Nugegoda ¹

¹RMIT University, Bundoora West Campus, School of Applied Sciences
Bundoora, Victoria, 3083, Australia,
²Inland Fisheries Service Tasmania, PO Box 575, New Norfolk, Tasmania, Australia 7140.
³National Centre for Marine Conservation and Resource Sustainability, Locked Bag, 1370, Launceston 7250, Australia.
E-mail contact: dayanthi.nugegoda@rmit.edu.au

1. Introduction

Cytochrome P450 aromatase is the only steroidogenic enzyme activating the synthesis of oestrogens from aromatisable androgens and plays a key role in neural development, sex differentiation, sexual/mating behaviours, reproductive cycles and also in other physiological functions [¹ and ²]. The aim of this study was to elucidate the action of selected Endocrine Disrupting Chemicals (EDCs) on the mRNA expression of aromatase isoforms in the Australian native Murray River rainbowfish.

2. Experimental design and expression analysis

The Australian native Murray River rainbowfish were purchased from a commercial aquarium fish wholesaler (Aquarium Industries, Epping, Melbourne, Victoria, Australia) and reared at 25±1 °C in 16:8 h light: dark regime in flow through aquaria with carbon filtered aerated water. Throughout the maintenance, water quality parameters including temperature, dissolved oxygen and pH were monitored. This study investigated the effect of two EDCs including exogenous oestrogen 17β-oestradiol (E2) and the weak oestrogen mimic Bisphenol A (BPA) on the expression of cyp19a isoforms in both sexes of adult Murray River rainbowfish. Reproductively active male and female fish were exposed to either 1, 3, 5 µg/L of E2 or 100, 500 µg/L of Bisphenol A for 96 h. In parallel, control and carrier controls were also run in parallel. 10 fish were exposed in 10 L of water with constant aeration and every 24 h two fish were sampled from each replicate for further analysis. The expression analyses of cyp19a isoforms in the brains and gonads of both sexes were studied using quantitative Real-Time PCR (qPCR). To normalize the data for the tested genes, gapdh mRNA was used as an endogenous control for all the tissues tested.

3. Results

cyp19a1a expression in the ovarian tissues was downregulated and inhibited with E2 exposure. Whereas, it was upregulated until 48 h and reduced at 72 and 96 h with BPA exposure. However, cyp19a1a expression was not detected in the tissues including testes and the brains of both sexes. The expression of cyp19a1b in the female fish brains was upregulated until 48 h and reduced thereafter with both EDCs. However, its (cyp19a1b) expression was suppressed with E2 and upregulated with BPA exposures in the brain of males. We also studied the expression of cyp19a1b in the gonads of both sexes where cyp19a1b in the ovaries was downregulated with exposure to both EDCs. Meanwhile, its expression in the testes was upregulated with E2 and suppressed with BPA throughout exposure.
4. Discussion and Conclusions

The results showed that E2 and BPA regulate expression of \textit{cyp19a} isoforms via both positive and negative feedback mechanisms. The results support the hypothesis that the expression of \textit{cyp19a} isoforms depends on the duration of exposures, tissues and sex of the fish. Collectively the results suggest that, E2 and BPA can have a disruptive effect on the steroidogenic pathways and hence sexual/mating behaviours, sex differentiation and reproductive cycles in this fish. Both aromatase isoforms can serve as a potential biomarker for detecting and monitoring of environmental exposures to EDCs. However, this study was conducted at transcript level; therefore further research is necessary to characterize the post transcriptional effect to understand the influence of these EDCs on the development, metabolism, reproduction and sex ratio of the fish. The results in this study reinforce the need of further study to understand the mechanisms underlying EDCs effects on key endocrine systems.

Key words: EDCs, \textit{Melanotaenia fluviatilis}, \textit{cyp19a} isoforms, qPCR

5. References


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A link between environmental contaminants in Southern Alberta Rivers and physiological consequences disrupting reproduction and metabolism in fish

Hamid R Habibi, Ava Zare, Julia Jordan, Suzanne Henderson, Aalim Weljie, and Leland J Jackson

Department of Biological Sciences and Institute of Environmental Toxicology, University of Calgary, Calgary, Alberta, Canada

E-mail contact: habibi@ucalgary.ca

1. Introduction

Anthropogenic chemicals related to municipal, industrial and agricultural sources have been detected in various rivers around the world. These environmental contaminants can potentially disrupt health in humans and wildlife. In 2005 Alberta Environment (AE) released a report that contained results of water analyses due to concerns of potential impacts of environmental contaminants on humans, livestock, aquatic organisms, and wildlife in Southern Alberta, Canada [1]. A more recent study, also demonstrated the presence of a number of organic contaminants in water collected from two rivers in Alberta, Canada [2,3]. The compounds detected included synthetic estrogens and industrial chemicals downstream of municipal wastewater effluents as well as natural hormones in agricultural areas. Greater concentrations of pollutants were measured at sites downstream of wastewater treatment plants and agricultural areas, which indicate cumulative inputs of such compounds in these rivers.

2. Materials and methods and experimental approach

In the present study, we collected water samples and fish from different sites on Bow, Oldman, and Red Deer Rivers at locations upstream and downstream of a number of major municipal wastewater treatment plants as well as agricultural sites. For field study, we collected longnose dace by electrofishing. Weight, length and sex as well as weight of internal organs were determined to to calculate gonadosomatic and hepatosomati indices and condition factor. Liver, testis, ovary and brain samples were removed and immediately frozen in liquid nitrogen in the field, then kept in a -80C freezer until quantitative RT-PCR analyses were conducted. Water samples were extracted as described previously [4]. Samples were spiked with five labelled internal standards (ring-13C6-nonylphenol, propane-d6-bisphenol A, di-n-octylphthalate-d4, 17-estradiol-17-acetate, and 2,2,3,4,4,6-d6-cholesterol). The organic phase were analyzed by ultratrace analytical gas chromatography–high-resolution mass spectrometry as outlined before [25]. To test the hypothesis that chemicals present in the three rivers tested cause disruption of health in fish, we performed controlled laboratory experiments in which goldfish in aquaria were exposed to the same concentrations of chemicals detected in the river system, individually and as mixtures. Multiple end points, including expression of various genes involved in gonadal development and differentiation were measured. Tissue samples were also used for metabolite extraction as described previously [5]. Extracted samples were used for quantitative 1H-NMR spectroscopy, using Bruker Advance 600 spectrometer (Bruker Biospin, Milton, Canada) with a 5 mm TXI probe at 298 K at 600.22 MHz frequency. Standard Bruker pulse sequence was used to obtain all one-dimensional 1H NMR spectra of aqueous samples and the residual water peak was irradiated during the relaxation delay of 1.0 s and during 100 ms of mixing time. 63536 data points over a spectral width of 12195 Hz with a 90˚ pulse width and 5s repetition time were acquired into 1024 scans. Standard Bruker pulse programs were applied to generate two-dimensional NMR experiments. The following 2D spectroscopy was performed to validate metabolite chemical shift assignments. The resulting H-NMR spectra were analyzed with Chenomx NMR Suite 6.0. The spectra for all samples were manually corrected for phase and baseline and then fitted, with reference to DSS peak. Metabolites were identified and quantified using Chenomx program and its reference literature. To validate the identity of metabolites, HSQC 2D spectrum for one of the samples was compared to its 3D 1H NMR spectra of aqueous samples and the residual water peak was irradiated during the relaxation delay of 1.0 s and during 100 ms of mixing time. 63536 data points over a spectral width of 12195 Hz with a 90˚ pulse width and 5s repetition time were acquired into 1024 scans. Standard Bruker pulse programs were applied to generate two-dimensional NMR experiments. The following 2D spectroscopy was performed to validate metabolite chemical shift assignments. The resulting H-NMR spectra were analyzed with Chenomx NMR Suite 6.0. The spectra for all samples were manually corrected for phase and baseline and then fitted, with reference to DSS peak. Metabolites were identified and quantified using Chenomx program and its reference literature. To validate the identity of metabolites, HSQC 2D spectrum for one of the samples was compared to its 3D 1H NMR spectrum metabolites. Thus the identity of metabolites was confirmed and used as a template for fittings of other samples. The log10-transformed data was used to generate the z-score plots, heat maps and GEDI diagrams. Individual metabolite z-scores were calculated based on a per-organ control mean and its standard deviation using the formula z-score=(treatment metabolite abundance – control mean)/standard deviation of control. Multivariate statistical data analysis was performed on log10-transformed relative abundances with SIMCA-P software. Unsupervised Principal Component Analysis (PCA) was performed on all of the data to identify most significant variances and potential outliers in the dataset. Orthogonal partial least squares discriminant
analysis (OPLS-DA) identified most significant variations between the treatment groups. Metabolites with Variable Importance in Projection (VIP) > 1 generated the significant differences between each treatment group and control. Metabolites whose VIP>1 and their corresponding z-scores were used to generate the plots and heat map.

3. Results and discussion

The objective of the present study was to investigate the presence and adverse developmental effects of environmental contaminants in Southern Alberta rivers by means of chemical analysis, field studies and controlled laboratory experiments. Organic contaminants (natural and synthetic steroids, organic compounds and pharmaceuticals) were detected at all sites sampled along the Oldman river, Bow river and Red Deer river. We used longnose dace (Rhinichthys cataractae) to investigate a link between exposures to environmental contaminants with biological response. A significant increase in female to male adult ratio from approximately 55% to 90% was observed in longnose dace caught down stream of certain municipalities. Significant increases in vitellogenin (VTG) expression were observed in male longnose dace in correlation with female-biased sex ratios, suggesting severe endocrine disruption of gonadal development likely due to presence of compounds with estrogen-like activities. We also observed significant changes in the expression of estrogen receptor (ER) subtypes, which responds to estrogenic compounds. In addition, CYP1A gene expression was elevated in fish caught close to urban locations, indication possible presence of organic pollutants interacting with arylhydrocarbon doxine receptors. The results are consistent with the hypothesis that exogenous factors resulted in sex changes in longnose dace and caused genotypic males to develop as phenotypic females. To test this hypothesis, we performed controlled laboratory experiments in which fish in aquaria were exposed to the same concentrations of a selected number of chemicals detected in the river system, individually and as mixtures. Multiple end points, including expression of various genes involved in gonadal development and differentiation were measured. The results demonstrate that different chemicals present in the Oldman River disrupt the gene expression profile of the liver, ovary and testis, and the action of these contaminants becomes significantly larger when present in mixtures, compared to the effect of compounds individually. Our findings provide clear link between environmental contaminants present in Southern Alberta rivers with disruption of health in fish.

To further investigate the mechanisms of endocrine disruption, we have applied 1H-NMR metabolomics as a tool to measure the concentrations of metabolites in the liver and gonad tissue extracts, and evaluated net metabolic dysregulation due to exposure. The results suggest significant dysregulation of amino acid, lipid, energy, carbohydrate, nucleotide and cofactor/vitamin metabolism. The effect of mixture of contaminants on liver was significantly different from all the individual treatments as shown in the heatmap (Fig. 1). However, in testes, the effect of the mixture resembled that of Bisphenol A and DEHP. The results provide novel information on the effect of EDCs individually and in mixture on global metabolism dysregulation in male goldfish and a framework for better understanding of the metabolic pathways affected by environmental contaminants in fish. The overall results demonstrate that environmental contaminants pose a risk to the aquatic environment in Southern Alberta Rivers.

3. Conclusions

The present study demonstrates severe health disruption of fish present in Southern Alberta Rivers due to cumulative impacts of contaminants related to municipal wastewater, agriculture and large cattle operations within Southern Alberta basin. These contaminants have hormone-like activity and pose a risk to the aquatic environment in Southern Alberta.

4. References


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EROD activity in peripheral blood lymphocytes: a non-invasive and relevant biomarker of exposure of dairy ruminant to PAHs and other POPs

Yann Guiavarch¹, Cyril Feidt¹, Guido Rychen¹

Research Unit on Animal and Functionality of Animal Products, Nancy University-Institut National de la Recherche Agronomique, ENSAIA, 2 avenue de la Forêt de Haye, 54505 Vandoeuvre-lès-Nancy, France

E-mail contact: yann.guiavarch@ensaia.inpl-nancy.fr.

1. Introduction

The use of biomarkers for evaluating the level of exposure of animals to environmental pollutants should be the first step prior to any exhaustive but often time-consuming and expensive analytical investigations (extractions in complex biological matrices followed by LC or GC-MS analysis). In this respect, EROD activity has been extensively used over the past decades as a non-specific biomarker of exposure of wild fauna to CYP450 1A1/1B1 inducing persistent organic pollutants (POPs) such as PAHs, PCBs, Dioxins and many other pollutants. In fish, EROD activity is measured in the liver or in the gill tissue. In birds and marine mammals, such as seals or penguins, it is often measured in liver. But EROD activity was also measured in duodenum and kidney of laboratory animals such as minipigs or rodents. To date, there is a total lack of information on the possibility to use this activity as a biomarker to evaluate the level of exposure of dairy ruminants to POPs such as PAHs. This could however be useful since POPs are often lipophilic molecules, which can therefore be transferred toward milk in a metabolized or native form with possible issues in terms of food chain. With such big farm animals (cows, goats or sheep), EROD activity measurements should of course be performed without slaughter. Since it was shown that EROD activity could also be detected in peripheral blood lymphocytes (PBL) of rodent and humans, we investigated the possibility to use this activity in PBL of dairy ruminants exposed to PAHs.

The objective of this brief presentation is to introduce kinetic and doses/responses results showing that EROD activity in peripheral blood lymphocytes can be used as a relevant and non-invasive biomarker of dairy ruminants exposure to a 40-day daily oral exposure to PAHs, using goat as a model species. In order to check whether this EROD activity in PBL may be used to properly evaluate EROD activity in the liver (which is much higher in absolute value), we studied the EROD activity induction in PBL of rats orally exposed to PAHs over a 28-day period, with simultaneous comparison of the EROD activity induction in the liver and in the brain. Results clearly demonstrate that EROD activity in PBL can be linearly correlated to EROD activity in the liver and in the brain, thus a priori strengthening the interest of using this activity as a relevant and non-invasive biomarker of exposure of dairy ruminant to CYP450 1A1/1B1 inducing POPs.

2. Materials and methods

EROD activity in PBL of goats

The goats were divided into two groups of three animals. After the acclimation period, goats were orally dosed with either 1 mg (0.02 mg/kg body weight) or 50 mg (1 mg/kg body weight) of a ternary PAH mixture consisting of phenanthrene (the most abundant 3-ring PAH in the environment), pyrene (a 4-ring PAH systematically and abundantly present in PAH mixtures) and benzo(a)pyrene (the reference PAH in terms of toxicity with a toxic equivalent factor of 1), over a 40 day period. Each animal served as its own reference and blood samples were collected at Day 0. Contaminated oil was directly administered into the mouth of the animal with a 2 mL syringe. At 10 day intervals, Blood samplings for immediate isolation of lymphocytes were also performed every 10 days. After isolation of PBL, EROD activity was determined fluorometrically by measuring resorufin production.

EROD activity in PBL, liver and brain of rats

Three groups of rat were orally exposed up to 28 days to 0, 6, or 600 µg/day of a mixture consisting of phenanthrene, pyrene and benzo(a)pyrene. Measurements of EROD activities in the three compartments were performed at day 0, 3, 7, 14, 21, 28 and 32 (4 days after the end of the exposure).
3. Results and discussion

3.1. EROD activity in PBL of goats

Constitutive EROD activity in lymphocytes was $0.5 \pm 0.3$ pmol resorufin/min/mg protein and was significantly induced over the entire exposure time, before stabilizing after 40 days at $6.30 \pm 1.3$ and $18.89 \pm 1.12$ pmol resorufin/min/mg protein with doses of 1 mg/day and 50 mg/day, respectively. Induction kinetics could be described using a logistic-like model (Figure 1) and approximate dose-response curves were proposed based on Hill or Michaelis-menten Models (Figure 2). Unlike what was previously observed in human PBL, a quite good homogeneity of EROD responses in different animals of the same herd is obtained certainly due to a much less pronounced genetic variability compared to humans (and also to similar diets).

![Fig.1 (n=3)](image1)

![Fig.2](image2)

3.2. EROD activity in PBL, liver and brain of rats

Significant inductions of EROD activity were observed in the three compartments and could also be accurately fitted versus time of exposure using a logistic model. The total activity in each compartments were 98% in the liver, 1% in brain and 1% in PBL. Approximate dose-response curves were established and correlations were observed between each compartment (PBL/liver (Figure 3), brain/liver, liver/PBL). These results are discussed at the light of results obtained with goats. Once more, EROD responses appeared to be quite homogeneous compared to what is observed in humans.

![Fig. 3](image3)

4. Conclusions

- EROD activity in PBL of dairy ruminants is a reliable and non-invasive tool to evaluate their level of exposure to PAHs under controlled exposure conditions.
- Experimental data achieved on PBL, Liver and brain of rats strengthen, a priori, the interest of using EROD activity in PBL to evaluate, in a non-invasive way, EROD activity in the liver (the main detoxifying organ).
- In terms of EROD induction, goat’s metabolism can face much higher level of exposure than what is observed in the most severe situations.

5. References

Predictors of disease susceptibility in quail (*Corturnix c. japonica*) exposed to environmental contaminants

Judit Smits¹ & Sukbir Nain¹

¹Faculty of Veterinary Medicine, University of Calgary, Calgary, Alberta, Canada T2N 4Z6
E-mail contact: judit.smits@ucalgary.ca

1. Introduction

We investigated the immune response, physiological markers, plus disease resistance to an infectious agent (*E. coli*), to characterize the biological costs of exposure to environmental contaminants. Experimental birds (*Coturnix c. japonica*) were orally exposed to three contaminants of global concern; lead (Pb), malathion, the most widely used organophosphate insecticide in North America[1], and perfluorooctanoic acid (PFOA), a widely used group of perfluoralkyl acids used in surfactants, lubricants, food packaging, polishes, and fire-retardants[2]. Quail were exposed for 8 wks to environmentally realistic concentrations; lead acetate (0, 5, 50ppm), malathion (0, 1, 10ppm) and PFOA (0, 1, 10ppm). Immunotoxicity tests were conducted at wk 5-6 of exposure; in wk 7 birds were challenged with subcutaneous injections of *E. coli* O2.

2. Materials and methods

The selected contaminants have been described as immunotoxic [2,3,4,5]. Immune function was tested through i) the T cell mediated PHA skin test, ii) the antibody mediated B cell response to vaccination with Newcastle Disease Virus, and iii) the innate immune response measured through; iiiia) the chemiluminescence assay and iiib) the expression of TLR-3 in the bursa of Fabricius. In the PFOA study only, thyroid hormones (T4, T3) were measured in plasma.

3. Results and discussion

3.1. Lead exposure

No evidence of Pb toxicity was observed. Of all immunotoxicity tests, only TLR-3 was different (increased) in the highest exposure group (Table 1). Mortality after bacterial challenge was lowest in the high exposure group, showing a hormetic (immunostimulatory) effect with Pb exposed birds having better survival (Table 2).

<table>
<thead>
<tr>
<th>Lead (Pb) exposure</th>
<th>PHA response (mm±SE)</th>
<th>Chemiluminescence (Absorbance Units±SE)</th>
<th>TLR-3 +ve cells ±SE</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 ppm</td>
<td>1.13 ± 0.05</td>
<td>2318 ± 109.4</td>
<td>1.4 ± 0.09³</td>
</tr>
<tr>
<td>5 ppm</td>
<td>1.0 ± 0.08</td>
<td>2312 ± 95.1</td>
<td>1.6 ± 0.18³</td>
</tr>
<tr>
<td>50 ppm</td>
<td>1.02 ± 0.04</td>
<td>2320 ± 80.8</td>
<td>2.3 ± 0.06³</td>
</tr>
</tbody>
</table>

Table 1. T cell proliferative (PHA) response, respiratory burst activity by phagocytes (chemiluminescence), and TLR-3 positive cells in bursae from quail exposed to lead.

<table>
<thead>
<tr>
<th>Health Effect</th>
<th>n</th>
<th>Control group</th>
<th>5 ppm Pb</th>
<th>50 ppm Pb</th>
</tr>
</thead>
<tbody>
<tr>
<td>Morbidity/Mortality</td>
<td>18</td>
<td>55.5% n=10</td>
<td>44.4% n=8</td>
<td>27.8% n=5</td>
</tr>
<tr>
<td>Bacteraemia</td>
<td>18</td>
<td>72.20% n=13</td>
<td>61.1% n=11</td>
<td>55.5% n=10</td>
</tr>
<tr>
<td>Airsacculitis</td>
<td>18</td>
<td>16.67 % n=3</td>
<td>11.11 % n=2</td>
<td>5.56% n=1</td>
</tr>
</tbody>
</table>
3.2. Malathion

In contrast to Pb, malathion was immunosuppressive with decreased circulating leucocytes (white blood cells WBC) and lymphocytes, decreased secondary antibody response, and increased mortality in the higher exposure group (Table 3). Histopathology revealed atrophy of the bursa of Fabricius, explaining the compromised antibody response.

<table>
<thead>
<tr>
<th>Malathion exposure</th>
<th>n</th>
<th>WBC</th>
<th>Lymphocytes</th>
<th>Secondary Immune Response</th>
<th>Mortality</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control group</td>
<td>18</td>
<td>Significance</td>
<td>35171 ±4229b</td>
<td>105 ±5.7b</td>
<td>22.2% n=4</td>
</tr>
<tr>
<td>1 ppm</td>
<td>18</td>
<td>32089 ±3489m</td>
<td>29400 ±4533m</td>
<td>103 ±11.4b</td>
<td>33.3% n=6</td>
</tr>
<tr>
<td>10 ppm</td>
<td>18</td>
<td>24733 ±1372s</td>
<td>21466 ±1281s</td>
<td>78 ±4.1s</td>
<td>50.0% n=9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>p=0.01</td>
<td>p=0.01</td>
<td>p=0.03</td>
<td>p=0.08</td>
</tr>
</tbody>
</table>

Table 3. Total WBC and lymphocyte count, secondary antibody response, and percent mortality after challenge with E. coli serotype O2 (1X10⁴ CFUs/mL)

3.3. PFOA

No clinical signs of PFOA toxicity were seen, and morbidity and mortality after bacterial challenge was not different among the groups. Both T4 and T3 were lower in the PFOA exposed birds (p<0.01). Although T cell response in the high dose group was lower (p<0.02) than low dose and control birds, the B cell and innate immune responses were not different. Although previous studies have shown that PFOAs are immunotoxic [6], the suppressed T cell response from PFOA did not translate into lowered overall lowered disease resistance.

4. Conclusions

This study of immunotoxic environmental contaminants provides evidence that an integrated examination of immune function using an infectious challenge is a better predictor of true immunocompetence than are individual tests of adaptive (specific) and innate (nonspecific) immune responses.

