Characterization and comparison of multi-drug resistant methicillin-resistant *Staphylococcus aureus* [MRSA] from recreational beaches and high touch surfaces at a university and surrounding community

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1. Introduction

*Staphylococcus aureus* is part of the normal flora and can be found in the nose and other areas of the body in 25-35% of humans. Over the last decade community-acquired methicillin-resistant *Staphylococcus aureus* (MRSA) has emerged as a major cause of disease in the general population with no health care exposure or known classical risk factors for MRSA infections. MRSA colonization is a known risk factor for developing MRSA infections and MRSA are spread from fomite to person and from person to person. However, few environmental reservoirs outside the healthcare setting and closed communities such as schools, prisons, and sports teams have been characterized. In a 2009 study we isolated and characterized 6 MRSA, 4 *S. aureus* and 21 methicillin resistant *Staphylococcus spp* from public marine recreational beaches. Five of the MRSA had properties suggesting that they were related to hospital isolates and one had not previously been identified in man. A MRSA isolate which carried macrolide and tetracycline resistance genes which could be transferred by conjugation to an *Enterococcus faecalis* recipient at a frequency of $10^{-8}$. The data suggested that there were multi-drug resistant MRSA in the beach environment and thus may be a reservoir for transmission of MRSA to beach visitors as well as a reservoir for macrolide and tetracycline resistance genes. The current study sampled and identified MRSA from local marine and fresh water recreational beaches which included sand, fresh and marine waters samples. In addition, MRSA was isolated from high touch surfaces on the University of Washington (UW) campus, in UW undergraduate housing, and the local community. The study illustrates that MRSA contaminates a variety of different community environments but more work is required to determine if exposure to these environments increases the risk of carriage and ultimate MRSA disease.

2. Materials and Methods

Sand, marine and fresh water samples (n=296) were collected from 2 marine and 1 fresh water recreational beaches in Seattle WA multiple times during the summer of 2010. Frequently touched public surfaces at the university including ATM, computer and vending machine keypads, bathrooms surfaces, locker and water fountain handles, elevator buttons, gym equipment and floors (n=294); undergraduate housing sites (apartments, houses, parent homes and dorm rooms) including couches, bathroom surfaces, kitchen, microwave touchpad, TV remote, and washing machines (n=85) and local community sites including ATM keypoints, public library touch screens and parking meters (n=130) were sampled with sterile swabs, and/or RODAC contact plates with Bacto® Staphylococcus Medium 110 supplemented with 10 ug/ml methicillin and 0.01% potassium tellurite from 2009-2010. Washing machines were sampled with sterile baby wash cloths. mStaphylococcus broth [1.5 X] (Difco Laboratories, Sparks, MD, USA) supplemented with a final concentration of 75 ug/L polymyxin B and 0.1% potassium tellurite (Sigma Co. St. Louis, MO USA) was added to the swabs and wash clothes. All the samples were incubated in 5% CO$_2$ at 36.5 °C until turbid and black. Isolates from broth and plates were biochemically verified as *S. aureus* and the mecA gene was verified for identification of MRSA.

The presence of type I-VII of mobile Staphylococcal Cassette Chromosome [SCCmec], multilocus sequence typing [MLST] of the allelic profile of seven housekeeping genes and the presence of aminoglycoside resistance gene, *aadD*; macrolide resistance genes, *erm*(A), *erm*(B) and *erm*(C), and *msr*(A); and tetracycline resistance genes, *tet*(M), *tet*(K), were determined by PCR assays and sequencing. Pulse field gel electrophoresis was done and the genetic relatedness of the MRSA isolates to USA300 was determined by Dice coefficient, UPGMA using the GelCompar II software according to the manufacturers’ instructions (Applied Maths, Inc., Austin, TX USA). Strains that had > 80% homology with USA300 were classified as USA300.

3. Results and discussion

3.1 Recreational beaches
Thirty-one MRSA were isolated; 22/144 (15.3%) fresh water and 2/56 (3.6%) marine water samples at levels ranging from 2-66 MPN/100 ml and 7/96 (7.3%) sand. Twenty-nine (93.5%) of the isolates carried other antibiotic resistance genes of which 24 (77.4%) were resistant to aminoglycosides, macrolides and tetracyclines; 24 (77.4%) carried one or both of the tetracycline resistance genes \([\text{tet}(K), \text{tet}(M)]\); 28 (90.3%) carrying \(\geq 1\) of the macrolide resistance genes \([\text{erm}(A), \text{erm}(C), \text{msr}(A)]\) and 29 (93.5%) carried the aminoglycoside resistance gene \([\text{aad}D]\). Twenty-one (67.7%) of MRSA were \(\text{SCCmec}\) type IV, but none were USA300 and 48% of the MLST types had previously been identified in livestock or squirrels but not humans.

3.2 University surfaces
Ten MRSA from 9 surfaces (3.1%) were isolated from ATM keypads, locker room handles, elevator buttons, and study lounge. All MRSA isolates carried \(\geq 1\) other class of antibiotic resistance gene; including 9 carried tetracycline, 7 macrolide and 2 aminoglycoside resistance genes. Four (40%) isolates were USA300 and 3 (33%) of the strains had MLST types that had previously been identified in livestock but not humans.

3.3 Student home surfaces
Ten MRSA were isolated from five (62.5%) of eight student homes and included dorm water fountains, microwave touch pads, couches, TV remotes, bathroom surfaces and washing machines. Four of the homes had multiple MRSA positive surfaces and two homes had USA300 representing 6 of 10 isolates. Nine of the isolates carried multiple other classes of antibiotic resistance genes including 8 which carried aminoglycoside, macrolide and tetracycline resistance genes and 1 carried aminoglycoside and tetracycline resistance genes. All isolates were ST types previously found in humans.

3.1 Community surfaces
Four (3.1%) MRSA were isolated from ATM keypads and public library touch screens. All four were multidrug resistant; 2 carried aminoglycoside, macrolide and tetracycline resistance genes, 1 carried aminoglycoside and tetracycline and the other macrolide and tetracycline resistance genes. One (25%) MRSA was USA300 and all isolates were ST types previously found in humans.

4. Conclusions
The highest level of MRSA positive samples [15.3%] were found in fresh water running into the marine beaches, where we frequently observed children playing during sampling, and at the fresh water beach on Lake Washington, while 11.7% of the surfaces from 5 of 8 undergraduate homes were MRSA positive. A surprising 98% of the 55 MRSA isolates were resistant to other classes of antibiotics and most likely represent reservoirs for these genes in the environment. In North America the majority of community acquired MRSA infections is due to USA300. This clone was found at the UW, in student housing and in the community but not in the recreational beach samples.

5. References


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Unique distribution of sulfonamide resistance genes, *sul*, in the 
Philippines aquatic environment

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1. Introduction

Antibiotic contamination has occurred in not only clinical but also the natural environment, which can eventually produce antibiotic resistant bacteria (ARB). Antibiotics and ARB are released from hospitals and animal farms to the environment through water. It is therefore critical to understand where released ARB ultimately end up in the environment, because they may act as reservoirs of antibiotic resistance genes that can spread among the natural microbial community. In Southeast Asian countries, intense squall and flooding events frequently occur and act as a disturbance of aquatic ecosystems, but they also can physically mix terrestrial and water-dwelling microbes. This work monitored the contamination status of antibiotics and ARB in water environments related to city activity. We found a unique distribution of *sul* genes in the environment.

2. Materials and methods

Sampling was performed in November, 2009, following catastrophic flooding in Manila City by Typhoon Ketsana (Ondoy). Surface water samples were taken at 4 sites in Laguna Lake, 2 sites in the Pasig River, and 4 sites in Manila Bay. Antibiotics, sulfamethoxasole (SMX), sulfamethazine, trimethoprim, oxytetracycline (OTC) and macrolides were quantified by LC-MS/MS. Total bacterial cell number, colony forming number and SMX and OTC resistant bacterial numbers were enumerated on nutrient agar plate incubated at 30˚C for 6 days. The sulfonamide resistance genes, *sul1*, *sul2* and *sul3*, were quantified by real time PCR (qPCR) using total DNA trapped on 0.2 µm pore filter. Copy number of each *sul* gene was normalized by 16S rRNA gene.

3. Results and discussion

3.1. Drug contamination

Most antibiotics expressed higher concentrations from river sites and the estuarine site relative to Laguna Lake and more marine sites, although OTC was not detected at all sites. Macrolide concentrations were also lower (Lyncomycin, 0.8-7.7 ng/L; Clarithromycin, nd-1.7 ng/L). High concentrations of SMX (47-94 ng/L) was detected in river and estuarine sites, whereas lake sites and marine sites indicated 27-41 ng/L and 8-18 ng/L, respectively. Sulfamethazine was relatively lower than SMX in all sites. This suggest that SMX is a major antibiotic in Metro Manila area including the animal and human medicine, which is similar to Indochina area [1].

3.2. SMX resistant bacteria

The SMX resistant bacterial rate in Laguna Lake sites showed 17-25%, river and estuarine sites showed 21-40% and 10-13% in marine sites with exception of 87% in a coastal marine site where received wastewater outflow. While the waterflow was very high because of the major typhoon event, the SMX-resistant bacterial rate was more than 10% among colony forming bacteria. The occurrence of SMX resistant bacteria was found in not only river but also seawater, although the SMX concentration was generally low due to dilution in the sea. The discrepancy between profiles of drug concentration and SMX resistant rate are of interest. ARB are thought to be
released from anthropogenic sources and reserved in coastal waters, even after disturbance by major flooding. Although the SMX-resistant bacteria rate in the rainy season in Indochina was 3-20% [2], the Philippine case was higher, even after flooding.

3.3. The sul gene distribution

Among sul genes in culturable bacteria, sul1 is major in Indochina [2]. All sul genes, sul1, sul2 and sul3, were detected in the present study from total DNA extracted from waters. These SMX resistance genes may have been derived from human activity. However, qPCR analysis of sul genes showed that seawater contained many more copies compared to the river and lake sites. This is the opposite result to the SMX-resistant bacteria rate obtained by colony count. Culturable bacteria being SMX resistant would be a minor component of the fresh water environment, whereas seawater bacteria being non-culturable and the majority should possess sul genes, which cannot be counted using the plate method. This study indicated that sul genes have a high number of copies in seawater bacteria despite them being relatively minor in lake and river bacteria (Fig. 1), suggesting that the ABR rate by colony method does not indicate real abundance and distribution in water environmental bacteria. Tetracycline resistance genes are known to have a similar copy number (10^{-3} copies per copy of 16S rDNA) in marine aquaculture sites even after farming has ceased [3], indicating qPCR monitoring is useful to know the real status and distribution of drug resistance genes. This study showed sul3 is more prominent than sul1 and 2 in Manila Bay. Continuous discharge of low-concentration drugs and ARB into the aquatic environment may convey the drug resistance gene to marine bacteria, which act to reserve ARB and antibiotic resistance genes in the microbial ecosystem.

4. Conclusions

Quantitative PCR and culture method revealed that the sul genes are conveyed by the non-culturable bacterial community in seawater, whereas culturable species can be detected in freshwater. Additionally, sul3 is a major gene in seawater of Manila Bay.

5. References


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Abundance of streptomycin and tetracycline resistance genes in apple orchards treated with streptomycin in comparison to untreated apple orchards

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1. Introduction
Antibiotic resistance is currently one of the greatest threats to human medicine. It is believed that the environment represents a large reservoir of antibiotic resistance genes and that anthropogenic effects enhance the development and spread of antibiotic resistance within the environment. The evolution and adaptation to the stress of antibiotics in relation to their use in medicine has occurred within the last 100 years. However, almost all of the antibiotics used as medicine have been extracted from soil bacteria, as a result, transferable antibiotic resistance is also thought to have originated either in the antibiotic producing bacteria or those co-existing in their environment. Since antibiotic resistance genes originated in environmental bacteria, an important question is: are changes in the natural ecosystems, mainly as the consequences of human activities, challenging the environmental microbiota in such a way that these modifications alter the resistance of human pathogens. Most studies of antibiotic resistance in the environment have focused on the PCR screening of extracted DNA for known resistance genes. These studies identify the presence or absence of resistance genes within the environment. However, in order to monitor the influence of anthropogenic effects on the abundance of resistance genes in nature we must also measure the abundance of these genes in nature.

Streptomycin is the only antibiotic authorized for use in plant agriculture within the EU and Switzerland. Its use is authorized on an annual basis for the prophylactic treatment of apple and pear orchards against the bacterial disease fire blight. Tetracycline is currently the only other viable alternative should resistance to streptomycin emerge in the fire blight pathogen; Erwinia amylovora. We have developed a multiplex qRT-PCR for the relative quantification of streptomycin and tetracycline resistance genes, with the 16S rRNA genes as the endogenous control. Using these multiplex qRT-PCRs we have monitored the abundance of streptomycin and tetracycline resistance genes in streptomycin treated and untreated orchards in 2010 and 2011.

2. Materials and methods
Sample of flowers, leaves and soil were collected from three orchard sites at each time-point (265 samples per year). The time-points consisted of prior to streptomycin spraying, one day after the streptomycin spraying, two weeks after streptomycin spraying and at apple harvest. The DNA extraction method and the relative abundance of resistance genes were detected as previously described [1].
3. Results and discussion
The abundances of \textit{strA} and \textit{strB} genes increased in the flower and leaf samples over time in comparison to the untreated samples in 2010 and 2011. However, the harvest samples contained a similar abundance of \textit{strA} and \textit{strB} genes to the samples prior to streptomycin spraying. There were no streptomycin influenced changes in the abundance of streptomycin resistance genes in the soil samples. The relative abundances of the tetracycline resistance genes \textit{tetB}, \textit{tetM} and \textit{tetW} were not affected by the treatment with streptomycin in the flower, leaf or soil samples. There were low fluctuations within the abundances of the streptomycin and tetracycline resistance genes within the samples isolated from the untreated orchards over time.

