

## 3<sup>rd</sup> NORMAN workshop

# New tools for bio-monitoring of emerging pollutants

Organised by

FP6 project NORMAN

IVM – Institute for Environmental Studies

VU University, Amsterdam

The Netherlands

29 to 30 October 2007

VU University

Conference room: Atrium

Amsterdam, The Netherlands

# Monday October 29

8:00	Registration in Atrium, VU University	
9:00	Welcome and Opening Remarks Pim Leonards	
9:10	Valeria Dulio	Overview of the NORMAN project
9:25	Keynote Richard Owen & Tamara Galloway	Biomonitoring tools for risk assessment of emerging pollutants - opportunities and challenges
10:15	— Coffee break	

## Session 1: Current approaches to biomonitoring in the field

Chair: Timo Hamers

10:45	Ron van der Oost	A bioassay-directed monitoring strategy to assess the risks of complex pollutant mixtures in drinking water
11:15	Minne Heringa	Measurement of genotoxicity in (drinking) water
11:45	Sander van der Linden	Profiling steroid receptor activity in effluents and surface water samples using CALUX
12:15	— Lunch	
13:30	Claudia Schmitt	Chronic biotests with <i>Potamopyrgus antipodarum</i> - Suitable tools for the detection of endocrine disruption in aquatic ecosystems
14:00	Bjørn Jenssen	Monitoring ecological significant effects of old and new persistent organic pollutants in protected top predators in the Arctic

## Session 2: Modern approaches for development of biomonitoring tools

Chair: Juliette Legler

14:30	Sabeth Verpoorte	The potential of lab-on-a-chip technologies for environmental biomonitoring
15:00	Karlijn van der Ven	A toxicogenomics approach to biomarker development in aquatic toxicology
15:30	— Coffee break	
16:00	Anders Goksøyr	A proteomics strategy for protein expression profiling and biomarker discovery in wildlife: effects of endocrine disrupting chemicals in frog ( <i>X. laevis</i> )
16:30	Juliette Legler	The pro's and con's of using genomics to assess emerging chemicals

# Tuesday October 30

## Session 3: Biomonitoring: from lab to field

Chair: Pim Leonards

9:00	Dick Roelofs	Soil invertebrates as a genomic model to study pollutants in the field
9:30	Kevin Chipman	Genomic stress responses to chemicals in the European flounder and identification of gene-sets predictive of origin of fish from the environment
10:00	Ludek Bláha	"From field to lab"and from "lab to field": chemical analyses, in vitro and in vivo bioassays in the study of endocrine disruptive effects observed in situ
10:30	—	Coffee break
11:00	Karen Kidd	Results from a whole lake experiment: Is the birth control pill an effective form of contraception for fishes?
11:30	Tjalling Jager	From molecules to populations: the causality of toxic effects
12:00	—	Lunch

## Session 4: Identification and measurement of emerging pollutants

Chair: Marja Lamoree

13:15	Marinella Farré	Monitoring of triclosan in waste waters by a high-throughput magnetic-particle immunoassay, confirmatory analysis by gas chromatography/mass spectrometry, and acute toxicity assessment
13:45	Kevin Thomas	Effects directed identification of emerging substances
14:15	Jana Weiss	Toxicity profiling in European river sediments with emphasis on the identification of thyroid hormone disrupting compounds
14:45	Jan Balaam	Identifying toxic emerging pollutants in European rivers and estuaries
15:15	<a href="#">Wrap-up</a>	
15:30	<a href="#">Closing</a>	

# Abstracts

## **Biomonitoring tools for risk assessment of emerging pollutants - opportunities and challenges**

Richard Owen<sup>1</sup> and Tamara Galloway<sup>2</sup>

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Biomonitoring tools (e.g. bioassays, biomarkers, community analyses) have enormous potential for reducing uncertainty in the risk assessment of both regulated and emerging chemical pollutants. Reducing uncertainty in risk assessment can support and justify the targeting of chemicals monitoring activity on a regional basis to those areas validated as being at high risk with high certainty. Conversely, increased confidence in risk assessments is critical for advocating the reduction of monitoring activity in areas designated as being at low risk (and thereby delivery of long term financial savings through a targeted, risk – based monitoring approach). The potential for use of biomonitoring tools such as biomarkers and bioassays to reduce uncertainty in chemicals risk assessments under legislative frameworks such as the Water Framework Directive has however yet to be fully realised.

We outline an integrated approach for incorporation of biomonitoring tools within the Water Framework Directive to reduce uncertainty in risk assessment of hazardous substances, both known and unknown. Data are presented for two field studies in which this approach is trialled, highlighting both the potential power of the approach and key challenges that the scientific and policy communities will need to address before it can be fully implemented.

## **A bioassay-directed monitoring strategy to assess the risks of complex pollutant mixtures in drinking water**

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**At present, the quality assessment of drinking water is mainly based upon the determination of water pollutant levels, and upon comparison of these levels with legislative threshold values. However, there is a growing awareness that focusing on chemical data alone is insufficient to assess the potential risks of all emerging pollutants. There is an increasing awareness that pollution-induced biological and biochemical effect-analyses are needed to evaluate or predict the impact of chemicals on human health. In this presentation an overview will be presented of the potential effect parameters that may be relevant for monitoring at different stages of the drinking water production process. In general, polar organic compounds will pose the highest threat for the drinking water quality since these substances may be able to pass the active carbon purification step. *In vivo* bioassays are already applied as biological alarm systems at the inlet of surface water used for the drinking water production. Emphasis in this overview will therefore be placed upon the use of *in vitro* assays as monitoring tools for the assessment of human health risks due to pollutants in the water, from source to tap. Potential biomarkers for the risk assessment of toxic substances in drinking water could be those indicative of genotoxic, carcinogenic, endocrine disruptive, immunotoxic, neurotoxic, reproductive or teratogenic effects. Potential bioassays for these effects will be presented. Moreover, detoxification biomarkers and potential risks of pharmaceuticals, such as the development of resistant bacteria due to antibiotics, are also of interest. Available methods to analyze these effects will be discussed. Future research will show which effects are most important for the quality assessment of drinking water. The relevance of endocrine disruption and genotoxicity have been demonstrated. Examples of the quality assessment of drinking water (from source to tap) using *in vitro* assays on endocrine disruption (ER CALUX) will be given. Low levels of estrogenic activity could be measured after various steps of the purification process, but no health risks were identified. In addition, a design to establish threshold values based upon the acceptable daily intake (ADI) of reference substances will be proposed, in order to use data of *in vitro* assays for the assessments of human health risks.**

## Measurement of genotoxicity in (drinking) water

Minne B. Heringa<sup>1</sup>, Stefan Voost<sup>1</sup>, Bart Wullings<sup>1</sup>, Evgeni Eltzov<sup>2</sup>, Robert Marks<sup>2</sup>  
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Dutch drinking water and its sources are continuously screened for the presence of contaminants to safeguard its good quality and safety. The present chemical analysis needs a complementary toxicity analysis to enable detection of unknown hazardous contaminants and to measure the total effect of the mixture. Because of the relatively low levels of contaminants in these waters, low-dose toxic effects, such as genotoxicity, are most relevant.

It is aimed to set up a system to test water samples for genotoxicity, comprising both a mutagenicity and a chromosome aberration test. This system should be as fast, sensitive and cheap as possible, and enable the analysis of fractionated water samples for contaminant identification. An overview will be given of the many available *in vitro* test systems for genotoxicity and why we chose to use the Ames II assay in combination with either the Comet or the Micronucleus assay. We adapted the Ames II assay, a liquid culture version of the classic Ames test that can be performed in multi-well plates, to make it more suitable for analysis of water samples:

- developed a method to check for cytotoxicity along with the Ames test and
- set up a new statistical evaluation of the detection limit

First results of the validation of the test with wastewater, surface water, groundwater and drinking water samples will be presented. Additionally, we have studied how water samples are best extracted and prepared for toxicity analysis as well as chemical analysis. In case of positive samples, we have our ToxPrint fractionation method to ease identification of the responsible compound.

A completely new way to analyze water for genotoxicity is by applying genetically modified bacteria that luminesce after exposure to genotoxic compounds. We will describe our current work in developing an on-line sensor with these bacteria to screen water for genotoxic compounds a.o.

## **Profiling steroid receptor activity in effluents and surface water samples using CALUX**

Sander van der Linden<sup>1</sup>, Minne Heringa<sup>2</sup>, Hai-yen Man, Leo Puijker<sup>2</sup>, Edwin Sonneveld<sup>1</sup>, Bram Brouwer<sup>1</sup> and Bart van der Burg<sup>1</sup>.