5. References


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Neuroanatomical and behavioral effects of early exposure to BDE-99 in an integrated avian laboratory and field model system

Margaret L. Eng¹, Tony D. Williams¹, Scott A. MacDougall-Shackleton², Robert J. Letcher³, and John E. Elliott⁴

¹Simon Fraser University, Burnaby, British Columbia V5A 1S6, Canada
²University of Western Ontario, London, ON N6A 3K7 Canada
³Environment Canada, Ottawa, Ontario K1S 5B6, Canada
⁴Environment Canada, Delta, British Columbia V4K 3N2, Canada
E-mail contact: mea10@sfu.ca

1. Introduction

Polybrominated diphenyl ethers (PBDEs) are a class of brominated flame retardants that have become ubiquitous in the environment. BDE-99 is one of the most abundant congeners, and is consistently found in avian tissue and egg samples throughout the world [1]. Previous avian studies have shown that early developmental conditions, such as nutrition and contaminant exposure, can have long-term neuroanatomical effects on the song control system [2, 3], which could in turn affect mating behavior and reproductive success. Contaminants may affect brain structure through various mechanisms, including altering stress hormone levels, or acting as sex-steroid disruptors [3]. BDE-99 has been demonstrated to cause neurotoxic and sex-steroid disrupting effects in mammals [e.g. 4, 5], and there is evidence that pentaBDEs can alter reproductive parameters in birds [6].

The objective of this study was to determine neuroanatomical and behavioral effects of early exposure to BDE-99 in an integrated avian laboratory and field model system, using the Zebra Finch (Taeniopygia guttata) and the European starling (Sternus vulgaris) as model passerine species. The zebra finch is a useful model to monitor effects of contaminants under controlled laboratory conditions, as they reach sexual maturity within 90 days, and readily breed in captivity. The European starling can be used as an ecological equivalent for comparative in situ investigations. Starlings are widespread, readily use nest boxes, feed on terrestrial invertebrates, and are easy to sample.

Because there is evidence that BDE-99 causes direct neurotoxicity and sex-steroid disruption, we predict that birds exposed to BDE-99 in the nest will have: (1) Smaller brain regions, (2) decreased song output and quality, and (3) Modified mating behavior.

2. Materials and methods

We conducted a pilot study to validate dosing. Nestling zebra finches were exposed to BDE-99 in safflower oil (0, 5, 25 or 100 ng/g bw/day; 10µl/g bw) for the duration of the 21-day nesting period. At 30 days of age, young were sacrificed. Plasma and adipose tissue was collected and analyzed for BDE-99 as well as lower brominated congeners (e.g., BDE-47 and BDE-28). PBDE determination was performed using GC/MS (ECNI mode).

We used a captive colony of zebra finches maintained in a controlled environment at Simon Fraser University in Burnaby, B.C.. Young were orally exposed to BDE-99 (0, 5, 25, 100 or 250 ng/g bw/day) for the duration of the 21-day nesting cycle. Birds were then raised to sexual maturity. Exposed males underwent two ten-minute mating trials over two days, using a different female for each trial. Songs from the trials were digitally recorded, and all mating behavior was recorded by an observer blind to treatment. We calculated various aspects of song quality (rate, phrase duration, # of syllables per song phrase, different syllables per song phrase). All behaviors repeatable within individuals across mating trials were used for further analysis. Following completion of mating trials, birds were sacrificed and brains were immediately collected.

In the breeding season of 2010, we monitored a nest box population of European starlings in Langley, B.C.. Young were orally exposed to 0, 25 or 250 ng BDE-99/g bw/day for the duration of the 21-day nesting cycle. In order to continue monitoring of experimental individuals, it was necessary to bring them into captivity prior to fledging (day 15). Birds were raised to maturity and sacrificed at the estimated peak of reproductive activity. Brains were immediately collected.

Brains were fixed in paraformdehyde, then cryoprotected and stored at –80°C. Using a cryostat, 40 µm coronal sections were cut and mounted onto microscope slides, then Nissl stained. All measurements were...
performed by an observer blind to treatment. Slides were examined with a bright field microscope equipped with a digital microscope camera. Images of sections of the telencephalon and the song-control regions, HVC, area X and RA, were captured. We used ImageJ software to trace the outlines of these regions and then combined these areas using the formula for the volume of a cone frustum to estimate the total volume of each structure.

All statistical analyses were done using SAS 9.1. Dose group differences in brain region sizes, behavioral traits and song characteristics were compared using PROC GLM. Categorical behaviors were compared using PROC FREQ. Repeatability of mating behavior was determined using PROC NESTED

3. Results and discussion

We measured PBDE concentrations in 20 zebra finch plasma samples (n = 5/dose) and 6 adipose samples. We found a strong dose-dependent relationship for plasma BDE99 levels at 30 days of age, with concentrations ranging from 1.6 ± 0.6 ng/g ww to 26.1 ± 5.9 ng/g ww. In addition, adipose tissue concentrations were significantly correlated with plasma levels (R² = 0.985, p<0.001).

We measured the neuroanatomy of 40 male zebra finches (n = 8 per dose group). We found no effect of dose on any of the song control nuclei (P > 0.1 for all regions). Whether a male sang or not was significantly affected by BDE-99 exposure, with higher treatment groups having a lower than expected proportion of birds that sang (P = 0.032). Although there was an effect on whether the birds sang or not, if the birds did sing, there was no effect of treatment on the song quality characteristics (P > 0.6 for all measures). All of the song quality traits were highly repeatable across trials (P < 0.0001 for all traits). Whether a male engaged in mating behavior had a similar response to whether a male sang or not, with higher treatment groups having a lower than expected proportion of males that interacted with the females (P = 0.02). In males that engaged in courtship behavior, the number of successful mounts was significantly higher in control birds than in the higher dose group birds (P = 0.036).

We collected a total of 18 male and 18 female European starling brains. Neuroanatomical measurements are currently underway.

4. Conclusions

In zebra finches, there was no significant effect on the volumes of the song control nuclei. There were negative effects on singing, but not song quality, and there were negative effects on mating behavior and success in zebra finches. In this study we found that zebra finches and European starlings can be successfully used as a model passerine system to link contaminant exposure with biological responses in passerine birds.

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CYP1A1 and CYP2B expression in mysticete skin biopsies from the Gulf of California: responses to contaminant or interspecies differences in detoxification ability?

Fossi, M.C. 1, Urban, J. 2, Maltese, S. 1, Coppola, D. 1, Panti, C. 3, Rojas-Bracho, L. 4, Jimenez, B. 5, Muñoz-Arnanz, J. 5, Marsili, L. 1

1 Department of Environmental Sciences, University of Siena, Via Mattioli 4, Siena, Italy.
2 Departamento de Biología Marina, Universidad Autónoma de Baja California Sur, La Paz, Mexico.
3 Department of Evolutionary Biology, University of Siena, Via A. Moro 2, 53100 Siena, Italy.
4 Programa de Mammifero Marino - Instituto Nacional de Ecología, C/o CICESE, Ensenada, Mexico.
5 Institute of Organic Chemistry, CSIC, Juan de la Cierva 3, 28006 Madrid, Spain.
E-mail contact: Fossi@unisi.it

1. Introduction
The main objective of this project was to investigate the inter-species differences in CYP1A1 and CYP2B expression and contaminant levels (OCs, PBDEs and PAHs) in three mysticete species, blue whale (Balaenoptera musculus), fin whale (Balaenoptera physalus) and Bryde’s whale (Balaenoptera edeni) of Gulf of California (Mexico) using skin biopsy as diagnostic tools. A suite of sensitive non-lethal biomarkers was applied, for the first time, to the three mysticete species in order to evaluate the toxicological status of this cetacean species in the Gulf of California and also to explore the role of migratory behavior and the feeding habits (zooplankton-eating species, versus fish-eating species) in the evolutionary process of the two isoforms of cytochrome. CYP1A and CYP2B have been previously detected in cetacean skin and induction of these isoforms was measured after exposure to lipophylic contaminants such as OCs, PAHs and PBDEs, both in vitro and in field studies [1,2,3].

2. Materials and methods
Sampling – Integument biopsies (epidermis, dermis and blubber) were collected from free-ranging Bryde’s whales (n=4) and fin whales (n=5) in the Gulf of California during the summer 2008 and blue whales (n=4) during the spring 2010 using biopsy darts launched with a crossbow (CITES Nat. IT 025IS, Int. CITES IT 007).

Organochlorine Compounds (DDTs and PCBs) were analyzed according to the U.S. EPA 8081/8082 method.

Low-Brominated (Tri- to Hexa-) BDEs were analyzed using GC-LRMS-ITD in the MS/MS operating mode, using the isotope dilution technique as described elsewhere [4].

Polycyclic aromatic hydrocarbons levels were quantified with High Performance Liquid Chromatography [5].

CYP1A1 - CYP2B western blot. Analysis of CYP1A and CYP2B was performed in integument biopsies by WB, using goat anti-rabbit CYP1A1 and CYP2B4 (Oxford MI, USA). Semi-quantitative analysis was performed with Quantity One software (BioRad) [6].

3. Results and discussion
This “multi-trial diagnostic tool”, applied to skin biopsies, underlined differences in POP levels and molecular biomarker responses between the three mysticete species of Gulf of California. Two main factors seem to regulate the expression of different CYP isoforms in the three species studied; the inductive power of POPs and the different evolution of the two cytochromes related to the different feeding habits of the mysticete species.

With regard the level of POPs higher levels of DDTs (Fig.1A) and PCBs (Fig.1B) were detected in the blue whale and fin whale (zooplankton-eating species) in comparison to the Bryde’s whale (fish-eating species). Particular concern can be generate from the high levels of PCBs detected in the migratory species blue whale, that could have bioaccumulated POPs during the migratory moves along the Californian coast. This contamination phenomenon can have induced both CYP1A1 (Fig.1C) and CYP2B (Fig.1D) in this species. Intermediate level both in term of OC levels and CYP expressions are detected in the Sea of Cortez resident.
species fin whale. On the opposite, extremely higher level of both CYP1A (Fig.1C) and CYP2B (Fig.1D) were detected in the fish-eating species, showing similar level of cytochromes to odontocete species resident in Sea of Cortez (Fossi, data not shown). Lower levels of OCs and high level of the CYP2B were detected in the Bryde’s whale specimens, suggesting a higher detoxification ability in the fish-eating species.

4. Conclusions

In conclusion these data show that two main factor can regulate the expression of the two CYP proteins in the mysticete species of Gulf of California: a) the inductive phenomenon linked to the presence of POPs of planar (CYP1A1) and globular (CYP2B) halogenated compounds in the blubber of blue whale; b) the role of evolutionary pressure related to the different dietary habits of the species (zooplankton-eating species fin whale and blue whale, fish-eating species Bryde’s whale). In particular, these preliminary evidences suggest a peculiar evolutionary process of the two isoforms of CYP in the fish-eating species (Bryde’s whale), which showed levels of both cytochromes similar to the odontocete species.

5. References


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**Glutathione-s-transferase protein and activity in epidermal tissue of humpback whales**

Michael Burkard1,4, Willa Huston2, Courtney Waugh3, Susan Bengtson Nash4

1Institute for Environmental Science, University of Koblenz-Landau, 76829 Landau, Germany
2Institute of Health and Biomedical Innovation, Queensland University of Technology, Kelvin Grove, QLD 4059, Australia
3The National Research Centre for Environmental Toxicology, The University of Queensland, Coopers Plains, QLD 4108, Australia
4Atmospheric Environment Research Centre (AERC), Griffith University, Nathan, QLD 4111, Australia

E-mail contact: burgikla@gmx.de

1. Introduction

Cetaceans (whales, dolphins, and porpoises) are particularly susceptible to accumulation of toxic burdens of lipophilic organochlorine compounds (OCs) due to their longevity and position at the top of marine food chains. Despite frequently reported elevated levels of blubber OC burdens in these species, the performance of chemical risk assessments is precluded by a lack of ancillary individual and toxicological information. Recently the International Whaling Commission (IWC) steering group on contaminants flagged the importance of driving research towards facilitation of cetacean chemical risk assessment[1].

Currently OC toxicology on mysticetes (filter-feeding whales) relies primarily on skin and blubber biopsies due to the rarity of stranding events of these large, often migratory species. As such advancements in mysticete toxicology must be underpinned by utilisation of these tissues for further molecular assessments.

Glutathione-s-transferase (GST) catalyses the conjugation of glutathione with various xenobiotics and therefore plays a major role in preventing oxidative stress[2]. GST has been detected across the animal kingdom and like many detoxification enzymes is substrate inducible and therefore a potential candidate for biomarker applications. To date no studies have reported the presence of GST in the skin, the most accessible tissue, of cetaceans.

Here we investigate the presence and activity of GST in the epidermal tissue of southern hemisphere humpback whales (*Megaptera novaeangliae*) and make a preliminary assessment of its applicability as a biomarker tool.

2. Materials and methods

Whales were biopsied off north Stradbroke Island, south east Queensland, Australia, at two time points on their annual migration; 1) northward (post-summer feeding) and 2) southward (end of fasting). Whale skin was stored at -80°C upon collection and the cytosolic fraction, with the microsomal fraction removed, was extracted and applied to the subsequent tests.

Whale skin cytosol was probed for GST protein by western blotting with a goat polyclonal GST primary antibody (Abcam) and anti-goat IgG secondary antibody (Sigma-Aldrich). Bands were detected by the ECL kit as per manufacturer instructions (GE Healthcare). GST activity was measured fluorometrically by the production of the GST-CDNB (1-chloro-2,4-dinitrobenzene) conjugation product via the method of Habig et al. (1974)[3].

3. Results and discussion

Western blotting verified for the first time the presence of GST protein in the skin of humpback whales (Fig 1). Following verification of the GST protein in a sub-set of animals, GST activity was measured in skin extracts of 33 individual animals. 22 of the animals were biopsied on the northward leg of their migration (19 male, 3 female) and 11 on the southward leg (6 males and 5 females). All extracts were analysed in triplicate and datasets showing a coefficient of variation of ≤35% were further compared.
The average measured activity in extracts was 1.44 U (1 unit = the amount of enzyme producing 1 µmol GST-CDNB product). No significant differences in activity were observed between the sexes or between migration cohorts, although expressed activity was observed to be lower in southward migrating (fasted) cohorts of both sexes (Fig. 2). This is in contrast to the expected increase in lipophilic OC exposure occurring at this time due to remobilisation of contaminant burdens along with stored lipid reserves. The production of reactive oxygen species is however a consequence of all metabolic processes and it is possible that at this late stage of the migration, following extended fasting, metabolism has been significantly depressed masking the expected increase in lipophilic contaminant exposure.

4. Conclusions
This study verified for the first time the presence of an active GST system in the skin of humpback whales. GST activities expressed were generally low and this combined with a relatively small sample number prevented quantitative examination of variation between sample cohorts. Future work will investigate the relationship between activity and OC contaminant burdens of the whales to further assess the suitability of the enzyme as a biomarker of OC exposure.

5. References

Acknowledgement - The authors would like to thank various field volunteers who assisted in the collection of tissue samples.
1. Introduction

Ecologists have been developing mechanistic models of ecological systems ever since the 1960s, when computers capable of numerically solving complex sets of equations first became widely available. The mechanistic detail possible in ecological modeling was necessarily limited by the primitive – by today’s standards – computers available to ecological modelers. Yet, within a few years it became clear that even with 1960’s technology the ability of modelers to write equations and define parameters greatly exceeded the ability of empirical ecologists to measure parameter values and perform experiments needed to validate the models. With the technological tools available to today’s ecological modelers, biological processes from the scale of the cell to the scale of an entire landscape can be linked together in a single model. However, the ability to develop such comprehensive models does not guarantee that the models will accurately simulate the behaviors of real biological systems, or that they can be used with confidence to support environmental risk management decisions. The fundamental limitations on ecological modeling that were recognized 40 years ago still remain, and are even more important now than they were at that time.

2. Case Studies

The purpose of this paper is to discuss those limitations, and propose steps that could be taken by today’s ecological modelers to ensure that mechanistic models achieve their full potential in ecological risk assessment. This paper will illustrate the above limitations through illustrative case studies of past modeling efforts and regulatory applications. The case studies will include theoretical explorations of the tradeoffs between various types of uncertainties affecting ecological models [2] and applications of models to two important environmental problems: assessing impacts of nuclear power plants on fish populations [3] and predicting potential ecological effects of toxic chemicals [4]. Discussion of the case studies will highlight both the scientific uncertainties inherent in the modeling process and the difficulties introduced by the differences between the scientific environment in which models are developed and the regulatory environment in which the models must be applied.

3. Recommendations

The above case studies provide valuable insights into the requirements for developing ecological models that can support regulatory ecological risk assessments. Based on these case studies, the paper will describe a generalized approach for ensuring that mechanistic models developed to support ecological risk assessments are not only properly tested and documented, but also suitable for implementation in regulatory applications such as pesticide registration and chemical risk management.

4. References

Comparison of bioassays with different exposure patterns: the predictive potential of mechanistic modelling

Elise Billoir¹,², Hélène Delhaye³, Carole Forfait², Bernard Clément³, Sandrine Charles² and Marie-Laure Delignette-Muller²

¹Pôle de Recherche ROVALTAIN en Toxicologie Environnementale et Ecotoxicologie, Bâtiment Rhovalparc - Entrée B, 1 avenue de la gare - BP 15173 - Alixan, F-26958 Valence Cedex 9, France
²Université de Lyon, F-69000, Lyon; Université Lyon 1; CNRS, UMR5558, Laboratoire de Biométrie et Biologie Évolutive, F-69622, Villeurbanne, France
³Université de Lyon, F-69000, Lyon; ENTPE; Laboratoire des Sciences de l'Environnement, rue Maurice Audin, F-69518, Vaulx-en-Velin, France

E-mail contact: elise.billoir@univ-lyon1.fr

1. Introduction

To test the effects of cadmium on laboratory aquatic microcosms, two experiments were performed, one in static conditions, one in flow-through conditions. In the first one, because of partitioning, the cadmium concentration in water was time-varying (TV). In contrast, the second one was designed so that the cadmium concentration in water remained close to constant (CST). In this paper, we addressed the following biological questions: did the flow-through conditions improve the organism development? Did they influence the sensitivity of organisms to cadmium? Focusing on the cladoceran Daphnia magna, we aimed at demonstrating the added value of mechanistic modelling compared with a classical analysis of data to answer these questions.

2. Materials and methods

In a previous study [1], we proposed a dynamic modelling framework inspired from DEBtox models (Dynamic Energy Budgets in toxicology) [2] to simultaneously analyze growth, reproduction and survival of D. magna in the context of time-varying exposure patterns. Here, we used this framework to analyze both TV and CST experiments, the constant exposure being considered as a particular case of time-varying exposure. The modelling approach was designed to take the exposure pattern into account. The principle was to distinguish the exposure and its impacts on the biological endpoints considered (daphnid survival, reproduction and growth). The exposure module of the model was adapted to TV and CST conditions, while impact models, accounting for effects due to exposure day after day, were the same for both experiments. Consequently, the results obtained in TV and CST cases were directly comparable.

Bioaccumulation kinetics was considered through a one-compartment model, linking the scaled internal concentration to the external one, with an elimination rate \( k_e (d^{-1}) \). Basing on a recurrent approach with a time step of one day, we then basically assumed that cadmium effects between times \( t-1 \) and \( t \) depended on the scaled internal concentration at \( t-1 \). More precisely, effects were assumed to occur when the scaled internal concentration exceeded a threshold concentration called \( NEC \): (no effect concentration, \( \mu g.L^{-1} \)), and to be proportional (coefficient \( k_r, \mu g^{-1}.L \)) to the excess above the \( NEC \). Two stress functions were distinguished: one accounting for lethal effects - in this case \( SC \) for Survival, and one accounting for sublethal effects - in this case \( GR \) for Growth and Reproduction. We named non-toxicological the parameters of control equations and toxicological those related to cadmium toxicity.

For each experiment (TV and CST), we used simultaneously the three types of data available (time-course of survival, growth and reproduction) to estimate all parameters by the mean of Bayesian inference. The principle of this inference technique is to update from chosen prior probability distributions to posterior probability distributions given the data.

3. Results and discussion

Concerning non-toxicological parameters, the point estimates obtained from TV and CST data sets differed (Figure 1), while estimate uncertainties were similar. We obtained a higher blank mortality rate \( (m) \), a lower maximum reproduction rate \( (R_{m}) \) and a lower maximum body length \( (L_{m}) \) in the TV case than in the CST one. These three results meant a better fitness of daphnids in the CST experiment than in the TV one,
independently of cadmium exposure. In accordance with our expectations, this may be attributable to the improvement of bioassay conditions due to water renewal in the CST experiment.

In contrast to non-toxicological parameters, for some toxicological parameters the estimate uncertainties obtained from TV and CST data sets differed (Figure 1). The elimination rate ($k_e$) was much more precisely estimated in the TV experiment than in the CST one, as well as lethal stress function parameters, NEC for survival ($NEC_s$) and intensity coefficient ($k_s$). Nevertheless, the point estimates were in accordance. From both experiments, similar posterior distributions were inferred for sublethal stress function parameters, NEC for growth and reproduction ($NEC_{GR}$) and intensity coefficient ($k_{GR}$).

Stress functions account for the impacts of cadmium in comparison to control conditions. The stress function parameters we inferred from the TV case and the CST case, independently, were in accordance, although control parameters were different in both cases. This therefore reveals the predictive potential of our modelling approach to describe cadmium impacts, whatever the exposure pattern (at least between the two exposure patterns tested). Given control conditions, our models might be able to predict the impacts of any scenario of cadmium exposure in D. magna.

4. Conclusions

The results obtained in this study confirm the potential of mechanistic modelling to make sense of ecotoxicological test results. In contrast to classical analyses (NOEC, EC), process-based modeling allows one to deal with various exposure patterns and to infer lethal and sublethal stress functions as responses to exposure. This study also reinforces the suitability of the Bayesian approach applied to process-based modeling. Considering all quantities as random variates, in the form of probability distributions, provides explicit and complete information for their comparison. The answers to our biological questions might be roughly summarized as follows: yes, flow-through conditions improved daphnid development, and no, it did not make the organisms less or more sensitive to cadmium. Finally, the similarity of cadmium stress functions inferred from time-varying and constant exposure conditions underlines the potential of our approach towards predictive ecotoxicology.

5. References


Juvenile food limitation: ecotoxicologists, be warned!