4. Conclusions
There were short term increases in the abundances of \textit{strA} and \textit{strB} genes associated with streptomycin treatment in the flower and leaf samples. However, the abundance of these resistance genes returned to pre-treatment level at harvest. Streptomycin treatment did not influence the abundance of streptomycin resistance genes in the soil samples nor did it influence the abundance of tetracycline resistance genes within the orchard samples. Thus, the streptomycin associated increases in resistance gene abundances are temporary.

5. References
1. Introduction

Antimicrobial resistance is a pestering problem, and the solution still remains to be found. Due to a supposed reservoir function of the environment, environmental contamination with antibiotics and/or resistant bacteria is a critical issue [1]. In addition, bacteria or resistance genes are hard to trace, once spread into water or soil. Therefore, it is the more necessary to monitor contaminated effluents before they enter the environment. Selection of antimicrobial resistant strains in the environment is unfortunate; however, main implications for human health should only be seen if such environmental strains i) are spread to humans and ii) are virulent in humans. Therefore, we investigated not only potentially selective factors for antimicrobial resistance in the environment, but, in parallel, the phylogenetic relationship between isolates of humans and pigs. Since resistance genes might spread independent of their original carriers [2], we also investigated the similarity of resistance gene profiles in phylogenetically closely related and distinct E. coli.

2. Materials and methods

Escherichia (E.) coli, enterococci, lactobacilli and clostridia were isolated from pig manure (n = 306) and sewage sludge (n = 111). In addition, E. coli were isolated from human stationary patients (n = 150). Bacterial resistance against 28 antimicrobials was assessed by microdilution following DIN 58940-83 for clostridia and DIN 58940-81 for the other investigated species. Contents of antibiotics (tetracyclines, sulfonamides) in manure were analysed by liquid chromatography-mass spectrometry. Contents of heavy metals were determined by atomic spectroscopic methods. The statistical association of potentially selective factors was investigated in a linear model. Factors analysed in the model were “chemical precipitation” for sewage sludge and “antibiotic contamination” as well as “heavy metal contents” for pig manure. The distribution of antimicrobial resistance genes (aadA, strA, strB, sulI, sulII, tetA, tetB, tetC, tetD, tetM) in E. coli was assessed by endpoint-PCR. The phylogenetic relatedness of the E. coli isolates was investigated by ERIC-PCR.

3. Results and discussion

3.1. Selection of antimicrobial resistance

3.1.1. Chemical precipitation

Chemical precipitation in sewage plants was significantly associated with increased bacterial resistance in sewage sludge. This association was observed for Escherichia coli, Enterococcus faecalis, Enterococcus faecium, Lactobacillus fermentum and Clostridium perfringens. At the same time, chemical precipitation was associated with higher bacterial counts for all investigated genera, except lactobacilli. Higher bacterial counts might be connected to ferric chloride which is regularly used as a chemical precipitant, since iron is an important growth stimulant for most bacteria [3]. Increasing bacterial counts multiply the observed effects of chemical precipitation on antimicrobial resistance, since the absolute number of resistant bacteria increases further.

3.1.2. Antibiotics

In manure samples which were analysed positively for tetracyclines and/or sulfonamides, significantly higher percentages of resistant and multiresistant bacteria were found. Some affected antimicrobial agents are reserve drugs, which are not approved for the treatment of animals. Thus, coselection seems to play a major risk factor for pig manure isolates to develop resistance.
3.1.3. Heavy metals

*E. coli* from manure samples with supermedian contents of copper (> 11.8 mg / kg manure wet weight) were significantly more often resistant against ampicillin and piperacillin, compared to isolates from manure samples with below-median contents of copper. *E. coli* from manure samples with zinc contents > 22.75 mg / kg manure wet weight were significantly more often resistant against ampicillin, doxycycline and piperacillin, compared to samples with zinc contents below 22.75 mg /kg manure wet weight (Fig 1).

3.2. Phylogenetic relation between environmental and human clinical *E. coli* with regard to resistance gene profiles

In general, different phylogenetic groups prevailed among isolates from pig manure and human hospitals. Isolates from sewage sludge resembled partly isolates from pig manure, partly isolates of human hospitals. Isolates of phylogroups B2 and D – which are often associated with human infection – were a rare exception in pig manure. However, isolates of phylogroup B2 were regularly found in sewage sludge.

Two closely (or maybe clonally) related *E. coli* isolates with identic ERIC-patterns were found in a sample of pig manure and a human stationary patient. Despite the close phylogenetic relatedness, both isolates carried different resistance genes. On the contrary, identical resistance gene profiles were present in phylogenetically distinct isolates from humans and pigs.

4. Conclusions

Chemical precipitation, antibiotic contamination, and heavy metal contents are associated with increased antimicrobial resistance of bacterial isolates. While there is a consensus on the need to reduce antimicrobials, copper and zinc are still used as feed additives, and the therapeutic use of zinc is rather increasing. Possibilities to reduce zinc and copper contents in the manure should be critically investigated for feasibility.

Environmental isolates of *E. coli* might be spread to humans. However, distinct gene profiles in clonally related strains indicate a major role of horizontal spread of genes in the distribution of antimicrobial resistance among *E. coli*.

5. References


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Occurrence and dissemination of antibiotic resistance genes in anthropic environments

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1. Introduction
Dissemination of antibiotic resistance genes is recognized to occur in various environmental compartments although it remains difficult to demonstrate in complex environmental matrices. Some environments have been defined as putative hot spots for gene transfer as they can sustain high microbial cell densities and combine both antibiotic and antibiotic resistance bacteria. In this study we compare the occurrence of class 1, 2 and 3 integrons (mobile genetic elements mobilizing different resistance cassettes according to their respective class) from wastewater, sludges, farm slurry and manure. Same or equivalent environmental matrices were also evaluated for their propensity to support the transfer of a model integron-bearing plasmid (pB10).

2. Materials and methods
2.1 Measuring the occurrence of integron
Farmyard manure and farm slurry were sampled from a cattle livestock of the experimental farm La Bouzule (Champenoux, France), and wastewater was collected in the wastewater treatment plant (WWTP) of Limoges. Total genomic DNA from farm samples and WWTP samples were extracted in triplicate from 250 mg of samples using the FastDNA® spin kit for feces (MP Biomedicals) and PowerWater DNA isolation kit (MoBio Lab. Inc), respectively. Class 1, 2 and 3 integrons were quantified by a multiplex qPCR and normalized to the 16S RNA-encoding DNA gene copy number so as to estimate the quantities of integrons per bacteria, according to Barraud et al., 2010 [1].

2.2 Evaluating the dissemination of plasmid pB10
Environmental matrices, sampled at farm La Bouzule or various WWTP, were inoculated with the donor bacteria Escherichia coli DH5α(pB10), and maintained in microcosms for one week. Aliquots were sampled daily and total DNA was extracted according to Bonot et al. (2010) [2]. DNA form the donor bacteria and the plasmid were quantified by qPCR and normalized to the 16S RNA-encoding DNA gene copy number so as to estimate the quantities of integrons per bacteria, according to Bonard et al., 2010 [1].

3. Results and discussion
3.1. Occurrence of integron
In farm samples, concentrations of class 1 and class 2 integrons were close to 10⁶ copies per gram of dry matter, while no class 3 integron could be detected. Prevalence of integrons was significantly more important in the farmyard manure than in the slurry. Concerning farmyard manure, prevalence of class 1 and 2 integron were equivalent (about 0.03 integrons per bacteria), as oppose to slurry where prevalence of class 1 integrons was 5 times higher (0.015) than class 2 integrons (0.003).
In WWTP influents, quantities of class 1 integrons were the most important ($10^{10}$-10$^{11}$ copies.L$^{-1}$) followed by class 3 integrons ($10^{8}$-10$^{10}$) and class 2 ($10^{8}$-10$^{9}$). Comparing influent and effluent we could show that WWTPs reduced the concentrations of integrons and bacteria by 2 log. Nonetheless, prevalence of class 1 integrons were not reduced (0.05-0.2 integrons per bacteria) as oppose to class 2 and class 3 integrons which significantly decreased by a 3 to 4-fold factor.

### 3.2. Dissemination of pB10 in complex environmental matrices

Plasmid pB10 did not appear to disseminate in manure microcosms from the Bouzule farm while maintaining steadily over the course of the experiment. In aerated activated sludge microcosms, pB10 did not persist because of an apparent loss of the donor bacteria. Nevertheless, the dissemination of the plasmid appeared as an increasing plasmid to donor ratio in microcosms setup with sludge from anaerobic digesters or fixed biofilm reactors stressing the importance of the sludge origin in the dissemination observed [4].

### 4. Conclusions

Integrons appeared to prevail in WWTP environment compared to farm samples. Less diversity was observed among integrons from farm samples (no class 3 integrons), signifying that a range of resistance cassette was not significantly represented. This could reflect a more homogenous anthropic selective pressure on antibiotic resistances. On a dynamic point of view, the transfer of the model plasmid pB10 could not be demonstrated in farm microcosms while it was seen in WWTP sludge microcosms. In the latter case transfer of pB10 occurred only under particular circumstances, anaerobic sludge and aerated biofilm, highlighting the role of the microbial community origin associated with a given WWTP process.

### 5. References


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Detecting evolutionary hot spots of antibiotic resistances in Europe (DARE) (COST Action TD0803)

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1. Introduction

Public and scientific news have repeatedly stated that bacterial resistance to antibiotics emerges as a major public-health problem. Infectious diseases cause about 13 millions deaths each year. This high mortality will increase as long as the evolution of new antibiotic resistances remains at the current rate. The key factor for the evolution of new antibiotic resistances is the ability of infectious organisms to adapt quickly to new environmental conditions. In addition, microbes can interchange genes, what has been named the shared genome, including genes that confer resistance to antibiotics.

Antibiotic resistances (ARs) are a great threat to human health, which has meant that research in this area has focused primarily on their role within clinical settings. This is in strong contrast to the research in nonclinical environments, such as the natural and urban environment, where this research has only recently received more attention. However, most antibiotics used for treatment in clinics are the product of environmental microorganisms. This strongly suggests that genes for antibiotic resistance must also have emerged in non-clinical habitats [1,2].

Here the first results of a European wide network COST Action are presented. The Action consists of 84 members of 18 European Nations and Israel which try to identify the gaps of knowledge, which need to be investigated in order to propose interventions to curb the spread of AR evolution and AR microbes within the environment [3].

2. Results and discussion

The members of the Action consist mainly of the following disciplines: Clinical research, animal culture, microbiology, evolutionary biology, ecology, bioinformatics, chemistry and civil engineering. The scientists of all these disciplines agreed that the most relevant hot spots for gene transfer and evolution of new ARs are the wastewater treatment plants (WWTPs) and animal farming where direct animal human contact occurs. Furthermore, the members agreed that in order to reach scientific breakthroughs it is pivotal to identify the most important organisms and antibiotic resistance genes (ARG), which will foster the evolution of new antibiotic resistances.

The Action decided on criteria, which should be used to identify the most relevant antibiotic resistances (3rd and 4th generation antibiotic) and accordingly the most relevant antibiotics. The AR gene should be mobile between genomes, the AR gene should exist at a detectable frequency within a bacterial community, The AR gene should show persistence within a bacterial community, The AR gene should code for “new” antibiotics.
According to these criteria the Action selected the following antibiotics (and resistances) as most relevant:

-β-lactams, Cephalosporins; Carbapenems, -vancomycin, macrolides, fluoroquinolones, aminoglycosidases.

Furthermore, the Action identified the following organisms as most relevant for the detection of gene transfer and antibiotic resistances within the environment. Also, the organisms under focus should be those which might act as carrier of ARGs, moving ARGs between clinics via the environment to pathogens or in a similar matter (WWTP, wastewater treatment plant, AH animal human contact):

Aeromonas (WWTP, AH), Enterococcus (WWTP,AH), Pseudomonas (WWTP), E. Coli (WWTP, AH), Acidenobacter (WWTP), Vibrio (AH), Salmonella (WWTP,AH), Staphylococcus MSRA (AH).

Last but not least the Action developed a sampling protocol, which should be employed in order to obtain a first geographic pattern on AR resistance in Europe, Such an epidemiological base information exists already for clinical and veterinary data, but is missing for environmental data.

In order to assess the risk of antibiotic resistance evolution a new approach of risk assessment is needed. The traditional risk assessment as usually employed by ecotoxicologists for pharmaceuticals or toxic chemicals cannot be applied. This is mainly due to the fact that antibiotics within the environment do not have primarily a lethal effect, they rather act as a katalysator for gene transfer and the evolution and rearrangement of resistance genes within the environment. In summary the Action has identified prioritized the need of investigations and corresponding funds. These are urgently called for as for example no systems exist which will allow to assess the risk of spread or evolution of new antibiotic resistances, although the requirements have become very clear.

3. Conclusions

The Action has invested considerable time and effort in order to identify current gaps of knowledge and has developed a priorisation of necessary knowledge to fill this gap as fast and efficiently as possible. In order to combat the evolution of new resistances it is necessary to obtain this necessary base knowledge. Only then will it be possible to make any reliable predictions for a risk assessment, on the effectiveness of current antibiotics and only then will it be possible to suggest appropriate measures, which should be implemented in effective rules and regulations for a national or European scale.

4. References


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Selective pressure of antibiotic pollution on bacteria of importance to public health

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1. Introduction

The occurrence of antibiotics in natural environments may favor the development and spread of antibiotic resistance. From an environmental health perspective, the selective pressure that antibiotic pollution may exert on bacteria of clinical importance is of particular concern. Several clinically relevant bacteria, such as E. coli and the enterococci, for example, occur and are able to grow in different environments (Topp et al. 2003; Moriarty et al. 2008), where in the presence of environmental concentrations of antibiotics they may face a selective pressure leading to a gradual increase in the prevalence of resistance.