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It is generally known that there are compounds present in the aquatic environment that can disturb endocrine processes. Although disruption of the endocrine process can be caused by different processes, important targets are the endogenous nuclear hormone receptors. Most research regarding the hormone receptor pathways focuses on compounds that bind to the estrogen and androgen receptor, but ligands for other hormone receptors may also be present in the aquatic environment, including the natural hormones, pharmaceuticals and industrial chemicals targeting these hormone receptors.

Since all compound targeting the same receptor can act in concert, even if present in very low concentrations, biological detection systems are important in determining the total biological activity for specific receptors in the aquatic environment. These bioassays have to be very specific, in order to discriminate between activities on the different endocrine receptors.

Recently, we expanded our existing panel of CALUX cell reporter gene assays, consisting of estrogen and androgens receptor assays, with assays responding to progestins, glucocorticoids and thyroid hormones. This panel was utilized to test different water extracts for hormone receptor activity. The sample preparation of water samples for analysis in toxicity tests is an important determinant for the outcome of these tests. Therefore, a comparison was made between solid phase extraction (SPE) and liquid-liquid extraction (LLE). The waters tested were municipal sewage treatment plant effluent, industrial effluent, raw hospital effluent, tap water and surface water. The results of these experiments will be presented.

## **Chronic biotests with *Potamopyrgus antipodarum* – Suitable tools for the detection of endocrine disruption in aquatic ecosystems?**

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Within the last years, the research on endocrine disruption in invertebrates increased and nowadays there are several examples reported in the literature. Especially prosobranch snails have been recommended as promising candidates regarding the *in vivo* assessment of endocrine active chemicals, because they are highly sensitive towards xeno-androgens (triphenyltin, tributyltin, methyltestosterone and fenarimol) and xeno-estrogens (bisphenol A, octylphenol, ethinylestradiol). The exposure of parthenogenic females of the “New Zealand mudsnail” *Potamopyrgus antipodarum* to the xeno-androgens Triphenyltin (TPT) and Tributyltin (TBT) resulted in a significant decrease in embryo production whereby EC<sub>10</sub> values of 30 ng TPT-Sn/kg and 37.8 ng TBT (as Sn)/L were determined. The exposure to the well known xeno-estrogens Octylphenyl (OP) and Bisphenol A (BPA) resulted in a significant increase in reproduction and EC<sub>10</sub> values of 4 ng OP/kg and 0.19 µg BPA/kg were calculated. Also, biological effect monitoring studies with *P. antipodarum* in several rivers or estuarine areas revealed the capacity of this biotest to detect an androgenic or estrogenic potential of sediment samples. Within the project MODELKEY, polluted and reference sampling sites from three European river basins (Scheldt, Elbe and Llobregat) were selected, based on available data on sediment and water contamination. An *ex situ* approach with *P. antipodarum* showed that half of the polluted sediments led to a significantly increased reproduction compared to their corresponding reference sites. Reasons for that could not be explained with the different physico-chemical characteristics of the tested sediments, thus chemical pollution could be assumed as being the main responsible factor. The main goal is now to interpret and explain those findings while bridging the gap between *ex situ*, *in situ* and effect directed analyses (EDA).

# **Monitoring ecological significant effects of old and new persistent organic pollutants in protected top predators in the Arctic**

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For Arctic top predators there are concerns that levels of persistent organic pollutants (POPs) are above threshold levels for biological effects. In general populations of Arctic top predators are low and they are protected. Thus, protection and ethics makes it very difficult to conduct invasive and destructive experiments and samplings to study effects of POPs in these species. One approach for monitoring effects of POPs in Arctic predators involves non-destructive sampling of tissues, blood or eggs, and relating indicators of biological responses (biomarkers) to contaminant levels. Due to logistic matters, it is often not possible obtain to non-destructive samples of tissues that can be frozen on liquid nitrogen for analyses of gene and protein expression. Thus, the most common approach has been to use only blood samples or eggs, or a combination of blubber biopsies and blood samples, and to identify associations between and biomarker levels and exposure. In Arctic top predators associations between POPs and biomarkers such as thyroid hormones, vitamin A, testosterone, estradiol and immunological variables have been identified. The factor that probably is the most limiting for a current use of the biomarker approach to identify and monitor effects of POPs in Arctic top predators, is the lack of information on reference values for biomarkers in these species. Such uncertainty makes it difficult to interpret if biomarker levels in exposed animals are within natural limits, or if the animals are clinically affected. However, by standardizing sampling with respect to species, age and season, by identifying reference values for biomarkers that are relevant for reproduction and health, and by determining to which extent these biomarkers are influenced by other environmental factors; it should be possible to perform long-term monitoring of effects of POPs in Arctic top predators.

# **The potential of lab-on-a-chip technologies for environmental biomonitoring**

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Microfluidic or lab-on-a-chip (LoaC) technologies represent a paradigm shift in ultra-small-volume liquid handling, by integrating sample processing into planar handheld devices containing networks of micrometer-dimensioned channels with lengths of micrometers to meters. Overall system volumes are on the order of nL, with the capability of pL sample analysis. During operation, fluids are shuttled from one region of the network to the other to undergo different processing steps. For high-throughput applications, arrays of parallel channels or other structures are possible. These systems offer unique advantages for automated, hands-off sample handling, enhanced speed of analysis, and portability. They are thus very promising for the development of single analytical modules incorporating all the necessary steps for real-time monitoring purposes in a variety of different contexts, from hospital to environment.

This presentation will consider a few examples of the use of lab-chip technologies for environmental biomonitoring. Some of our own work in the mid-90s focused on the colorimetric detection of ammonia, using the Berthelot reaction to convert ammonia to a coloured product. The reaction between ammonia and reagents was carried out in a microfluidic system primarily to reduce the reagents required, with an eye to developing a system which could operate autonomously for months at a time. More recent examples both from our lab and others involve automated nucleic acid analysis in nL volumes for high-speed identification of pathogenic species. The potential of lab-chip systems for these types of applications will be explored in this presentation.

# **A toxicogenomics approach to biomarker development in aquatic toxicology**

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Every toxic compound exerts its initial effect by interacting with its primary lesion (binding to molecule, receptor, transporter), leading to a chain of events orchestrated by the cell to maintain its homeostasis and to ensure its survival. By measuring toxicological effects at the molecular level, better insight into the mode of action of a chemical can be achieved, which can eventually lead to improved risk assessment of this chemical. Thanks to the rapid progress in the development of molecular biological techniques, tools have been developed that may increase our understanding of how chemicals can have an impact on the environment. Moreover, these new methodologies enable environmental toxicologists to monitor gene expression changes resulting from toxicant exposure even in species whose genome is still poorly characterized.

In our laboratory, a range of gene expression analysis techniques - DNA microarrays, Reporter assays, Real-time PCR – and proteomics is applied to investigate the impact of anthropogenic compounds in the environment. The compounds studied include pharmaceuticals, endocrine disrupters and industrial (high-production-volume) chemicals. Gene expression profiles and signatures are used not only for mode of action studies but also for classification of chemicals and risk assessment. In this presentation examples are given how this “toxicogenomics” approach is used to elucidate molecular modes of action of single compounds and to unravel modes of action of whole effluent toxicity and impact assessment. Our results demonstrate the benefit of toxicogenomic tools in a “systems toxicology” approach, involving the integration of adverse effects of chemicals and stressors across multiple levels of biological organisation.

# ***A proteomics strategy for protein expression profiling and biomarker discovery in wildlife: effects of endocrine disrupting chemicals in frog (*X. laevis*)***

**Anders Goksøyr<sup>1,2</sup>, Christina C Tølfesen<sup>1</sup>, Anneli Bohne Kjersem<sup>1</sup>, Tina Søfteland, Torbjørn Midtun<sup>1</sup>, Ralph Urbatzka, Werner Kloas<sup>3</sup>, Bjørn Einar Grøsvik<sup>1,4</sup>**

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Environmental contaminants may affect endocrine functions in many wildlife species and negatively impact reproduction and development by mimicking the action of natural hormones. It is also suggested to be one of the factors contributing to the worldwide decline of amphibians. To be able to assess the effects of chemical induced endocrine disruption and its impact on wildlife species there is a need to develop suitable monitoring tools. We have used a proteomics strategy based on two-dimensional gel electrophoresis (2-DE) and mass spectrometry (MS) to identify novel biomarkers for endocrine disrupting compounds (EDC) in frog and various fish species. In controlled aquaria exposures African clawed frog (*Xenopus laevis*) was treated with selected model compounds: 17  $\alpha$ -ethynylestradiol (EE2), tamoxifen (TAM), 17  $\alpha$ -methylidihydrotestosterone (MDHT), flutamide (FLU), and with water from Lambro, a polluted tributary to the River Po (Italy). Plasma and liver samples were analyzed by two-dimensional electrophoresis (2-DE) and Delta 2D image analysis software, and differentially regulated proteins were identified by mass spectrometry based methods (MALDI-TOF and LC-MS/MS) and subsequent Mascot searches in the NCBIInr database.