Elke ZIMMER¹, Tjalling JAGER¹, Virginie DUCROT²

¹ Dept.of Theoretical Biology, Vrije Universiteit, de Boelelaan 1085, NL-1081 HV, Amsterdam, the Netherlands
² INRA, Equipe Ecotoxicologie et Qualité des Milieux Aquatiques, UMR985 Ecologie et Sante des Ecosystemes, Agrocampus Ouest, 65 rue de Saint Brieuc, 35000 Rennes, France
E-mail contact: elke.zimmer@falw.vu.nl

1. Introduction

It is in the nature of standard ecotoxicological tests that they are as simple as possible. They are conducted generally under standardised laboratory conditions for the species of interest; conditions which have been proven to maintain the species in a good condition. Since we do not know exactly what the organisms eat in their natural habitat, and some even change their diet during the life-cycle, it is likely that the chosen food is not optimal for them throughout the whole life-cycle. However, if we do not know exactly what situations we investigate in the laboratory toxicity tests, how can we trust our extrapolations to populations in the field?

One simple way to test for the nutritional state of organisms is to scrutinise their growth curve. It has been found already nearly a century ago [1] that the change in length over time of most organisms follows a certain shape under optimal feeding conditions: growth is continuous, linear in the beginning, and approaches to the maximum size asymptotically (equivalent to one-compartment accumulation curves, see Fig. 1). Therefore, a deviation of the growth curve of an organism from this Von Bertalanffy pattern points at deviation from the optimal condition of the organism and urges further investigation. One well established theory for metabolic organisation, that predicts Von Bertalanffy growth under constant conditions in the standard version, is the Dynamic Energy Budget (DEB) theory ([2]). By using DEB theory for the interpretation of life-history data, deviations from the optimal conditions, for example in terms of food limitation in the juvenile stage ([3]), can be detected. For ecological risk assessment, it is essential to detect only effects that are caused by the chemical of interest, and to exclude any side effects following from experimental conditions. In fact, if the organisms are stressed due to experimental conditions, this stress might interact with the effect of the chemical, and lead to a misinterpretation of the chemical's toxicity.

Fig. 1: The Von Bertalanffy growth as predicted for L. stagnalis using the standard DEB model (left, black line). The measured growth (data V. Ducrot, red) deviates from the Von Bertalanffy pattern. By including a food limitation of the juveniles depending on body length in the standard model (right, black line), the model fits the data.

We investigated the effects of food limitation on predictions for toxic impacts on the population growth rate using data from life-cycle experiments with Lymnaea stagnalis. Under controlled laboratory conditions(see [4]), the survival, growth, development and reproduction of L. stagnalis were monitored in clean water conditions for a year, starting from freshly laid egg-clutches. Even though the animals were fed ad libitum with lettuce, the growth curves deviated from the Von Bertalanffy pattern as predicted by DEB theory under constant experimental conditions (see Fig.1). Additional partial life-cycle experiments with different food sources (i.e. fish flakes) clarified that juveniles can grow much faster on other food sources than on lettuce, which indicates that they are not under optimal conditions in the chosen laboratory setup. New born pond snails are believed to feed on periphyton in nature, and it is likely that they smoothly change their diet to leaved plants during development. Therefore, we implemented food limitation as a function of length in the standard DEB model.

In ecological risk assessment, focus is placed on the estimation of population level effects. Does the food limitation in a short part of the life-cycle of a test organism affect interpretation of the toxic effects, and does
this have consequences for extrapolation to the population level? In a simulation study, we investigated how an initial food limitation in juveniles affects the interpretation of toxicity data, and distorts the extrapolation of toxic effects to the population level.

2. Simulation studies

A set of standard DEB parameters for *L. stagnalis* was estimated from data on growth and reproduction at different food levels (unpublished data, V.Ducrot). The standard DEB equations ([5]) were used to simulate the life-cycle of the snail, from which the population growth rate was computed using the Euler-Lotka equation (e.g. [3]). Two different feeding scenarios were studied: (i) snails were fed *ad libitum* during the whole life cycle (i.e. like assumed in the standard DEB model and toxicity tests) and (ii) a food limitation occurred in juveniles, as a function of length (like observed during the tests). From the DEB perspective, a potential mechanism of effect of toxicants is the increase of somatic maintenance costs, which results in an increase of the somatic maintenance rate coefficient $k_M$. This would result in a decrease of the maximum length of the organism, but does not have a direct effect on reproduction (only indirectly, as body size determines feeding rates). In the simulation study, we investigated the relevance of accounting for juvenile food limitation when analysing ecotoxicity data by comparing the population growth rates simulated under both scenarios (see Fig. 2).

![Figure 2: The growth curves of *L. stagnalis* as predicted from the standard DEB model (left, black solid) with the extension with the food limited juveniles (left, red solid). With an increasing $k_M$, the population growth rate for the standard DEB model (right, black line) goes to zero for much higher values of $k_M$ comparing to the food limited juveniles (right, red line)](image)

3. Conclusions

The simulated growth curves show a clear difference in the effect on somatic maintenance costs (due to the theoretical toxicant) between the standard DEB predictions and the food-limited juveniles. Under non-limiting food conditions the snails have enough energy to reach the adult stage and reproduce, in spite of higher somatic maintenance costs. The food-limited juveniles do not start to reproduce during the simulation time; their initial growth is severely impeded because of the interaction between the food limitation and the effects of the toxicant. The extrapolation to the population level also shows that the food limitation in juveniles leads to greater effects of somatic maintenance on the population growth rate, and to extinction at much lower effects on this parameter. This result points to the conclusion that extrapolations to population-level effects from ecotoxicological experiments with organisms that are (unknowingly) not held under optimal conditions may lead to an overestimation of toxicity. We therefore strongly recommend to closely investigate the organism’s food requirements (especially for juveniles) under control conditions to avoid those overestimations.

4. References


The importance of density dependence and intra-specific interactions in population models for use in ecological risk assessment

Charles R.E. Hazlerigg1,2, Kai Lorenzen1, Charles R. Tyler2, James R. Wheeler3 and Pernille Thorbek3

1 Imperial College, Silwood Park, Ascot, Berkshire, SL5 7PY, UK.
2 Ecotoxicology and Aquatic Biology Research Group, University of Exeter, Hatherly Laboratories, Prince of Wales Road, Exeter, EX4 4PS, UK.
3 Environmental Safety, Syngenta Ltd., Jealott’s Hill International Research Centre, Bracknell, Berkshire, RG42 6EY, UK.
E-mail contact: c.hazlerigg07@imperial.ac.uk

1. Introduction

The range of endpoints used to assess the effects of test substances in the laboratory cover mortality, growth and development. Under certain circumstances various biomarkers and reproductive success are also measured. However, population level processes (e.g. density dependence, behavioural interactions) may mean that statistically significant effects from laboratory based studies may not necessarily reflect their real-world importance for population abundance and biomass. Population models for the fathead minnow and roach have been developed to try to address this issue1-2. However, whilst the literature is extensive for zebrafish (Danio rerio) biology and ecotoxicology, offering great potential for developing population models, it has so far been overlooked. We have formulated an individual-based zebrafish population model using results derived from a survey of wild fish, semi-natural experiments and meta-analysis data. We use it here to investigate the population level importance of three lifecycle based ecotoxicology endpoints: sex ratio, growth and fecundity.

2. Materials and methods

The model, written in Netlogo (CCL, Northwestern University), mimics a 6m² pond (Fig. 1(a)). Zebrafish development is divided into four life-stages (eggs, larvae, juveniles and adults) with life history characteristics altered with each time-step (Fig. 1(b)). Density dependence is included for growth and survival and parameterised from experimental results. Exposure scenarios causing a 10, 50 and 90% decrease in growth rate and fecundity were investigated. Sex ratio scenarios tested for a 10, 30, 70 and 90% proportion of the population developing into males. Each simulation ran for 3000 days, with each scenario repeated 10 times.

Figure 1: (a) View of spatially explicit model. Green patches are vegetation where juveniles have decreased predation. Red patches are breeding-grounds for reproduction, where eggs are safe from cannibalism. Blue patches are open water. (b) Model scheduling and overview of each process.
3. Results and discussion

3.1. Sex ratio

- Sex ratio changes in the hatched eggs resulted in altered sex ratios in the mature population (Fig. 2(a)). However, the abundance of mature adults in the population significantly increased with increasing male sex ratio bias (ANOVA $F_{3,36} = 9.46$, $P < 0.001$, Fig. 2(b)), likely caused by the earlier maturation of male individuals compared with female individuals.

![Fig 2: Changes in population sex ratio (a) and abundance of mature adults (b) under different male proportions of hatched young - 0.1 (black), 0.3 (red), 0.7 (green) and 0.9 (blue).](image)

3.2. Growth and reproduction

- Growth depression resulted in significantly reduced abundance of mature females with a 10% growth rate decrease resulting in a 30.3% lower mature female abundance. A 50+% decrease in growth rate resulted in population extinction within the 3000 day simulation.

- Fecundity depression resulted in significantly increased abundance of mature females in the population (ANOVA $F_{3,36} = 16.93$, $P < 0.001$), likely caused by increased young survival through density dependent processes at lower population densities.

4. Conclusions

- Although feminisation of fish by endocrine disruptors is commonly found\(^3\), sex ratio bias is not necessarily a cause for concern at the population level in its own right.

- Populations are sensitive to changes in the growth rate of individuals, with small reductions in individual growth rates resulting in large reductions in population abundance.

- Fecundity reductions often observed via endocrine mediated effects\(^4\) may be compensated for in wild populations due to density dependent processes.

5. References


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Towards good modelling practice: TRACE, a standard for documenting ecological modelling in chemical risk assessment

Amelie Schmolke¹, Pernille Thorbek², Donald DeAngelis³ and Volker Grimm¹

¹ Helmholtz Centre for Environmental Research – UFZ
Dept. of Ecological Modelling
Permoserstraße 15, 04318 Leipzig, Germany
² Syngenta, Environmental Safety
Jealott's Hill International Research Centre
Bracknell, Berkshire RG42 6EY, UK
³ USGS/Florida Integrated Science Centers
and Dept. of Biology, University of Miami,
PO Box 249118, Coral Gables, FL 33124, USA
E-mail contact: amelie.schmolke@ufz.de

1. Introduction

Ecological models are becoming increasingly important in the context of chemical risk assessments [1]. Due to the interaction of numerous factors, and the extent of temporal and spatial scales of concern, empirical approaches often are too limited to inform decisions or regulations that are aimed at the population or ecosystem level. Ecological models have the potential to bridge this gap. Several key mechanisms can be included in an ecological model simultaneously, and existing knowledge and experimental data can be extrapolated to larger temporal and spatial scales. However, no general guidelines exist for the development and use of ecological models. Such guidelines for good modelling practice would be essential for quality assurance of ecological models in the context of chemical risk assessments, and would provide a tool for regulatory agencies to assess the usefulness of models in specific contexts.

In the scientific literature, numerous publications introduce guidelines or advice for the development, evaluation and application of ecological models in decision support contexts [2]. However, such advice is not easy to implement in actual modelling projects. Here, we present the first step towards the implementation of good modelling practice: a standard framework for the documentation of ecological models and the underlying modelling process [2]. This documentation framework covers model development, evaluation, and application. The elements in the document framework were compiled through an extensive literature review, and discussions with experts from academia, industry, and regulatory authorities. The documentation will enable decision makers, who do not need to be experienced modellers, to assess the quality and suitability of models for the specific problem at hand. Modellers are given a systematic tool that helps them organising the modelling process in a comprehensive and efficient way.

2. Preparation of the document

We present the framework for the documentation of ecological modelling projects. Each issue listed in the documentation framework should be addressed by the model authors in the given section. If a step in the modelling process has not been conducted, it should still be addressed in the documentation, i.e. modellers will have to address why they did not conduct a step in the modelling process, or why a specific point does not apply to their modelling approach. The main stages of the modelling process, i.e. development, evaluation, and application, only loosely reflect the time-line of a modelling project, e.g., verification may be conducted before calibration. We want to emphasise that the final document should correspond to one version of the model.

3. Documentation framework

3.1. Model development

Problem formulation: context in which the model will be used, and which audience is addressed; specification of the question(s) that should be answered with the model; statement of the domain of applicability of the model including the extent of acceptable extrapolation; assessment of the availability of knowledge and data; specification of acceptable model outputs.
**Design and formulation:** description of the conceptual model; description and justification of the modelling approach employed; its level of complexity; and the entities and processes represented in the model; the most important simplifying assumptions; detailed description of the actual model.

**Implementation:** documentation of methods and tools (software, operation systems, standard procedures) used; description of model implementation, and where and how the model has been stored.

**Parameterization:** list of all parameter values used in the model, and their data sources; how parameter values were obtained or calculated from data; uncertainties associated with each parameter.

**Calibration:** documentation of the data set(s) used for calibration; which parameters were calibrated; what optimisation method was used.

### 3.2. Model evaluation

**Verification:** assessment of whether the model and its implementation are working according to their specifications; documentation of what tests have been conducted.

**Sensitivity analysis:** exploration of the model behaviour by varying parameters over plausible ranges; documentation which parameter combinations have been tested; justification of the parameters used and their ranges/ combinations applied.

**Validation:** comparison of model and/or submodel outputs with empirical data that have not been used for parameterisation or calibration; documentation of data sources; what parts (submodels) have been validated; what validation methods were applied.

### 3.3. Model application

**Results:** outputs that are used to inform decisions; description of conducted simulation experiments; statistics applied to model outputs; how outputs fostered the understanding about the system modelled.

**Uncertainty analysis:** uncertainties in model outputs used for recommendations; description of variance, noise, and bias in empirical data; determination of stochasticity in the model; description of model uncertainty which can be assessed through application of different models or submodels.

**Recommendation:** description how the initial question could be answered; summary of conclusions drawn from model; clarification of extrapolations (in time and space) that were/may be applied.

### 4. Conclusions

A guideline for good modelling practice in chemical risk assessment is urgently needed. In the scientific literature, all necessary advice can be found, but no help for the implementation of such advice is provided. We introduce a documentation framework for comprehensive documentation of the whole process of ecological modelling. A document that applies the proposed framework will foster acceptance of modelling approaches to chemical risk assessments. It provides a standardised, comprehensive and transparent checklist and reference for stakeholders and potential peer reviewers to assess the quality and usefulness of a model for the problem at hand. Thus, the proposed model documentation framework is the basis for the compilation of an actual guideline for good modelling practice in risk assessment contexts.

### 5. References


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Development of a reliable and robust method for the detection of ng/L concentrations of Lipid – Soluble Metal Complexes in natural waters

Daniel Kilgore¹,², Dr Simon Apte², Dr Grant Hose¹, Dr Mark Taylor¹

¹ Department of Environmental Science, Macquarie University, Sydney, NSW 2109
² Centre for Environmental Contaminants Research, CSIRO Land and Water, Sydney, NSW 2234
E-mail contact: daniel.kilgore@csiro.au

1. Introduction

In natural waters trace metals are present in a wide range of physico – chemical forms or species [1]. It has been demonstrated that the toxicity of these trace metals to aquatic organisms is related in many cases to the activity of the free metal ion species [2]. However, several studies have shown that the toxicity and bioavailability of Lipid - Soluble Metal Complexes (LSMC), formed by the reaction of metals with synthetic and natural organic ligands, may exceed that of the free metal ion [3,4,5]. This increased toxicity is due to the ability of the neutrally charged LSMC to passively diffuse directly across the cell membrane [6]. Given the increased toxicity of LSMC, it is surprising that very few studies have been conducted that seek to identify and quantify LSMC in aquatic systems. This is mainly due to the fact that a reliable, robust and sensitive method for the determination of LSMC in waters has not yet been developed. In this research, a sensitive method for the determination of ng/L concentrations of lipid soluble cadmium, copper, nickel, lead and zinc complexes in waters was developed. This method was used to determine the concentration of LSMC in a number of aquatic environments.

2. Materials and methods

Solvent extraction is a commonly used technique for the detection of ultra – trace concentrations of metals in waters. Extraction into an appropriate solvent allows for preconcentration of the analyte/s of interest and has the ability to remove or reduce matrix effects that may be present when analysing the water sample directly. This research has used the technique of solvent extraction to extract LSMC from collected fresh and saline waters. 1 – octanol was chosen as the solvent as it has a similar dielectric constant to that of a cells lipid bilayer [5]. Initially a shake flask method was employed to extract the LSMC followed by back extraction with acid. Method validation work revealed incomplete back extraction of some LSMC and entrainment of natural colloids into the solvent layer. The method was adjusted and LMSC preconcentration was attained by using octanol filled dialysis cells. Vacuum distillation prior to back extraction with nitric acid was used ot isolate the metals from the octanol phase. By utilising dialysis membrane as a physical barrier between the solvent and the water sample, natural colloids were not able to diffuse into the solvent allowing for the detection of only LSMC from the water sample. By initially using 250 mL of sample and having a final back extract volume of 5 mL a preconcentration factor of 50 is achieved. In addition to this the use of a class 100 cleanroom has allowed for detection limits in the low ng/L. Given the preconcentration factor and the sensitive limits of detection LSMC concentrations in a range of aquatic environments were able to be determined.

3. Results and discussion

3.1. Method Limits of Detection

The developed method was validated using metal – APDC complexes as a model LMSC. Their formation and stability are relatively well understood under typical laboratory conditions. The LSMC was spiked into a water sample and the percent recovery was determined using the developed method. The detection limits of the method are presented in table 1 below followed by the method validation data in table 2. The detection limits were calculated from 3 sigma of the standard deviation of 3 method blanks.

<table>
<thead>
<tr>
<th></th>
<th>Cd (µg/L)</th>
<th>Cu (µg/L)</th>
<th>Ni (µg/L)</th>
<th>Pb (µg/L)</th>
<th>Zn (µg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit Of Detection</td>
<td>0.002</td>
<td>0.003</td>
<td>0.005</td>
<td>0.001</td>
<td>0.013</td>
</tr>
</tbody>
</table>

Table 1: Method Limits Of Detection
<table>
<thead>
<tr>
<th>Spike Recovery Mean (n=6)</th>
<th>Cd (%)</th>
<th>Cu (%)</th>
<th>Ni (%)</th>
<th>Pb (%)</th>
<th>Zn (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>100</td>
<td>105</td>
<td>107</td>
<td>98</td>
<td>108</td>
</tr>
<tr>
<td>Standard Deviation (n=6)</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>1</td>
<td>5</td>
</tr>
</tbody>
</table>

Table 2: Method validation spike recovery results

The detection limits of the method are more than adequate to allow for the detection of LSMC in natural waters. The method was also tested using two other laboratory prepared LSMC, The Oxine– metal and Potassium Amyl Xanthate – metal complexes with similar results.

### 3.2. Analysis of Natural Waters

Once the method had been validated a number of aquatic environments from around the Sydney area were sampled for LSMC analysis. These environments included contaminated sites likely to contain high concentrations of organic ligands that form LSMC as well as relatively pristine sites that are likely to contain only naturally occurring LSMC. LSMC were detected in a number of samples and the concentration ranged from 0.001 µg/L to 0.118 µg/L. In many cases these concentrations were higher than the predicted free metal ion concentrations and comprised between 0.21% and 23.4% of the total dissolved metal concentration. Analysis of different aquatic environments revealed that LSMC are present at a number of sites and that consideration of the LSMC concentrations and not just the free metal ion concentration is an important issue.

### 4. Conclusions

A robust, reliable and sensitive method for the detection of LSMC in waters was developed. The detection limits of the method allowed for the determination of LSMC in a range of aquatic environments. Detection of LSMC at a number of site tested indicates that the issue of LSMC and their toxicity to aquatic organisms requires further attention. Analysis of further aquatic environments will allow for a better understanding of the distribution of LSMC and also identification of environments likely to contain high LSMC concentrations. In addition to this further work should be performed into the toxicity of LSMC to aquatic organisms at environmentally relevant concentrations.

### 5. References

Effect of organic complexation on copper accumulation and toxicity to the estuarine red macroalga Ceramium tenuicorne: A test of the free ion activity model

Erik Ytreberg¹, Eklund Britta¹, Kuria Ndungu ¹

¹ Department of Applied Environmental Science, Stockholm University, Stockholm Sweden
E-mail contact: erik.ytreberg@itm.su.se

1. Introduction
The bioavailability of copper in synthetic media appears to be controlled by the free metal ion concentration, as predicted by the free ion activity model (FIAM) [1]. The FIAM follows from a simple premise: that equilibrium exists between the free metal ions in solution and the metal ions bound to transport enzymes (or other physiologically active sites) on cell membranes i.e. the biotic ligands [2]. Although current regulations for water quality criteria (WQC) and copper toxicity to biota are still based on total dissolved copper concentrations, there are ongoing efforts to incorporate metal speciation in WQC and toxicity regulations via the biotic ligand model (BLM) [2]. The BLM is derived from the FIAM and takes into consideration the properties of natural water such as dissolved organic carbon (DOC), water hardness, and pH to account for the influence of competition between cations for the biotic ligand and the reduction of metal bioavailability by natural ligands (part of DOC) [2]. So far, BLMs have been developed for copper for selected biota in freshwaters but there is currently no BLM available for biota in marine waters.

The aim of this study was to investigate how copper speciation (due to organic complexation) affects accumulation and toxicity to the red macroalga Ceramium tenuicorne, indigenous to the Baltic Sea.

2. Materials and methods
The laboratory copper uptake and toxicity experiments were designed to simulate DOC variability in estuarine and coastal environments like the Baltic Sea. We used a Nordic reference fulvic acid (FA) with the assumption that a significant fraction of the natural organic matter (NOM) in estuarine and coastal environments is terrestrial FA.

Theoretical chemodynamic studies on metal uptake by algae [3] and preliminary field studies seem to indicate that copper uptake and accumulation by algae might be better related to the labile or weakly complexed copper fraction rather than the free copper concentration as predicted by FIAM [4]. Our hypothesis was that at [Cu²⁺] typical of estuarine and coastal waters (i.e. 10⁻¹²-10⁻¹⁵ M) [5], the intracellular copper accumulation by C. tenuicorne would be better correlated to the concentration of weakly complexed copper concentration.

A full characterization of the copper speciation in the growth media and especially the capacity of the weak copper complexing ligand sites in the Nordic FA were performed. We chose a competitive ligand equilibration adsorptive cathodic stripping voltammetry (CLE-ACSV) method that utilizes salicylaldoxime (SA) as the added competitive ligand for its (CLE-ACSV) enhanced sensitivity over direct measurements of free copper concentration using either ISE or even anodic stripping voltammetry [6].

3. Copper accumulation by the macroalga C. tenuicorne
The free copper concentrations in the artificial seawater (ASW) test media containing 4 and 8 mg/L FA ranged from less than 0.1 pM to about 1 pM. The corresponding free copper concentrations, in the absence of FA, were considerably higher i.e. 1.5 to 5.9 nM (modeled by visual MINTEQ). The concentration of the weakly complexed copper (i.e. copper species exchangeable with 1 µM of the CLE-ACSV competing ligand, SA) in the water containing FA was an order of magnitude lower (table 1) to that without added FA, i.e. ca. 1 % of the total copper concentration. The intracellular copper concentration in the algae exposed to this water (no FA added) ranged from 0.9 to 3.1 µmol/g dw. The corresponding intracellular copper concentration in the algae exposed to ASW containing 4 and 8 mg/L of FA was similar and ranged from 0.6 to 2.8 µmol/g dw. The copper concentration accumulated by the algae is despite the fact that the free copper concentration in the waters the latter algae were exposed to was 4-5 orders of magnitude lower. Thus the intracellular copper concentration seems to be better related to both the total copper concentration and also to the weakly complexed copper, rather than the free copper concentration [Cu²⁺].
Table 1: Copper accumulation by *C. Tenuicorne* as a function of copper speciation in 10 salinity artificial seawater. \([\text{Cu}^{2+}]\) in ASW test solutions without added FA were calculated from Visual MINTEQ program.