In this study we use bacterial species sensitivity distributions (SSDs) derived from a comprehensive set of minimum inhibitory concentration (MIC) distributions of antibiotics to model bacterial sensitivities and characterize the selective pressure that antibiotic pollution may exert on bacteria of importance to public health in the environment. This is done under the premise that antibiotics will primarily increase the prevalence of resistance by favoring the selection of resistant phenotypes via the inhibition of sensitive ones. Although there is evidence to suggest that sub-inhibitory concentrations of antibiotics may indirectly favor resistance (Hoffman et al. 2005), the use of bacterial inhibition as an assessment endpoint provides a standardized response across taxa that can be directly linked to a selective pressure favoring an increase in the prevalence of resistance.

2. Materials and methods

MIC distributions for ciprofloxacin, erythromycin and tetracycline were obtained from the European Committee on Antimicrobial Susceptibility Testing (EUCAST) MIC and Zone diameter distribution website (http://www.srga.org/eucastwt/wt_eucast.htm; accessed November 2010). Bacteria represented in these MIC distributions were placed in a wider phylogenetic context by conducting a brief phylogenetic analysis. Mantel correlograms were used to explore the correlation between evolutionary distances and pairwise differences in median MICs between taxa for each antibiotic dataset. Only genera for which there was evidence to suggest that under certain conditions they could grow in an environmental compartment were used to derive SSDs.

SSDs were derived by bootstrap regression (Grist et al. 2002) using two endpoints from the MIC distribution of each species. These endpoints were representative of the median sensitivity of the wild-type population (MIC50) and the lower limit of antibiotic sensitivity (NOEC), respectively. To each of 5000 bootstrap resamples a log-logistic model was fitted by maximum likelihood estimation of the distribution parameters and direct optimization of the log-likelihood function. The distribution parameters were used to get replicate estimates of antibiotic concentrations associated to a potentially affected fraction (PAF) between percentiles 0.01 and 0.99 at 0.01 step intervals. From these, the bootstrap estimate and 95% bootstrap confidence intervals were calculated.

We determined the PAF of bacterial genera by all three antibiotics at the aquatic and soil VICH phase I action limits (International Cooperation on Harmonization of Technical Requirements for Registration of Veterinary Products [VICH] 2002) and at measured environmental concentrations (MEC). This was complemented with a direct comparison of MEC and VICH action limits with the wild-type cutoff value (COwT) of species represented in the MIC distributions of each antibiotic. Although ciprofloxacin is not used in veterinary medicine, it is the major active metabolite of enrofloxacin in different species, and it was used as a representative of the fluoroquinolones when comparing PAFs with VICH phase I action limits.

3. Results and discussion

3.1. Inhibitory effects at environmental concentrations

Upper 95% bootstrap confidence intervals indicate that concentrations of ciprofloxacin, erythromycin and tetracycline measured in river sediments are ≥ the MIC50 of 49%, 4% and 3% of the bacterial genera, respectively. Concentrations of these three antibiotics in the sediments of a swine feces lagoon are
estimated to be ≥ the MIC$_{50}$ of 84%, 13% and 47% of the bacterial genera. Concentrations of tetracycline measured in farmed soil are ≥ the MIC$_{50}$ of 23% of genera. MECs of ciprofloxacin in swine feces lagoon sediment are above the CO$_{WT}$ of 14 bacterial taxa belonging to 8 genera of predominantly enteric bacteria (Figure 1). Concentrations of tetracycline measured in liquid manure are above the CO$_{WT}$ of all but one bacterial taxa and concentrations measured in swine feces lagoon sediments are borderline with the CO$_{WT}$ of Staphylococcus and Streptococcus (Figure 1). MECs of erythromycin are below the CO$_{WT}$ of all taxa (Figure 1).

![Figure 1](image_url)

**Figure 1. Minimum inhibitory concentrations ≥ the CO$_{WT}$ for bacterial taxa in the MIC datasets of ciprofloxacin (a), erythromycin (b) and tetracycline (c). Colored symbols represent the CO$_{WT}$ in different genera. Dashed lines extend up to the maximum MIC beyond the CO$_{WT}$. Colored horizontal lines represent antibiotic concentrations as defined in the legend. The x-axis is an index representing the number of bacterial taxa in each dataset.**

3.2. Inhibitory effects at VICH phase I action limits

PAFs at the VICH phase I aquatic action limit suggest that it is protective of any major inhibitory effects on bacteria, although a minority of sensitive individuals could be inhibited in up to 10% of genera (i.e., upper 95% confidence interval in ciprofloxacin NOEC SSD). Concentrations of ciprofloxacin and erythromycin at the VICH phase I soil action limit, on the other hand, are estimated to be ≥ the MIC$_{50}$ of 65 and 15% of bacterial genera, respectively. The VICH phase I soil action limit is below the erythromycin and tetracycline CO$_{WT}$ of all species, indicating that at this concentration these antibiotics are not expected to inhibit 100% of the wild-type population in any species. The ciprofloxacin MIC distributions (Figure 1), on the other hand, show that the VICH phase I soil action limit is above the CO$_{WT}$ of 5 bacterial taxa and borderline with that of 9 other taxa.

4. Conclusions

Our results indicate that concentrations of antibiotics measured in different environments due to their use in human and veterinary medicine and used as action limits in ERA may be high enough to exert a significant selective pressure on bacteria of importance to public health. The PAF of bacterial genera at concentrations of antibiotics measured in river sediments, liquid manure and farmed soil suggest that these environments are likely to be hot-spots for the development of antibiotic resistance. Antibiotic resistance hampers the effective treatment of infectious diseases and its emergence in the clinic, the community and the environment needs to be minimized. The explicit consideration of antibiotic resistance in the ERA of antibiotics along with efforts to reduce the input of antibiotics into the environment are crucial to maintain background resistance levels.

5. References

Monitoring and management of antibiotic resistance of veterinary drugs in Germany

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Background

The use of antibiotic drugs in veterinary therapy results in a target conflict of demand for therapy versus antimicrobial resistance. Advantages such as to secure animal health of companion and farm animals, avoidance of economical loses in animal husbandry, supporting animal welfare and prevention of bacterial zoonoses which threat human health are opposed by the risk of the enhancing antimicrobial resistance. Indeed, every use of antimicrobials may lead to the development or promotion of resistances in animals and is also susceptible to affect security of human health.

Factors supporting the development of resistance are: Missing background information, therapy of whole herds, frequent use of broad-spectrum antibiotics, sub-therapeutical dosing, inadequate duration of therapy, prophylaxis, inadequate metaphylaxis, prioritization of economical interests, missing antibiograms to adjust therapy, application in case of trivial infections, lacking sense of responsibility and other factors such as resistance development in the environment, plasmid transfer of resistance genes or import of resistance from foreign husbandries.

In theory, a de-escalating therapy should be chosen to fight bacterial diseases. Such strategy implies an adjustment to a better targeted therapy at the arrival of diagnostic findings. In reality, often a broad and expensive initial therapy is initiated and continued (“never change a winning team”) without adjustment despite sensitive pathogens.

A management approach to minimize the risk of antibiotic resistance development is the responsible use of antimicrobials on the basis of the „ONE HEALTH Principle“ (animals + humans = one health) of the WHO and EU (animal health strategy 2007-2013). This strategy needs information on the use of antibiotics and information on the current status and development of antimicrobial resistance.

Monitoring and Management of antibiotic resistance in Germany

This information is obtained from monitoring programs conducted and improved in Germany for more than 10 years.

These include:
- National resistance monitoring - animal pathogens
- National food monitoring program
- Monitoring of resistance for zoonosis germs
- National Monitoring of resistance for commensal germs

Additional information is obtained from the collection of postmarketing data (ADRs, PSURs etc.) and since 2011 from data of antibiotic disposal to be registered by law.

The German National Antimicrobial Resistance Monitoring

The German National Antimicrobial Resistance Monitoring (GermVet) is conducted since 2001 performed as annual multicenter studies. It uses isolates from diseased animals for both livestock and companion animals taken from representative random sampling. Additionally, samples from inconspicuous animals and food of animal origin are investigated. The regional distribution of the random sampling is in accordance with the livestock density of the federal states. The study design includes the choice of bacterial isolates (random sampling plan), no determination of "copy strains", no antimicrobial pre-treatment and acquisition of "meta-data, (additional information to MIC-data). Bouillon-Microdilution is used as a quantitative method for sensitivity testing. Evaluation of results is performed applying clinical breakpoints according to CLSI (M31-A3) making results comparable to human resistance studies.
Results from the monitoring program

Current results from the monitoring indicate that the situation of resistance is divers between farm and companion animals. Resistances are frequently detected but therapy is still secured. Thus, mastitis-pathogens from diary show only low resistance rates. No vancomycin-resistant Enterococcus spp. has been identified so far. High resistance rates are observed with calves, but are getting lost with age. ESBL (Extended Spectrum β-Lactamases) resistance may pose problems more serious than MRSA in the future but for mastitis ESBL resistance has not been found with Klebsiella spp. so far. To date, good susceptibility is observed with pathogens from respiratory diseases. The results of GermVet are also compiled together with German resistance data from human medicine the GERMAP 2010 report which is available online at http://www.p-e-g.org/econtext/germap.

Risk management mitigation measures for antibiotic resistance

Mitigation measures for antibiotic resistance comprise restriction of indications, setting of additional requirements for application, setting of maximum residue limits (MRLs) and withdrawal times, elaboration of guidelines for the prudent use of antibiotics and as overall strategy the so-called national action plan for antibiotic resistance (DART) of the German Government.

Examples for minimisation measures are those for tetracycline containing veterinary drugs in 1997 including the ban of prophylactic application (in the meantime banned for all antibiotic uses in veterinary medicine), limitation of indications and pre-therapy preparation of an antibiogram. Restriction of indication (no application with trifle infections) was stipulated in 1998 for enrofloxacin. In the same year, additional requirements such as the investigation of resistance data became obligatory for danofloxacin. In 2010 and 2011 EU Commission referrals (RL 2004/28, Art. 35 Referral) restricted the uses of fluoroquinolones and cephalosporines (3rd and 4th generation) which are now only to be used as so called „second-line“ antibiotics including preparation of antibiograms, prudent use according to antibiotic guidelines and no use in case of trifle infections.
Zebrafish eleutheroembryos provides a suitable vertebrate model for screening thyroid gland disrupting chemicals

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1. Introduction

The finding that even mild hypothyroxinemia early in pregnancy can produce irreversible neurological effects in the offspring has raised concern about some widely distributed chemicals, collectively called thyroid gland function disruptors, impairing the synthesis of thyroid hormone. Consequently, there is a need for the development of simple methodologies suitable not only for screening the potential goitrogenic effect of chemicals but also for estimating the potential hazards associated with exposure to individual chemicals and mixtures impairing thyroid function.

Recently, we developed a simple, rapid zebrafish eleutheroembryo bioassay for assessing the potential of chemical pollutants and drugs to disrupt thyroid gland function [1]. This bioassay used a thyroxine immunofluorescence quantitative disruption test (TIQDT) to measure impairment of the thyroid function as a decrease in the intrafollicular T4-content (IT4C). Under the EU directive on the protection of animals used for scientific purposes, the TIQDT assay is an alternative method compliant with the 3R principles (relative replacement of animal tests).

The purpose of this study was to assess (1) the suitability of TIQDT on zebrafish eleutheroembryos as a predictive vertebrate model of thyroid gland function in Tier 1 screening batteries for detecting molecules that impair TH synthesis; and (2) the use of TIQDT for quantitative estimation of the thyroid disrupting potency and hazard of chemicals, which would provide a useful source of information for risk assessment. Thyroid gland functionality was evaluated with TIQDT after exposure to 25 selected molecules by measuring IT4C in over 4700 individual zebrafish eleutheroembryos.

2. Materials and methods

Thyroxine immunofluorescence quantitative disruption test (TIQDT) on zebrafish prefeeding larvae was used to evaluate the effect of single and combined chemicals on thyroid gland functionality. Whole-mount in situ hybridization (WISH) was used to analyze the effect of chemicals on the level of transcript of target genes in

Figure 1: Screening of thyroid gland function activity of chemicals and drugs using TIQDT. Red bars indicate thyroid gland function disruptors, yellow bars are false positives and green bars indicate compounds with no effect on the thyroid function. Chemicals with no bars (Amitrol-ETU) were thyroid gland function disruptors inducing a total abolition of the IT4C.
the thyroid follicular cells. Initially, chemicals were screened at the maximum tolerated concentration. Finally, concentration–response for the effect on the thyroid gland function and the survival were performed for selected thyroid gland function disruptors.

3. Results and discussion

This study demonstrated that zebrafish eleutheroembryos provided a suitable vertebrate model, not only for screening the potential thyroid disrupting effect of molecules, but also for estimating the potential hazards associated with exposure to chemicals directly impairing thyroxine (T4) synthesis. Amitrole, potassium perchlorate, potassium thiocyanate, methimazole (MMI), phloroglucinol, 6-propyl-2-thiouracil, ethylenethiourea, benzophenone-2, resorcinol, pyrazole, sulfamethoxazole, sodium bromide, mancozeb, and genistein were classified as thyroid gland function disruptors (Figure 1). Concordance between TIQDT on zebrafish and mammalian published data was very high for those chemicals with a direct-effect on the thyroid gland function and the physiological relevance of T4-intrafollicular content was clearly higher than regulation at the transcriptional level of \(tg\) or \(slc5a5\). Moreover, concentration-response analysis provided information about the thyroid disrupting potency and hazard of selected positive compounds (Figure 2). Finally, the effect of perchlorate, but not MMI, was completely rescued by low-micromolar amounts of iodide.

![Figure 2: TIQDT provides information about thyroid disrupting potency (EC50) and hazard (Thyroid Disrupting Index, TDI: LC50/EC50) of chemicals and drugs.](image)

4. Conclusions

TIQDT performed on zebrafish eleutheroembryos is an alternative whole-organism screening assay that provides relevant information for environmental and human risk assessments.