The most marked effect of EDC exposure in frog was seen in the protein pattern of plasma from EE2 treated animals. Plasma from the remaining treatment groups showed a protein pattern very similar to that of control with no clear EDC induced alterations. In liver, altered protein expression was observed in response to all test compounds used. In a partial mapping of the *Xenopus laevis* plasma proteome, a total of 179 proteins were analyzed by MS. Of these, 123 were successfully identified, 55 by MALDI-TOF and 68 by LC-MS/MS. Multiple protein identities were sometimes obtained from the same protein spot. The majority of the identified protein spots represent more abundant plasma proteins belonging to 19 different protein families. Differential expression analysis identified the egg-yolk protein vitellogenin (Vtg), an established biomarker for estrogen exposure, and the serpin like protein Ep45, as being up-regulated by EE2 exposure. Plasma proteins showing reduced levels were abundant plasma proteins, among them albumin, serotransferrin and fibrinogen. In the *X. laevis* liver proteome, MALDI-TOF MS and subsequent Mascot searches in the NCBIInr database successfully identified 193 out of 241 analyzed protein spots yielding a success rate for identification of 80 %. 131 of the 193 proteins were found to represent unique protein spots, 22 of which were found to contain more than one protein. Collectively EE2, TAM, MDHT, FLU and LAM affected 86 % of the 501 protein spots detected in the *X. laevis* liver proteome. 106 of the differentially

regulated proteins represented identified proteins with known function; among these were catalase, carbamoyl-phosphate synthase I and proteins of the aldehyde dehydrogenase, heat shock protein 70 and protein disulfide isomerase families. Used either singly or in combination, these proteins may be interesting as putative biomarker candidates for EDC with (anti)estrogenic and (anti)androgenic activity.

Similar studies with carp (*Cyprinus carpio*) and Atlantic cod (*Gadus morhua*) exposed to various contaminants and effluents have yielded similar results, pointing at interesting biomarker candidates, although identification success rate has been much less (generally around 50%).

In application of proteomics to ecotoxicology the lack of sequence information on non-model organisms pose a challenge to protein identification. E.g. for blue mussel (*Mytilus edulis*) and cod (*Gadus morhua*) there are at present no more than 874 and 1585 protein sequences in the NCBI database. For organisms such as zebrafish (*Danio rerio*) however, where about two thirds of the genome have been sequenced (see [http://www.sanger.ac.uk/Projects/D\\_rerio/](http://www.sanger.ac.uk/Projects/D_rerio/)), more than 55869 sequences exist. An important aspect in proteome analysis projects, and also in biomarker discovery, is to link identified proteins to molecular functions, biological processes and to determine subcellular locations as well as involvement in intracellular pathways using information on gene ontology (GO), and pathway tools/databases such as the Kyoto Encyclopedia of Genes and Genomes (KEGG). However, for organisms other than the classical model organisms such as mice and rat, most of the sequences in the databases also have a low level of annotation. In addition many of the proteins in the databases represent hypothetical proteins of unknown identity and function.

Although they are complementary techniques, proteomics, contrary to DNA microarray technology, also permit differential expression to be studied in tissues that are not transcriptionally active. In this study most attention was paid to the estrogenic responses induced by EE2, and several proteins were found to be differentially regulated. Compound specificity is vital to the implementation of these responses as biomarkers for EDC with estrogenic activity. Although receptor cross-talk may be expected to complicate biomarker development, diagnostic efficacy may be improved using a multimarker approach. Studies with other estrogen agonists such as E2, DES, 4-nonylphenol or bisphenol A may help elucidate modes of action (MOA) that are common to estrogens. Equally important is the study of EDCs with other MOA i.e. androgenic, anti-androgenic, anti-estrogenic as well as those acting through other non-traditional nuclear receptor pathways. Such studies may not only provide information on biomarker candidates towards these specific compounds, but may also help answer which proteins represent a common stress response and hence which responses are not specific for estrogen and estrogen mimics.

In a future validation of biomarker candidates more quantitative methods should be employed to assess intra- and inter-individual variation, time and dose-response. The focus should be on reproducibility and sensitivity. Specific antibodies are currently being tested to assess such relationships. In this context both standard quantitative ELISA assays as well as novel protein array technology should be explored as formats for high-throughput standardized screening. The advance in protein array technology and antibody-based proteomics opens new avenues to large scale protein expression profiling and in elucidating protein function and networks of interacting proteins.

The studies being reported was supported by EC grant QLK4-2002-02286 'EASYRING' (FP5), Total E&P, and the Norwegian Research Council.

## **The pro's and con's of using genomics to assess emerging chemicals**

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Genomics, in particular transcriptomics, enables the global analysis of the expression of multiple (sets of) genes involved in cellular responses. A genomics-based approach has been successful in a number of fields, such as drug discovery, where genomics is used to screen candidate drugs for specific mechanisms of action, or in medical sciences, where genomics has been pivotal in revealing mechanisms and markers of complex diseases. In the application of genomics in the field of toxicology (toxicogenomics), it has been established that “almost without exception, gene expression is altered during toxicity, as either a direct or indirect result of a toxicant exposure” (Nuwaysir et al., 1999, *Mol Carcinog.* 24(3):153-9). In theory, the measurement of gene expression levels upon exposure to a chemical can provide both information about the mechanisms of action of chemicals, and a “genetic signature” from the resulting pattern of gene expression. The question is: has genomics actually delivered all it has promised when it comes to assessing emerging chemicals in the environment? Can we indeed use genomics to unravel mechanisms of action? Can we distinguish gene signatures of toxicants in complex mixtures and environmental samples? And is the high cost involved in performing microarrays justified in terms of providing us with useful and usable new information? In my presentation I will attempt to provide a balanced overview of the advantages and disadvantages of this exciting new technology and its application in the field of environmental toxicology.

## Soil invertebrates as a genomic model to study pollutants in the field

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Stress is a major component of natural selection in soil ecosystems. The most prominent abiotic stress factors in the field are temperature extremes (heat, cold), dehydration (drought), high salinity and specific toxic compounds such as heavy metals. Organisms are able to deal with these stresses to a certain extent, which determines the limits of their ecological amplitudes. Functional genomic tools are now becoming available to study stress in ecologically relevant soil organisms. We specifically developed genomic tools to study transcriptional responses and adaptation to stress in the Collembolans *Orchesella cincta* and *Folsomia candida*. A microarray was developed for *F. candida* that contains more than 5.000 probes representing unique cDNA expressed sequence tags. This chip was used to study transcription responses to chemical and physical stresses in soils. For instance, 1.000 genes were differentially up-or down-regulated due to cadmium exposure, and more than 100 genes due to mild heat shock. General response pathways, such as the heat shock response, were identified in both stress treatments. Also, specific stress response genes related to Cd toxicity were up-regulated, e.g. glutathione S-transferases, UDP-glucuronosyltransferases, and membrane transporters (e.g. ABC-transporters). These genome-wide expression patterns not only give more insight in the molecular mechanism of stress responses, but may also be used as diagnostic tool to assess the biological quality of soils.

# **Genomic stress responses to chemicals in the European flounder and identification of gene-sets predictive of origin of fish from the environment**

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Genomic technologies enable a global assessment of the health status of an organism through an understanding of the functional pathways that are responding to pollutant exposure and have the potential to discover new biomarkers.

Flounder taken from different sites in Northern Europe (and of different pollution status) can be distinguished according to their hepatic gene expression profile using microarray and associated bioinformatic approaches. This demonstrates that the transcriptional state of liver is informative of the complex set of influences that characterize gene expression at different sites.

“Stress genes” that respond to a range of chemical exposures have also been identified using the same technologies after treatment of flounder under laboratory conditions. Gene expression in liver tissue at different times after chemical exposures was compared to the relevant time-matched control groups to generate lists of genes whose expression was altered. As well as providing potential novel individual biomarkers, gene ontology (GO) analyses using Blast2GO allowed the identification of pathways modulated by toxicant stress; for example chaperones, protein synthesis and degradation, cytoskeleton, apoptosis and cell cycle pathways were modulated by cadmium.

Using a multivariate variable selection coupled with a statistical modeling procedure (GALGO). We demonstrate that signatures associated with exposure to individual chemicals can predict geographical site but that the accuracy is limited to specific sites. However, by combining the signatures derived from laboratory exposure to individual chemicals very accurate models for classification of all the different environmental sites was achieved. The findings demonstrate that the expression of stress genes contributes to differences in fish at different environmental sites and that the application of machine learning techniques to biomarker discovery can greatly enhance our ability to understand the complex patterns of response to environmental exposure.