<table>
<thead>
<tr>
<th>FA (mg/L)</th>
<th>Total Cu (nM)</th>
<th>Weakly bound Cu (pM)</th>
<th>([\text{Cu}^{2+}]) (nM)</th>
<th>Intracellular Cu (µmol/g alga-dw)</th>
<th>w/s (g alg-dw cm(^{-2}))</th>
<th>(J_w) (mol min(^{-1}) cm(^{-2}))</th>
<th>(J_{w-diff}) (mol min(^{-1}) cm(^{-2}))</th>
<th>(J_{w-diff}) (mol min(^{-1}) cm(^{-2}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>16</td>
<td>1500</td>
<td>0.9</td>
<td>1.7 x 10(^{-14})</td>
<td>2 x 10(^{-14})</td>
<td>1 x 10(^{-14})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>25</td>
<td>3000</td>
<td>1.5</td>
<td>1.7 x 10(^{-14})</td>
<td>3 x 10(^{-14})</td>
<td>3 x 10(^{-14})</td>
<td></td>
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<tr>
<td>0</td>
<td>61</td>
<td>5900</td>
<td>3.1</td>
<td>1.7 x 10(^{-14})</td>
<td>5 x 10(^{-14})</td>
<td>5 x 10(^{-14})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>16</td>
<td>0.1</td>
<td>0.6</td>
<td>1.7 x 10(^{-14})</td>
<td>1 x 10(^{-14})</td>
<td>8 x 10(^{-19})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>27</td>
<td>0.3</td>
<td>0.9</td>
<td>1.7 x 10(^{-14})</td>
<td>2 x 10(^{-14})</td>
<td>2 x 10(^{-18})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>53</td>
<td>1.4</td>
<td>1.4</td>
<td>1.7 x 10(^{-14})</td>
<td>2 x 10(^{-14})</td>
<td>7 x 10(^{-18})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>24</td>
<td>0.1</td>
<td>0.9</td>
<td>1.7 x 10(^{-14})</td>
<td>2 x 10(^{-14})</td>
<td>8 x 10(^{-19})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>47</td>
<td>0.3</td>
<td>2.1</td>
<td>1.7 x 10(^{-14})</td>
<td>4 x 10(^{-14})</td>
<td>8 x 10(^{-19})</td>
<td></td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>104</td>
<td>1.3</td>
<td>0.8</td>
<td>1.7 x 10(^{-14})</td>
<td>5 x 10(^{-14})</td>
<td>7 x 10(^{-18})</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

From the above observation, it appears that at low \([\text{Cu}^{2+}]\), copper uptake by *C. tenuicorne* might be limited by the diffusive flux of the free \(\text{Cu}^{2+}\) to the cell membrane [3]. Such a situation would be characterized by an uptake flux that exceeds the diffusive flux of the free \(\text{Cu}^{2+}\). The corresponding gradient in the diffusion layer would then lead to dissociation of sufficiently labile complex species which then effectively contribute to the supply of \(\text{Cu}^{2+}\) to the biointerface [3]. To test this hypothesis, we calculated the diffusive flux of the free \(\text{Cu}^{2+}\) and the weakly bound copper species and compared them to the uptake flux. The transport of \(\text{Cu}^{2+}\) to the cell \((J_{\text{Cu-diff}}, 1 x 10^{-18} to 7 x 10^{-18} \text{ mol min}^{-1} \text{ cm}^{-2})\) appeared to be 3-4 orders of magnitude smaller than the uptake of copper \((J_{\text{Cu}}, 1 x 10^{-14} to 5 x 10^{-14} \text{ mol min}^{-1} \text{ cm}^{-2})\) in ASW with added FA (4-8 mg/l, table 1). This indicates that the bulk solution might not supply enough free copper ions to the algal cells. The consequence is that labile or weakly bound copper in solution provides the metal to the cell wall. For the range of weakly bound copper concentrations encountered in this study (0.1-1.4 nM), the estimated diffusion flux ranges from about \(1 x 10^{-15}\) to \(5 x 10^{-14} \text{ mol min}^{-1} \text{ cm}^{-2}\) (table 1), which is comparable to the estimated uptake flux of copper \((1 x 10^{-14} to 5 x 10^{-14} \text{ mol min}^{-1} \text{ cm}^{-2})\).

4. Conclusions

Growth inhibition of the macroalgae occurred at relatively high free copper concentration (EC-50 of ca. 10 nM modeled), in ASW without added FA), in agreement with previous toxicity studies on other algae [7]. It is thus likely that copper toxicity to *C. tenuicorne* and other algae does not occur before diffusion limitation is reduced [4], although toxicity inside the cell might still be controlled by the free copper concentration [4]. Thus although equilibrium free copper concentration from model calculations or actual measurements of metal speciation in coastal marine waters (having similar dissolved organic matter to the ones in this study) might show very low free copper concentrations, the intracellular and hence copper toxicity, might not be correlated to this concentration. Rather, the sum of this (free copper concentration) and some of the weakly complexed copper might determine copper accumulation and probably its eventual toxicity to macroalgae in marine waters.

5. References

Toxicity of metal mixtures to *Daphnia magna*: A test of a multi-metal, multi-site biotic ligand model using Cu and Zn

Joseph S. Meyer\(^1\), James F. Ranville\(^2\), Mandee Pontasch\(^2\), Robert C. Santore\(^3\), Joseph W. Gorsusch\(^4\), and William J. Adams\(^5\)

\(^1\) ARCADIS, Lakewood, CO 80401 USA
\(^2\) Department of Chemistry and Geochemistry, Colorado School of Mines, Golden, CO 80401 USA
\(^3\) HydroQual, Inc., East Syracuse, NY 13057 USA
\(^4\) Copper Development Association, Webster, NY 14580 USA
\(^5\) Rio Tinto, Lake Point, UT 84074 USA

E-mail contact: Joseph.Meyer@arcadis-us.com

1. Introduction

For decades, a simplistic summation of toxic units based on concentrations of dissolved metals has been used to semi-quantitatively predict the toxicity of metal mixtures. However, that approach tends to over-predict toxicity [1]. In its place, we have been developing a mechanistic model based on tissue residues of metals and the concept that (1) some metals act interchangeably (i.e., they have the same mechanism of toxicity, and thus their toxic units can validly be summed -- referred to as dose additivity) but (2) some groups of metals act independently (i.e., they have different mechanisms of toxicity, and thus their toxic units can not validly be summed -- referred to as response additivity). For example, Ag and Cu impair Na homeostasis; Cd and Zn impair Ca homeostasis; and Ni impairs Mg homeostasis in *Daphnia*, but impairs respiration instead of ionoregulation in fish [2-4]. Therefore, Ag and Cu should be modeled together using dose additivity, and Cd and Zn should be modeled together also using dose additiviity (but with a separate relationship from Ag and Cu); whereas the Ag-Cu pair, the Cd-Zn pair, and Ni should be modeled together using response additivity.

To calculate tissue residues and thereby predict toxicity across wide ranges of water quality, we have been developing a multi-metal, multi-site biotic ligand model (MMMS BLM) that concurrently accounts for metal-metal competition for binding on dissolved ligands in the water and at sites of toxicity on organisms. The HydroQual BLM (http://www.hydroqual.com/wr_blm.html), which incorporates Windermere Humic Aqueous Model (WHAM) V for metal-organic complexation, is used to perform the multi-metal speciation calculations. The MMMS model of acute toxicity proposed by Niyogi and Wood [2] is the basis for defining sites of toxic action (i.e., the biotic ligands) for the metals, although we allow for the possibility that all metals can bind to any of the biotic ligands regardless of whether they cause toxicity at that site.

For the initial test of the MMMS BLM, we exposed *Daphnia magna* to Cu alone, Zn alone, and various mixtures of Cu and Zn in which either (1) Cu concentration was varied and Zn concentration was held constant or (2) Cu concentration was held constant and Zn concentration was varied. We then compared observed mortality to the response-additive mortality predicted from results of the Cu-only and Zn-only toxicity tests.

2. Materials and methods

The *D. magna* were cultured in moderately hard reconstituted (MHR) water [5] and were tested in MHR water to which dissolved organic carbon (DOC) was added as Suwannee River fulvic acid at 3 mg DOC/L. Standard 48-h lethality tests begun with <24-h-old neonates were conducted for all single-metal and metal-mixture tests [5]. Temperature, pH, alkalinity, and concentrations of Cu, Zn and major inorganic anions and cations were monitored during the tests. Because the concentration-response curves for individual metals can vary from test to test, we always conducted a Cu-only and a Zn-only test concurrent with each Cu-Zn mixture test.

Concentration-response data from the metal-only tests were fitted to a maximum likelihood probit regression using ToxCalc 5 (Tidepool Scientific, McKinleyville, CA), and the mortality predicted for Cu alone and Zn alone in each Cu-Zn exposure combination was calculated from the fitted probit slope and intercept, as follows:

\[
m = \text{NORMSDIST}(\text{slope}\times \log_{10}(C) + \text{intercept} - 5)
\]

(Eqn. 1)

where \(m\) = the predicted mortality proportion due to Cu or Zn alone, NORMSDIST = a mathematical function in Excel\(^®\) that returns the value of the standard normal distribution, slope = the probit slope for the concurrent
Cu-only or Zn-only test, intercept = the probit intercept for the concurrent Cu-only or Zn-only test, and C = the Cu or Zn concentration in the Cu-Zn exposure combination. Then, the predicted response-additive mortality of each Cu-Zn mixture (m\textsubscript{Cu-Zn}) was calculated as:

\[
m_{\text{Cu-Zn}} = 1 - (1 - m_{\text{Cu}})(1 - m_{\text{Zn}})
\]  

(Eqn. 2)

where \(m_{\text{Cu}}\) = the predicted mortality proportion due to Cu alone, and \(m_{\text{Zn}}\) = the predicted mortality proportion due to Zn alone. Metal concentrations used in these calculations were either dissolved metal concentration (as measured by inductively coupled plasma emission) or free-metal-ion concentration (as calculated using the BLM metal-speciation mode, in which WHAM-predicted metal-metal competition was allowed when calculating binding of Cu and Zn to sites on the fulvic acid).

3. Results and discussion

In all 13 Cu-Zn mixture tests, the toxicity of Cu-Zn mixtures always appeared to be either synergistic (i.e., the observed mortality was greater than the predicted mortality) or additive (i.e., the observed mortality equalled the predicted mortality) when based on dissolved metal concentrations, whether Cu was varied while Zn was held constant, or vice versa. However, in the same tests, the toxicity of the Cu-Zn mixtures always appeared to be either antagonistic (i.e., the observed mortality was less than the predicted mortality) or additive when based on free-metal-ion concentrations. Therefore, opposite conclusions about the nature of metal-metal interactions (either synergistic, additive, or antagonistic) could be reached, depending on whether the concentrations are viewed from a dissolved-metal perspective or from a free-metal-ion perspective.

A residual concern is that the cloak of non-additive toxicity must be invoked if only the exposure-solution chemistry is considered, whether viewing the mixture from a dissolved-metal perspective or from a free-metal-ion perspective. However, the MMMS BLM can obviate the need to invoke non-additive toxicity by accounting for potential metal-metal competition in binding at the biotic ligands. This is possible even though Cu and Zn do not have the same mechanism of action (and thus do not share the same site at which toxicity is caused), if Cu is allowed to compete for binding at the Zn-toxicity ligand and Zn is allowed to compete at the Cu-toxicity ligand. Therefore, inferred tissue residues (the output of the MMMS BLM) can be used to impose additivity and thus simplify the interpretation of this type of metal-metal interaction, when viewed from a tissue-residue perspective.

4. Conclusions

This research has revealed several apparent metal-metal interactions that otherwise might lead to conclusions that metals interact in non-additive ways, yet simple geochemical speciation in the BLM can explain these interactions and reconcile the apparent non-additive toxicity by taking a tissue-residue perspective instead of a dissolved-metal or a free-metal-ion perspective. Therefore, our preliminary results demonstrate that a MMMS BLM could be an effective tool to help regulatory agencies implement more appropriate methods to regulate metal mixtures than the current default, overly conservative toxic-units approach.

5. References


Acknowledgement – The Copper Development Association and Rio Tinto funded this research. The authors thank Katie Dahl and Samantha Smith for assisting with the toxicity tests and chemical analyses.
Effects of chronic nickel exposure on algae, zooplankton and snails in a semi-realistic microcosm

Udo Hommen\textsuperscript{1}, Christoph Schäfers\textsuperscript{1}, Heinz Rüdel\textsuperscript{1}, Burkhard Knopf\textsuperscript{1}, Chris Schlekat\textsuperscript{2}, Emily C. Rogevich\textsuperscript{2}

\textsuperscript{1}Fraunhofer Institute for Molecular Biology and Applied Ecology, Auf dem Aberg 1, 57392 Schmallenberg, Germany
\textsuperscript{2}Nickel Producers Environmental Research Association (NiPERA), 2605 Meridian Parkway, Durham, North Carolina 27713, USA

E-mail contact: udo.hommen@ime.fraunhofer.de

1. Introduction

In the EU Existing Substances Risk Assessment for Nickel and Nickel Compounds [1], chronic NOEC or EC\textsubscript{10} values were available for 31 aquatic species. HC\textsubscript{5} (Hazardous Concentrations for 5 \% of species) were calculated from site specific Species Sensitivity Distributions (SSD) using chronic Ni Biotic Ligand Models (BLMs) to consider pH, DOC, and hardness for prevailing water chemistry conditions for seven European scenarios. Based on the resulting HC\textsubscript{5} values, ranging from 7.1 to 43.6 $\mu$g Ni/L, Predicted No Effect Concentrations (PNECs) of 3.6 to 21.8 $\mu$g Ni/L were calculated [1].

In order to analyze the ecological relevance of these PNECs, a microcosm study was conducted where a freshwater community containing plankton and snails was exposed to five Ni concentrations over four months under semi-realistic environmental conditions.

2. Materials and methods

The study was conducted in 14 microcosms (1 m\textsuperscript{3}) with a natural sediment layer of 20 cm and an overlaying water volume of 750 L, located in a greenhouse at the Fraunhofer IME in Germany.

After a pre-treatment period to establish stable populations, a Ni solution (NiCl\textsubscript{2}) was added at target concentrations of 6, 12, 24, 48 and 96 $\mu$g/L. Each Ni concentration was replicated in two microcosms, while four microcosms served as untreated controls. To maintain constant Ni exposures, appropriate amounts of Ni solution were added throughout the exposure period of four months on a nearly daily basis.

Total and dissolved Ni in the water was determined at least twice a week. Nickel in sediment was measured monthly while Ni in biota (periphyton, macrophytes and snails) was measured at the end of the study. Water quality parameters (e.g. pH, DOC, alkalinity), phytoplankton, zooplankton (both: abundance of taxa), periphyton (chlorophyll a) and snails (abundance) were monitored at regular intervals.

3. Results and discussion

3.1. Exposure

The mean measured total Ni concentration in the microcosm water for all samples (unfiltered water, taken before the addition of Ni solutions) was 91\% of the nominal values. If the Ni concentrations measured immediately after the Ni additions were considered, the mean total Ni was 105\% of nominal. Dissolved Ni concentrations were only slightly lower than the total concentrations (97\%).

At the end of the study, Ni accumulated in and on periphyton and macrophytes, by mean factors of approximately 4 000 and 2 500 respectively (based on wet weight). However, the mean accumulation factor for the snails was below 500.

3.2. Effects

No pronounced or long-term effects were observed up to 24 $\mu$g/L for phytoplankton community structure, abundance of populations and chlorophyll a levels (e.g. Figure 1). Temporary (i.e., occurring on single sampling periods) significant deviations from controls were found for three of 141 algal species at 24 $\mu$g/L. The effects at 48 and 96 $\mu$g/L were mainly due to effects on Cryptophyceae. Also some other taxa showed pronounced effects at the two highest treatment levels, but these effects were only temporary in most cases.
The Ni exposure had no persistent effects on periphyton pigment concentrations but biomass measurements at the end of the study indicated an indirect (positive) effect at 48 and 96 µg/L.

Community analysis of the zooplankton data set indicated deviations from controls at the two highest treatment levels into two directions: reduced abundances at 48 µg/L and increased abundances at 96 µg/L which was mainly caused by the rotifer *Keratella quadrata*, the most dominant species. Other zooplankton taxa showed only temporary and slight deviations from the controls at 48 or 96 µg/L. No treatment related effects were found up to 24 µg/L.

No effects on snails (dominated by *Lymnaea spec.*.) were found up to 24 µg/L while at 48 µg/L and 96 µg/L snails became quasi extinct which likely explains the higher periphyton biomass found here.

4. Conclusions

The intended chronic Ni exposure of the community over four months was achieved. Uptake and accumulation of Ni via the food seems not to be relevant for the snails.

In total, exposure up to 24 µg Ni/L over 4 months had no ecologically adverse effects on phytoplankton, periphyton, zooplankton and snails. At 48 and 96 µg/L clear effects were found on the phytoplankton, namely Cryptophyceae, and the snails. These effects might have affected the zooplankton, i.e. rotifers, and the periphyton.

Thus, the study-specific NOEC is considered to be 24 µg Ni/L (LOEC 48 µg Ni/L).

The remaining uncertainty for extrapolation from the NOEC to the field is considered small:

- The community in the microcosms included species closely related to the most sensitive species according to laboratory single species tests, i.e. the snail *Lymnaea* and phyllopoda like *Daphnia*.
- Phytoplankton species of different classes were present and one group, Cryptophyceae, not tested in the laboratory before, was found to be the most sensitive algae group.
- The abiotic conditions in the systems, (i.e. the mean pH (8.6), hardness (100 mg CaCO₃/L), and DOC (2.9 – 5.4 mg/L)) represent conditions of high Ni bioavailability. For these conditions, the BLM predicted HC₅ is between 4.2 and 6.8 µg/L [2], and thus, close to the lowest HC₅ of the EU scenarios [1].
- The study-specific NOEC of 24 µg Ni/L is a factor of 3.5 to 5.7 above the HC₅ values derived for the given environmental conditions. In addition, Peters et al. [3] showed that bioavailability-based HC₅ values for Ni are protective for macroinvertebrates communities in the field.

Thus, the HC₅ value seems to be adequately protective for freshwater systems.

5. References

1. Introduction

Selenium is a naturally occurring essential element showing a very narrow margin between essentiality and toxicity. So far, the main focus of the extensive research on the environmental fate and effects of Se has been the aquatic environment. The diet appears to be the primary exposure pathway to aquatic organisms and toxicity is primarily manifested as reproductive impairment in egg-laying vertebrates due to maternal transfer. Tissue concentrations in reproductive organs have been demonstrated to be a better predictor of toxicity than total or dissolved aquatic concentrations. Behaviour, bioaccumulation and toxicity of Se in aquatic systems seem to be largely species- and/or site-specific, and therefore, deriving a single, conservative aquatic water quality criterion may not be appropriate for all ecosystems. Currently, chemical regulations such as the European REACH regulation are however generally based on the derivation of a single threshold concentration for each environmental compartment (e.g. water, sediment, soil). For Se, these standard risk assessment procedures may hamper a meaningful evaluation. However, since REACH and similar regulations are pushing the limits with regard to timely submission of chemical safety reports, they also force difficult substances as Se to be assessed in a pragmatic way using the scientific knowledge available.

2. Materials and methods

In an extensive literature review, all relevant studies on ecotoxicity and environmental fate and behaviour of Se are screened for the quality (reliability) of the data reported. All available reliable data for inorganic selenium compounds were used in a read-across approach for the assessment of direct toxicity of inorganic Se to aquatic or terrestrial organisms. However, because in the environment inorganic Se can be transformed into organic forms, data for the Se containing amino acids seleno-methionine and seleno-cysteine were also taken into account for the evaluation of dietary toxicity to higher organisms (birds, mammals, reptiles and fish). In order to correct for differences in solubility among Se compounds, only reliable data based on measured dissolved Se concentrations were taken into account for evaluation of the toxicity of Se to aquatic organisms. For toxicity of Se to terrestrial organisms, no effect of solubility was expected since data were only available for the highly soluble sodium selenite and sodium selenate salts. Predicted No Effect Concentrations (PNEC) were calculated according to the REACH Guidance documents [1]. Background Se concentrations are significant compared to the toxicity thresholds proposed for both freshwater and soil (Table 1). Therefore, the added risk approach is preferred and all toxicity thresholds were based on added Se concentrations, without the natural background concentration in the exposure media.

<table>
<thead>
<tr>
<th>Environmental compartment</th>
<th>Background concentration</th>
<th>PNEC added</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh surface water (µg Se/L)</td>
<td>0.32 (median) 0.85 (90th percentile)</td>
<td>2.67 (direct toxicity) 0.21 (secondary poisoning)</td>
</tr>
<tr>
<td>Agricultural soil (mg Se/kg dw)</td>
<td>0.35 (median) 0.59 (90th percentile)</td>
<td>0.1 (selenite) 0.04 (selenate)</td>
</tr>
</tbody>
</table>

3. Results and discussion

3.1. Aquatic environment

For aquatic organisms, reliable data were identified for H₂SeO₃, Na₂SeO₃, SeO₂ or Na₂SeO₄ and comparison in toxicity among these Se substances did not yield consistent or significant differences. Therefore, results for all four substances were used in a read-across approach. Chronic toxicity data were available for 11 freshwater organisms, representing 8 taxonomic groups. The lowest toxicity data were observed for fish.
Species-mean NOECs were calculated for each substance and the lowest value for each species was selected for the species sensitivity distribution (range: 10 – 4570 µg Se/L). A HC5 of 8.0 µg Se/L was derived. An assessment factor of 3 was selected based on an uncertainty analysis on this HC5, resulting in a PNEC for freshwater organisms of 2.7 µg Se/L. No reliable data were identified for the toxicity of Se substances to sediment-dwelling organisms.