5. References


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All mixed up: Phenotypic plasticity in a genotypic world

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1. Introduction

Xenopus laevis has a ZW system of genetic sex determination with female heterogamety. Until recently, it was not possible to distinguish between genetic male and female X. laevis. The discovery of DM-W, a female-specific sex-linked gene located on the W chromosome, now allows molecular determination of genetic sex, or genotype, in X. laevis [1]. Many amphibians, including X. laevis, are sensitive to exposure to steroid hormones near the time of sexual determination and differentiation. Exposure to adequate doses of sufficiently potent estrogens during this sensitive period causes disordered sexual development and male-to-female phenotypic sex reversal of genetic males [2]. However, because there was historically no way to assign genetic sex, conclusions about the effects of estrogens on sexual development of X. laevis have been drawn only after lengthy grow out and back-breeding experiments [2] or inferred based on altered sex ratios in treated groups [3].

The current study was designed to utilize a model estrogen, 17α-ethynylestradiol (EE2), to cause disordered sexual development in X. laevis, and to identify the genetic sex of impacted animals by presence or absence the sex-linked gene DM-W. Based on results previously published in the literature, the concentrations of EE2 used in the study, 0.1, 1, or 10 µg EE2/L, were expected to cause low, medium, and severe effects (sex reversal), respectively. Because the concentrations were chosen based upon their ability to elicit phenotypic effects, they are not in the range of environmentally relevant concentrations.

2. Materials and methods

During the 96 d exposure, animals were monitored daily to determine time to metamorphosis. At exposure termination, tissue samples were removed and gross phenotypic morphology of the gonads was determined by use of a dissecting microscope. Gonads were preserved in formalin, and then processed for histological sectioning and hematoxylin and eosin staining. Histological phenotype was examined, and individuals were classified as male, female, intersex, or abnormal male (Figure 1). Some individuals from the EE2 treatments exhibited abnormal amounts of a proteinaceous fluid that was hypothesized to be vitellogenin in and around the kidneys and gonads. For this reason, an immunoassay to identify this protein as vitellogenin was developed by use of standard immunohistochemistry protocols and a sea bream anti-vitellogenin antibody. A genomic DNA sample isolated from the leg of each froglet was used to determine genetic sex as per the multiplex PCR assay detailed by Yoshimoto et al. [1].

![Figure 1: Histological sections of gonads exhibiting the four categories of phenotypic sex observed](image-url)
3. Results and discussion

3.1. Time to metamorphosis

The average number of days required to reach metamorphosis ranged from 65 d in control animals to 88 d in animals grown in water which contained 10 µg EE2/L. Metamorphosis of individuals in all of the EE2 treatments was significantly delayed compared to control and solvent controls (Figure 2). Delays to metamorphosis in various species of amphibians after exposure to sufficiently potent estrogens has been observed prior to the current study [4,5]. The mechanism(s) by which estrogenic substances inhibit larval development is not clear [4], but immunohistochemical data gathered showed that many individuals were over-expressing the vitellogenin protein, which is a plausible cause for delayed metamorphosis. We hypothesize that frogs exposed to EE2 were partitioning energy resources needed to successfully complete metamorphosis into manufacturing an unnecessary protein and failed to metamorph in a timely manner.

Figure 2: Average number of days required to reach metamorphic climax for X. laevis exposed to EE2

3.2. Genotypic and phenotypic sex ratios

The overall M:F genetic sex ratio for the current experiment was 49:51. Phenotypic sex ratios were significantly different from controls in all EE2 treatments, with differences due to altered development of animals with male genotypes. When exposed to 0.1 or 1 µg EE2/L, a majority (72%) of genetic male animals in both treatments were classified as phenotypic abnormal males, 11 and 22%, respectively, were intersex, and 17 and 7%, respectively, were normal males. When exposed to 10 µg EE2/L, 42% of genetic males developed as abnormal males, 8% were abnormal males, 33% were intersex, and 16% developed as phenotypic females. The exact mechanisms responsible for sexual differentiation in X. laevis are not completely clear but the process probably involves a gene cascade orchestrated by the sex-linked gene DM-W. Endogenous estrogens do not seem to play a major role in sexual differentiation [6], and exposure to exogenous estrogens causes sex reversal by inhibiting normal processes in male development. Near the time of sexual differentiation, the primordial germ cells of animals with a male genotype would normally begin to migrate from the cortex to the medulla of the primordial gonad, but treatment with estradiol inhibits this response in a dose-dependent manner [7]. These demasculinizing effects are probably due to the ability of tadpoles at this stage of development to respond to hormones via receptors present on primordial germ cells, even though they do not normally express great concentrations of endogenous hormones.

4. References

Androgen-induced kidney hypertrophy in the European bullhead (Cottus sp.): a potential biomarker of androgen exposure

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1. Introduction

Some studies have demonstrated the presence of androgenic pollutants in continental and estuarine aquatic ecosystem and the exposure of organisms that live there through the use of biomarkers ([1, 2]). It is relevant to develop tools to assess effects of androgens in wild fish. To assess the early effects of contamination of aquatic environments in fish, the European bullhead (Cottus sp.) appears as a relevant model. Previous studies have demonstrated the presence in the kidney of a syaloglycoprotein comparable to the spiggin in stickleback ([3, 4]). Thus, this species is likely to have biomarkers of androgenicity. In addition, its use in a biomonitoring context is relevant. This benthic fish is widely distributed in clean and contaminated continental European aquatic ecosystems.

The present work was designed to identify an androgeno-regulated signal in the kidney of the European bullhead (Cottus sp.) and to characterise response patterns prior to laboratory and field applications to address androgenic potential of chemicals.

2. Materials and methods

In a first step, wild bullheads were electrofished in a reference French river during breeding and non breeding season. Observation of microscopic sections of gonads allowed to confirm the sex of the fish and to check reproductive status. Analysis of histological sections of kidney allowed the calculation of the Kidney Epithelium Height (KEH) as described in the stickleback.

In order to check if the hypertrophy of the nephtics channels could be specifically inducible by androgens in bullheads, bullheads from the field outside the breeding season were exposed during 14 or 21 days to different concentrations of androgens (0.5 or 5 µg/L of Trenbolone (Tb), 1 or 10 µg/L of 11-ketotestosterone (11-KT), 0.5 or 5 µg/L of Spironolactone (Sp), 0.5, 5 or 50 µg/L of dihydrotestosterone (DHT)), estrogen (0.05 or 0.5 µg/L of Ethinyl-estradiol (EE2)) or heavy metal (1 or 10 µg/L of Cadmium (Cd)) dissolved in dimethyl sulfoxide (DMSO 0.001% per aquarium) or only to the solvent for the controls. After exposure, gender and reproductive status were determined and KEH was calculated.

To characterise the potential of KEH in bullhead in field experiments, 20 wild adult fish were electrofished out the breeding period, in 9 streams located in the North of France and characterized by various contamination levels. After sampling, gender and reproductive status were determined and KEH was calculated.

3. Results

3.1 KEH validation in bullhead
During breeding period, male bullheads exhibited an increased KEH value of 20.96 ± 3.57 µm compared to female (16.96 ± 1.28 µm). No gender difference was recorded in non-breeding bullheads.

3.2 Characterization of the KEH method in monitoring conditions and in the field

Results showed that androgens are able to induce kidney hypertrophy. Trenbolone induced a dose-dependent hypertrophy (KEH= 16.59 ± 0.81 µm at 0.5 µg/L and 21.01 ± 1.76 µm at 5 µg/L vs 15.58 ± 0.85 µm for the controls) as the higher concentration of 11-KT (KEH = 17.55 ± 1.48 µm at 10 µg/L) and DHT (17.43 ± 1.77 µm at 50 µg/L). SPI and Cd have no effect on kidney ultrastructure. In bullheads exposed to EE2, a trend to decrease of KEH was observed (data not shown).

In wild bullhead, the response of KEH don’t allow to discriminate sites since the KEH mean is 16.03 ± 1.25 µm for the references sites and 16.17 ± 1.08 µm for the contaminated sites (data not shown).

4. Conclusion

This work highlights an androgen-regulated signal in the kidney of the European bullhead. However, kidney hypertrophy appears as few sensitive and time consuming indicators. Also, identification of associated protein and/or RNA modifications could provide a relevant biomarker of androgen exposure.

5. References


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Endocrine disrupting compounds as potential obesogens: musk compounds as a case study

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1. Introduction

Since the discovery of leptin and other adipokines, it became clear that the adipose tissue is not only a storage place for excessive fat but a real endocrine organ, making it possibly sensitive to endocrine disrupting compounds (EDCs). Recently 'the environmental obesogen hypothesis' stated that environmental pollutants such as EDCs could play a role during the development of metabolic diseases such as obesity1, 2, broadening the endocrine disrupting concept to a more physiological disruption concept including endocrine, neural and metabolic disruption. This hypothesis could, together with an increased caloric intake, a sedentary lifestyle and genetic predisposition, give potential complementary explanation to the recent epidemic proportions of obese and diabetic patients.

Musk compounds are a group of synthetic EDCs used in a variety of personal care products and therefore often daily used. Synthetic musks enter the systemic circulation mainly through dermal absorption and have been detected in human adipose tissue due to their high lipophilicity (Log kow = ± 5)3, 4, 5, 6. Musk compounds can be divided into three different classes based on their structure: i) nitromusk fragrances and ii) polycyclic and iii) macrocyclic musk compounds.

During this study we evaluated the potential obesogenic properties of three musk compounds belonging to the different classes of musks: the nitromusk Musk xylene; the polycyclic musk Tonalide® and the macrocyclic musk Ethylene Brassylate.

2. Materials and methods

3T3-L1 cell line
3T3-L1 cells (American Type Culture Collection, Manassas, VA) were maintained in standard medium as described previously7 by Masuno et al. (2005).

For differentiation assays, cells were seeded into 6- or 24-well plates for RNA extraction and Adipored staining respectively, and grown until confluence. Standard medium of 2 days post-confluent cells was replaced by mature medium containing Foetal Bovine Serum instead of Newborn Calf Serum and test compounds were added in a concentration gradient. As a positive control, 2 days post-confluent cells (day 0) were stimulated for 48h in mature medium containing an MDI hormonal cocktail (0.5 mM isobutyl methylxanthine, 0.25 µM dexamethasone and 10 µg/mL insulin) and another 8 days in mature medium containing only insulin. Effects on differentiation were evaluated at day 10.

- **Intracellular lipid measurements: Adipored staining**
  On day 10, the intracellular lipid content was measured by performing an Adipored Assay (Lonza, Walkersville, MD) following the manufacturer's indications.
- **RNA extraction and gene expression analysis using real-time polymerase chain reaction**
  RNA extraction was performed using RNeasy kit form Qiagen, according to manufacturer's instructions. A starting amount of 1 µg RNA was transcribed to first strand cDNA according to Revert Aid TM H Minus First strand cDNA synthesis kit for RT-PCR (Fermentas). Real-time PCR reaction master mix was used following the manufacturer's instructions (Brilliant® II SYBR® Green QPCR mastermix, Agilent Technologies, Santa Clara, CA). According to the equation of Pfafff the expression values of the target gene adipocyte specific protein 2 (aP2) were normalized by a comparison to the internal control gene TATA binding protein (TBP), resulting in relative expression ratios. Comparison of several household genes (18S, RPLP0 and TBP) favoured TBP because of its stable expression during differentiation and different exposure conditions.
3. Results and discussion

3.1. Adipored screening of Musk compounds

The test compounds were evaluated for their potential to induce adipogenesis using the 3T3-L1 cell line, a model in vitro cell system for the study of adipogenesis. These cells are fibroblastic and can differentiate into adipocytes after a ten-day exposure with an adipogenic cocktail (isobutylmetylxanthin, dexamethasone and insulin; MDI). We have tested the effect of musk compounds on the induction of differentiation using the Adipored assay. This fluorescent staining enables the quantification of the lipid droplets associated with the phenotype of mature adipocytes. Musk xylene and Ethylene Brassylate didn’t induce the differentiation of the 3T3-L1 adipocytes. However, Tonalide® induced a dose-dependent increase of lipid droplet formation after ten days of exposure (Figure 1A).

3.2. Gene expression

To further confirm the effect of Tonalide® on the differentiation of adipocytes, the expression of an adipocyte specific marker gene adipocyte specific protein 2 (aP2) was measured. The cells were exposed at three different concentrations of Tonalide® and RNA was extracted at three different time-points during differentiation. Tonalide induced a time-dependent increase of the aP2 expression during differentiation (Figure 1B). A more extended dose response in the lower concentration range will be performed to further confirm these results.

![Figure 1](https://example.com/figure1.png)

Figure 1. Concentration- and time-dependent induction of 3T3-L1 cell differentiation by the musk Tonalide®. A. Dose response relation of lipid content (Adipored Assay) in 3T3-L1 cells exposed to Tonalide during 10 days. B. Dose and time response relation of aP2 expression after Tonalide® exposure. Data are represented as mean values of three independent experiments and analyzed for significant differences compared to solvent control (One Way ANOVA, *p≤0.05; **p≤0.01; ***p≤0.001).

4. Conclusions & Future work

The Adipored assay as well as the expression of the adipocyte specific marker gene aP2 show that Tonalide® is the only musk compound, from the three tested, inducing the differentiation of adipocytes in vitro.

In the next step, microarray analysis and RNAi technology will be applied to unravel the adipogenic mechanisms of Tonalide®.

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5. References

[3.] Kannan K, et al. (2005); Polycyclic musk compounds in higher trophic level aquatic organisms and humans from the United States Chemosphere. 61 (5): 693-700.
[6.] Moon HB, et al. (2011); Occurrence and accumulation patterns of polycyclic aromatic hydrocarbons and synthetic musk compounds in adipose tissues of Korean females Chemosphere.
[7.] Masuno H, et al. (2005); Bisphenol a accelerates terminal differentiation of 3T3-L1 cells into adipocytes through the phosphatidylinositol 3-kinase pathway Toxicol Sci. 84 (2): 319-27.
[8.] Pfaffl MW. (2001); A new mathematical model for relative quantification in real-time RT-PCR Nucleic Acids Res. 29 (9): e45.
Development of gene expression biomarkers in cetaceans skin biopsies exposed to bisphenol A (BPA) and perfluorooctanoic acid (PFOA): new tools for emerging contaminants assessment

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1. Introduction

Emerging contaminants in marine wildlife are poorly investigated, in particular in those species considered at risk as cetaceans. Currently, one of the main toxicological issues, in the management and conservation of the marine environment, is the study of the potential impact of compounds released from plastics, such as bisphenol A (BPA) and industrial derivates such as perfluorooctanoic acid (PFOA). On this regard, the assessment of toxicological risk in wildlife requires the development of sensitive biomarkers, including those based on the use of in vitro systems. BPA is one of the most distributed compounds in the world, both in the aquatic and terrestrial ecosystems [1] and the EPA has already defined it as endocrine disrupting chemical since act as agonist or antagonist for endocrine receptors [2]. The perfluorinated compounds are used as surfactant and in the surface treatments, they are persistent and it has been shown that they can act as endocrine disruptors as well.