This work was funded by the NERC, CEFAS and EU.

## **"From field to lab" and "from lab to field": chemical analyses, *in vitro* and *in vivo* bioassays in the study of endocrine disruptive effects observed *in situ***

Ludek Bláha<sup>1</sup>, Klara Hilscherová<sup>1</sup>, Edita Mazurova<sup>1</sup>, John P. Giesy<sup>2</sup>, Rita Triebkorn<sup>3</sup>,  
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There is an increasing concern about ecotoxicological consequences of endocrine disruptive compounds accumulated in freshwater sediments. In this contribution we report results from a complex integrative assessment of contaminated pond sediments from the Ostrava-Karviná industrial region, Czech Republic, which harbor an unusual intersexual population of the narrow-clawed crayfish *Pontastacus leptodactylus* (Decapoda, Crustacea).

With the aim to understand possible causes of endocrine disruptive effects at these localities, we have combined chemical analyses (series of toxic metals, PAHs, PCBs, OCPs etc.) with a set of *in vitro* bioassays (arylhydrocarbon receptor-, estrogen receptor- and androgen receptor- mediated effects, effects on steroidogenesis) as well as laboratory *in vivo* experiments with two species of invertebrate organisms (aquatic amphipod *Gammarus fossarum* and prosobranchian snail *Potamopyrgus antipodarum*).

Chemical analyses revealed high concentrations of polycyclic aromatic hydrocarbons in sediments, indicating that these compounds along with their derivatives significantly contribute to the observed high arylhydrocarbon receptor (AhR-) mediated activities determined with *in vitro* luciferase assay using H4IIE.luc cell line. Treatment of the sediment extracts with sulphuric acid completely diminished the AhR-mediated activity, which corresponded with the low concentrations of the measured persistent organic pollutants (polychlorinated biphenyls and organochlorine pesticides - PCBs and OCPs) and also documented that polychlorinated dibenzo-p-dioxins or furans did not significantly contribute to the high overall AhR-mediated activity. Extracts of sediments from the contaminated sites also caused significant estrogenic and antiandrogenic responses in the recombinant reporter mammalian and yeast bioassays. Sediment extracts significantly modulated expressions of several genes involved in steroidogenesis. These effects were determined by quantitative real time PCR assay with a novel *in vitro* bioassay with human H295R cell line. The most pronounced effects were up to 10-fold inductions of the CYP11B2 gene, and suppressed expressions of 3 $\beta$ HSD2 and CYP21 genes. Endocrine disruptive potencies determined with *in vitro* techniques were confirmed also with long-term *in vivo* ecotoxicological assays. Multiple reproduction parameters of *G. fossarum* as well as *P. antipodarum* were affected in laboratory exposures to contaminated sediments (and/or their extracts).

In summary, combinations of chemical analyses and multiple *in vitro* and *in vivo* techniques allowed mechanistic insight into the chemical-induced endocrine disruption in intersexual crayfish observed in the field. Our results also for the first time demonstrated suitability of the H295R cell bioassay for investigations of

complex sediment samples, and demonstrated significant effects of organic compounds on steroidogenesis as a novel mechanism of endocrine disruption. The research is supported by the Ministry of Education of the Czech Republic (project No. 0021622412 "INCHEMBIOL"), and by European Commission (6th FWP project ECODIS, No. 518043).

## **Responses of a freshwater food web to whole-lake additions of a potent estrogen**

### **Results from a whole lake experiment: Is the birth control pill an effective form of contraception for fishes?**

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Considerable evidence now exists that fish are being adversely impacted by estrogens and their mimics in municipal wastewater treatment plant (MWTP) effluents. However, it is not known whether the responses observed at the organism level, such as the production of egg protein precursors (vitellogenin) in male fish downstream of MWTPs, are indicative of problems at the population level. To investigate this unknown, an estrogen-addition experiment was conducted at the Experimental Lakes Area (ELA) in northwestern Ontario, Canada, from 1999-2006. This study examined the effects of whole-lake additions of the potent synthetic estrogen 17 $\beta$ -ethynylestradiol (EE2) on the fish, zooplankton, benthic invertebrate, algal, and microbial communities. EE2 concentrations in surface waters of the experimental lake were maintained at concentrations of ~5-6 ng/L through weekly additions during the summers of 2001 to 2003. Population-level data were collected for all trophic levels, and several tissue- and biochemical-level endpoints were examined in lake trout (*Salvelinus namaycush*), white sucker (*Catostomus commersoni*), pearl dace (*Semotilus margarita*) and fathead minnow (*Pimephales promelas*). Benthic invertebrate, zooplankton, microbial and algal communities were not affected by the EE2 additions. In contrast, male and female fishes showed induced vitellogenin production (up to 9000 fold) and delayed gonadal development when compared to reference lake fishes, and male pearl dace and fathead minnow developed intersex after one and three seasons of EE2 additions, respectively. After the second summer of additions, a recruitment failure was observed for the fathead minnow and this, in turn, resulted in a collapse of the population. The longer-lived, multiple spawning species (pearl dace and lake trout) also declined in abundance but their populations were not impacted as quickly or to the same extent as the fathead minnow, suggesting that life history strategies are important in determining risk of a species to environmental estrogens. In addition, the population-level responses for the lake trout were more likely related to a loss of prey species than due to a direct effect of the EE2. In summary, this experiment showed that chronic exposure to low concentrations of a potent estrogen mimic can affect the sustainability of fish populations and that the shortest-lived fish species are at greatest risk.

# **From molecules to populations; the causality of toxic effects**

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What processes link toxicant concentrations in the environment to whole-organism and population effects? This is one of the fundamental questions in ecotoxicology; a question that is tackled with a variety of approaches. The classical (and still dominant) approach would be to derive a concentration that is associated with no significant effects (NOEC) or a specific percentage of effect (EC<sub>x</sub>). These statistics have some nasty practical problems, e.g., their values change with exposure time and differ between endpoints. As such, these statistics do not help to elucidate the causality of toxicity. Another school in ecotoxicology focuses at the molecular level, e.g., identifying receptors for the toxicants and observing toxicant effects on gene expression. Even though these approaches are focussed on causality, they only consider the first step in the chain of processes. Molecular mechanisms, by themselves, are not sufficient to predict whole-organism responses on life-history parameters such as feeding, growth, age and size at first reproduction, and offspring production. It is these life-history parameters, and the relationships between them, that determine the population response to toxicants. If we ultimately want to understand and predict responses at the population level, we need a theory at the individual level, which explains feeding, growth, development, reproduction as well as the functional links between these processes. In this presentation, I will discuss how energy-budget theory can provide a link between molecular-level responses and the individual's life-history, and how changes in different part of the energy budget have different consequences at the population level.

# **Monitoring of triclosan in waste waters by high-throughput magnetic-particle immunoassay, confirmatory analysis by gas chromatography/mass spectrometry, and acute toxicity assessment**

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**The EPA registers triclosan as a pesticide, giving it high scores as a risk to both human health and the environment. It is a chlorinated aromatic, similar in molecular structure and chemical formula to some of the most toxic chemicals on earth: dioxins, PCB's, and Agent Orange. High-throughput screening methods are necessary to carry out efficient monitoring programs that may help to prevent certain health diseases linked to water consumption. For this purpose, a new commercial sensitive magnetic particle-based immunoassay to determine Triclosan [5-chloro-2-(2,4-dichlorophenoxy) phenol] was evaluated in front of chromatographic methods for the determination of Triclosan and their main metabolite, methyl Triclosan in wastewater.**

This enzyme-linked immunosorbent assay (ELISA) is based in rabbit polyclonal antibodies, and could detect Triclosan in standard solution (25% methanol/H<sub>2</sub>O v/v) at 20 ppt and its metabolite, methyl-Triclosan, at 15 ppt.

A previous evaluation of the immunoassay was carried out according to a three steps approach, consisting in cross-reactivity studies, matrix effects studies, and recoveries in different water matrices.

The percentage of cross reaction of different structurally related compounds such as chlorophenols, polybrominated diphenyl ether (PBDEs), and dioxins was evaluated.

A matrix effects study was carried out using different influents and effluents of wastewater from different plants, river water, well water, and tap water, and recovery studies for the different types of water showing good recovery percentages (which results were over the 80%), with an excellent repeatability, and low coefficient of variation (CV%<10).

The Triclosan ELISA was applied to the analysis of more than 70 real samples from 8 wastewater treatment plants. Water samples were prepared to contain 25% methanol and analyzed directly without any sample extraction or pre concentration by the Triclosan ELISA kit, and in parallel after solid phase extraction followed by gas chromatography-mass spectrometry (SPE-GC-MS).