3.2. Terrestrial environment

A clear difference in both adsorption to soil and toxicity to terrestrial organisms was observed between selenite and selenate, with selenate showing significantly lower sorption and higher toxicity to terrestrial invertebrates and plants. Therefore, data could not be combined and separate soil threshold concentrations were derived for selenite and selenate. For both substances, data were available for plants, invertebrates and micro-organisms. Reliable chronic NOEC values vary between 1 and ≥10 mg Se/kg and between 0.4 and ≥20 mg Se/kg for selenite and selenate, respectively, with plants being most sensitive to both Se substances. Applying an assessment factor of 10, resulted in a PNECsoil of 0.1 and 0.04 mg Se/kg dry weight for selenite and selenate, respectively.

3.3. Secondary poisoning

Reliable chronic NOECoral data are available for various Se substances (Na2SeO3, Na2SeO4, H2SeO3, Se-methionine) and 24 vertebrate species (Table 2). The lowest NOECoral of 1.0 mg Se/kg diet was observed for both chicken and mouse. According to the standard REACH Guidance [1], an extra assessment factor of 30 has to be applied to the lowest NOECoral for derivation of the PNECoral. It was however judged that selection of the lowest NOECoral value already represents a conservative approach and that therefore no further assessment factor should be taken into account.

### Table 2: Range in NOECoral values reported in literature for aqueous and terrestrial vertebrates

<table>
<thead>
<tr>
<th>Species</th>
<th>Exposure period</th>
<th>NOECoral (mg Se/kg dw food)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fish (6 species)</td>
<td>56 – 182 days</td>
<td>3.7 – 89.8</td>
</tr>
<tr>
<td>Reptiles (2 species)</td>
<td>98 – 300 days</td>
<td>≥14.7</td>
</tr>
<tr>
<td>Birds (6 species)</td>
<td>7 – 735 days</td>
<td>1.0 – 40.0</td>
</tr>
<tr>
<td>Mammals (10 species)</td>
<td>3 – 360 days</td>
<td>1.0 – 300</td>
</tr>
</tbody>
</table>

A generic, conservative bioaccumulation factor for Se in aquatic organisms of 4721 L/kg dw was calculated as the 90th percentile of the reliable bioconcentration and bioaccumulation data for 8 fish species (range: 26 – 4787 L/kg dw) and 4 groups of invertebrates (range: 1149 – 4688 L/kg dw). This results in a PNECaquatic for indirect effects of 0.21 µg Se/L, which is significantly lower than the PNEC proposed for direct effects to aquatic organisms. No reliable data were available for bioaccumulation of Se in terrestrial invertebrates.

4. Conclusions

For aquatic organisms, no significant nor consistent differences in toxicity were observed across inorganic Se substances, allowing a single PNEC value for Se based on read-across among all toxicity data. In contrast, selenate (SeO₄²⁻) is significantly more toxic to terrestrial organisms compared to selenite (SeO₃²⁻).

An added risk approach is preferred because the PNECaquatic values suggested are within the range of typical Se background concentrations and the border between essential and toxic Se levels is very narrow.

This hazard assessment confirms the dietary exposure pathway as the most important route of toxicity in the aquatic environment.

Using an added approach, the current PNECs can be used in a first-tier risk assessment, however, site-specific evaluations should be encouraged, taking into account the dietary route. Tissue-based criteria in combination with predictive models translating tissue-based to water-based PNECs may offer a potential for further refinement.

5. References


Acknowledgement – This work was funded by the Selenium & Tellurium consortium
Life-cycle traits of *Porcellio dilatatus* exposed to different Cd species: effects on survival and reproduction

Carla Filipa Calhôa¹, Amadeu M.V.M. Soares¹ and Susana Loureiro¹

¹CESAM & Department of Biology, University of Aveiro, Campus Universitário de Santiago, 3810-193 Aveiro, Portugal
E-mail contact: fcalhoa@ua.pt

1. Introduction

Terrestrial isopods are saprophytic detritivores that inhabit the upper layer of the soil and surface leaf litter, and play an important role in maintaining the structure and fertility of soils [1,2]. The woodlouse species *Porcellio dilatatus* (Crustacea) has been often chosen as a model species as it is an important representative of the invertebrate soil fauna [3,4]. Moreover they have been widely used for the examination of metal accumulation and toxicity testing because of their extraordinary capacity to accumulate high concentrations of metals from the environment predominantly in their hepatopancreas [5,6].

The most widely used toxicological endpoints in isopod testing are survival, growth, food consumption processes, and reproduction. Using long-term exposure tests and reproduction as a response endpoint to test sublethal effect of chemicals is crucial to understand and transpose those effects to the community and population levels.

Previous dietary studies on the assimilation of Cd in the terrestrial isopod *Porcellio dilatatus* [3] indicated that the Cd speciation dictate the assimilation efficiency (AE) of Cd in plant-isopod food chain. The use of Cd-cysteinate in this study provides an experimental tool to explore the bioavailability of Cd that is complexed within biological tissue.

In this study, the effects of cadmium speciation on the life-trait of *Porcellio dilatatus* upon a long term exposure was investigated. The main goal of this study was to determine differences in toxicity of two species of Cd [Cd(Cys)₂ and Cd(NO₃)₂] on the survival and reproductive effort.

2. Materials and methods

Isopods were selected from cultures of *P. dilatatus* that have been maintained for more than 3 years in the laboratory on a substrate of sand in plastic containers at 20°C with a 16:8 h (light:dark) photoperiod. A mixture of lettuce leaves and gelatine was selected as a suitable food substrate to be used as the exposure route [7,8]. Small portions of the mixture (gelatine discs) weighing approximately 9 mg (dry wt) were made and were stored frozen at -20°C until required.

Three treatments (diets) were established for this long term exposure test to evaluate the toxicity of metal speciation to the terrestrial isopod *P. dilatatus*.

- Cd(Cys)₂ contaminated food - gelatine contaminated with Cd-cysteinate mixed with non-contaminated leaves of *L. sativa*;
- Cd(NO₃)₂ contaminated food - gelatine contaminated with Cd nitrate mixed with non-contaminated leaves of *L. sativa*.
- Control food - gelatine mixed with non-contaminated leaves of *L. sativa*.

A total of 50 non-gravid females were selected and separated into a test box for one month to guarantee that they were not pregnant when the test started. After this one month period a total of 30 males were also selected. At this stage (T0) 30 females and 30 males were exposed individually in test boxes for a period of 28d (T1), and exclusively fed on gelatine discs according to the treatments previously. Survival was checked every two days.

After this 28d period of individual exposure, one male and one female were paired randomly per box for mating, using 10 replicates per treatment. Female reproductive cycle and survival was followed for 54 days (T2), being monitored 3 times a week. The percentage of females that successfully reached pregnancy and the percentage of inconclusive pregnancies were recorded. The number of juveniles born per female and their individual weight were also registered.
3. Results and discussion

3.1. Survival

From the survival data, there was an unexpected difference between sexes in both exposures. During the 82 days of exposure, there was no mortality on control females while 20% of the control males died during the test. In the Cd(Cys)₂ treatment 70% of females and 90% of males have died at the end of the test. As for the Cd(NO₃)₂ treatment mortality was of 10% and 70%. Males exposed to Cd(Cys)₂ died earlier than those exposed to Cd(NO₃)₂, showing higher acute toxicity under a long-term exposure.

3.2. Pregnancy and abortions

In the control all females successfully reached pregnancy and delivered mancae within the test period. Only 30% of the females fed with Cd(Cys)₂ become pregnant but none produced any manca; two of them because have died and the one that survived did not deliver any manca, due to inconclusive pregnancy. In the Cd(NO₃)₂ treatment half of the females successfully reached pregnancy but only 80% of these were able to carry it till the end.

3.3. Number of juveniles and individual juvenile weight

In the control there were more juveniles delivered but the individual weight was lower than in the Cd(NO₃)₂ treatment, that produced fewer juveniles but with higher individual weight.

The decrease on the number of mancae delivered per female in the Cd(NO₃)₂ treatment but with increase on juveniles’ individual weight might be a response for their investment in quality rather than in quantity of juveniles, producing a higher quality and fit offspring.

4. Conclusions

Different species of Cd affect survival and reproduction of terrestrial isopods in different ways. As far as we are aware, the present study is the first toxicity test demonstrating that metal speciation affects reproduction. Cd(Cys)₂ showed to be more toxic in this long term exposure and to jeopardize completely the reproduction effort of isopods.

Although long-term studies are time consuming they can provide crucial and useful information to understand life-cycle traits of terrestrial isopods under contamination scenarios, which cannot be achieve under short-term exposure tests.

5. References


Acknowledgement - This work was supported through the project TROPHA-Trophic assimilation of inorganic and organic pollutants in terrestrial invertebrate, financed by Fundação para a Ciência e Tecnologia (FCT/POCTI/BSE/48757/2002), and a PhD fellowship awarded to C. F. Calhôa (FCT/SFRH/ BD 18942/2004), also from FCT.
Solid-phase applications for biosensor deployment in predicting the biotoxicity of heavy metals in soils

Wei Ma¹, Graeme I. Paton¹,²

¹Biological Interactions in Soils, Cruickshank Building, Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen, AB24 3UU, UK
²Remedios Limited, Balgownie Drive, Aberdeen, AB22 8GW, UK
E-mail contact: wei.ma@abdn.ac.uk

1. Introduction

Over the past decades, biosensors have been shown for use in a wide range of applications [1]. The current tendency to carry out environmental field monitoring has driven the development of biosensors as new analytical tools able to assess the biological effects and toxicity which are of great interest [2].

Among inorganic pollutants, heavy metals have been a focus of this study. Bioavailability is the major factor controlling the toxicity of heavy metals in soil. Until now, our assessment of bioavailability has been restricted to measuring the aqueous soluble fraction of samples and the biosensor responses. This fails to consider the complex environment of the soil solid phase which is likely to host most of the labile and bioavailable pollutants and be the more dynamic in both space and time to perturbations.

In this study, a range of novel solid phase devices were compared to assess the reproducibility of given assays and their relationship with aqueous phase assays. The aims of this study were:

- To optimisation of the biosensing system and characterisation of the biosensor responses to various environmental conditions.
- To evaluate the bioavailable fraction of heavy metals using different solid-phase extraction technologies.
- To compare and predict the biotoxicity of heavy metals in soils using both aqueous-phase and solid-phases technologies.

2. Materials and methods

2.1 Strains

Biosensors which were used are listed below in Table 1.

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Biosensor</th>
<th>Promoter</th>
<th>Reporter Gene</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cu</td>
<td>P. fluorescens DF57-Cu15</td>
<td>Cu-inducible</td>
<td>luxAB</td>
</tr>
<tr>
<td></td>
<td>P. fluorescens DF57-40E7</td>
<td>Constitutively marked</td>
<td>luxAB</td>
</tr>
<tr>
<td>Zn</td>
<td>E.coli HB101 pUCD607</td>
<td>Constitutively marked</td>
<td>luxCDABE</td>
</tr>
</tbody>
</table>

Table 1: Description of biosensors used in this research

2.2 Soil sources

Four coarse-textured soils from north-east Scotland (Brechin, Culbin, Insch and St. Fergus) were used in this research [3].

2.3 Resin preparation

Amberlite IR120-plus (16-50 mesh, wet) cation-exchange resin was obtained from ALDRICH. Na⁺ form of the original resin was totally converted to the Ca²⁺ form because the greater affinity of the resin for divalent cations. The target analytes can be “screened and concentrated” overcoming some of the sensitivity constraints of biosensors.

2.4 Filter device
5mL of Serum Filter (Sera-Separa, Evergreen Scientific) was fitted in 15mL centrifuge tubes (Corning®) to collect the freely permeable fractions of soil solution.

2.5 Chemical analysis and bioluminescence measurement

Concentrations of Ca$^{2+}$, Na$^{2+}$, Cu$^{2+}$ and Zn$^{2+}$ in solution were measured using flame atomic adsorption spectroscopy (FAAS-Perkin Elmer A Analyst 100 Spectrometry). Bioluminescence of the biosensors in test solutions was detected by a Jade luminometer (Labtech International, UK) as relative light unit (RLU).

3. Results and discussion

3.1 Optimisation of biosensors

Optimisation of biosensors was operated under different conditions such as different washing media (Davis mineral medium, modified mineral medium and ringer solutions), different Cu and Zn concentrations, different Cu induction times, different n-decanal addition times and different pH values. The pH of the Cu solutions was below 6.5 which is due to the hydration reaction may occur during the process between Cu$^{2+}$ from prepared CuSO$_4$ solution and OH$^-$ from Milli-Q water.

3.2 Optimisation of solid-phase technologies

Filtration devices were prone to considerable variability while physical syringes enabled sample equilibration and higher consistency. The performance of the biosensor was not solely determined by the extraction procedure used but was influenced by the presence of a suitable osmotic buffer and the solubility of the heavy metals. This approach was complemented by the application of inducible biosensors for metals (Cu and Zn).

4. Conclusions

In general, the toxicity and bioavailability of metals in solid phase was lower than in aqueous phase demonstrating the need to measure both phases and develop a relationship between them and the likely mobility and partitioning of the target analytes. Chemical speciation work has further verified that biosensors respond to the free ion fraction of the measured metals.

5. References

1. Introduction

Lead toxicity data derived from soils freshly spiked with Pb salts may overestimate Pb toxicity under field conditions because of a reduced mobility and lower soil solution ionic strength in field contaminated soils. Lead is strongly immobilized in soil by adsorption or precipitation which can increase due to ageing [1]. However, isotope exchange studies have suggested that ageing of Pb is not be very pronounced, 45%-78% (mean 58%) of Pb in different field contaminated soils is exchangeable within a few days only [2]. In addition to ageing, the soil solution ionic strength and pH in freshly spiked soils can explain the differences in toxic thresholds for Pb between freshly spiked and field contaminated soils. Leaching of freshly spiked soils reduces the confounding effects of increased ionic strength and increases critical toxic concentrations of Pb [3]. This suggests that ageing effects in soils leached soils should not be very large. This study was designed to quantify the difference in Pb toxicity between soils dosed with Pb(NO₃)₂ left to age 5 years outside with natural leaching, soils freshly dosed with PbCl₂ and soils freshly dosed with PbCl₂ leached with artificial rainwater. Toxicity was assessed with plant growth (*Lycopersicon esculentum* L.).

2. Materials and methods

Three control soils with varying soil characteristics (soils A, B and C, eCEC= 23, 14 and 9 cmolc/kg, respectively) were dried, sieved at 4 mm and dosed with Pb(NO₃)₂ up to 7 different concentrations (0-8000 mg Pb/kg). Five kg samples of each treatment were incubated outside in flower pots with free drainage for 5 years. The same soils were spiked with PbCl₂ to the same Pb concentrations (0-8000 mg Pb/kg). Half of these freshly spiked soils were leached with artificial rainwater with 2 pore volumes and drained overnight. The leached soils were dried and sieved again. In addition, pH was matched across all rates after leaching using CaO.

Total Pb concentrations were determined with an *aqua regia* digestion, followed by determination with ICP-OES (Perkin Elmer 3300 DV). Pore waters were extracted by centrifugation of incubated soil. After filtration (0.45 μm) the pore water extracts were measured by ICP-OES.

The air dried soils were preincubated for a week at 70% of pF 1.9 before plant growth assays. Tomato seeds were transferred to pots (20 seeds per pot; 4 replicates/treatment and dose) which were placed in a growth cabinet (Weiss, 18' SP+/5 Ju-Pa) with a 14h/10h day/night cycle, air temperatures of 20°C during the day and 15°C during the night and a relative humidity of 75% throughout. Water loss was restored daily. Plants were thinned to five plants per pot after one week and harvested after 15 days. Dried (60°C, overnight) plant yield was determined per pot.

The data were statistically examined through the application of a log-logistic dose-response model fitted with the Marquardt method (SAS version 9.1). No Observed Effect Concentrations (NOECs) are the highest Pb concentration in the soil at which no significant adverse effects were observed compared to the control soil and were determined by ANOVA.
3. Results and discussion

![Figure 1: Yield of tomato shoots (as % of the control treatment) in Pb²⁺-salt spiked soils: ◊ spiked, □ spiked + leached, Δ aged with indicated loglogistic fit.](image)

Effects of Pb on plant growth in aged soils were absent for soil A and B but in soil C a significant decrease in plant yield was detected from a concentration of 4000 mg Pb/kg onwards. The difference can be explained by the lower CEC value of soil C (Table 1), making Pb more bioavailable.

In the freshly spiked soils significant effects of Pb were observed. Leaching of the spiked soils resulted in significantly (p=0.05) higher EC50 values of Pb in soil compared with the EC50 values of Pb of the spiked but unleached soils. Lead in aged soils is still less toxic than in spiked + leached soils, suggesting that ageing does reduce toxicity in contrast with the starting hypothesis. Pore water Pb concentrations (not shown) did neither explain the differences in toxicity among the 3 treatments.

<table>
<thead>
<tr>
<th>soil</th>
<th>pH</th>
<th>CEC (cmol/kg)</th>
<th>Pb (mg/kg)</th>
<th>Pb (mg/L)</th>
<th>EC50</th>
<th>95% Confidence limits</th>
</tr>
</thead>
<tbody>
<tr>
<td>spiked</td>
<td>6.9-7.4</td>
<td>14</td>
<td>136-8700</td>
<td>0.02-1.1</td>
<td>3600</td>
<td>2400 - 4800</td>
</tr>
<tr>
<td>A spiked + leached</td>
<td>7.2-7.4</td>
<td>15</td>
<td>135-7200</td>
<td>0.01-22</td>
<td>5300</td>
<td>4400 - 6300</td>
</tr>
<tr>
<td>spiked + aged</td>
<td>7.0-7.3</td>
<td>14</td>
<td>188-7000</td>
<td>0.7-26</td>
<td>&gt; 6800</td>
<td>- -</td>
</tr>
<tr>
<td>B spiked + leached</td>
<td>5.5-6.1</td>
<td>25</td>
<td>52-6400</td>
<td>0.1-4.8</td>
<td>5600</td>
<td>4600 - 6600</td>
</tr>
<tr>
<td>spiked + aged</td>
<td>6.5-6.8</td>
<td>22</td>
<td>102-5700</td>
<td>0.02-0.16</td>
<td>&gt; 5600</td>
<td>- -</td>
</tr>
<tr>
<td>C spiked + leached</td>
<td>4.8-6.2</td>
<td>8.4</td>
<td>21-6600</td>
<td>0.01-310</td>
<td>1400</td>
<td>1100 - 1700</td>
</tr>
<tr>
<td>spiked + aged</td>
<td>6.5-6.8</td>
<td>8.7</td>
<td>22-3100</td>
<td>0.01-3.6</td>
<td>2400</td>
<td>1900 - 2900</td>
</tr>
<tr>
<td>spiked + aged</td>
<td>6.5-6.8</td>
<td>8.2</td>
<td>280-6400</td>
<td>0.02-6.1</td>
<td>5100</td>
<td>4300 - 5800</td>
</tr>
</tbody>
</table>

Table 1: Some soil characteristics of the selected soils.

4. Conclusions

Soils spiked with a lead salt are poor indicators for field contaminated soils. To extrapolate toxicity values obtained with laboratory spiked soils to field relevant situations a leaching/ageing factor is needed. This study explain that both processes contribute to the observed discrepancies.

5. References


Acknowledgement - This work was financed by the Lead Development Association International
Trace metal fate and uptake by vegetables grown in close proximity to traffic in Toronto, Canada

Clare LS Wiseman

Centre for Environment, University of Toronto, 33 Willcocks St, Toronto, Ontario, Canada
E-mail contact: clare.wiseman@utoronto.ca

1. Introduction

Urban areas are recognized to be “hot spots” for contaminants, due to the presence of a high concentration of pollutant sources such as industry and traffic, combined with the legacy of historical, land-use related contamination common to cities such as Toronto (Diamond and Hodge 2007). As a result, the safety of growing food in urban areas has become a widespread cause of concern, especially given its increased popularity. In addition to other urban contaminants, the presence of trace metals in soils such as Pb, Cd, Ni and Zn, and their potential for plant uptake, is of concern to residential gardeners, local community organizations and public health agencies. This has led many municipalities in Canada to examine ways to support urban gardening initiatives, while ensuring the possible risks of contaminant exposures are minimized. Toronto Public Health, for instance, has been working on a soil management protocol to guide the safe cultivation of contaminated soils (Archbold 2010, personal communication). Existing knowledge regarding the processes regulating the bioavailability of metals under natural (field) conditions and their uptake by plants for a wide spectrum of cultivated species has been identified as a primary limiting factor in the development of reliable soil quality guidelines. Much of the information about metal dynamics in soils, and the role of various physicochemical characteristics incorporated in more advanced fate models, has been derived from controlled studies conducted in the lab, using artificially amended and aged soils. Recent evidence has demonstrated that metals present in field soils, which have undergone natural aging and leaching processes, are comparatively much less likely to be bioavailable (Antunes et al. 2010; Smolders et al. 2009). This has led some to question the validity of applying metal fate models such as the terrestrial Biotic Ligand Model to guide the development of soil quality guidelines. In the absence of more data obtained from field observations, such models are predicted to yield overly conservative soil quality guidelines, which may unnecessarily discourage urban agricultural activity. This highlights the need to further examine the effects of metal aging in soils under natural conditions to ensure that soil quality guidelines are more reflective of the risks posed through trace metal behaviour and fate in the urban environment. Especially, there is a need to examine the metal dynamics and speciation of lesser known elements such as V, Ba, Ce and Sb. These metals are of concern in urban environments with heavy traffic, a primary emitter of these elements, where many community gardens and initiatives tend to be located.

This research examines the fate of traffic-related trace metal emissions and their uptake by plants grown in close proximity to traffic in Toronto, Canada. Preliminary results obtained from the first phase of this long-term study to examine trace element fate and stabilization processes in aging field soils over time are described. As part of this, initial data regarding trace metal concentrations in bulk vs. plant rhizosphere soils, as an indicator of bioavailability, is discussed.

2. Materials and methods

Plants were cultivated at several locations with predicted variable metal inputs over the growing season in 2010 (i.e. oregano (O. heracleoticum), eggplant (S. melongena) and beets (B. vulgaris)). At one location, which is situated at a busy intersection on the St. George campus location of the University of Toronto (UofT), the top 30 cm of soil was replaced with an organic, triple mix soil to control for previous contamination of the roadside environment (verge dimensions: 15 ft x 5 ft). The same soil was used to cultivate plants in containers at the other two locations. In addition, oregano (O. heracleoticum) plants have also been planted at three other sites located close to several major roads, which have some of the highest traffic densities in Toronto (planted in existing soil). Soil and plant tissues were sampled during all phases of growth, to assess variability in metal accumulation in tissues as a function of growth stage. Prior to drying in an oven at 60 °C, all harvested herbs and vegetables are halved, with one portion then being washed 3 times with distilled water (to assess surficial deposition vs. uptake through roots). Plant tissue samples have also been taken from other vegetation growing in close proximity to the cultivated locations (e.g. tree bark, weeds). In October 2010, whole plants were sampled (i.e. eggplant (S. melongena) and oregano (O. heracleoticum)), together with soils in direct contact with the plant roots. The plant parts, including roots, stem, leaves, fruit and/or leftover buds and flowers were also sampled. Plant tissue (whole eggplant (S.
melongena) and beets (B. vulgaris)) and soil samples provided by a farm located in a rural area outside of Toronto (Simcoe region) were also analyzed to gain an impression of concentrations in urban vs. rural locations.