To develop new gene expression biomarkers in cetaceans, in order to investigate biological response to these two emerging compounds, we exposed skin biopsies of three odontocetes species (sperm whale, killer whale, and bottlenose dolphin) to BPA and PFOA. We selected two potential biomarker genes such as the peroxisome proliferator-activated receptors α and γ (PPARα and PPARγ). The PPARs belong to a superfamily of ligand-dependent nuclear receptor (PPARα, β and γ) which regulates physiological processes of lipids homeostasis, inflammation, adipogenesis, reproduction, etc. [3]. PPARα and PPARγ seem to be modulated by the presence of BPA and PFOA respectively.

2. Material and Methods

2.1. Slices

Subsamples of skin biopsies from sperm whale (Physeter macrocephalus), killer whale (Orcinus Orca), and bottlenose dolphin (Tursiops truncatus) were exposed for 24 h to increasing concentration of BPA (0,1 μg/ml, 1 μg/ml, 10 μg/ml e 100 μg/ml) and PFOA (0,1 μg/ml e 1 μg/ml) plus vehicle (ethanol for BPA and methanol for PFOA) as experimental control.

2.2. PPARα and PPARγ gene sequencing

Total RNA was isolated from slices, the RNA reverse transcribed in cDNA and the partial coding sequence of the two genes of interest were sequenced in the three species after specific primers design.

2.3. Gene Expression Assays

After the selection of several primer pairs designed on the three species sequences for both genes, quantitative Real-Time PCR assays were performed to quantify PPARα, PPARγ, and also other two genes of interest such as estrogen receptor alpha (ERα) and E2F1 transcription factor (E2F1) mRNA levels previously developed [4]. Gene expression was normalized to two reference genes: YWHAZ and GAPDH [5] and data analyzed using ΔΔCt method [6].

3. Results and discussion

The two genes of interest (PPARα, PPARγ) were sequenced in three odontocetes species (sperm whale, killer whale, and bottlenose dolphin). The mRNA levels were quantified in response to the two different treatments in the treated slice samples. All the four genes (PPARα, PPARγ, Erα, E2F1) are modulated by the treatments in all the three species. In particular, the results of this set of experiments, revealed that the BPA treatments up-regulate the expression of the genes PPARα and PPARγ showing a dose-response...
Increasing the BPA concentration increases the bottlenose dolphin, killer whale and sperm whale slices mRNA levels, as well as for E2F1 apart from the killer whale (Fig 1a). On the opposite, the PFOA exposure shows a down-regulation of the PPARα and PPARγ both in sperm whale and killer whale slices, while ERα and E2F1 are poorly modulated by PFOA in both species (Fig 1b).

Concerning the inter-specific responses to the BPA treatment, the sperm whale exhibit the highest up-regulation of the PPARγ gene compared to the other to species. On the other hand, killer whale shows a dose-response of ERα gene expression after PFOA exposure, suggesting the role of the compound as endocrine disruptor with estrogenic activity.

**Fig. 1. Levels of gene expression (±SD) of PPARα, PPARγ, ERα, and E2F1 normalized to YWHAZ and GAPDH in BPA and PFOA treated slices (a - BPA - = ethanol, BPA + = 0.1 μg/ml, BPA ++ = 1 μg/ml, BPA +++ = 10 μg/ml e BPA ++++ = 100 μg/ml; b - PFOA - = methanol, PFOA + = 0.1 μg/ml, PFOA +++ = 1 μg/ml).**

**4. Conclusions**

These data represent the first evidence of emerging contaminants effect on cetaceans based on an *in vitro* experiment and suggest the potential use of these diagnostic markers as early warning signal of exposure to plastic released compounds and emerging contaminants in marine wildlife monitoring.

**5. References**

1. Introduction

Endocrine disrupting chemicals (EDCs) have become a problem of concern due to their potential to cause abnormal reproductive development, reduced fecundity and changes in population sex ratios – all with dire consequences to reproduction and balance of ecosystems. Most of the known endocrine disrupting chemicals are estrogenic, affecting particularly reproductive functions and produce estrogen-like effects.

While chemical analysis methods have been widely used to identify known EDCs in water, these methods are merely used to detect ‘target’ chemicals, and they do not reveal mutual and synergistic effects of the complex mixtures of these EDCs. EDCs in environmental samples normally occur as complex mixtures, making it difficult to associate any effect to single chemicals; thus it is becoming a standard to test whole effluent extracts to evaluate estrogenic or androgenic activities by using in-vitro assays.

Research endeavors aimed at the design of waste water treatment plants that clean EDCs are raising hopes that a combination of proper treatment methods could reduce their concentrations before they reach aquatic systems (Auriol et al 2006, Lee et al 2008). While there is little research concerning water pollution by EDCs in developing countries, such as Zimbabwe, the urban population in these countries is increasing quite fast yet the necessary expansion of sewer systems have remained relatively static. Furthermore their lack of properly maintained sewage treatment infrastructure and poor enforcement of waste disposal legislation is resulting in contamination of water bodies with untreated domestic and industrial effluent that could put these countries at greater risk of ecological mayhem.

The objective of this study is to measure the total estrogenic and androgenic potencies of treated sewage effluents and water from polluted sources in the industrialised, semi-arid city of Bulawayo, Zimbabwe as an indicator of potential adverse effects of inadequate waste water management practices in developing countries.

2. Materials and methods

- Treated effluent water samples were collected from Aisleby Sewage Treatment Plant (AIS) and Thorngrove Sewage Treatment plant (THORN) were most of Bulawayo’s industrial and domestic effluent is treated. Water samples were also colleted from Umguza Dam, Khami Dam Matsheumhlope River (MAT), all peri-urban water bodies which receive effluents. Umguza Dam receives raw industrial effluent and effluent from AIS and THORN STPs while Khami Dam receives mainly domestic sewage.
- Organic pollutants in water samples (500ml) were extracted by SPE (C18) cartridges
- Extracts were eluted with methanol and dried under nitrogen gas.
- Dried extracts were serially diluted in yeast culture medium and tested for estrogenic and androgenic activity using the yeast estrogen screen (YES) assay (Routeledge and Sumpter 1996) and yeast androgen screen and (YAS) assay respectively.
- 17β-estradiol equivalence quantities (EEQ) and dihydrotestosterone (DHT) equivalence quantities (DHTEQ) of the water samples were calculated.

3. Results and discussion
Yeast estrogen screen (YES) assay

Figure 1: Relative estrogenic activity of 17β-estradiol (E2) against the log concentration (serially diluted from 1.46 ng/L to 3000 ng/L). Response curves of the water samples, represent sample concentrating factors of 0.04 – 166.7. The sudden drop in absorbance seen in AIS and MAT samples at higher concentrations is due to lysis of yeast cells.

Yeast androgen screen (YAS) assay

Figure 2: Relative androgenic activity of DHT plotted against the log concentration diluted from 12.2 ng/L to 25 000 ng/L.

<table>
<thead>
<tr>
<th>Sample</th>
<th>EEQ (ng/L)</th>
<th>DHTEQ (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Umguza</td>
<td>236.5</td>
<td>Not detectable</td>
</tr>
<tr>
<td>Khami</td>
<td>8.5</td>
<td>Not detectable</td>
</tr>
<tr>
<td>MAT</td>
<td>2.2</td>
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<td>THORN</td>
<td>32.8</td>
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<tr>
<td>AIS</td>
<td>55.3</td>
<td>93.1</td>
</tr>
</tbody>
</table>

Table 1: EEQ and DHTEQ of water samples using the YES and YAS assays respectively. EEQ for Umguza Dam (236.5 ng/L) is remarkably high relative to literature reports.

4. Conclusions

- The results could indicate the potential adverse effects of inadequate waste water management practices in developing countries.
- Estrogenic EDCs are remarkably high compared to androgens, concurring with literature.
- We recommend ‘follow-up’ field based studies to determine the effects of these xeno-estrogens on reproductive health of fish and other aquatic organisms in these peri-urban water bodies.

5. References


Acknowledgement – We research was sponsored by the International Science (ISP) Programme, Uppsala University, Sweden. The permission granted by Bulawayo City Council to do this study is appreciated.
Assessment of exposure to estrogenic contaminants (PAHs and Alkylphenols) in bile extracts of red mullet from the Western Mediterranean Sea: an integrated chemical and biological approach

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1. Introduction

Many published studies link exposure of fish and other wildlife to environmental endocrine disrupting chemicals (EDCs) with adverse health consequences. Assessment of exposure to potentially estrogenic compounds such as polycyclic aromatic hydrocarbons (PAHs) or alkylphenols (APs) cannot be adequately done by measuring concentrations in fish tissue as these compounds are easily metabolized by fish. However, metabolites of these two groups of compounds can be chemically detected and quantified in fish bile. Interactions of estrogenic EDCs with estrogen receptors is believed to be an important mechanism of endocrine disruption in fish. Red mullet is being used as target species for pollution monitoring programmes in the Mediterranean Sea.

In this work we have identified and measured the concentrations of major metabolites of PAHs and APs in field collected bile extracts of red mullet (Mullus barbatus) from Mediterranean Spanish waters. In addition we have applied the estrogen receptor-mediated, chemical-activated luciferase reporter gene-expression assay (ER-LUC) to measure total estrogenic activity in the same bile extracts of male fish. By integrating the results of both analytical chemical and bio-analytical approaches, we have attempted to explain the measured ER-LUC activity by the calculated potencies based on chemical analysis of hydroxylated PAH and AP metabolites.

2. Materials and methods

Red mullets were sampled in the autumn of 2010 at coastal sites near Barcelona, Delta del Ebro, Valencia, Marine Reserve of Palos Cape (used as a reference site in this study) and from 3 sites located in the Mar Menor Lagoon (Murcia, SE Spain) (Figure 1). For each site, fish bile samples were pooled per sex. Hydroxylated-PAHs (1-naphthol, 9-phenantrol, 9-fluorenol, 1-pyrenol, 1OH-BaP and 3OH-BaP) and alkylphenols (4-n-nonylphenol (NP) and 4-tert-octylphenol (OP)) were quantified by gas chromatography–mass spectrometry in electron ionization mode (GC-EI-MS) [1]. For the assessment of estrogenic activity, the ER-LUC assay was carried out, using stably transfected BG1Luc4E2 human ovarian cancer cells (containing a luciferase reporter gene under transcriptional control of an estrogen-responsive element (ERE)) [2].

Figure 1: Sampling sites of red mullet (Mullus barbatus) along the Spanish Mediterranean coast.
For comparison of the estrogenic potency of estradiol (E2) and the compounds chemically quantified in bile samples, cells were exposed to the same individual compounds (1-naphtol, 9-fluoreno1, 9-fenantrol, 1-pyrenol, 1OH-BaP, 3OH-BaP, OP and NP).

3. Results and discussion

3.1. Chemical analysis of bile extracts

Concentrations of hydroxylated-PAHs (ΣOH-PAHs) in fish bile samples from Mar Menor sites and Barcelona were markedly higher than those from the other sites (29-folds higher in MM3 than in Palos Cape). 1-Pyrenol, considered a general indicator of PAHs exposure in fish, was detected in all samples with maximum levels in Barcelona (51 ng/g). 1-OH-BaP and 3-OH-BaP were identified in less than 25% of the samples, with levels in all cases below the detection limits. 1-Naphtol, an indicator of recent exposure to petrogenic compounds, was not detected in any of the bile samples. The concentrations of NP showed a similar spatial pattern as those of 1-pyrenol. However, OP concentrations in bile samples were higher at Palos Cape (reference site) and along the Spanish coast near Valencia than at the other coastal sites. The NP concentrations were always one order of magnitude higher than the OP concentrations.

3.2. Biliary estrogenic activity in male fish

Preliminary results of the ER-LUC assay showed that highest values of estrogenic activity were found in bile extracts of Mar Menor Lagoon. OP has the highest estrogenic potency, followed by NP, 3OH-BaP, 1OH-BaP and 1-Pyrenol when the BG11uc4E2 cell line is used.

![Figure 2: Comparison of the estrogenic potency of 4-tert-Octylphenol and 4-n-nonylphenol in the BG11uc4E2 cell line.](image)

4. Conclusions

Analysis of biliary metabolites indicated geographical differences of exposure to EDCs in red mullet from different Spanish Mediterranean sites that were consistent with the results of bio-analytical analysis. Biliary estrogenic activity was elevated in those fish that were highly exposed to 1-pyrenol, 4-n-NP and 4-tert-OP. These findings warrant further study on potential endocrine disruptive effects in red mullet and other fish species dwelling in the areas where the highest exposure to EDCs was demonstrated. This work demonstrates the usefulness of combining the ER-LUC assay with biliary chemical measurements in biomonitoring programmes of marine contamination.