In addition, the toxicity of the samples as well as the Triclosan extracts were assessed using the bioluminescence inhibition of *Vibrio fischeri*.

Very good agreement was obtained between analytical approaches, and good concordance with the toxicity studies.

Since the environmental point of view this study is of great interest given the frequency with which Triclosan and methyl Triclosan found in wastewater, the permanence of Triclosan and methyl Triclosan after water treatments.

# **Toxicity identification evaluation (TIE) and effects-directed analysis**

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(EDA) techniques are increasingly being used to identify unknown cause(s) of effects. Combined with biological effects based monitoring these tools can provide valuable information on the chemical(s) that are actually exerting the measured effect(s). The presentation will provide an overview of TIE/EDA and demonstrate through the use of case studies a number of examples where the combination of biological effects measurements and TIE/EDA have identified substances that would have otherwise slipped the net if a 'priority substance' approach would have been used (e.g. steroid estrogens and androgens). However, the causal agent may not always be identified or it becomes prohibitively expensive to identify the cause of effect in a highly complex mixture. The 'unknowns' identified during TIE/EDA studies may be more important than those substances identified since they are likely to be poorly characterised with very little information available. Here lies a major challenge for environmental scientists. In addition, this presentation will provide a realistic assessment of where TIE/EDA techniques are, how they can be effectively used to complement existing strategies and where there are limitations.

## **Toxicity profiling in European river sediments with emphasis on the identification of thyroid hormone disrupting compounds**

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In areas influenced by anthropogenic activity, samples can contain a complex mixture of substances that can exhibit toxic effects, such as endocrine effects, in organisms. Effect Directed Analysis (EDA) studies employ bioassay-directed fractionation techniques to be able to identify those fractions containing toxic compounds, and hence perform a toxicity profiling of the sample. The samples presented here are selected after a toxicity screening of river sediments within the Modelkey EU-project (SSPI-CT-2003-511237-2).

The selected “hot” samples come from 3 river systems, Llobregat (Spain), Scheldt (Location Schijn, Belgium) and Elbe (Location Most and Prelouce, Czech). The bioassays used are TTR-binding assay (radioligand based) and (anti-) AR-CALUX®. The aim of the EDA is to identify endocrine disrupting compounds responsible for the bioassay responses. This is performed in several steps; bioassays on whole extract, first, second and third fractions, chemical analysis, identification of compounds (known and unknown), analytical confirmation and finally toxicity confirmation. The presentation will contain methodology and data gained so far.

# Identifying Toxic Emerging Pollutants in European Rivers and Estuaries

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Identification of emerging pollutants is a complex area of research, with many differing approaches. Techniques can be applied to target specific contaminants of interest, or *in-vitro* and *in-vivo* bioassays can be used in order to assess overall toxicity of a sample. This toxicity may be acute or chronic, and endpoints include oestrogenic, androgenic and antibiotic activity as well as aryl hydrocarbon agonist potency. Combining both biological and chemical methods using effect directed analysis (EDA) techniques, complex environmental samples can be simplified and compounds causing a range of toxicities can be elucidated.

This presentation will focus on methods used and results obtained from sediments and water samples from European estuaries within the MODELKEY project. Samples were extracted using SPE (water) and ASE (sediments) and then EDA was used in order to assess the estrogenic, anti-androgenic and anti-biotic activity of the samples. Samples were fractionated using HPLC and positive fractions screened using GC-MS techniques. Preliminary results will be discussed.

# Posters

# ChiroChip, a DNA-microarray-based screening method for the assessment of chemical-induced effects in *Chironomus riparius*

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The aim of the project is to establish a molecular screening method which allows the testing of chemical substances in the laboratory as well as the effect monitoring of pollutants in aquatic ecosystems. With the help of the cDNA-microarray and quantitative PCR assays, the differential gene expression after exposure to pollutants will be determined. Test organism is the midge *Chironomus riparius* (Arthropoda: Insecta: Diptera), which is used worldwide in laboratory test for the assessment of chemicals and for the biomonitoring of surface waters. The sediment test with chironomids is one of the most important and most frequently applied biotests and is a prescribed enhancement of the test programme for sediment pollutants. The chironomid sediment test is also important in the area of environmental surveillance and monitoring. To this date, no cDNA arrays exist for *Chironomus*, but a relevant project with zebrafish has started in Germany and other projects are dedicated to the development of arrays with daphnids, which thus covers two species of the aquatic triade (algae, daphnids and fish). The first cotoxicological and genomic results will be presented here.

## Compounds with specific modes of action in river sediments - temporal and spatial variations also in relation to floods

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The contamination of river sediments is frequently assessed from single time sampling reflecting just the actual situation. However, the sediments comprise a dynamic system especially in areas with occurrence of floods. Two year study has been conducted to reveal the spatial and temporal variability in the concentrations of commonly studied contaminants, but namely of the chemicals with specific modes of action in river sediments, in a model study area where 10-50 year flood occurred in 2006. Parts of the selected model study area (Zlin region in the Czech Republic) are being regularly flooded and the water and sediment quality has been impacted by historical industrial and agricultural activities. Thus there is a great potential for contamination by various types of pollutants and also a risk of redistribution of the contamination during floods. Recent floods in March/April 2006 caused by very fast melting of extensive snow cover raised up to the 50-year flood level and the culmination flow reached up to 45-times the average flow rate, which lead also to flooding of industrial facilities, and material damages. Sediments were repeatedly collected – in spring and autumn before floods (2005) and the same seasons after floods (2006) from fourteen sampling sites from part of river Morava and its tributary Drevnice. Sampling sites include relative unpolluted areas and industrial places.

River sediment samples were characterized for content of organic carbon and detailed grain size distribution. The concentrations of polycyclic aromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs) and organochlorine pesticides (OCPs) and heavy metals were determined in these samples.

Battery of *in vitro* bioassays using recombinant yeast and human cell systems was applied to evaluate the extracts of sediments from four consequent sampling representing two different seasons. These bioassays show an integrative measure and enable to identify the sites with continuously increased potencies for specific toxicity and also the potential effect of relatively frequent floods on their occurrence. Several bioassays have been applied as a specific bioanalytical tools for detection of the presence of contaminants with dioxin-like and hormone-like activity in the complex environmental mixtures. Since these effects can be induced by numerous compounds that are not routinely analyzed the bioassay served as a tool to assess their overall presence in the sediments and the season- and flood-related changes.

The total potencies of the sediment extracts for cytotoxicity, dioxin-like, (anti)estrogenic and (anti)androgenic potency were assessed along with analysis of known pollutants. *In vitro* measurements of the dioxin-like activity of extracts using a transgenic H4IIE-*luc* cell line transfected with luciferase gene under control of Ah-receptor. Hormonal activity was measured using recombinant yeast bioassay (anti/androgenicity) and transgenic MVLN cell line (estrogenicity). Yeast line stably expresses human androgen receptor (AR) linked to a reporter gene and MVLN cell line contains luciferase gene under control of estrogen receptor (ER).

All applied bioassays have shown very good responsiveness to the complex mixtures of contaminants present in the sediments and enabled to point out the sites with continuous presence of contaminants affecting the specific receptor pathways. Even though there has been great variation in the absolute concentration equivalents of compounds with specific mode of action related to season and occurrence of floods, sites with reoccurring high

estrogenic or dioxin-like activities could be clearly identified. There was a decrease of dioxin-like activity at most sites after floods. Most sediment samples repeatedly showed estrogenic activities, except of the spring 2006 after floods, where there was no activity or it was strongly decreased. At the same time, sediment samples from some sites repeatedly showed moderate antiestrogenic activity. But the seasonal variations (temporal trends) were less pronounced in the case of antiestrogenic activities. Samples of the river sediments showed no androgenic activities, while antiandrogenic activity was detected in samples from all sites in most of the seasons. The overall antiandrogenic activity also decreased after floods. The results clearly reflected the continuous presence of estrogenic and antiandrogenic compounds in the studied rivers. Some samples had also shown cytotoxic effects in the highest concentrations.

Despite the seasonal variation, there was a good correlation in the results between the individual seasons. Some sites have shown relatively stable values across seasons, while there has been much greater variation at several other sites. The dioxin-like and estrogenic activity correlated well with the total concentrations of PAHs, suggesting the role of these pollutants and their derivatives in the observed dioxin-like and estrogenic activities. The data matrix also showed significant interrelation between dioxin-like activity, antiandrogenicity and content of organic carbon, silt and concentration of PAHs and PCBs, which documents significance of abiotic factors in accumulation of pollutants with specific mode of action. However, these relationships have been partly distorted by the floods.