To digest soil and plant tissue samples for analysis using a quadrupole ICP-MS (Thermo Scientific X Series II) at the Dept. of Geology (UofT), the following steps were taken in a clean room (Class 100): 1. 4 ml of aqua regia is added to approximately 0.5 grams of sample in covered Teflon beakers (OmniTrace Ultra grade HCl and HNO₃ from EMD) and then heated to 95ºC for ca. 60 minutes on a heating plate. 2. An approximate 3 to 5 ml of concentrated HF (OmniTrace Ultra, EMD) is added and the sample heated at a continuous temperature of 95 ºC for a minimum of 60 minutes, to ensure completed dissolution, 3. Optima grade (Fisher) H₂O₂ is added to samples with high organic contents until effervescence subsides, and 4. A final 2-3 ml HNO₃ is added to samples, which are then evaporated to near dryness. Samples are then diluted to a volume of 25 ml with Milli-Q water, centrifuged and transferred to Teflon tubes. A multielement standard solution, two Certified Reference Materials and one reagent blank was measured with each sample series (i.e. CRM 1570a Trace Elements in Spinach Leaves and CRM 2709a San Joaquin Soil (NIST)).

3. Results and discussion

Cr, V, Ni, Zn, Sb, Cd, Ba, Mn, Cu and Pb concentrations were found to be highest in the soils taken from the roadside locations, with the greatest concentrations occurring in soil taken from the oregano (O. heracleoticum) sites located along the roads with the highest traffic volumes. These soils are also found to have levels of Zn, Pb and Cd, which substantially exceed the guidelines for agricultural soils established by the CCME (2010) (i.e. guidelines of 200 mg Zn/kg, 70 mg Pb/kg and 1.4 mg Cd /kg). The soil concentrations of Mo, Cd, Sb, Pb, Ti, V, Cr, Co, Ni Cu and Zn at the St. George Campus roadside location were observed to increase between 2 and 8 times, since the top soil was remediated in May 2010. This indicates a rapid accumulation of a variety of trace elements in the top soil of roadside soils during the initial phase of the monitoring program. Clearly, traffic is a major contributor to trace element concentrations measured at this site. Compared to the bulk soil, levels of all elements, with the exception of Ba, were found to be substantially less in the rhizosphere of eggplant (S. melongena) grown at the St. George roadside location (e.g. Cr was 11 times lower, while V was 2.5 times lower than that in bulk soils (sampled in Oct. 2010)). Interestingly, metal concentrations measured for the farm soil from the Simcoe region were comparable to those observed for the heavy traffic locations, suggesting that metal contamination is not necessarily restricted to urban cities.

4. Conclusions

Traffic is a major source of trace element emissions to the roadside environment, which raises concerns regarding the suitability of using underutilized urban spaces such as boulevards for the cultivation of edible produce. This research indicates a rapid accumulation of trace metals in remediated soils in close proximity to traffic. At the same time, the rhizosphere soil of cultivated eggplant (S. melongena) had significantly lower trace metal concentrations compared to bulk soil measurements. The physico-chemical differences in the bulk soil compared to the rhizosphere soil, and the associated differences in trace metal concentrations, requires further attention in attempts to develop soil quality and soil management guidelines. Finally, soil contamination is clearly not restricted to urban areas, as is often assumed, but may be more a reflection of regional contaminant levels.

5. References


Acknowledgement - The author wishes to thank the Centre for Urban Health Initiatives, University of Toronto, for providing the seed grant for this study.
Metals incorporation and physiological changes in Ulva spp. as responses to the nocturnal pulse of metals from sediment in eutrophic systems – a field transplantation experiment

Patrícia Pereira¹,², Hilda de Pablo¹, Carlos Vale¹ and Mário Pacheco²

¹Instituto Nacional de Recursos Biológicos (INRB/IPIMAR), Av. Brasília, 1449-006 Lisboa, Portugal
²CESAM/Departamento de Biologia da Universidade de Aveiro, Campus de Santiago, 3810-193
E-mail contact: patbio@ipimar.pt

1. Introduction

Coastal lagoons with symptoms of eutrophication often present low oxygenated waters, particularly during the night [1]. Under these conditions, sediment could release additional quantities of metals to the overlying water [e.g. 2, 3]. Consequently, inhabitant organisms may accumulate higher levels of metals exported from the sediment, which could be on the basis of adaptative responses such as those related with the protection against oxidative stress. Field and laboratory studies have proposed the macroalgae Ulva spp. as a good biosentinel organism [4]. This ability could be particularly valuable to monitor water quality in eutrophic coastal lagoons due to the massive development of this macroalgae. Keeping this in view, it is relevant to clarify if Ulva spp. transplanted in short-time exposures (during 24 hours) respond to the increase of metal availability both in terms of metals accumulation and changes of its physiological status.

2. Materials and methods

A field transplantation experiment was performed with Ulva spp. in three short-time exposures (between 15:30-00:30; 00:30-07:30; 07:30-15:30) along a period of 24 hours in summer 2007 (Figure 1). This study was carried out in a coastal lagoon previously considered to be a paradigm of eutrophic conditions and moderately contamination by metals (Óbidos lagoon, Portugal) [3,5]. In summer, dissolved oxygen in a confined branch of the lagoon (Barrosa branch) could fluctuate pronouncedly along a day-night cycle [3].

Ulva spp. was collected at a reference site and transplanted to Barrosa branch in order to evaluate the uptake of metals and oxidative stress effects in real field conditions. For comparison proposes, identical conditions were applied to the macroalgae at a site near the reference (Lower lagoon) (Figure 1). After each in situ exposure period algae was shock frozen in liquid nitrogen. Ulva spp. was analysed for metal levels (Mn, Fe, Pb, Cu, Ni, Cd), antioxidants and lipid peroxidation. Water quality (including metal levels in water) was also characterised along the 24-hours cycle.

3. Results and discussion

3.1. Variation of physicochemical parameters and metals availability along day-night

Dissolved oxygen at Barrosa branch varied between 40% and 190% saturation levels, being the most elevated values registered at daylight hours and lower oxygenation during the night until dawn (7:15 am).

Figure 1: Experimental design
Increase of ratios to Al of particulate Mn, Fe and Pb during the night revealed an additional enrichment of these elements in the suspended particulate matter, since Al varied within a narrow interval (mean values 4.9 - 5.0%). Thus, this enhancement pointed to a supplementary input of metals from the sediment occurring during the night.

### 3.2. Metal levels and oxidative stress in transplanted *Ulva* spp.

*Ulva* spp. translocated to the Barrosa branch incorporated significantly higher levels of metals in comparison to Lower lagoon, indicating that the algae respond within hours to the higher metal availability (Figure 2). At Barrosa branch, metal levels in *Ulva* spp. also increased significantly during the night. Fe and Mn (more redox sensitive) were incorporated for longer exposure periods than Pb, Cu and Cd probably due to the higher quantities released to the water column.

An induction of SOD and an inhibition of CAT were recorded in *Ulva* spp. transplanted to Barrosa branch (between 0:30 and 7:30) (Figure 2), eventually as a response to the higher incorporation of Mn, Fe and Pb. A tendency for peroxidative damage was also observed in macroalgae transplanted to the Barrosa branch.

![Figure 2: Manganese levels and SOD activity in transplanted Ulva spp. to Barrosa branch and Lower lagoon](image)

4. **Conclusions**

Current results demonstrated that availability of metals in the water column may increase during the night in eutrophic confined areas of coastal lagoons, highlighting the importance of assessing chemical conditions over day-night cycles. Accordingly, aquatic organisms under such conditions would be exposed to an additional metals load. In particularly, *Ulva* spp. incorporated metals and exhibited physiological changes within hours exposures, reflecting the pulse of metals from sediment during the night.

5. **References**


**Acknowledgement** - Patricia Pereira (SFRH/BD/17616/2004) benefit from a PhD grant from the “Fundação para a Ciência e a Tecnologia” (FCT).
Kinetics of mercury bioaccumulation in the polychaete *Hediste diversicolor* and in the bivalve *Scrobicularia plana*, through a dietary exposure pathway

Patrícia Gonçalves Cardoso¹, Eduarda Pereira², Tiago Fernandes Grilo¹,³, Armando Costa Duarte² and Miguel Ângelo Pardal³

¹IMAR – CMA - Marine and Environmental Research Centre, Department of Life Sciences, University of Coimbra, PO Box 3046, 3001-401 Coimbra, Portugal  
²CESAM – Centre for Environmental and Marine Studies, Department of Chemistry, University of Aveiro, 3810-193 Aveiro, Portugal  
³CEF – Centre for Functional Ecology, Department of Life Sciences, University of Coimbra, PO Box 3046, 3001-401 Coimbra, Portugal  
E-mail contact: gcardoso@ci.uc.pt

1. Introduction

There has been a great concern throughout the world about metal pollution and its impact on living organisms. Metals deposit in aquatic systems and marine invertebrates can be exposed to them from both dissolved and particulate fractions (Metian et al., 2009). Dissolved metals can be accumulated by direct adsorption while metals associated with particles can be accumulated following ingestion and digestion of food. Delineating the routes of metal uptake is extremely important for understanding metal bioaccumulation and toxicity and for setting appropriate water and sediment quality criteria.

Macrobenthic organisms such as the bivalve *S. plana* and the polychaete *H. diversicolor* are considered excellent indicators of biotic integrity. These organisms play an important role in the food web since are located between the primary producers (e.g., algae) and the secondary consumers (e.g., carnivorous fish) being suitable for evaluating environmental impacts due to contaminants.

To the best of our knowledge, an actual assessment of the relative contribution of different exposure pathways on the bivalve *S. plana* and on the polychaete *H. diversicolor,* for mercury has not been carried out yet. Therefore, the main aims of the present study were: 1) to determine the kinetics of mercury bioaccumulation of the polychaete *H. diversicolor* and the bivalve *S. plana,* exposed to dietary pathway through a mesocosm laboratory experiment and 2) to infer about the relative contribution of different exposure pathways (e.g. sediment and food) on mercury accumulation of these two macrobenthic species.

2. Materials and methods

2.1 Experimental set-up

The experiments were performed in acid-washed glass containers of 3 litres (Ø: 14 cm). Sediment mesocosms were established by transferring 2000 g (dry weight-DW) of homogenised substrate to each glass container. Then, 1.5 L of filtered estuarine water (salinity - 30) was added to each container, always maintained under oxic conditions. In each of the glass containers 5 individuals of *H. diversicolor* and 3 individuals of *S. plana* were introduced, according to the mean densities observed in the field. Daily, it was added to the containers an amount of 0.06 g DW of contaminated alga for the treatments I and II and the same biomass of non-contaminated alga given to the control. The alga corresponded to the food resource and to the only source of mercury contamination. For each of the three studied conditions (reference – non-contaminated alga, low and high metal contamination) six different sampling times were chosen (t₁ – 2 days; t₂ – 5 days; t₃ – 9 days; t₄ – 15 days; t₅ – 23 days; t₆ – 31 days). At each contamination condition and sampling times three replicates were carried out. At the pre-defined times, the organisms (*H. diversicolor* and *S. plana*) were removed from the glass containers, rinsed with estuarine water and placed in constantly aerated clean estuarine water at 20°C for 24 hours to allow depuration, being afterwards dissected in the case of *S. plana* and then freeze-dried for later mercury analysis.

3. Results

3.1 Mercury bioaccumulation rates
Total mercury concentration accumulated by the two macrobenthic species showed similar kinetics, a linear pattern of mercury accumulation throughout the experiment, reaching higher values in the treatment that corresponded to a higher contamination. In addition, we could also observe that the polychaete *H. diversicolor*, despite starting the experiment with a lower Hg concentration, it reached at the end of the experiment higher values than the bivalve *S. plana*. Total mercury accumulated for both species seems to be proportional to the metal concentration in the food, since in the treatment II, the mercury accumulated is approximately 3 times higher than in treatment I, which is in accordance with the mercury concentration in the food. Attending the daily total mercury bioaccumulation rate, we can observe that for *H. diversicolor*, the increment was regular for both treatments (treatment I – 0.0015 µg g\(^{-1}\) per day; treatment II – 0.0039 µg g\(^{-1}\) per day) and higher than for *S. plana* (treatment I – 0.0007 µg g\(^{-1}\) per day; treatment II – 0.0029 µg g\(^{-1}\) per day).

4. Conclusions

Both studied species presented a similar model of Hg bioaccumulation kinetics, a linear pattern of accumulation through time being the mercury accumulation in the organisms proportional to the mercury concentration in the food. Moreover, the mercury bioaccumulation rates were higher in the polychaete *H. diversicolor* than in the bivalve *S. plana*, which can be related to their feeding strategies, ingestion rates and assimilation efficiencies. Comparing the effect of different exposure pathways (food versus sediment) on the mercury bioaccumulation rates, we may infer that mercury uptake via food (i.e. particulated macroalgae) is a major pathway of metal bioaccumulation for the polychaete *H. diversicolor*, while for the *S. plana* it seems to be the sediment. Moreover, the mercury bioaccumulation process through the dietary pathway, revealed to be faster than through sediment exposure, especially for the polychaete, which can represent a non-negligible risk for Humans.

5. References


1. Introduction

In the aquatic environment anoxic, organic rich sediments usually act as an important sink for metals and metalloids, representing an enormous mass of metal in potentially reactive, bioavailable and toxic form. This is due to the high affinity of organic matter and Acid Volatile Sulfides (AVS) with inorganic particles. AVS is operationally defined as the amount of sulfides volatilized by the addition of 1 N HCl and formed through the decomposition of organic matter under anaerobic conditions (Di Toro et al., 1990). In their reaction with metals, AVS can form thermodynamically stable metal sulfide precipitates, which results in a decreased concentration of free metal ions and therefore reduced metal bioavailability. The metals which are associated with AVS are called Simultaneously Extracted Metals (SEM). SEM is generally determined as the metal fraction which describes the sum of molar concentrations of toxicologically important, cationic metals (Cu, Pb, Cd, Zn and Ni) which are extracted together with AVS.

Recent water quality improvements in freshwater ecosystems are generally accompanied with an increase in oxygen concentrations (Karr and Dudley, 1981). These elevated oxygen levels can affect the redox chemistry of the sediment drastically and lead to a rapid breakdown of organic matter and AVS (Teuchies et al., in press), resulting in an increased flux of metals to the overlying surface water. However, to which extent these metals will be released from the sediment and what effect this release will have on metal bioaccumulation and toxicity in aquatic invertebrates is not yet understood.

The main objective of the present study was to evaluate the effect of improved oxygen concentrations in overlying surface water on the bioavailability, accumulation and toxicity of sediment-bound metals in the aquatic invertebrates Lumbriculus variegatus and Asellus aquaticus.

2. Materials and methods

2.1 Experimental setup

A 60 days lab experiment with natural metal-polluted sediment containing high amounts of organic matter and AVS (table 1) was conducted in 2 different experimental containers. In each set-up 120 individuals of Lumbriculus variegatus and 60 individuals of Asellus aquaticus were spread among 6 different tubes in the sediment (Lumbriculus) and 6 different cages in the water (Asellus). The oxygen level in the surface water (artificial OECD water) of the first container was set at 95 % saturation, while the second container was held at 40 % oxygen concentration. Oxygen levels were monitored and held constant using an O2-stat system (Consort, Belgium).

<table>
<thead>
<tr>
<th>Trace metals (µmol/g)</th>
<th>Ag</th>
<th>Cd</th>
<th>Co</th>
<th>Cr</th>
<th>Cu</th>
<th>Ni</th>
<th>Pb</th>
<th>Zn</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.018</td>
<td>0.011</td>
<td>0.141</td>
<td>1.12</td>
<td>1.66</td>
<td>0.369</td>
<td>0.525</td>
<td>13.1</td>
<td></td>
</tr>
<tr>
<td>(± 0.002)</td>
<td>(± 0.001)</td>
<td>(± 0.013)</td>
<td>(± 0.114)</td>
<td>(± 0.175)</td>
<td>(± 0.030)</td>
<td>(± 0.075)</td>
<td>(± 1.26)</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Overview of the most important sediment characteristics and trace metals measured in the sediment before the start of the experiment. Average values and standard deviations are presented (n=6).

2.2 Sampling and metal analysis

At 6 different time points (after 0, 2, 5, 12, 32 and 54 days) sediment cores were sampled. SEM-AVS together with organic matter and the total metal content of the sediment was measured at 4 different depths (0-1; 1-4; 4-8 and 8-15 cm). At each sampling moment metal bioavailability in both water and sediment (4 different depths) was measured using Diffusive Gradients in Thin films (DGT; Zhang et al., 2002). In addition metal accumulation together with toxicity endpoints like energy reserves (glycogen, lipids and proteins) and the induction of metallothioneins (MT) were analyzed in the invertebrate species. Total and dissolved metal concentrations in the surface water were measured daily.

Trace metals (As, Cd, Co, Ag, Cr, Cu, Zn, Ni and Pb) and cations (Ca, Mg, K, and N) were measured in DGT, sediment (total as well as SEM), surface water and invertebrate samples. All metal analyses were performed...
using High Resolution Inductively Coupled Plasma Mass Spectrometry (HR-ICP-MS; Thermo Scientific, Finnigan element 2, Waltham, MA, USA).

3. Results and discussion

Figure 1 presents the SEM\textsubscript{Tot}-AVS concentrations measured in the upper layer (0-1 cm) of the sediment in the 2 treatments. From this figure it is clear that the elevated oxygen concentrations in the surface water (95 % O\textsubscript{2}) significantly affect the SEM\textsubscript{Tot}-AVS levels in the sediment, which leads to an increased metal bioavailability in the sediments pore water (SEM\textsubscript{Tot}-AVS > 0 after 54 days). The AVS levels in the low O\textsubscript{2} treatment show no significant difference in time. Already after 2 days a significant difference in SEM\textsubscript{Tot}-AVS between the treatments is noticed.

![SEM\textsubscript{Tot}-AVS and Glycogen content Asellus aquaticus graphs](image)

The glycogen content of *Asellus aquaticus* in time is showed in figure 2. The glycogen content of the organisms in the high oxygen treatment does not significantly differ between the sampling dates. Only after 54 days a significant difference in the glycogen content between the treatments is noticed. However, this difference is due to the increased glycogen content of the low oxygen treatment. Furthermore no significant correlations could be found between SEM\textsubscript{Tot}-AVS in the sediment and the glycogen content in *Asellus aquaticus*. These results indicate that after almost 60 days of elevated oxygen concentrations in the surface water, metal release from the sediment has not increased enough to induce toxic effects in water-inhabiting invertebrates like *Asellus aquaticus*.

Further work of this study will investigate the metal concentrations in DGT and the accumulation in both *Asellus aquaticus* and *Lumbriculus variegatus*. These results will reveal to which extent metals can be mobilized and accumulated from water and sediment after increased oxygen exposure.

4. Conclusions

- SEM\textsubscript{Tot}-AVS levels in the upper layer of the sediment significantly increase after exposure to elevated oxygen concentrations in the surface water.
- This increased metal bioavailability does not affect the glycogen levels in the water-inhabiting invertebrate *Asellus aquaticus*.
- The breakdown of AVS in the sediment does not lead to an increased metal toxicity in the surface water after 54 days.
- Ongoing work: DGT concentrations and metal accumulation in aquatic invertebrates.

To which extent sediment-bound metals can be mobilized and accumulated after elevated oxygen exposure?

5. References


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Marine diatom *Thalassiosira pseudonana* towards a system biology for the water quality assessment

Raquel N. Carvalho¹, Stephanie K. Bopp ², Alina Burchardt¹, Fabrizio Sena¹, Teresa Lettieri¹

¹ Joint Research Center, Institute for Environment and Sustainability, Rural, Water and Ecosystem Resources Unit, T.P. 300, Via E. Fermi 2749, 21027 Ispra (VA), Italy
² EFSA-European Food Safety Authority, Largo N. Palli 5/A, 43100 Parma, Italy
E-mail contact: teresa.lettieri@jrc.ec.europa.eu

1. Introduction

Diatoms are unicellular, photosynthetic, eukaryotic algae that inhabit marine and fresh waters worldwide. These phytoplankton organisms play an important role in the carbon cycle, being responsible for about 40% of the total carbon fixation in oceans.

Molecular or morphological changes in these species under ecological stress conditions are expected to serve as early indicators of toxicity and predict the global impact on the entire ecosystem. *Thalassiosira pseudonana* is a marine diatom whose genome sequence became recently available [1]. This breakthrough opened the doors for ecotoxicological studies using molecular tools applied to *T. pseudonana* as a model aquatic organism.

Genomics and proteomics tools have been used in *T. pseudonana* to study the effects of exposure to common aquatic pollutants. The purpose of such studies is to provide i) a system biology approach to identify the key pathways linked to the exposure compounds, ii) selection of molecular biomarkers of exposure as early identification of water quality endangerment, iii) a more comprehensive substitute to mere water chemical analysis.

Polycyclic aromatic hydrocarbons (PAHs) have a ubiquitous distribution in aquatic environments worldwide, arising both from natural as well as anthropogenic sources, and are considered a major threat to marine and freshwater ecosystems. Several PAHs have been classified as potential carcinogens to humans and were included in the priority list of the European Union’s Water Framework Directive (2000/60/EC). We have used a protocol for diatom exposure, followed by gene expression and protein expression analysis, as pilot studies for a system biology approach in environmental monitoring.

2. Materials and methods

Diatom cells have been exposed to the PAH compound Benzo[a]pyrene (BaP) at a concentration which inhibits the growth by 30%. Exposed diatom cultures have been harvested for total RNA [2] and protein extraction (Carvalho and Lettieri, unpublished data). Extracted RNA has been analyzed by DNA Microarray analysis or quantitative Real Time Polymerase Chain Reaction (PCR) for selected genes. Quantitative proteomics has been performed by using iTRAQ labeling (Applied Biosystems) for the identification and quantification of protein regulation upon exposure to BaP.

3. Results and discussion

3.1. Gene expression profile analysis

A DNA microarray was designed, customised in our laboratories, and manufactured by Agilent (Santa Clara, USA). Eleven thousand genes were spotted in four probe groups, one probe group contained genes that we already observed being regulated by quantitative Real-Time PCR [2] and was used as quality control.

We identified more than 500 regulated genes which were mainly involved in processes e.g. oxidative stress, transcription regulation, lipid transport and metabolism and biosilification process.