5. References


Acknowledgement - This work has been supported by the Spanish Inter-Ministerial Science and Technology Commission through ‘DECOMAR’ project (CICYT, CTM2008-01832) and by the Spanish Ministry of Environment through ‘2-ESMARME’ project.
Investigation of endocrine disruption in Australian aquatic environments – stage 1

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1. Introduction

This project uses an integrated approach consisting of multiple in vitro and in vivo bioassays, in situ sampling and trace chemical analysis to compare endocrine disruption at 73 sites in mainland Australia. Most previous studies on endocrine disruption in Australia have been localized to specific areas and often focused on wastewater treatment plants (WWTPs). There is, however, increasing anecdotal evidence that other point sources such as industrial discharges and non-point sources (e.g., agricultural run-off) may be significant contributors of EDCs to the Australian aquatic environment. For example, the estrogenic activity measured downstream of a wastewater treatment plant (WWTP) discharge in New South Wales (NSW) was found to have half of the estrogenic activity of water sampled upstream of the discharge point. In another study, estrogenic activity in two of three reference sites indicated that some of the waterways in Sydney are receiving EDCs from yet undetermined sources [1]. In this study, sites were selected to include waterways receiving a variety of point and non-point inputs, such as wastewater discharge, agricultural run-off, industrial effluent, urban drains and pristine reference sites.

The primary objective of this project is to determine the extent and significance of endocrine disruption in Australian aquatic ecosystems. This objective is being addressed using a step-wise integrated testing strategy in the following stages:

1. In vitro bioassay techniques, specifically the estrogen receptor (ER), androgen receptor (AR), progesterone receptor (PR) and glucocorticoid receptor (GR) CALUX assays to determine the estrogenic and anti-estrogenic, androgenic and anti-androgenic, progesterone and glucocorticoid activity, respectively, of water extracts generated with a standard solid-phase extraction (SPE) protocol.

2. In parallel, identify and quantify EDCs present in the same water extracts using liquid chromatography tandem mass spectrometry (LC/MS-MS).

3. Use several stock solutions representative of a selection of exposure concentrations found in the environment for in vivo laboratory fish exposures using one native species (rainbowfish, Melanotaenia fluviatilis) and one widespread pest species (mosquitofish, Gambusia holbrooki) and assess the amount of endocrine disruption by measuring plasma blood vitellogenin concentrations (rainbowfish) or vitellogenin mRNA (mosquitofish) along with an androgenic biomarker (being developed).

4. Use in situ techniques to sample fish from polluted aquatic environments identified in stage 1 and assess the extent of endocrine disruption using the same endpoints previously mentioned as well as gross gonadal histopathology.

5. Finally, derive a risk assessment using all of the information gathered from steps 1 to 4 to determine potential risks to aquatic ecosystem health.

2. Materials and methods

2.1. Water sampling and extraction

Duplicate 1 L discrete water samples were taken quarterly over a year from each of the 73 study sites. Samples were preserved by dropping the pH to 2 and immediately shipped on ice to a central laboratory...
where samples were concentrated by solid-phase extraction (SPE) using Oasis HLB cartridges. The preservation and extraction techniques were fine-tuned during a preliminary experiment and shown to produce reliable results for known EDCs such as estrogen and androgen hormones, plasticisers and pesticides. Standard water quality parameters (e.g., pH, temperature, conductivity, hardness) and catchment information were also recorded. Sample extracts were split into two aliquots, one for \textit{in vitro} bioassay analysis and the other for chemical analysis.

### 2.2. Sample Analysis

The ER-, AR-, PR- and GR-CALUX bioassays were used to measure endocrine-active compounds. Estrogenic and androgenic activity has been measured in both agonistic and antagonistic modes, as anti-estrogens can have androgenic-like effects and anti-androgens can have estrogenic-like effects in whole animals. The ER- and AR-CALUX bioassays have been shown to be well correlated with \textit{in vitro} responses in rats [2].

In parallel with the \textit{in vitro} bioassays, high-pressure liquid chromatography – tandem mass spectrometry (HPLC/MS-MS) analyses was carried out to characterize the chemicals responsible for the biological activity and determine the most likely contaminant source (e.g., run-off contaminated with pesticides, industrial wastewater or treated sewage discharge, urban storm water run-off, dairy wastewater). Chemical analysis focused on estrogens and estrogen mimics (such as estrone, estriol, 17β-estriadiol, 17α-ethynylestradiol, bisphenol A, 4-nonylphenol and 4t-octylphenol), androgens and androgen mimics (such as testosterone, androstenedione, androsterone, etiocholanolone and 17α-trenbolone), pharmaceuticals and personal care products (such as carbamazepine, fluoxetine, sertraline and triclosan) and pesticides (atrazine, dieldrin, 2,4-D, endosulfan and glyphosate).

### 3. Results and discussion

#### 3.1. Sample Analysis

\textit{In vitro} analysis is currently underway, and ER-, AR-, PR- and GR-CALUX data will be presented during the conference. Preliminary data indicates the presence of estrogenic compounds in at least 11 of the sample sites and little to no androgenic activity at any sites sampled.

Chemical analysis from the first sampling event has identified estrogen mimics (bisphenol A, t-octylphenol), the pesticide atrazine, industrial compound \textit{tris}(2-carboxyethyl)phosphine (TCEP) and numerous pharmaceuticals and personal care products (atenolol, dilantin, paracetamol, sulfamethoxazole, trimethoprim, triclosan, fluoxetine, carbamazepine, ibuprofen, salicilic acid, p-hydroxy atorvastatin, o-hydroxy atorvastatin, fluoxetine, \textit{N},\textit{N}-Diethyl-meta-toluamide (DEET), primidone, naproxen and gemfibrozil). Hormone analysis is currently underway.

### 4. Conclusions

Thus far at least 11 sites containing estrogenic compounds have been found in Australia, and numerous potentially endocrine disrupting compounds have been identified. Full details will be presented during the conference.

### 5. References


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Applying tissue-burden based quality benchmarks to assess the ecological risks of endocrine disrupting organotin compounds in Hong Kong waters

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1. Introduction
Organotin compounds (OTs), such as tributyltin (TBT) and triphenyltin (TPT), have been widely used as effectual biocides and antifouling agents in freshwater and marine environments since the 1960s [1]. These chemicals, however, have caused widespread adverse endocrine disrupting effects on marine organisms such as induction of imposex (i.e., superimposition of male sexual characteristics – penis and vas deferens – on females) in over 200 species of neogastropods, and growth inhibition and deformities in oysters [2]. Being one of the most toxic chemical groups contaminating our marine ecosystems over the past 40 years, OTs have been forced into a global ban on their application in antifouling systems on seagoing vessels since September 2008 [3]. It is, therefore, anticipated that a reduction of OT pollution after the global ban will lead to recovery of marine neogastropod populations. In Hong Kong, active ecological risk assessments for OTs have been conducted before the global ban, through evaluating the imposex status and levels of TBT contamination in the intertidal rock shell Thais clavigera [4-5]. No similar survey, however, has been commenced since 2006 and no study has ever focused on TPT, which is also very toxic to marine organisms and can cause imposex in T. clavigera [6]. This study aims to (1) investigate the contemporary imposex status in T. clavigera in Hong Kong waters after the global ban of OT-based antifouling systems; (2) determine the OTs levels, including the phenyltin compounds (PTs), in the tissues of T. clavigera; and (3) perform a probabilistic ecological risk assessment based on the tissue burden of butyltin compounds (BTs) and PTs in T. clavigera.

2. Materials and methods
About 40 Thais clavigera adults were collected at each of the 29 sites along the coast of Hong Kong between May and October 2010. These sites were previously visited by Leung et al. and Qiu et al. [4-5]. Samples, after being frozen at -20°C, were identified for sex and level of imposex development using Vas Deferens Sequence Index (VDSI) and Relative Penis Size Index (RPSI). For each site, samples were pooled as triplicate before chemical analysis (Fig. 1). Tissue concentrations of six OT species (i.e., mono-BT, di-BT, tri-BT, and mono-PT, di-BT and tri-PT) were quantified using gas chromatography-mass spectrometry (GC-MS) following the method of Guðmundsdóttir et al. [7].

A probabilistic ecological risk assessment was conducted, using the Monte Carlo approach, by computing the distribution of risk quotients (RQs; Fig. 2).

Fig. 1: Procedures of organotin analysis [7].

Fig. 2: Equation for calculating the risk quotient (RQ) [4].
3. Results and discussion

3.1. Imposex status

_Thais clavigera_ were found in 28 out of 29 sites. Imposex occurred in every female across all sites. Mean VDSI ranged from 2.61 to 5.73, while RPSI ranged from 1.19 to 94.66. Imposex levels of _T. clavigera_ in Hong Kong remained high at sites close to shipping facilities such as marinas and cargo ports. At certain clean sites, the contamination seemed to be even worse than before as shown by a 100-fold increase of RPSI in Kong Tau Pai when compared with the data collected at the same location in 2004 [4].

3.2. Tissue burden

TPT, which is primarily used as pesticides and fungicides in agricultural land, was the most predominant and abundant residue among all OTs, ranging from 227.9 to 11108.0 µg kg⁻¹ dry weight (dw) in _T. clavigera_ samples. They accounted for 46.0% to 98.5% of all OTs in their tissues. TBT was the second most abundant residue, ranging from 5.8 to 422.0 µg kg⁻¹ dw (0.4% to 14.5% of total OTs). TPT, however, was 6–36 folds more than that of TBT in the tissues of _T. clavigera_.

TBT was still detectable in local _T. clavigera_ showing persistent OT contamination in the marine environment of Hong Kong even after the global ban of OT-based antifouling systems. Our results also suggested possible illegal uses of such antifoulants on vessels or submerged mariculture facilities.

3.3. Risk assessment

11.1% of _T. clavigera_ across all sites in Hong Kong waters were at risk with RQ > 1 due to exposure to PTs, suggesting that the animals are still under considerable threats associated with this pollutant. TPT is a major environmental concern which deserves immediate actions to control its use and release, and to remediate its pollution in the marine environment of Hong Kong and the Pearl River Estuary.

4. Conclusions

Organotin pollution, especially from phenyltin compounds, is still threatening Hong Kong’s marine environments. The rock shell _Thais clavigera_ in Hong Kong is still showing 100% imposex, while they have very high concentrations of organotins in their tissues. Immediate actions are required to control the use and release of these compounds to better protect the marine life.

5. References


Acknowledgement - The authors thank the undergraduate helpers in assistance of sampling work. The chemical and data analyses conducted at HKU were supported by the Area of Excellence Scheme under the University Grants Committee of Hong Kong Special Administrative Region Government, China (Project No. AoE/P-04/2004) and by the Research Grants Council through a General Research Fund (Project No. HKU 7034/07P). We also thank Helen and Jessie for their technical support. Kevin Ho would also like to thank The University of Hong Kong to partially support his PhD study.
Polycyclic aromatic hydrocarbons and endocrine disruption: the role of junctional intercellular communication

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1. Introduction

Male reproductive function in animals and humans is considered to be a system highly sensitive to many chemicals and physical agents generated by industrial or agricultural activities [1]. Recently, many disturbing trends have been observed in male fertility, such as decreasing sperm counts, deteriorating semen quality and increasing frequencies of malformations of testis and incidence of testicular cancer [2]. Endocrine-disrupting Chemicals (EDCs) are discussed as possible cause of these adverse trends in male reproductive health [3].

In addition, to estrogen- and androgen-mediated processes, there is a strong evidence that testicular cell-to-cell communication mediated by gap junctions, termed Gap Junctional Intercellular Communication (GJIC), is involved in testicular development, regulation of hormone release, cell differentiation, initiation, and maintenance of spermatogenesis [4]. Thus, inhibition of GJIC during critical stages of development may result in male reproductive dysfunction leading to infertility. Indeed, many chemicals known to be EDCs modulate GJIC and/or impair connexin expression in gonadal or non-gonadal cells [5-7]. However, there is limited information on the detailed role of GJIC in adverse reproductive effects caused by specific EDCs.

2. Materials and methods

To determine whether selected chemicals may cause their toxic effects through closing gap junction channels, the inhibition of GJIC was studied in testicular cells using a simple in vitro assay: a scrape-load dye transfer technique [8]. In this assay, a gap junction permeable tracer, luciferase yellow, is injected into the cells with a simple scrape using a scalpel (Figure 1). The distance at which the fluorescent dye diffuses during a certain period from the scrape line is indicative of GJIC level.

![Figure 1: Intercellular communication of Leydig cells in control (A.) and after treatment with 40nM TPA (tetradecanoyl phorbol acetate; B.). The diffusion of the lucifer yellow dye (LY, MW = 457.25) as compared with the nondiffusable rhodamine dextran (RhD, MW = 10,000) was examined as a measure of GJIC by fluorescence microscopy. Scale (-) = 50 μm](image)

3. Results and discussion

Recent studies indicate that anthropogenic air pollutants can possibly impair reproduction of human and wildlife. It has been reported that Polycyclic Aromatic Hydrocarbons (PAHs) on airborne particulate particles compromised sperm functions and altered endocrine hormone levels in exposed animals [9-10]. Our study addressed the endocrine-disrupting potential of air pollution as a source of compounds that may alter male fertility. The inhibition of GJIC by PAHs and air samples was assessed in testicular cells in this experiment, in order to determine whether PAHs could cause endocrine disruptive effects by closing gap junction channels.

It has been previously reported that three- and four-ringed PAHs with a bay region or bay-like region inhibited of GJIC in a rat liver epithelial cell line with oval cell characteristics [11]. These methylated anthracenes are predominant PAHs in cigarette smoke. Consistent with these previous observations, 1- and
9-methylanthracene, which have a bay-like region, inhibited GJIC in Leydig cells, whereas the linear isomer, 2-methylanthracene, had no effect on GJIC (Table 1). Inhibition occurred within 30 min. Surprisingly, PAHs did not cause GJIC inhibition in Sertoli cells.

<table>
<thead>
<tr>
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<th>Leydig cells</th>
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<td><img src="image4" alt="Structure" /></td>
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</table>

*Table 1: Inhibition of GJIC of testicular cells after in vitro treatment with PAHs ( + GJIC inhibition, - no GJIC inhibition).*

4. Conclusions

In conclusion, our *in vitro* study indicates that a much overlooked class of compounds may contribute to endocrine-disrupting potential of air pollution. This biological effect of methylanthracene depends on the ring position of the methyl group and the type of testicular cells.