Samples from industrial places as well as areas without any obvious major sources induced significant responses in assays for the dioxin-like and hormone-like activity. Thus the disperse nonpoint regional sources seem to contribute significantly to the pollution of the studied area.

The project was supported by GACR 525/05/P160 and by the Ministry of Education of the Czech Republic (project No. 0021622412 "INCHEMBIOL").

## Effect monitoring: measuring toxic potency of water

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### Summary

Bioassays represent powerful tools for the assessment of environmental quality. This paper focuses on the measurement of the toxicity of surface water samples towards a battery of several different aquatic organisms. The toxicity is determined by isolating toxic organic chemicals from the surface water by use of a concentration procedure, and by exposing the organisms to the concentrates thus obtained. Subsequently this method evaluates the results statistically, to yield a measure for the degree to which the water represents a risk for the aquatic ecosystem. This risk is expressed in terms of 'toxic potency' which represents the fraction of the ecosystem that is affected in any way by the environmental conditions. New developments in this method incorporate the use of specific bioassays to measure the effects of groups of compounds with a specific mode of action, for example antibiotics or endocrine disruptors.

### Introduction

The EU Water Framework Directive wants the European member states to report on the state of the aquatic environment on a regular basis. Surface waters must have both a 'good ecological quality' and a 'good chemical quality'. To assess the status of a water body, member states must have a fair view of the degree to which chemical concentrations exceed the standards. However, any regular evaluation of the surface water quality by means of measuring the chemicals present is impracticable, because of the countless number of substances that may occur in water samples. Furthermore, analytical methods for the measurement of many chemicals are lacking, whereas in many instances standards have not (yet) been derived. An evaluation as to whether chemicals are present in acceptable concentrations or not is therefore practically impossible. In addition, individual measurements of substances do not account for any possible additive effect of chemicals that are present concomitantly in the same sample. Ecological observations on aquatic systems do give information on the impact of the water quality on flora and fauna, but unfortunately under many circumstances such observations may prove rather insensitive, due to a relatively long period of time that may elapse between the actual moment of exposure to the chemicals and the moment at which the effect can be observed. Moreover, there could be other stressors than chemical substances involved, like eutrophication or hydromorphological changes. Bioassays render the opportunity to measure the total effect of the presence of all chemicals in a water sample and to deduce from such measurements on a set of different organisms, representing a model for the ecosystem, the potential effect, *i.e.*, the ecological risk of the water quality towards the aqueous ecosystem.

### Methods

The procedure of sampling and of preparation of the samples has been described earlier [1, 2]. In short, it is based on a selective concentration of organic compounds from surface water samples. The toxic effect of the concentrated organic compounds is determined with bioassays. The concentration step is necessary to be able to make a

dilution range of the sample and to exclude disturbing factors, like pH or humic acids, which could affect the bioassay results. An evaluation of the toxic endpoints thus obtained yields a quantitative approximation of the ecosystem risks.

Samples of surface water (60 liters) were taken at regular monitoring sites at regular intervals during a year. The samples were taken immediately to the laboratory by cooled transport. Immediately upon arrival of the samples at the laboratory a 1:1 mixture of well-purified XAD-4 and XAD-8 resins was added, after which the organic compounds in the samples were allowed to adsorb onto the resin under continuous agitation during 48 h at room temperature. Thereafter the loaded resin was isolated by sieving, and dried subsequently overnight at room temperature. The organic compounds were transferred from the resin to acetone by elution, which yielded a 1000-fold concentrate (60 ml).

### **Bioassays**

Bioassays were performed on diluted samples of the 1000-fold concentrate. For this, a dilution series was made and test organisms were exposed to the dilutions using standard ecotoxicological protocols. Inherent to this dilution method, high toxicity of water samples was found when effects were found in the relatively less concentrated samples, whereas a toxic effect of 'clean' water only was observed in the more concentrated samples. Endpoints of the toxicity tests were expressed as  $EC^{f}_{50}$  or  $LC^{f}_{50}$ , where the suffix 'f' stands for the use of concentration factors instead of the usual substance concentrations. The bioassays were chosen such, that (i) only a small volume of the original concentrated sample is sufficient to obtain results of the tests, (ii) results can be obtained in a relatively short period of time, and (iii) the technique by which the bioassay is done is relatively simple (e.g., because it is commercially available).

Five bioassays were selected. The first test is the Microtox test [3], which measures luminescence of the bacterium *Vibrio fischeri* as a measure of the energy state of the organism. It is determined in a luminometer after exposure of the bacteria during 5 or 15 minutes to dilutions of a sample. The  $EC^{f}_{50}$  is taken as the concentration factor that decreases light emission by 50%.

The second test is a PAM fluorescence test [4] with green algae. Changes in chlorophyll fluorescence by *Pseudokirchneriella subcapitata* in response to pulses of light are a measure of the efficiency of photosynthesis by this alga. It is measured in a fluorescence spectrophotometer (Water-PAM, Heinz Walz GmbH, Effeltrich, Germany) after 4.5 hours of exposure to dilutions of the sample. The  $EC^{f}_{50}$  is taken as the concentration factor that decreases fluorescence yield by 50%.

The third test is a Daphnia IQ test [5]. The water flea *Daphnia* expresses its  $\beta$ -galactosidase enzyme activity by cleaving 4-methylumbelliferyl  $\beta$ -D-galactoside which yields the fluorescing umbelliferyl determinant. Fluorescence of the daphnids upon irradiance with ultraviolet light was observed by eye. The  $EC^{f}_{50}$  is taken as the concentration leading to 50% inhibition of fluorescing daphnids after 1.25 hour exposure.

The fourth test is the Rotox test [6]. Mortality of the rotifer *Brachionus calyciflorus* in response to the samples was expressed as  $LC^{f}_{50}$ , the concentration factor leading to 50% mortality after a 24 hour exposure.

The last test is the Thamnotox test [7]. Mortality of the crustacean *Thamnocephalus platyurus* in response to the samples was expressed as  $LC^{f}_{50}$ , the concentration leading to 50% mortality after a 24 hour exposure.

### **Risk assessment**

Since the bioassays represent different types of species in morphology and trophic level, it was assumed that from their endpoints a species sensitivity distribution could be deduced, which was specific for the types of toxic substances in the samples and for the aquatic ecosystem. Classically, species sensitivity distributions are valuable tools in risk assessment, because they represent the number of species that may be affected at a given toxicant concentration. When dealing with toxicant mixtures, such a distribution can be set up comparably from concentration factors instead of substance concentrations.

The acute endpoints from the bioassays were fitted onto a distribution curve. The resulting cumulative species sensitivity distribution curve was extrapolated to a chronic no effect one by assuming an average acute-to-chronic ratio of 10 [1]. The potentially affected fraction in the original water sample was inferred from the value of this curve when the concentration factor =1. This parameter was defined as the toxic potency of the surface water sample. Mathematical and statistical descriptions of the method, as well as a discussion on its uncertainties, have been presented elsewhere [1].

### **Results and Discussion**

During the year, surface water samples were taken in bimonthly intervals. This was done at several regular monitoring sites in The Netherlands, in the catchment areas of the Rhine, Meuse and Scheldt rivers. In many instances the surface waters appeared to be more toxic in summer than in winter.

This course in time of the toxicity is typical for the effect on algae, but not necessarily for the other organisms we examined in our bioassays. The cause of this phenomenon is not known. Chemical analysis of the surface water shows, of course, fluctuations in substance concentrations due to differing loads of water over the seasons. However the occurrence of specific toxic compounds in summer, for instance pesticides, cannot be excluded. For daphnids a trend was found that was reverse to that of the algae, and for the other test organisms the outcome of the assay was rather indifferent towards seasonal changes.

With the sets of endpoints obtained from each bimonthly sample, risk assessment was done by fitting them to a sensitivity distribution curve, as described in the Risk assessment section. From this calculation it appeared that in the summer season of 2002 (May – August) the surface water was risky for approximately 10% of the aquatic species, whereas in the cold and wet winter season (October – March) the water was much less toxic for the aquatic organisms.

Since 1996 the state of the surface waters in The Netherlands has been monitored using the bioassays presented in this paper. By now their use has proved to be a solid method to assess the ecological risk imposed by the chemicals concentrated from the surface water. Being integral, it is an attractive method for monitoring the chemical state of the environment and it is complementary to the chemical analysis of the individual substances.

At the moment studies are undertaken to incorporate specific bioassays for groups of compounds with specific modes of action. For example, very promising results were obtained from using a multi bacteria test to measure the specific effects of antibiotics. Bioassays may play an important part within the monitoring system demanded by the Water Framework Directive. Under circumstances when a good ecological state is not observed in a surface water system, the results of the bioassay method presented here may indicate whether any (known or unknown) substances may cause a relevant effect on the ecosystem. Thus, bioassays are valuable tools helping water management

authorities in focusing on measures to improve the water quality in order to attain a good ecological state.