Particularly interesting was the down regulation of the Silicon transporter 1 (ST1). This gene encodes for an enzyme which is responsible for the uptake of silica from the media into the cell of diatoms. We had previously observed a down regulation of other genes involved in silica shell formation [2], suggesting that one of the pathways affected by Bap exposure is the biosilification process.
3.2 Protein Profile analysis

The proteomics' profile analysis showed that around 16% of the total identified proteins were regulated and one fourth of them confirmed the gene expression changes.

In the table 1 is the list of the confirmed regulated proteins, including ST1, which was downregulated as also observed in DNA microarray analysis. To verify the effect on silica uptake upon exposure to BaP, we measure the silica in diatom media after 24 hours exposure to BaP. We clearly observed that in BaP-treated diatom cells the uptake was reduced down to 30% with respect to the control [3].

<table>
<thead>
<tr>
<th>Name</th>
<th>GO</th>
<th>Regulator factor (protein level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mitochondrial chaperonin</td>
<td>Chaperone</td>
<td>1.48</td>
</tr>
<tr>
<td>N-acetylornithine aminotransferase</td>
<td>Metabolic process</td>
<td>1.28</td>
</tr>
<tr>
<td>Proteasome subunit alpha type</td>
<td>Protein catabolism</td>
<td>1.21</td>
</tr>
<tr>
<td>Predicted protein</td>
<td>Unassigned</td>
<td>1.21</td>
</tr>
<tr>
<td>Silicon transporter</td>
<td>Membrane transport</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Table 1: list of proteins which regulation is also confirmed at gene expression level

Despite the low protein identification rate achieved in our studies, we were able to select a set of 19 proteins with changes in expression. Our data shows that the lipid metabolism and biosilification processes are involved, but also unveils other potential pathways involved in the diatom exposure or in toxicity responses to BaP.

4. Conclusions

Our data show that one of the processes affected by BaP is the biosilification processes and we could link the gene-protein and physiology state of diatom cells upon exposure to the pollutant. These studies are a good example of a system biology approach in an aquatic ecosystem. This approach in diatoms will help to elucidate pathway/metabolic processes involved in the mode of action of pollutants and to identify molecular biomarkers of exposure to be later on integrated into environmental monitoring for water quality assessment.

5. References


Acknowledgement - The authors thank “IP & Technology Transfer Unit” (EU-JRC) for funding the project to design and customise the diatom DNA Microarray. We thank Joaquin Pinto Grande and Bruno Paracchini for technical assistance.
Analysis of microcystin algal toxins in Lake Maggiore water (N-Italy) by SPE-UPLC-MS-MS

Robert Loos1, Veljo Kisand2 and Bernd M. Gawlik1

1Institute for Environment and Sustainability (IES), Joint Research Centre (JRC), Via E. Fermi, TP 290, Ispra (VA), I-21020, Italy
2Institute of Technology, University of Tartu, Nooruse 1, Tartu, EST-50411, Estonia
E-mail contact: Robert.Loos@jrc.ec.europa.eu

1. Introduction
Algal blooms can generally occur in nutrient rich calm fresh waters and low salinity marine areas like the Baltic Sea. Usually these blooms form in mid to late summer and can carry on into the fall, and tend to float near the water surface. These algal blooms potentially affect water quality as well as the health of human and natural resources.

A special type of cyanobacteria (blue-green algae) can produce toxins, called microcystins, which are cyclic peptides. Currently, more than 60 structural variants of microcystins have been identified. The most important (most often analysed and found) species is microcystin LR.

People (and other mammals) can get sick from microcystin toxins if they have direct contact with a toxic algal bloom by swallowing water, or by having skin contact. Microcystin poisoning can cause breathing problems, stomach upset, nausea, vomiting, diarrhea, headache, fever, allergic skin reactions (a rash or skin blisters), and even liver damage.

2. Materials and methods

Sampling: Water sampling in several lakes in North Italy was performed from the river shore using 1 liter Schott-Duran glass bottles and a 3 m telescopic sampling stick. Sampling locations at the Lake Maggiore were: Angera, Arolo, Ispra, Ranco, and Cerro. Moreover, three other smaller lakes (Varese, Monate and Comabbio) were investigated.

Sample preparation: Extraction of 400 ml water at neutral pH using Oasis HLB 200 mg cartridges with automated solid-phase extraction (SPE) using an AutoTrace® System; elution with 6 ml methanol and evaporation of the extracts to 500 µl.

UPLC-MS-MS triple-quad analysis: Acquity UPLC BEH C18 column, particles 1.7 µm; 50 x 2.1 mm; eluents: water and acetonitrile (both 0.1% acetic acid), gradient start with 90% water to 90% acetonitrile; injection volume 5 µl; Waters Aquity UPLC coupled to an AB Sciex 5500 Qtrap MS-MS.
3. Results and discussion

3.1. SPE - UPLC-MS-MS method development

Ultra performance liquid chromatography (UPLC) triple quadrupole mass spectrometry (tq-MS) is very well suited for the analysis of microcystins. The sensitivity for these cyclic peptides is however relatively low, due to their difficult electrospray ionization. Top LC-MS instruments are necessary.

It was shown that microcystins can be detected in the positive or negative ionization modes. The specific MS-MS transitions for microcystin LR are m/z [M+H]+ 995.5 > 213, 135, and 553, or in negative ionization mode by [M-H]- 993.5 > 265, 283, and 128, respectively.

Very high SPE recoveries of around 97 % (n=4) could be achieved (at neutral pH). UPLC-MS-MS sensitivity is in the low µg/L concentration range, and overall method detection limits (including SPE enrichment) are lying in the low ng/L concentration range.

3.2. Analysis of microcystins in lake water

On 27 August 2010, the Local Health Authority (ASL) Varese reported the presence of algal blooms along the whole Lake Maggiore lake shore, and issued the advice to swimmers to avoid contact with these algae and not to swallow or digest water.

Some water samples from Lake Maggiore, Monate, Varese and Commabio taken at the end of August and beginning of September 2010 were analysed. Microcystin LR was detected in all samples at low ng/L concentration levels. The highest levels were found with ~ 350 ng/L at the north side of Ranco (Via Alberto), where the water is relatively shallow and calm. In this sample, also microcystin RR (1039.5 > 620) and LF (986.8 > 135) were detected (without standards available). Contamination depends strongly on weather (wind) conditions and currents. No microcystin LR was found in JRC tap water.

![Figure 1: Lake Maggiore water samples (2 September 2010)](image)

4. Conclusions

A very sensitive method for the analysis of presence of microcystins in water, based on solid-phase extraction (SPE) followed by ultra performance liquid chromatography (UPLC) triple quadrupole mass spectrometry (tq-MS) has been developed. New analytical results show that algal toxins are present in Lake Maggiore water. Their formation is favoured by high summer temperatures, nutrient rich (eutrophication) and calm water conditions. Risk to humans is relatively low, provided that direct contact with algae is avoided. In the future, however, contamination might increase due to changing climatic conditions. More spatially and temporally spread monitoring and research on other algal toxin is necessary. Analysis of microcystins is difficult due to availability of chemical standards and their low stability.

5. References


Trophic transfer of microcystins from *Lymnaea stagnalis* (Gastropoda Pulmonata) to *Gasterosteus aculeatus* (Teleostei Gasterosteidae) and impact on the fish

Emilie Lance¹, Anais Petit¹, Wilfried Sanchez², Myriam Bormans¹, Claudia Gérard¹

¹ UMR CNRS 6553, University of Rennes 1, 263 Avenue du Général Leclerc, 35042 Rennes Cedex, France
² National Institute for Industrial Environnement and Risks, Rue Taffanel, 60550 Verneuil en Halatte, France
E-mail contact: emilie.lance@univ-rennes1.fr

1. Introduction

Due to eutrophication of freshwaters, the frequency of cyanobacteria proliferations is increasing worldwide. From 40 to 75% of cyanobacterial blooms produce hepatotoxins [e.g. microcystins (MCs)], endotoxins released in water during the cell lysis and which constitute a real threat for target organisms as gastropods (intoxication by absorption of toxic cyanobacteria or dissolved toxins). MCs mainly accumulate in the liver (or digestive gland) of metazoans where they interact reversibly (free MCs) or irreversibly (covalently bound MCs) with phosphatase proteins, leading to tissue destruction. Our previous experiments of chronic exposures to MC-producing cyanobacteria (*Planktothrix agardhii*, 5 and 33 µg L⁻¹) demonstrate that the gastropod *Lymnaea stagnalis* ingested toxic cells and accumulated free and bound MCs at concentrations up to 69.9 µg total MCs g DW⁻¹, among them up to 67% of bound MCs (Lance et al., 2006, 2009). Although 90% of free MCs were eliminated after a 3-week depuration period [probably by detoxification processes involving glutathione enzymes (Wiegand et al., 1999)], the elimination of bound MCs [probably during the Ppase renewal] was lower (from 0 to 59%) and their proportion among total MCs increased (up to 90%) during depuration (Lance et al., 2009). Consequently, *L. stagnalis* can be a MC-vector for numerous higher consumers (e.g. crayfish, leeches, aquatic insects, fish, and waterfowl), which in turn are consumed by aquatic or terrestrial predators (e.g. fish, amphibians, musk rats, and birds).

The three-spined stickleback, *Gasterosteus aculeatus*, a euryhalin fish from the temperate areas of the Northern hemisphere, is omnivorous and can ingest snails depending on their availability (Bruslé and Guignard, 2001). As they frequently stay in the littoral zone where cyanobacteria accumulated after wind events and gastropods co-occur, intoxication of fish via ingestion of MC-intoxicated gastropods is environmentally relevant. Numerous studies (for review: Malbrouck and Kestemont, 2006) reported MC accumulation in fish associated with liver pathology and behavioral changes (e.g., increased ventilation rates) following toxic cyanobacteria consumption, but more rarely following MC-intoxicated preys. The trophic transfer of MCs in freshwater ecosystems between omnivorous or planktivorous fish to carnivorous ones has been suggested in the field (for review: Ibeling and Chorus, 2007). The aim of this study is to evaluate if free and bound MCs accumulated in *L. stagnalis* tissues after toxic cyanobacteria consumption are transferred to *G. aculeatus*, and what are the consequences on this latter in terms of MC accumulation, histopathology, oxidative stress response and behavioural changes.

2. Materials and methods

*L. stagnalis* gastropods were previously exposed to toxic *P. agardhii* (producing 33 µg MCs L⁻¹) during 5 weeks. Then, half of the snails (group 1) were sacrificed and the other half (group 2) were placed in depuration during 3 weeks. When *G. aculeatus* were fed with intoxicated gastropods their digestive gland contained two different total MC concentrations (with group 1 > group 2) and different proportions of bound among total MCs (i.e. 64% and 94% of bound MCs respectively for the groups 1 and 2). Fish were fed daily on digestive glands from the two intoxicated groups during 4 days (i.e. intoxication period), then on healthy digestive glands during 4 days (i.e. depuration period). The impact of intoxication on the three-spined stickleback was investigated by measuring: 1) the accumulation and elimination of MCs in several organs (liver, muscle, kidneys, gills), 2) the potential oxidative stress response via the activity of three detoxifying enzymes: glutathione peroxidase, glutathione-S-transferase and superoxide dismutase, 3) the histopathology of the liver, target organ of MCs, 4) the behavioural changes in locomotory activity.

3. Results and discussion

MC accumulation in *G. aculeatus*

Fish accumulated free MCs (i.e. up to 3.9 ± 0.1 µg g⁻¹ DW) in the liver, kidneys, muscles, and gills after 4 days of daily consumption of MC-intoxicated *L. stagnalis* digestive glands (Table 1). But there was no
biomagnification since free MC concentration in the fish liver was similar or 1.7 times less important than in the gastropod digestive gland. MC elimination after depuration was complete in the gills, almost complete in the liver and kidneys (92%), but partial in the muscles (from 6 to 58%) (Table 1). Moreover, the total MC content in the fish was probably higher because we disregarded bound MCs (these analyses are still ongoing). MC accumulation in fish muscles suggests a risk of MC transfer through the food web, possibly leading to human contamination.

### Table 1: Free MC accumulation (µg.g⁻¹ DW) (mean ± SE) in different organs of three-spined sticklebacks fed on high (“intox” snail”) or low (“depur” snail”) MC-contaminated snail digestive glands at the end of intoxication and depuration periods.

<table>
<thead>
<tr>
<th>Fish treatments</th>
<th>Intoxication period</th>
<th>Depuration period</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Snail group 1</td>
<td>Snail group 2</td>
</tr>
<tr>
<td>Fish organs</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver</td>
<td>0.33 ± 0.12</td>
<td>3.96 ± 0.14</td>
</tr>
<tr>
<td>Gills</td>
<td>0.16 ± 0.05</td>
<td>2.54 ± 0.11</td>
</tr>
<tr>
<td>Kidneys</td>
<td>0.36 ± 0.04</td>
<td>1.69 ± 0.15</td>
</tr>
<tr>
<td>Muscles</td>
<td>0.17 ± 0.08</td>
<td>1.05 ± 0.09</td>
</tr>
</tbody>
</table>

3.2. Impact on G. aculeatus: liver histopathology, biotransformation/oxidative stress enzymes, and locomotory activity

MC accumulation in the fish is a consequence of toxin absorption through digestive tract during digestion, resulting in MC distribution in the entire body via the blood flux that may impair homeostasis (for review: Malbrouck and Kestemont, 2006). After ingestion of MC-intoxicated digestive glands, fish exhibited a slight oxidant stress (increased activity of the anti-oxidant glutathione peroxidase) in the liver, associated with pathology (i.e. few disintegration of the parenchyma architecture localized near the blood vessel). Moreover, they moved during a significantly lower time compared to control fish, probably due to a reallocation of energy towards metabolism and excretion of MCs.

4. Conclusions

The presence of free MCs in several organs of G. aculeatus after consumption of intoxicated gastropods suggests the possible MC transfer in aquatic food web. The negative impact on fish appeared limited probably due to both low intensity and duration of intoxication. Risks are expected to be higher in the field due to i) extended period of chronic MC exposure corresponding to cyanobacteria proliferations from April to October in temperate regions; and ii) numerous co-occurring other stresses (e.g., pollution, hypoxia) probably reducing MC detoxification capacity of fish. Further investigations on transfer and toxicity of bound MCs are needed as they are less easily eliminated than free MCs and can persist in organisms after the bloom collapse, then representing a higher health hazard.

References

Is the invasive *D. polymorpha* better adapted to cyanotoxin exposure than the native *U. tumidus*?

Vanessa Burmester¹, Claudia Wiegand²

¹Leibniz-Institute of Freshwater Ecology and Inland Fisheries, Müggelseedamm 301, 12587 Berlin, Germany
²University of Southern Denmark, Institute of Biology, Campusvej 55, 5230 Odense
E-mail contact: Burmester@igb-berlin.de

1. Introduction

In anthropogenic impacted aquatic environments, biodiversity is altered due to loss of habitat and resources, and due to introduction of pollutants and nutrients (eutrophication). Both, decline of biodiversity and establishment of invasive species have been observed. Eutrophication is already responsible for a shift of biodiversity towards phytoplankton development, in particular cyanobacteria, which are hazardous due to the production of cyanobacterial toxins. Amongst these cyanotoxins, microcystin is the most common in freshwater. Physiological adaptations to pollutants and cyanobacterial toxins enable organisms to live in contaminated waterbodies. Main detoxification enzymes for the cyanotoxin microcystin are the glutathione S-transferases (GST) [1].

Freshwater mussels such as *Dreissena polymorpha* and *Unio tumidus* are non-selective filter feeders, with high filtration activities, thus may accumulate cyanotoxins during cyanobacterial blooms [2]. In contrast to the indigenous *U. tumidus*, the invasive *D. polymorpha* seems to be a moderate sensitive species able to develop sustainable population in contaminated water courses. The population of Unionidae is endangered by water pollution, shoreline construction and competition with *D. polymorpha*. Competition is for food, as the habitats are different: the unionids need soft sediment, whereas *D. polymorpha* attaches to any kind of hard substrate, including shells of unionids. By that, food availability decreases for the unionid, and moreover, the additional weight causes the unionid to sink deeper in sediment [3].

This study compares the two species with regard to their detoxification capacity for microcystin-LR. A further aim was to correlate the detoxification attempts to the physiological costs for the organisms.

For this, the activities of biotransformation (glutathione S-transferase, GST), antioxidant enzymes (superoxide dismutase, SOD and catalase, CAT) and energy reservoirs (glycogen-, lipid- and glutathion content) were compared in the invasive *D. polymorpha* and the native *U. tumidus* in response to cyanotoxin exposure.

2. Materials and methods

*D. polymorpha* (22-25 mm shell length) and *U. tumidus* (50-61 mm shell length) were collected in July from Lake Küstrinsee (Germany). The mussels were acclimated for 14 days in the laboratory in artificial tank water (AFW) at the respective mean temperature measured in the field (20 ±0.5 °C), and fed twice daily with freeze-dried Spirulina sp. powder. Light and dark phases were set to 14:10 h.

Mussels were exposed for 24 h and 7 d to microcystin-LR at 10 µg L⁻¹ and 50 µg L⁻¹. Each mussel was exposed in 100 ml medium (toxin in AFW or AFW for control).

After exposure, mussels were rinsed briefly, and whole mussel tissue, digestive gland and gills (n = 5) of *D. polymorpha* were removed on ice. *U. tumidus* were dissected on ice and digestive gland, gills, mantle, foot (n = 5) isolated from other organs. The samples were immediately frozen in liquid nitrogen and stored at -80°C until determination of enzyme activities (GST, CAT and SOD) and energy reservoirs (Glykogen-, Glutathion- and Lipidcontent). Tissue of each mussel was analyzed individually.

Statistical differences in enzyme activity and energy reservoirs between treatments and control were calculated by ANOVA. Significance levels were evidenced by Duncan`s Test at p<0.05. (StatSoft, Inc. 2000).

3. Results and discussion

In *D. polymorpha* the sGST activities were significantly elevated for the entire exposure period in whole tissues, but also tendentially elevated in digestive gland and gills, compared to controls, increasing with exposure concentration and duration. *D. polymorpha* seems to biotransform the microcystin-LR with increase in MC-LR concentration or exposure duration. In a previous study, clearance of this toxin was evidenced for
concentrations up to 100 µg L⁻¹, without harming the *D. polymorpha*, and the parent compound as well as biotransformation products were excreted into the medium [2]. High activities of the P-glycoprotein (MXR), found in that study, together with the activities of the sGST of this investigation evidence a strong biotransformation capacity of *D. polymorpha* for the cyanobacterial toxin. Contrarily, the sGST activity decreased in the digestive gland, gills, foot, mantle of *U. tumidus* with exposure duration, compared to control animals, and to an even lower value in the higher exposure scenario. Due to high variation between the individuals (samples were not pooled) these changes in enzymatic activities failed statistical significance. Nevertheless, it seems that with longer exposure duration this species is impaired to perform biotransformation of the toxin via the GST system.

CAT activities were not significantly changed during the whole period in both species. It responded in *D. polymorpha* with decreased activities after short time exposure to 10 µg L⁻¹, and with slight increased activities at the higher concentration. Catalase is the most efficient antioxidant enzyme, hence toxin concentration might not have been sufficient to provoke increase in activity. Similar, the response of antioxidant enzymes of *U. tumidus* exposed to industrial impacted streams revealed minor changes for catalase and superoxide dismutase, but reduction in activities of glutathione peroxidase and glutathione reductase [4]. If those enzymes would indicate oxidative stress in the mussels exposed to cyanobacterial toxins will be investigated further.

The glycogen content decreased in both species indicating the requirement for energy due to the stress caused by the MC-LR exposure. The decrease in the glycogen content in the whole mussel tissue of *D. polymorpha* was statistically significant for the highest concentration after 24 h. Also in the digestive gland and the foot of *U. tumidus* there was a concentration dependent decrease. In all but one exposure scenario, the short time depletion of the glycogen storage was higher than the long time usage from this energy reservoir, confirming its role as fast energy supply.

4. Conclusions

*D. polymorpha* detoxifies MC-LR but at the expense of energy. The results suggest that *U. tumidus* is less able to detoxify MC-LR via biotransformation enzyme GST. No oxidative stress occurred, but MC-LR caused an enhanced requirement for energy in both mussel species, possibly for detoxification (*D. polymorpha*) or other stress reactions (*U. tumidus*).

Compared to *U. tumidus* the invasive *D. polymorpha* seems to be better adapted to cyanobacteria contaminated waterbodies. This, in addition to the usage of habitats, the impact on Unionidae by attaching on them, indicates a contribution to an ecological benefit for *D. polymorpha* in comparison to *U. tumidus*.

Further investigations will compare the microcystin accumulation, and the efficiency of microcystin-LR biotransformation and excretion in both species.

5. References


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The use of single ecosystem function for a sustainable removal of cyanobacterial toxins from water

Claudia Suseth Romero¹ and Stephan Pflugmacher¹

¹Berlin Institute of Technology, Institute of Ecology, Department Ecological Impact Research and Ecotoxicology, Franklinstr. 29, 10538 Berlin, Germany

E-mail contact: claususeth@gmail.com

1. Introduction

Cyanobacterial blooms are a worldwide problem due to the eutrophication of water bodies. Most of the cyanobacteria known today are producing toxins, from which the microcystins are the most common detected. Over 70 congeners of microcystins have been isolated (Carmichael, 1997). Microcystins are known to be potent inhibitors of protein phosphatase 1 and 2A and also known to be tumor promoters. During senescence of cells the toxins are released into the water in high amounts. The microcystins itself are very stable molecules and highly persistent in the water (DeFigueiredo et al 2004).

In many countries, namely the semi-aride and aride ones, freshwater lakes are used for aquaculture, spray irrigation of agricultural plants and also as a source for drinking water (Nimptsch et al. 2008). There are several studies on how to remove cyanobacteria respectively their toxins from the lake water. Some of these studies are using clay particles, or hey bale to get rid of the cyanobacteria. But in most cases the toxins themselves stay in the water body.

Aim of our research is to identify and use single ecosystem functions, which can help in remediation of cyanotoxins from lake water in a sustainable way. The single ecosystem function are e.g. the biology of aquatic macrophytes and their ability to take up toxic substances.

2. Materials and methods

15 different aquatic macrophytes were used in exposure experiments with three different microcystins (MC-LR, MC-RR and MC-LW) in a concentration of 50ug/L. Static renewal exposure was done for one week. After this week, plants were carefully rinsed with water and extracted using 70% methanol.

Fig. 1 Examples of the various macrophytes used in this research

Figure 2 Chemical structure of the micocystin congeners used in this research
Analysis of microcystin within the plant material was done using LC-MSMS techniques and reference substances from Alexis GmbH (Grünberg, Germany)

3. Results and discussion
The experiment showed, that the ability of aquatic macrophytes to take up cyanobacterial toxins, namely microcystin congeners, depends on the plant used. Some macrophytes have the ability to take up nearly all toxin, such as Ceratophyllum demersum and Vesicula dubyana, whereas other macrophytes such as Potamogeton crispus is only able to take up 30 % of the whole toxin used in the exposure. These differences seems to be dependent on leave amount and surface.