5. References

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Improving the *in vivo* predictive value of *in vitro* estrogenicity bioassays by combining *in vitro* results with metabolism characteristics of estrogenic compounds

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1. Introduction

Concerns about the possibility of estrogenic compounds to cause endocrine-disrupting effects have led to the development of several *in vitro* assays allowing detection of endocrine disrupting compounds (EDCs) in e.g. environmental samples [1]. Among these, stably transfected reporter gene *in vitro* bioassays such as the ER-Calux bioassay (T47D or U2OS cell-based) or the transcriptional activation assay (using HeLa-9903 cells) provide rapid and sensitive, effect-directed tools for screening samples for the presence of estrogenic compounds. A limitation of these *in vitro* bioassays, however, is that they do not take the ADME (absorption, distribution, metabolism and excretion) characteristics of compounds into account, which can hamper the *in vivo* predictive value of these bioassays. Our aim was to alleviate this limitation by taking ADME characteristics into account when comparing *in vitro* results with *in vivo* outcomes, with metabolism as a first characteristic studied.

2. Materials and methods

We compared the intrinsic estrogenicity of a suite of selected (xeno-)estrogenic compounds relative to the reference ethinylestradiol (EE2) as determined with the transcriptional activation assay, to literature-derived *in vivo* estrogenic responses obtained with the uterotrophic assay using subcutaneously exposed rats. A methodology was applied in which the *in vitro* ER-activation potencies of individual compounds were used to calculate the dose of these compounds which would have equivalent *in vivo* uterotrophic responses as the reference compound EE2. Equivalency factors relative to EE2 were calculated that not only take the *in vitro* estrogenic potency of the test compound relative to EE2 into account, but also the extent of hepatic clearance relative to EE2, in order to compensate for differences in metabolism. To this end, incubations of test compounds with rat liver fractions (with specific co-factors) were performed in order to determine the hepatic clearance via microsomal NADPH-dependent reactions, via glucuronidation (microsomes with UDPGA), and via sulfonation (cytosol with PAPS). The decrease in substrate was analyzed by HPLC-UV and used to derive the overall hepatic clearance. EE2 equivalent doses adjusted for hepatic clearance were calculated by multiplying the EE2 equivalent *in vitro* ER-activation potencies with the EE2 equivalent hepatic clearance.

3. Results and discussion
3.1. Hepatic clearance

Figure 1 shows the hepatic clearance of EE2, as well as for the model compounds bisphenol A (BPA), genistein (Gen), and nonylphenol (NP) as determined in incubations with rat tissue fractions for NADPH dependent reactions (NADPH), glucuronidation (UDPGA) and sulfonation (PAPS).

3.2. Effect of adjusting the EE2EQ doses for differences in hepatic clearance on the EE2EQ dose response curve

Figure 2A shows the uterotrophic response reported for EE2, Bisphenol A (BPA), nonylphenol, and genistein at different subcutaneous doses as reported by Kanno et al. [2,3]. Figure 2B shows the EE2 equivalent dose-response curve based on conversion of the applied doses to EE2 equivalent doses by taking the intrinsic estrogenic potency relative to EE2 into account. The intrinsic estrogenic potencies, acquired with the Transcriptional Activation (TA) assay using hER-HeLa-9903 cells, were obtained from literature [4]. Figure 2C shows EE2 equivalent dose-response doses calculated taking both the in vitro derived estrogenic potencies relative to EE2, as well as the hepatic clearance relative to EE2 into account.

Adjusting for the differences in hepatic clearance relative to EE2 (Figure 2C) results in 1.7 and 2-fold lower EE2 equivalent doses for genistein and bisphenol A, respectively, compared to the EE2 equivalent doses given in figure 2B. As a result, the adjusted EE2 equivalent dose-response curves obtained for these compounds shift towards the dose-response curve of EE2. For nonylphenol the EE2 equivalent doses are the same after adjusting for hepatic clearance relative to EE2, because this compound is cleared at a similar rate by the liver as EE2. Still the in vivo uterotrophic responses of BPA, genistein and nonylphenol are much lower than would be expected based on their EE2 equivalency (about a factor 10). This suggests that the observed in vivo uterotrophic effects induced by EE2 cannot be explained by a lower hepatic clearance alone. Other factors, such as differences in serum protein binding could also play a role.

4. Conclusions

The studies demonstrate that combining the results for estrogenic activity determined with in vitro bioassays with compound-specific kinetic characteristics for hepatic clearance does improve the correlation with the in vivo effect doses obtained with the rat uterotrophic assay, although not sufficiently. Therefore the outcome of the present work is a first step to increase the in vivo predictive value of in vitro estrogenicity bioassays, which can eventually help to derive an in vitro effect-based benchmark dose needed for risk assessment of samples containing estrogenic compounds. The methodology described is currently extended with a broader range of different compounds, which includes alkylphenols, benzophenone derivatives, isoflavones, phenyl methanes and steroids. In addition also differences in serum protein binding are assessed. In order to extend our methodology with absorption characteristics, the oral bioavailability of selected model compounds is being studied in vitro with Caco-2 cell monolayers in a transwell model simulating the intestinal transport barrier, allowing a comparison to data from the rat uterotrophic assay using orally exposed rats.

5. References

1. Introduction
Fish histopathology experiments are usually conducted with multiple fish in each of multiple tanks within a water control and several test concentrations. Responses are typically ordered severity scores on each subject with values 0 for no effect, 1 for minimal effect, through 4 or 5 for severe effect. For a 1-generation study, Mann-Whitney or Dunn nonparametric comparisons of treatment groups to control are typically done, where the data are either replicate means or medians, or else replicates are ignored and all subjects are analyzed as independent observations. The mean score is statistically unsound and both it and the median score ignore much important information. Furthermore, tests on median scores make no allowance for varying numbers of fish of each sex in different vessels. Ignoring replicates and treating all subjects as independent ignores the experimental design and frequent observation that fish in the same tank tend to have responses correlated differently from fish in different tanks. Such pairwise approaches also ignore the expectation that severity of effect tends to increase with increasing concentrations. A step-down Jonckheere-Terpstra test, applied either to replicate medians or ignoring replicates, uses this presumed monotone concentration-response but otherwise suffers from the same limitations as pairwise methods. Alternatives exist, such as polychotomous or multicategory logit models, but these can be challenging to implement or interpret.

2. Materials and Methods
The Cochran-Armitage test has long been used to analyze incidence data where there is an expectation that severity of effect tends to increase with increasing concentrations, but it has no method for incorporating replicate information or adjusting for varying replicate sizes. Rao and Scott modified this test for incidence data that does allow replicates to be modeled and adjusted for varying sizes.

A conceptually simple new test, the Rao-Scott Cochran-Armitage by Slices, or RSCABS, is proposed for severity score data such as found in histopathology. It is based on the Rao-Scott modified Cochran-Armitage test further modified to incorporate a fixed number of scores or ordered response values, so that it incorporates the replicate vessel experimental design, allows for varying numbers of fish per vessel, and incorporate the expected monotone concentration-response, while retaining the individual subject scores and revealing the severity of any effect found statistically significant. A statistical protocol is proposed for analyzing both 1-generation and multi-generation histopathology studies.

The methodology and protocol have been applied to the histopathology data from three multi-generation studies from recent joint work by Japan and the US under the auspices of the OECD Validation Management Group for Ecotoxicity. Results from RSCABS for these three studies are compared to analyses by Mann-Whitney, Dunn’s, step-down Jonckheere-Terpstra, and chi-squared tests. The theoretical properties of RSCABS are based on the earlier work of Rao, Scott, Cochran, and Armitage and extended by a power analysis specifically developed for RSCABS.

Generations were compared using the Cochran-Mantel Haenszel after removing treatment effects, and by Mann-Whitney and Chi-squared tests within each treatment.

3. Results and Discussion
As a rule, RSCABS found (1) all the Jonckheere-Terpstra results, (2) all Mann-Whitney and Dunn results where the high concentration was significant, (3) and sometimes found results not found by Mann-
Whitney, Dunn, or Jonckheere-Terpstra. In all cases, RSCABS showed how severe were the effects found, something not indicated by the alternative procedures. RSCABS did not find results for responses where changes in severity were observed only in low or intermediate test concentrations but not in higher concentrations.

4. Conclusions
RSCABS is shown to have distinct advantages over alternative methods for analyzing histopathology responses within a single generation, both in terms of sensitivity and information provided on the severity of effects found statistically significant. This advantage applies whenever severity of effect tends to increase as test concentration increases. Alternative methods are useful for comparing generations. Software has been developed, which requires no programming expertise to use, to carry out the RSCABS and alternative approaches within and between generations. While the illustrations are from fish studies, the methodology is general and can be applied to histopathology studies for other species and to non-histopathology data having analogous structure. Where replicate vessels are not present or subjects are individually housed, the method still applies.

5. References

Stability of functionalized gold nanoparticles in water systems with different composition

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1. Introduction

Many engineered nanoparticles (ENPs) have been functionalized for intended applications. This functionalization will influence their fate and behavior in aquatic systems when they are released. The exposure and transport of functionalized engineered nanoparticles (FENPs) are strongly influenced by their aggregation state in aquatic environments. Thus the aggregation kinetics has become the focus of current researches [1, 2]. However, in these studies, the environmental parameter (e.g. pH or electrolyte) was separately studied in simplified conditions, which is limited for fully delineation of the behavior of FENPs. Therefore the objective of this study is to investigate the combined influences of ionic strength and pH on aggregation kinetics of FENPs in the absence and presence of NOM. In the study, two types of gold nanoparticles (GNPs) were employed as the models of FENPs. Early stage aggregation rates were determined from the samples in the matrices by using time-resolved dynamic light scattering measurements. The results display the colloidal stability of FENPs at different hydrochemical conditions and might be included into exposure modeling.

2. Materials and methods

A citrate-coated GNPs purchased from British Biocell International (BBI) and a 11-mercaptoundecanoic acid (MUA)-coated GNPs synthesized by the Chemistry Department of the University of Alberta (Canada) were employed as the models of FENPs.

Aggregation rate as a function of pH and electrolyte concentration was investigated in the absence and presence of (50 mg/L) NOM with four different electrolytes (NaCl, CaCl₂, CaSO₄ and MgCl₂). Time-resolved dynamic light scattering measurement (Zetasizer Nano-ZS) was used to determine the aggregation rates of samples in matrices. Then, we used Inverse Distance Weighting (IDW) algorithm with Suffer 9.0 (Golden software, Inc) to interpolate the extracted aggregation rates into contour maps. Each contour map is supported by 20 individual data points (aggregate rates).

![Aggregation rate of citrate-coated GNPs as a function of CaCl₂ concentration and pH in the (a) absence and (b) presence of NOM (50 mg/L); aggregation rate of MUA-coated GNPs as a function of CaCl₂ concentration and pH in the (c) absence and (d) presence of NOM (50 mg/L). Fast aggregation rates (instability) are shown in dark shading, while slow aggregation rates (stability) are shown in bright shading.

3. Results and Discussion

3.1 The influence of electrolyte and pH on citrate-coated GNPs

In the absence of NOM. The aggregation rates of citrate-coated GNPs were elevated by the increasing of CaCl₂ concentration (Fig. 1a) due to the compression of electrical double layer through screening of surface charge. But adjusting suspension pH did not influence the aggregation rate of citrate-coated GNPs. In the study, the investigated pH range (4 to 8) is above the most acidic pKₐ of the COO⁻ (3.13) on the citrate [3]. Thus the
particles were electrostatically stabilized by the negative charge on gold surface. The similar results were also observed on citrate-coated GNPs in matrices for NaCl, CaSO₄ and MgCl₂.

**In the presence of NOM.** The addition of NOM increased the stability of citrate-coated GNPs in the presence of CaCl₂ (Fig. 1b), which is due to the electrosteric forces caused by the NOM replacement of citrate on gold surface. This stabilizing effect of NOM on citrate-coated GNPs also occurred in the matrices for NaCl, CaSO₄ and MgCl₂.

### 3.2 The influence of electrolyte and pH on MUA-coated GNPs

**In the absence of NOM.** The increase of CaCl₂ concentration enhanced aggregation rates for MUA-coated GNPs. (Fig. 1c); adjusting suspension pH to acidic or basic range also increased the aggregation rates in the presence of CaCl₂. Due to the pKₐ of MUA is between pH 4.3 and 5.4 [4]. At pH < 5, the negative charge on gold surface is significantly reduced by protonation, and then attachments occur. At pH > 7, in the presence of Ca²⁺, the elevated negative surface charge induced bridging effect of divalent cation (Ca²⁺) between MUA-coated GNPs, which resulted in the increased aggregation rates. The similar results were also observed on MUA-coated GNPs in the matrices for CaSO₄ and MgCl₂. But since mono charged Na⁺ cannot show a bridging, the elevated aggregation rates only occurred at acidic range in the presence of NaCl.

**In the presence of NOM.** Adding NOM also increased the stability of MUA-coated GNPs in matrix for CaCl₂ (Fig. 2b). Since 11-mercaptopoundecanoic acid has a much stronger bonding to the gold surface than citrate [5], it is very unlikely that the replacement of MUA by NOM occur. Therefore we assumed that the Ca²⁺ may have facilitated NOM adsorption onto the surface of MUA-coated GNPs by neutralizing the surface charges of both MUA-coated GNP and NOM. The similar results were also observed on MUA-coated GNPs in the matrices for CaSO₄ and MgCl₂. However, the addition of NOM did not obviously increase the stability of MUA-coated GNPs in the matrix for NaCl. This reveals that NOM was not effectively adsorbed onto the surface of MUA-coated GNPs since mono charged Na⁺ cannot facilitate the adsorption of NOM to the gold surface.

### 4. Conclusion

When the FENPs released into the water systems, the fate and transport will be influenced by electrolyte, pH and NOM concentration in the water system. For citrate-coated GNPs, pH in the aquatic environment may not impact their colloidal stability in the absence of NOM, but the electrolyte concentration does. The presence of NOM in aquatic environment will increase the stability of citrated-coated GNPs. For MUA-coated GNPs, the aggregation kinetics is influenced by the electrolyte concentration, the pH and the charge of the cations in the suspension. Adding NOM may increase the stability of MUA-coated GNPs due to adsorption of NOM onto the gold surface through neutralizing the surface charges on the surfaces of GNPs and NOM by divalent cations (e.g. Ca²⁺ or Mg²⁺). However, in monovalent cation (e.g. Na⁺) dominant water systems, the presence of NOM will not significantly increased stability of MUA-coated GNPs.