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# environmental fate of selected pharmaceuticals: sorption and desorption by sediment

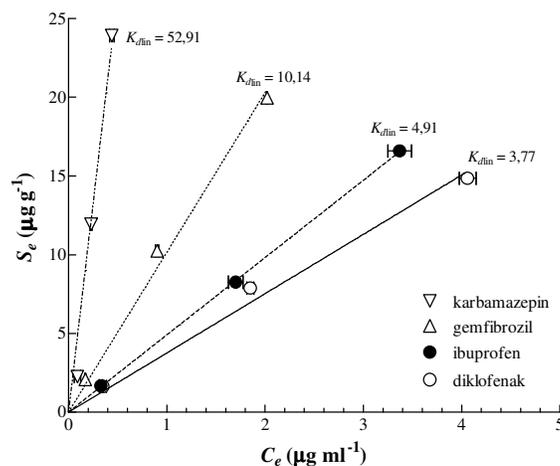
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This study is dedicated to laboratory investigation of sorption-desorption behavior of four pharmaceutically active substances (diclofenac, ibuprofen, gemfibrozil, and carbamazepine) in one river sediment. Batch equilibration method was used at three initial concentrations in aqueous solution. The results of the experiments show that distribution coefficients were either relatively low for diclofenac and ibuprofen or higher for gemfibrozil and carbamazepine. Distribution coefficients (mean  $K_d$  values for three initial concentrations), as measured by the batch experiments, were  $4.21 \pm 0.58$  for diclofenac,  $4.98 \pm 0.29$  for ibuprofen,  $11.28 \pm 1.22$  for gemfibrozil, and  $44.82 \pm 14.89$  for carbamazepine. Based on sorption data, migration potential of the pharmaceuticals in surface waters would decrease as follows: diclofenac > ibuprofen > gemfibrozil >> carbamazepine. During desorption significant hysteresis was observed. Only  $2.44 \pm 1.05\%$ ,  $3.67 \pm 0.35\%$  and  $1.79 \pm 1.17\%$  of the initially sorbed ibuprofen, gemfibrozil and carbamazepine were desorbed during 48 h, respectively. Diclofenac exhibited the highest desorption ( $15.01 \pm 2.59\%$ ). Generally, the results indicate that significant amounts of the pharmaceuticals may be eliminated from surface waters via sorption by sediment and that observed low desorption would further limit their migration in surface waters.



Sorption isotherms of selected pharmaceuticals measured for the river sediment (OC = 0,36%), lines are fittings of the Henry equation,  $K_{dlin}$  – linear distribution coefficient,  $S_e$  – sorbed amount of chemical in sediment,  $C_e$  – equilibrium concentration of chemical in water.

# Biomarker Studies on Samples from the German Environmental Specimen Bank

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Biomarkers are physiological and/or biochemical measures which react upon impacts of chemicals or physical influences and which can be quantified on different organisational levels of an organism (bio-molecule, cell, tissue, organ) as measure for an exposure, an effect or a susceptibility (Arbeitskreis Bioindikation, 1996). Therefore biomarkers are of great interest for a comprehensive environmental monitoring since these can be first indicators of a biological answer of an organism to changing environmental conditions. On a first level the presence of a compound in an organism itself can be seen as a biomarker (bioaccumulation marker; van der Oost et al., 2003). This approach is an advantage to measurements of persistent compounds in environmental media like water or soil since such data are a proof for the bioavailability of a certain chemical. However, especially relevant are effect biomarkers which are directly correlated to an exposure to a toxic compound (e.g. imposex effect of triorganotin compounds) or other stressors.

In recent years numerous effect biomarkers like biotransformation enzymes, stress proteins, or endocrine parameters have been identified (an overview was presented in a recent review by van der Oost et al., 2003). Well known are those biomarkers which are applied for the detection of the exposure of organisms to pollutants (e.g. induction of cytochrome P450 1A as biomarker for the presence of PAH, PCDD or PCB). Other biochemical measures used are DNA adducts as biomarker for gene toxicity. Only recently molecular-biological methods became available which allow biomarker measurements on DNA and RNA levels. These enable the analysis of gene activation or changes in the expressed protein levels in response to changes of environmental factors or the presence of chemicals (toxicogenomics). Since changes in levels of cellular products like enzymes, structural proteins or hormones are closely connected to the activities of the respective genes there is a large potential for the use of this concept in monitoring programs.

The environmental specimen bank program in Germany provided so far data from retrospective monitoring for several target compounds. Examples are time series of monitoring data for organotin compounds in archived marine biota samples (Ruedel et al., 2003) or freshwater fish (Ruedel et al., 2007), and for synthetic musk fragrances in freshwater fish (Ruedel et al. 2006). This kind of biomonitoring of target substances in organisms is a valuable tool to improve the understanding of the exposure of ecosystems and biota to chemicals (bioaccumulation marker). However, the resulting concentration data only give information about the concentrations in the tested organisms themselves but not whether any exposure directly influences the organism, e.g. by damaging biological structures or changing the gene expression. Now research within the specimen bank program is intensified to couple the exposure data generated to possible effects on a molecular level especially as a response to chronic exposures. The material seems appropriate for this purpose since all materials are deep-frozen with liquid nitrogen directly after sampling. Detailed method descriptions are available for sampling, dissection, and homogenization by cryo milling (Umweltbundesamt 1996). Afterwards sub-samples (approx. 10 g portions into individually labeled 20 mL-vials) are stored in the gaseous phase above liquid nitrogen at temperature below -150°C. This low temperature ensures that chemical processes in the samples are minimized. Atmospheric

oxygen is excluded through the inert gas layer resulting from the evaporation of liquid nitrogen so that oxidative reactions are prevented. Since the temperature is below the glass transition temperature of water of approx. -135°C physical processes are stopped (e.g., no growth of ice crystals which may change the morphology of the sample material).

The aim of this study was to analyze the possible usage of molecular biomarkers in cryogenically archived samples taken from the German ESB, and to execute a feasibility study. As organisms fish were used. For the German ESB bream from currently 17 sampling sites are archived annually since 1992. The present study shows that the analysis of molecular biomarkers in archived ESB samples can successfully be applied for a retrospective monitoring. By comparison of gene sequences with zebrafish (*Danio rerio*), specific genes could be identified which are regulated in bream (*Abramis brama*) by different stressors. It was possible to detect genes which are expressed in an agent-specific or agent-unspecific way. *HSP* (heat shock protein) was identified as a marker to be regulated agent-unspecifically. *HSP* is over-expressed fast by the influence of different stressors and can therefore be used as a health indicator. Agent-specific genes like *metallothionein* and *vitellogenin* represent genes which are influenced by heavy metals or endocrine disrupters, respectively.

If it is possible to identify appropriate molecular biomarkers even in other ESB sample organisms, an effective tool will be available which extends the retrospective monitoring from bioaccumulation markers to effect biomarkers including the detection of changes in the genome.

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# Determination of Indoor Exposure to Air Pollution

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## Introduction

Exposure biomarkers are frequently used in epidemiological studies to determine the global effects of exposure of the population to pollutants. In Flanders, large humane biomonitoring studies have been carried out in the recent years (2001-2006). Exposure biomarkers are however intermediates between the occurrence of the exposure and later biological effects (M.C. Madden, J.E. Gallagher, Biomarkers for Exposure, Air pollution and Health, 2000). Therefore, information of the exposure should be known when designing human biomonitoring programs. In Flanders, a project has been funded to measure the indoor exposure of children to air contaminants. (FLIES : Flanders Indoor Exposure Survey, 2005-2007).

Air pollutants present in the indoor environments are the result of (1) product emissions such as furniture, consumer products, cleaning materials, and (2) infiltration of ambient substances. Knowledge on the relative importance of these two terms is crucial for stipulating a good indoor air quality policy.

The aim of the study was (1) to make an inventory of indoor concentrations in Flemish dwellings and other micro-environments in which people spend a large part of their time, (2) to assess the effect of traffic density on indoor concentrations and (3) to determine the personal exposure of children and, (4) to differentiate the personal exposure in an ambient and non-ambient fraction. Children were selected as sensitive groups because they experience a higher air pollutant load given their 50 % larger body-weight rescaled or 35 % larger long-surface rescaled exposure than adults.

The selection of the investigated pollutants was based on 2 criteria : 1) only pollutants with possible outdoor sources were selected (biological agents and ozone not retained) and 2) only pollutants for which health effects have been proven in the past (e.g. based on WHO health criteria). Terpenes, pesticides and bromated flame retardants were not measured in the first Flemish Indoor Exposure Survey because of too specific.