4. Conclusions
To use single ecosystem functions, such as the uptake of aquatic macrophytes in order to eliminate cyanobacterial toxins from water, is dependent from the species used in correlation with leave amount and surface. This has to be taken into account, when developing “Green Liver Systems” for water purification.

5. References
Effects of slurry from sulfadiazine (SDZ) treated pigs on the structural diversity of microorganisms in rhizosphere soil

Rüdiger Reichel¹, Rosendahl, Ingrid², Amelung, Wulf², Thiele-Bruhn, Sören¹

¹ Soil Science, Faculty of Geography/Geosciences, University of Trier, Germany
² INRES-Soil Science and Soil Ecology, University of Bonn, Germany
reichel@uni-trier.de, thiele@uni-trier.de

Introduction

Considerable amounts of antibiotics such as sulfonamides are administered to livestock. The majority of administered sulfonamides are excreted unchanged or as metabolite and finally reach the soil environment via manure, along with sulfonamide resistant microorganisms (Heuer et al. 2008). The broad-spectrum bacteriostatic action of sulfonamides such as sulfadiazine (SDZ) has been demonstrated to be more pronounced when microbial growth stimulating C-sources (e.g. glucose or manure) are introduced together with SDZ (Hammesfahr et al. 2008, Thiele-Bruhn & Beck 2005). The bioaccessible antibiotic concentration in bulk soil is determined by its sorption, sequestration, bound residue formation and (bio)degradation. However, the chemical, physical and consequently biological properties in proximity to plant roots are basically different from the un-rooted bulk soil; rhizosphere microbial communities differ significantly from those of bulk soil in higher activity, faster growth (Tate 2000) and altered structural diversity (Smalla et al. 2001). Several studies gave evidence that concentrations of organic contaminants dissipated or were degraded due to rhizosphere effects (Yoshitomi & Shann 2001, Chaudry et al. 2005). The effects of antibiotics on microbial community structures in rhizosphere soil as a hot-spot of soil microbial activity and redistribution of contaminants among environmental compartments are generally not well understood. We hypothesized, that compared to bulk soil, antibiotic effects are dissimilar in different soil micro-compartments and even more pronounced in soil affected by the presence of maize roots.

Material and methods

A controlled greenhouse mesocosm experiment was executed using topsoil (Luvisol), contaminated with SDZ by a single application of slurry from medicated pigs. The slurry was thoroughly mixed with soil in an agricultural relevant ratio of 1:25 (w/w) and pre-grown maize plants were transplanted. Composite bulk soil samples were collected on days 0, 6, 13, 27, 41 and 60. Rhizosphere soil samples were taken on days 13, 27, 41 and 60. The extracted maize plants were shaken vigorously to remove the bulk soil. Soil still adhering to the root was defined as rhizosphere soil (D’Costa et al. 2006). The roots were cut into pieces and mixed to get a composite rhizosphere soil sample. The easily extractable bioaccessible and residual fraction of SDZ was extracted and determined according to Förster et al. (2009). Quantitative effects on soil microbial community structure were analyzed using phospholipid fatty acid (PLFA) markers assigned to bacteria, gram-positive gram-negative bacteria and fungi. Lipids were extracted according to Zelles and Bai (1993) and gas chromatographically analyzed. Qualitative effects on community structures were analyzed using DNA from manure and soil samples extracted according to Heuer and Smalla (2007). Amplification of 16S rDNA gene fragments was done using the total community primers (Heuer et al. 1997), pseudomonas and ß-proteobacteria primers (Milling et al. 2005). The silver stained gels were analyzed and compared based on relative molecular weight calculations, which were derived from standard lanes. The band patterns were exported as binary data for further multivariate statistical analysis.

Results and discussion

The correspondence analysis of DGGE band profiles of control and SDZ treatments (Fig. 1) showed clear group separation. Influencing factors were: time related influences > manure treatment > bulk and rhizosphere affiliation. In contrast to the hypothesis, structural effects, likely caused by SDZ, seemed to be more pronounced in bulk than in rhizosphere soil. Total PLFA yields from rhizosphere soil were significantly higher compared to those from bulk soil, indicating the rhizosphere effect. Ratios of gram-positive to gram-negative PLFA markers (gram+/-gram-) are illustrated in Fig. 2. Compared to the control, the corresponding SDZ bulk soil showed a shifted gram+/-gram- ratio fluctuation during the time course. Hence, SDZ contamination possibly has changed population structures of gram-positive bacteria and especially may have influenced the balance between gram-positive and gram-negative bacteria populations. The bioaccessible SDZ concentrations (µg kg⁻¹ dm +/- STDEV) dissipated in bulk soil from 21.02 +/-1.5 at day 0 to 0.97 +/-0.26 at day 60. In the rhizosphere soil the concentrations declined from 1.83 +/-0.43 at day 13 to 0.22 +/-0.25 at day 60. It can be stated that antibiotic
effects on the total microbial community structure were much clearer in bulk soil compared to rhizosphere soil. This finding was mirrored by a stronger dissipation, and thus lower bioaccessibility of SDZ in the rhizosphere. While antibiotic effects were temporally significant in bulk soils, effects in rhizosphere could not be revealed, using endpoints such as total community DGGE or PLFA. However, antibiotic effects in rhizosphere soil were detected when investigated on specific community levels, e.g. pseudomonas. These results show that it is not sufficient to investigate homogenized soil material but the heterogeneity of soils must be considered in ecotoxicology testing, which reveals a diverse effectiveness of contaminants.

References
**Figure 1.** Correspondence analysis of total bacterial community 16S rDNA DGGE mesocosm profiles from rhizosphere soil (RS) of sulfadiazine (SDZ) and control (CTRL) treatments (4 replicates) at time points -1, 0, 6, 13, 27, 41 and 60 d. Samples not matching the general grouping are marked with small captions.

**Figure 2.** Gram-positive to gram-negative fatty acid ratios from bulk soil (BS) of control (CTRL) and sulfadiazine (SDZ) treatments (n = 4) at incubation times 0, 6, 13, 41 and 60 d. Results of rhizosphere soil (RS) are shown for the time points 13, 41 and 60 d. Lines point up bulk soils trends. The table below summarizes the significance (sig.: significant, ns: not significant) among antibiotic and control treatments (Kruskal-Wallis-Test at p < 0.05).
Evaluation of soil compaction effects on soil organisms and soil biological processes in soils

Berndt-Michael Wilke\textsuperscript{a}, Anneke Beylich\textsuperscript{b}, Hans-Rudolf Oberholzer\textsuperscript{c}, Stefan Schrader\textsuperscript{d}, Heinrich Höper\textsuperscript{e},

\textsuperscript{a} Berlin University of Technology, Institute of Ecology, Franklinstr. 29, D-10587 Berlin, Germany
\textsuperscript{b} IFAB Institut für Angewandte Bodenbiologie GmbH, Sodenkamp 59 D-22337 Hamburg, Germany
\textsuperscript{c} Reckenholz-Tänikon ART Research Station, Reckenholzstrasse 191, CH-8046 Zürich, Switzerland
\textsuperscript{d} Johann Heinrich von Thünen Institut, Bundesforschungsinstitut für Ländliche Räume, Wald und Fischerei, Institut für Biodiversität, Bundesallee 50, D-38116 Braunschweig, Germany
\textsuperscript{e} Landesamt für Bergbau, Energie und Geologie, Geozentrum Hannover, Stilleweg 2, D-30655 Hannover, Germany

E-mail contact: bmwilke@tu-berlin.de

1. Introduction

Soil compaction is a worldwide environmental problem of increasing importance occurring in arable and grassland as well as in forest soils. It is caused by the use of heavy machinery, but also by livestock trampling and human leisure activities. Generally, soil compaction affects soil physical properties by increasing soil bulk density, and hence reduces the volume and the connectivity of pores.

Up to now investigations have mainly focused on effects of soil compaction on soil physical parameters and on plant growth. Threshold values for soil physical properties have been proposed by soil physicists in order to identify deleterious effects of soil compaction on plant growth, crop yield as well as on the air and water regime of soils. So far, no such values were identified with respect to adverse affects of soil compaction on soil organisms and on soil biological processes. A general demand on threshold values for conservation purposes is crucial to guide policy makers in decision-making. Thus the scope of our study was to find out whether threshold values of soil structure parameters proposed by soil physicists correspond to harmful impacts on soil organisms and biological processes in soils.

2. Materials and methods

In order to get evidence on threshold values we screened in total 240 peer-reviewed papers in relevant scientific journals published in the years 1963 – 2007 for data on effects of soil compaction on soil organisms and soil biological processes. The results presented in these papers were compiled in a data base. In total, 640 data records on microorganisms and microbial activity and 332 data records on soil fauna were evaluated. In order to identify relationships between changes in physical and biological soil parameters and to derive threshold values, the whole data base was evaluated by regression analysis.

3. Results and discussion

Out of the overall 240 papers, however, only 54 were suitable for our purposes. The main criteria for the selection of data were:

- Data on the relevant physical soil parameters, especially (effective) bulk density and macropore volume, are presented or can be derived
- uncompacted control treatments are included
- original and raw data are given,
- effects of compaction can be distinguished from other effects (e.g., effects of different tillage systems)

3.1. Soil fauna

The soil zoological parameters investigated in field studies were mainly abundance and biomass, while laboratory experiments investigated predominantly burrow formation and cast production of earthworms in meso- and microcosms prepared with sieved soil compacted artificially. Considering all data, no clear relation between relative change of zoological parameter and increase of bulk density is observed.
Furthermore, a differentiation into field and laboratory studies gives no better relation ($r^2$ near 0.00). The threshold values proposed by soil physicists could neither be verified nor falsified by our analyses on soil fauna data. Apart from high variability of general soil properties and the soil physical parameters measured, possible reasons are (1) the assessment of different zoological parameters that might react differently to soil compaction and (2) various methods to induce soil compaction (e.g., livestock trampling or wheeling might have different effects on soil physical parameters due to kneading and shearing forces than artificial compaction of soil columns).

3.2. Soil microorganisms

In our data set, 29 data records originating from six scientific papers on C-mineralisation in field experiments or field studies were compiled. In spite of the important effects of compaction on physical soil properties (bulk density, air capacity), effects on C-mineralisation were very variable, with changes ranging from -47 to +51 %. About half of the cases exhibited a negative effect. The overall relation between the compaction-induced changes in C-mineralisation and bulk density resulted in a significantly positive regression, which means that C-mineralisation is more likely enhanced with increasing bulk density or with soil compaction, if compaction results in higher bulk densities.

For the evaluation of compaction effects on C-mineralisation in laboratory experiments, 68 data records from nine papers were available. In 11 cases the proposed threshold value for the effective bulk density (1.7 g cm$^{-3}$) and in 7 cases the threshold values for air capacity (5 and 7 Vol.%) was reached. The correlation coefficient ($r^2$) between changes in C-mineralisation (in % of control) and effective bulk density or bulk density was 0.32 or 0.17, respectively. All compaction treatments resulting in an effective bulk density of more than 1.70 g cm$^{-3}$ lead to a decrease of CO$_2$-production.

Compaction lead to similar effects on microbial biomass than on C-mineralisation: Whereas in field experiments compaction induced a rather slight increase of the biological parameters, in laboratory experiments a clear decrease was found. Above a compaction level indicated by an effective bulk density of more than 1.7 g cm$^{-3}$, all effects on microbial biomass were negative.

4. Conclusions

Negative and positive effects occurred with slight compaction as well as with strong compaction for soil zoological as well as for soil microbiological parameters. Apart from compaction itself, hydrology and oxygen levels were of major importance for adverse effects, especially for microbiological parameters. A verification of the thresholds for soil compaction published so far was not possible based on the data evaluated.

In order to provide a scientifically meaningful data base for the assessment of soil compaction effects on soil biodiversity, related functions and processes, we recommend considering the following abiotic parameters as essentials:

- Site properties (land use, climate, exposure)
- Soil properties (soil type, texture (clay, silt and sand fraction in %), (effective) bulk density, soil organic matter content, pH-value)
- Soil moisture (water content / water tension)
- Pore volume, macroporosity
- Air and/or water conductivity

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Biomarkers and energetic reserves in isopods: the effects of long-term exposure to dimethoate.

Nuno G. C. Ferreira, Miguel J. G. Santos, Rui Morgado, Amadeu M.V.M. Soares and Susana Loureiro

Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal
E-mail contact: nunoferreira@ua.pt

1. Introduction

The potential impact of a stressor in ecosystems requires the observation of effects at different levels of biological organization, starting at the molecular level and ending at the population/community level (Moore et al., 2004). At a molecular level, biomarkers have been use as efficient analysis tools due to their sensitivity, quickness and accurate relationship between toxicant exposure and respective biological response (Morgan et al., 1999). Depletion of energy reserves and energetic metabolic costs can be used as other organizational level paramater. Due to the stress induced by xenobiotics, metabolic changes can induce the depletion of energy reserves, resulting in negative effects on growth or even reproduction (de Coen 2003).

Isopods as macrodecomposers play an important role in the decomposition process, vegetal litter fragmentation and re-cycling process of nutrients (Ferreira et al. 2010, Zimmer et al., 2003; Loureiro et al., 2006). The use of the terrestrial isopod species Porcellionides pruinosus have been described as a good test-organism for soil contamination or changes in their habitat (Jansch et al., 2005; Loureiro et al., 2005; Loureiro et al., 2009).

Organophosphorous pesticides (OP) are one of the most extensively used pesticides, being applied in a great variety of agricultural produuts. OPs and carbamate pesticides are generally acutely toxic and exert potentially effects on non-target organisms (de Coen et al., 2003). One of the most used OP is dimethoate since successfully combines a selective toxicity to insects through a systemic action. It acts in the enzyme acetylcholinesterase (AChE), inhibiting the degradation of acetylcholine, which will produce extensive cholinergic stimulation and neurotoxicity (de Coen et al., 2003).

The main goal of this study was to evaluate the long-term effect of dimethoate to several enzymatic biomarkers and energy reserves in the terrestrial isopod Porcellionides pruinosus during a 28-day exposure period followed by a 14-day recovery period. For this purpose organism were exposed to two dimethoate concentrations and two different exposure temperature. The results obtained here will help to understand the process that undergoes the exposure and recovery of the tested organisms along with possible attempts to achieve a homeostasis status.

2. Materials and methods

Test organisms were obtained from culture box (25ºC, 16h:8h ligh:dark photoperiod), weighted (15-25mg) and checked for abnormalities; organisms moulting or pregnant females were discarded. Organisms were exposed to soil contaminated with dimethoate at 0.4 mg/kg soil (real case scenario) and 10 mg/kg soil (below EC50 value) and to two different temperatures: 20ºC and 25ºC with a 16:8 h (light:dark) photoperiod. A control per temperature was also included. Exposures were performed in plastic boxes, containing aprox 2cm of natural LUFA 2.2 soil layer (Speyer, Germany), with 5 replicates with 40 isopods each. Food consisting of alder leaf disks (Ø 10 mm, ± 20 mg) were supplied during exposure. After 28 days of exposure, isopods were changed to clean soil and recovery was assessed.

Four organism from each box were collected at time 0h, 24h, 48h, 96h, 7-days, 14 -days, 21-days, 28-days (exposure period) and 35-days, 42-days (pos-exposure period).

The biomarkers glutathione S-transferases (GST), glutathione peroxidase (GPx), catalase (CAT) and lipid peroxidation (LPO) were measured in a pool of two full-body organisms as each replicate. Another organism was divided into head and body to test acetylcholinesterase (AChE) and lactate dehydrogenase (LDH), respectively, each part corresponding also to a replicate. A total of five replicates was obtained as final measurement of each biomarkers.

For the energy reserves (lipids, carbohydrates and proteins) and electron transport system, one organism was used. Each organism corresponded to a replicate, in a total of five replicates for each measurement.
Mortality was also reported at the same periods of time where organisms were collected.

3. Results and discussion

3.1. Biomarkers

As expected a strong inhibition was observed in acetylcholinesterase as the main target of the pesticide. Although a previous study had stated that organisms have low survival chances under neurotoxic regimes i.e. where AChE inhibition is higher than 80%, we observed a 7 day survival period with AChE inhibitions higher than 90% (Ludque et al. 1975).

Other biomarkers as for example lipid peroxidation presented significant differences when compared with the control at periods where high mortality rates were counted.

3.2. Energy Reserves and Cellular Energy Allocation (CEA)

Energy reserves content, energy consumption and cellular energy allocation rate significantly fluctuated along time exposure and recovery.

4. Conclusions

The results show not only correlations between several biomarkers, the energy reserves and mortality, but also with other detoxification processes not related with neurotoxicity. The present work showed that several sub-individual biomarker activity can be used as early warnings for Environmental Risk Assessment, and that sub-individual effects can be linked with ecologically relevant parameters (e.g. mortality and energy reserves content)

5. References


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Development of a new plant-based biotest to assess trace element phytoavailability in contaminated soils: Selection of target-plant species for standardisation

Laure Lemal¹, Matthieu N. Bravin², Philippe Hinsinger¹ and Emmanuel Doelsch²

¹ INRA, UMR 1222 Eco&Sols (INRA-IRD-SupAgro), Place Viala, F-34060 Montpellier, France
² CIRAD, UPR Recyclage et risque, F-34398 Montpellier, France
E-mail contact: matthieu.bravin@cirad.fr

1. Introduction

While the concept of contaminant bioavailability in soil has been recently defined in the international standard ISO/DIS 17402 [1], its application at an operational level still requires the identification, the development and the standardisation of a set of tools targeted to various organisms (e.g. plants, soil microorganisms and fauna, Human beings…). This is especially a concern for the assessment of trace element (TE) phytoavailability in contaminated soils as TE remain a major contaminant in European soils and higher plants are of primary interest for their role in human food and animal feed.

Phytoavailability of TE can be estimated with either chemical or biological methods. While chemical methods are usually the cheapest, are easy to perform and some of them are already standardised at an international level, chemical methods strictly measure TE availability in soils and thus have to be correlated with biological measurements before to be used as phytoavailability indicators. In addition, chemical methods are per se not designed to address the diversity of responses observed among different plant species or cultivars. Alternatively, a few biological methods are already standardised at an international level. However, biological methods were mainly designed to assess TE phytotoxicity while bioaccumulation remains a sound issue for a range of TE. The determination of TE accumulation in shoots is usually not sufficiently sensitive to assess TE phytoavailability compared to the amount accumulated in the whole plant (roots included). Moreover, the biological methods which are based on soil-grown plants require a tedious washing procedure to reliably measure TE accumulated in the roots. Thus, there is still a need to develop biological methods in order to properly assess TE phytoavailability, particularly in term of bioaccumulation.

Accordingly, the ongoing NormaRHIZO research project was designed to provide a strong scientific input in the development of a new plant-based biotest, the RHIZOtest, in the scope of standardisation [2]. The RHIZOtest is notably based on a complete physical separation between plant and soil compartments enabling an easy, fast and clean recovery of the roots. The present abstract introduces the first step of the project focused on the selection of the target-plant species suggested for the standardisation of the RHIZOtest.

2. Materials and methods

Three agricultural soils exhibiting a fairly large range of pH and a high concentration in several trace elements (TE) (Table 1) were selected for this experiment. Concentrations of TE in soils were due to anthropogenic activities for soils 1 and 3, while high concentrations in soil 2 were attributed to the natural pedgeochemical background.

<table>
<thead>
<tr>
<th>pHCaCl₂</th>
<th>Total Cd</th>
<th>Total Pb</th>
<th>Total Zn</th>
<th>Origin of trace element concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil 1</td>
<td>5.3</td>
<td>4.5</td>
<td>141</td>
<td>563 Steel industry</td>
</tr>
<tr>
<td>Soil 2</td>
<td>5.9</td>
<td>5.6</td>
<td>172</td>
<td>1408 Pedogenic background</td>
</tr>
<tr>
<td>Soil 3</td>
<td>7.1</td>
<td>1.7</td>
<td>163</td>
<td>432 Waste water spreading for decades</td>
</tr>
</tbody>
</table>

Table 1: Soil characteristics (TE, trace elements)

Ten plant species commonly cropped over the world and likely exhibiting distinct behaviours of TE uptake were tested: alfalfa (Medicago sativa), barley ( Hordeum vulgare), bread wheat (Triticum aestivum), cabbage (Brassica oleracea), fescue ( Festuca arundinacea), lettuce (Lactuca sativa), rape (Brassica napus), ryegrass ( Lolium perene), sorghum (Sorghum bicolore), tomato (Lycopersicon esculentum).
The RHIZOtest was deployed for each plant species and soil according to Bravin et al. [2]. Briefly, plants were first grown (“preculture period”) from seeds for two weeks in hydroponics in a cylinder closed at the bottom with 30-µm polyamide mesh to favour the development of a planar mat of roots. Plants were then firmly pressed for 8 days on the top of a thin soil layer (“test culture period”).

Plants were harvested at the end of test culture period, digested and TE concentration (arsenic, cadmium, copper, lead and zinc) were determined in plant shoots and roots. Phytoavailability of each TE was then calculated as the flux of TE to the plants during the test culture period, according to Bravin et al. [2].

3. Results and discussion

Excepted alfalfa, cabbage and wheat, plant species exhibited an adequate and homogeneous growth for both roots and shoots for the three soils. This means that for seven out of ten plant species the RHIZOtest enables a fair and unbiased assessment of trace element (TE) phytoavailability between soils.

As expected, plant uptake flux of TE significantly varied among the ten plant species tested. For example, Pb and Zn uptake flux varied respectively by 3- and 4-fold among the ten plant species cropped on the soil 3 (Figure 1). This confirms the need to account for a broad range of diversity in the ability of plant species to uptake TE. However, TE phytoavailability also broadly varied among TE (Figure 1) and soils.

![Figure 1: Lead and zinc uptake flux of in the ten plant species cropped on the soil 3 (see Table 1).](image)

In order to classify the ability of the ten plant species to assess a rather low or high TE phytoavailability, TE uptake flux measured for each combination (plant species/TE/soil) was transformed in semi-quantitative variable by ordination and scoring. Scores were added to give a global classification of plant species. According to the precautionary-like principle, we selected the three plant species (barley, rape and fescue) from which we estimated the highest TE phytoavailability among the three soils and the five TE investigated.

4. Conclusions

This study supports the requirement of biological methods that enable to encompass the biological diversity in the assessment of trace element (TE) phytoavailability that chemical methods are not able to take into account. Further development of the RHIZOtest will be to validate the scope of the method in term of physical-chemical properties and level of contamination of soils for the three selected species. Such kind of validation procedure for a biotest is the unique opportunity for achieving operational methods based on a hard scientific background that could be standardised for the assessment of TE phytoavailability.

5. References


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