## Methods

The monitoring campaign was performed in January-February 2006 in the eastern part of Flanders in 50 dwellings of volunteers. The dwellings were selected based on the proximity to traffic: 12 houses in rural background (RB) areas (< 50 cars passing/day), 26 houses in urban background (UB) area (< 500 cars/day) and 12 houses in urban hot spot (HS) area (>15000 cars/day). The emphasis on dwellings in UB areas is because 75 % of the Flemish houses are of this type. In each dwelling two indoor locations, namely living room and bedroom, and two outdoor locations, namely front door and back door (if applicable) concentrations were sampled. The set of 50 houses existed of a heterogeneous mix of dwelling types. Additionally, indoor

and outdoor air was sampled at schools or daycares, transport and sport and leisure infrastructures ( all together 23 locations).

Information regarding possible indoor sources and dwelling characteristics was obtained by means of a detailed questionnaire. The second part of the questionnaire investigated the children's time patterns.

Except for particulate matter, all pollutants were measured for 7 days by diffusive sampling techniques. Particulate matter was measured by Grimm dust, Buck or Aeromini monitors (filter and optical techniques). The measurements were performed in line with the ISO-16000-x series



**Figure 1: Indoor Measurement Set-Up**

The children's personal exposure was calculated based on time activity patterns obtained through the questionnaires and concentrations measured in each micro-environment.

The statistical analyses was performed using the statistical tool package Statistica (version 7). The non-parametric Kruskal-Wallis Anova test and non- parametrical correlation analyses were applied because of not normally distributed datasets.

## **Results**

A summary of indoor and outdoor concentrations in 50 Flemish dwellings is given in Table 1. Concentrations show a very high variability between different houses (n=50), both indoors and outdoors. The most abundant gases in both indoor and outdoor environments were toluene, NO<sub>2</sub>, formaldehyde and acetaldehyde.

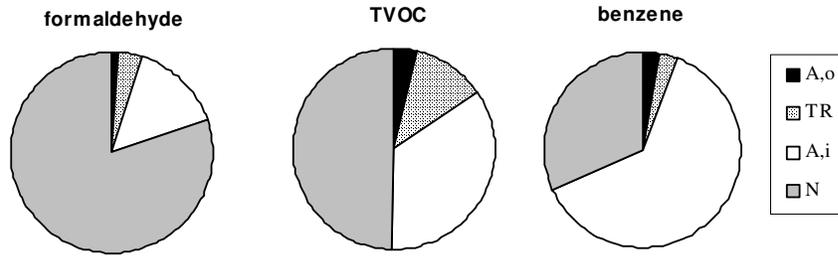
Statistics in Table 1 include both living room and bedroom concentrations and front door and backdoor concentrations. On average, the ratio of bedroom to corresponding living room concentration was near 1 (average ratio varied from 0,80 for acetaldehyde to 1,51 for styrene). In general, backdoor concentrations were slightly ( $\pm 0,9$  fold) lower than front door concentrations. This suggests that the dwellings act as a barrier for pollutants that are mainly formed at the street.

**Table 1: Indoor (living room and bedroom) and outdoor (front door and backdoor) concentrations in 50 Flemish dwellings**

	Dwelling Indoor (n=100)			Dwelling Outdoor (n=86)		
	median	mm	max	median	Min	max
		<i>µg/m<sup>3</sup></i>			<i>µg/m<sup>3</sup></i>	
MTBE	0,54	0,12	33,47	0,35	0,08	1,27
Benzene	2,14	0,70	23,71	1,58	0,69	3,21
Trichloroethene	0,16	0,03	1,21	0,12	0,03	0,26
Toluene	8,13	1,29	122	3,06	1,12	12,27
Tetrachloroethene	0,26	0,06	52,13	0,20	0,03	6,48
Ethylbenzene	1,09	0,20	7,37	0,50	0,16	1,38
m-+p-Xylene	2,33	0,43	17,52	1,12	0,31	3,56
Styrene	0,21	0,01	2,95	0,06	0,01	1,94
o-Xylene	0,86	0,14	6,14	0,41	0,12	3,48
1,2,4- Trimethylbenzene	1,99	0,17	16,94	0,64	0,14	3,32
p-Dichlorobenzene	0,07	0,03	7,11	0,03	0,03	0,09
TVOC	491	138	2793	218	136	396
NO <sub>2</sub>	21,3	7,1	122,1	40,9	1,9	71,2
Formaldehyde	23,7	1,4	124,1	3,4	2,2	64,9
Acetaldehyde	21,8	1,1	65,2	13,8	0,4	63,2
PM10	11,7	2,3	58,0	33,8	17,8	73,7

The total exposure of children is calculated based on median concentrations in each microenvironment and average time patterns. Typical children's exposures reached 1,9-2,7 µg benzene/m<sup>3</sup>, 0,17-0,23 µg trichloroethene/m<sup>3</sup>, 6,3-7,5 µg toluene/m<sup>3</sup>, 0,24-0,48 µg tetrachloroethene/m<sup>3</sup>, 0,9-1,4 µg ethylbenzene/m<sup>3</sup>, 2,0 – 2,9 µg m+p xylene/m<sup>3</sup>, 0,10-0,70 µg styrene/m<sup>3</sup>, 0,8-1,1 µg o-xylene/m<sup>3</sup>, 1,6-2,3 µg 1,2,4-trimethylbenzene, 0,1-0,9 µg p-dichlorobenzene, 410-522 µg TVOC/m<sup>3</sup>, 19-34 µg NO<sub>2</sub>/m<sup>3</sup>, 15-25 µg formaldehyde/m<sup>3</sup>, 10-27 µg acetaldehyde/m<sup>3</sup> and 8-13 µg PM10/m<sup>3</sup>. These ranges of typical exposure refer to variation in typical exposure for different age classes (0-2,5 years, 2,5-6 years, 6-12 years, 12- 18 years) and proximity of the houses to traffic density (UB, RB, HS). The differences in exposure between different age classes and traffic density classes were rather small. It was assessed that the highest exposed children (P<sub>95</sub>) were 2-4 fold higher exposed than the median, typical exposed children for most gases. Exceptions were tetrachloroethene (x11), and p-dichlorobenzene (x 50). These extremes were related to the extremely large range of indoor concentrations (Table 1).

The distribution of exposure over ambient (A: A<sub>i</sub> + A<sub>o</sub>;; with A<sub>o</sub>, exposure to ambient pollutants directly in outdoor environment and A<sub>i</sub>, exposure to ambient pollutants that have infiltrated indoors), indoor source-related (N) and exposure during transport (TR) is given in Figure 2 for TVOC and for the 2 components with a extreme A,N, TR distribution



**Figure 2: Distribution of typical exposure to formaldehyde, TVOC and benzene over ambient, non-ambient (indoor sources) exposure and exposure during transport.**

### **Conclusion**

Children’s exposure to air pollutants is mainly determined by indoor dwelling exposure given the majority of time spent indoors. Variation in exposure between children is mainly due to variations in indoor dwelling concentrations (up to 100-fold), rather than variations in children’s time budgets, which are limited.

This study shows that the contribution of infiltrated outdoor air pollution to personal exposure is different among the investigated pollutants. This is a point of attention in ambient air quality policies, to include the indoor exposures more explicit. Recommendations for precautionary measures to reduce or avoid indoor exposure to certain gases, for example to formaldehyde, are difficult to make based on this study because only a few clear source concentration - exposure relationships were found. For this, work on short-term and long-term emission sources and their relation to concentrations, using various time average measurements should be performed. This is best placed in the context of a product policy.

## **Toxicological profiling: use of in vitro and in vivo bioassays to characterize hot spots for Effect Directed Analysis in the Elbe, Scheldt and Llobregat rivers**

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The use of bioassays in combination with chemical fractionation, separation and identification techniques, also called Effect Directed Analysis (EDA), is a powerful tool for the identification of environmental key toxicants in (aquatic) ecosystems. As a first step and in order to select sites for a comprehensive EDA study in the Modelkey project (EU contract number GOCE 511237), a number of locations in the river basins of the Elbe, Scheldt and Llobregat were selected for toxicological profiling of water and sediment using a vast array of bioassays corresponding to a broad range of toxicity syndromes.

Combination of the results will generate a fully integrated set of data for each location. Based on these data, choices will be made for further study of specific locations, enabling the design of a tailor-made EDA approach for each location with the ultimate aim of identifying the key toxicants responsible for the observed toxicities.

For toxicological profiling, the in vitro assays used covered endocrine disruption, genotoxicity and antibiotic resistance. In addition, in vivo bacteria, algae and daphnia tests and several sediment contact tests have been included.