### 5. References:


Application of Field-Flow-Fractionation for the analysis of engineered nanoparticles in complex matrices

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1. Introduction

Engineered nanoparticles (ENPs) are increasingly introduced into consumer products and many of these products are likely to release ENPs into the environment. For example, silicon dioxide nanoparticles (SiO2NPs) are used as a food additive and the use of silver nanoparticles (AgNPs) is rapidly growing because of the antimicrobial properties of silver. Those products could release nanoparticulate silver into the aquatic and terrestrial environment via waste water treatment (effluent and sludge). Those two examples show the complexity and the diversity of the matrices in which ENPs are likely to occur. However, methods for the detection, quantification and characterization of ENPs in complex media like sludge, soil or food matrices are still missing.

Field Flow Fractionation (FFF) is one of the most promising techniques currently developed for these tasks. It is a separation technique for macromolecules and particles based on hydrodynamic principles. Besides, FFF is a versatile tool for the analysis of engineered nanoparticles in complex samples due to the availability of different subtypes like Flow-FFF and Sedimentation-FFF, a wide variability of run conditions, a relatively broad accessible size range, and the possible coupling with sensitive detection systems like ICP-MS. [1].

The strategy adopted for the analysis of ENPs in complex matrices must be adapted to the type of the ENPs and the nature of the matrix. Therefore, different methodologies have to be specifically developed for the analysis of AgNP in the background of natural nanoparticles and SiO2NP in food matrices. These new developments have been performed on reference samples and are presented here.

2. Materials and method

The method development for the extraction of AgNPs from soil and sewage sludge was done by spiking a known amount of a well characterized AgNP (NM300 from the OECD repository) to a reference soil (Refesol soil 01-A) and dried sewage sludge samples from model waste water treatment plants. Extractions of the particles from the matrix were performed using solutions containing different surfactants at various pH supported by ultrasonication. Dissolved Ag-species were separated with centrifugal ultrafiltration and total silver concentrations determined by ICP-MS. Total silver concentrations in the soil and sludge samples were determined by microwave assisted acid digestion and ICPMS analysis. Concerning the SiO2NPs, a known amount is spiked to tomato soup. Extractions of the particles from the matrix were performed using microwaved assisted acid digestion followed by a re-stabilization step in a surfactant mixture at high pH.

Two subtypes of FFF were used, Flow-FFF and Sedimentation-FFF. The FFF-systems were coupled to UV-DAD spectrometry, 3D-fluorescence detection, online static and dynamic light scattering and ICP-MS. AgNP and SiO2NP dispersions and a natural colloidal soil extract (containing a typical assembly of natural nanoparticles found in the environment) were used to develop and evaluate suitable separation conditions for the different samples.

3. Results and discussion

Extractions of AgNPs from a spiked soil showed the highest efficiencies of ~25% using solutions containing a surfactant mixture at pH 9. The size distribution of silver determined with FFF showed two peaks (Fig. 1). The second peak can be identified as free AgNPs extracted from the soil matrix while the first peak eluting in or close after the void peak is probably formed by small complexes of dissolved Ag with surfactant molecules or humic substances. Extraction efficiencies were much higher using solutions with highest pH compared to surfactant-based extractions. Nevertheless, for pH 11 high concentrations of dissolved silver were found in the filtrates of the centrifugal ultrafiltration and no peak from single particles could be found in the FFF-fractograms.
No particulate Si background was identified in the tomato soup (Fig. 2). The $^{28}$Si elemental size distribution of acid digested SiO$_2$NP spiked to fresh tomato soup is very similar to the one of pure SiO$_2$NPs. This result shows that the extraction did not affect the size distribution of the SiO$_2$NP. The $^{28}$Si mass recovery is $\sim$ 67%. This could be explained by a partly dissolution of the SiO$_2$NP during the digestion procedure or an interaction of the SiO$_2$NP with the background of the tomato soup.

4. Conclusions

It is possible to detect and quantify AgNPs in a soil extract and SiO$_2$NP in food matrices using Flow-FFF and conventional ICP-MS as a sensitive elemental detector. The analysis of model systems used in the method development showed no intensive interaction of the engineered silver nanoparticles with the natural nanoparticles of the colloidal soil extract but some interaction of SiO$_2$NP with the tomato soup background cannot be excluded. Fractionations of colloidal extracts of soil samples spiked with AgNPs showed the presence of free particles as well as the formation of dissolved silver species which are probably complexes of silver with humic substances. Those dissolved species were not observed in the case of SiO$_2$NP. The next step, concerning AgNPs, would be to apply this methodology to mixed samples of sewage sludge and agricultural soils to determine the size distributions and concentrations of silver nanoparticles probably released into the soil and investigate extraction efficiencies across different matrices. Concerning the SiO$_2$NP, the next step is to test the reproducibility of the results and improve the recovery in order to reach a fully quantitative method.

5. References


Acknowledgement – European Commission under the 7th Framework Programme (NANOLYSE project).
1. Introduction

We aim at understanding the fate of silver nanoparticles in river biofilms. This work focusses on the effects of the extracellular matrix of biofilms on silver nanoparticles (Ag NPs) and introduces new possibilities to visualize nanoparticles in environmental samples.

River biofilms, or periphyton, are the main producers of biomass and oxygen in freshwater ecosystems and serves as a filter. Disturbance of these communities may thus adversely affect the local ecosystem. Ag NPs dispersed in river water will first interact with the extracellular matrix (EM) of periphytic organism before the latter are actually exposed. EM components may change the physical and possibly chemical properties of the nanoparticles and thus influence the interaction with and effects on the organisms. To understand the interaction of Ag NPs with periphytic organisms it is thus essential to know if and how they are modified by the EM.

2. Materials and methods

2.1. Culturing of autotrophic biofilms, extraction and characterization of EM

Autotrophic biofilms are colonized on glass slides in indoor channels in a flow-through system fed with water from river Chriesbach (Switzerland). Illumination is provided by fluorescent light tubes with a spectrum similar to daylight at 100 µE/m²s in a 12h:12h light:dark cycle. Three week old biofilms are transferred into 2mM NaHCO₃ (pH 7.6) containing protease inhibitors. The biomass is removed by sonication, centrifugation, and filtration. The extract is analyzed for cell lysis (Glucose-6-phosphate dehydrogenase assay), protein content (SDS-PAGE and Coomassie staining), TOC/DOC content (photo-oxidation followed by UV detection), and DOC size distribution.

2.2. Incubation of extracellular matrix with silver nanoparticles and silver nitrate and sample characterization

Ag NPs (primary particle size: 30nm, stabilized with carbonate) and silver nitrate (AgNO₃) are diluted in 2mM NaHCO₃ (pH 6-8.6) in a total volume of 10mL containing 10mg/L DOC/EM and stirred at 400rpm in the dark or the light (see 2.1). Samples without EM and without nanoparticles/silver nitrate serve as controls. The samples are characterized at several time points to assess the presence or absence of NPs and the size (by dynamic light scattering (DLS), NP tracking analysis (NTA), electron microscopy (EM)), stability (electrophoretic mobility), Plasmon resonance (UV-VIS), dissolution (ICP-MS of ultrafiltrates (3kDa) after acidic digestion), and chemical composition (ESRF) of the NPs present.

2.3. Characterization of nanoparticles within biofilms

Intact biofilms are rinsed with NaHCO₃ and incubated with Ag NPs and silver nitrate in the dark or the light (same conditions as in 2.1). NPs within the biofilms are visualized and characterized using confocal laser scanning microscopy (CLSM) in reflection mode and NTA.
3. Results and discussion

3.1. Weathering of silver nanoparticles depends on light, pH, and biofilm EM

Dispersions of Ag NPs were exposed to EM from biofilms under varying conditions mimicking different natural states (+/- light, varying pH due to photosynthetic activity) over the course of one week. Nominal Ag concentrations were 0.5 mg/L and 5 mg/L. The mean particle diameter was 40nm (DLS) and remained stable in NaHCO₃ at pH 6, 7.6, and 8.6 over one week in both light and dark. Electrophoretic mobility, plasmon resonance, and free Ag⁺ concentration remained constant over time. The addition of EM lead to slightly larger particle diameters but dispersions remained stable in the dark. Exposure of Ag NP dispersions containing EM to light lead to pH-dependent weathering over time. Among others, the particle size increased and the size-dependent plasmon resonance signal broadened (pH 8.6) or disappeared (pH 6, pH 7.6) (Fig. 1). We are currently determining the composition of the NPs over time.

Figure 1: UV-VIS light absorption of AgNP dispersions after 1 week in the light. Dashed curves show a typical Plasmon resonance signal.

- Ag NPs may remain stable in the absence of organic material but may weather quickly in the presence of EM and light.

As a fraction of the nanoparticles (<1%) is dissolved and thus present as ionic silver, we wanted to assess the influence of the ions on the weathering process.

3.2. De novo synthesis of silver nanoparticles in the presence of EM from biofilms

Silver nitrate was incubated under the same conditions as silver nanoparticles and characterized using the same methods. There was no significant nanoparticle formation in the dark in the absence of EM and a low rate of particle formation in the light. In the presence of EM in the light, de novo particle formation was observed within minutes and at a slower rate in the dark. The size of the resulting particles was pH dependent. The presence of a Plasmon resonance signal shows that at least parts of the NPs are elemental Ag. We are currently measuring the chemical composition of the newly formed particles.

- Nanoparticles may form from Ag⁺ in the presence of EM material.
- As part of the newly formed particles are elemental silver, reduction must have taken place, either by interaction with photons (photoreduction) and/or with EM.

3.3. Silver nanoparticles can be visualised and characterized in intact biofilms

We have developed a new approach using confocal light scanning microscopy (CLSM) and NTA to characterize NPs in biofilms. The size of Ag NPs was thus determined in intact biofilm samples. We are now going to measure the size change of particles over time and also try to track the formation of Ag NPs in biofilms exposed to Ag⁺. The chemical identity of the NPs in the biofilms will be analysed via CLSM-Raman Spectroscopy.

4. Conclusions

Ag NPs, Ag⁺, and EM components from river biofilms form a dynamic system which is influenced by pH and presence/absence of light. Nanoparticles may increase in size, dissolve, and form de novo. Our results indicate that wherever there is Ag⁺ and EM in the environment, nanoparticles may form. This is essential information for the assessment of the potential environmental effects of AgNPs.
Trophic transfer of gold nanoparticles in a water food chain

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1. Introduction

The risk of nanoparticles is becoming an issue. The nanoparticles are released into water environment and they may cause adverse effect to ecosystem. They are ultrafine particles and can be easily penetrated through cell membrane. Furthermore, they may transfer in a water food chain. Previous studies reported the trophic transfer of quantum dot, gold nanoparticles, and titanium dioxide nanoparticles in an aquatic food chain [1, 2, 3, 4, 5]. To understand their potential risk of gold nanoparticles regarding food chain transfer, we investigated a trophic transfer of gold nanoparticles (AuNPs; average 10 nm) in a basic water food chain. The low and high trophic level organisms of this study were green algae *Chalmydomonas reinhardtii* and invertebrate *Daphnia magna*.

2. Materials and methods

- Gold nanoparticles were obtained in an aqueous colloidal state from Sigma Aldrich (St. Louis, MO, USA). The particle sizes ranged from 8.2~12.0 nm (average 10 nm).
- *Chalmydomonas reinhardtii* (UTEX 2244) was chosen as food source (low trophic level) and *Daphnia magna* was selected as high trophic level organism.
- *Chalmydomonas reinhardtii* (1 × 10^5 cell/ml) was initially exposed to gold nanoparticles directly. The exposure method was modified OECD guidelines for the testing of chemicals No. 201 [6]. After 48 hours, algae cells were collected and washed to remove gold nanoparticles.
- 2-day old *Daphnia magna* were fed by the exposed algae cells (2.5 × 10^5 cell/ml) during 24 hours.
- The bioaccumulation of each species and ionization of gold nanoparticles in the exposure medium were measured by ICP-AES.

3. Results and discussion

3.1. Algae exposed to gold nanoparticles; Direct exposure

- Growth rate of *Chalmydomonas reinhardtii* was not inhibited at 1 mg/L of AuNPs.
- The bioaccumulation of gold nanoparticles in *Chalmydomonas reinhardtii* were about 1.5 times higher than that in control group.
- Dissolution of gold nanoparticles in test condition is negligible. Therefore, effect of free gold ion was not considered in this study.

*Fig. 1. TEM image of gold nanoparticle*
3.2. Trophic transfer of gold nanoparticles from algae to invertebrate; food exposure

- Mortality and adverse effect of *Daphnia magna* fed by algae, which was previously exposed to gold nanoparticle, was not significant.
- The bioaccumulation of gold nanoparticles in *Daphnia magna* were about 1.9 times higher than that of control group.
- The bioaccumulation was confirmed, however, biomagnification was not observed.

4. Conclusions

The results showed that gold nanoparticles can transfer from *Chalmydomonas reinhardtii* to *Daphnia magna* via food exposure. This study demonstrated that nanoparticles can be transferred from the algae to invertebrates in aquatic ecosystem.

5. References


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Effect of non-ageing and ageing ceria nanoparticles suspensions on fresh water micro-algae

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When assessing the hazards properties of nanomaterials in the environment, the main research challenges are numerous. Firstly, determining if nanomaterials are more or less toxic than the bulk forms of the same materials and the extent to which toxicity is governed or influenced by the physico-chemical properties of the nanoparticles. Secondly, it appears necessary to study the effect of nanomaterials and nanoparticles throughout their life cycle including both initial forms and physico-chemically modified form (i.e. aggregated or agglomerated forms) resulting from an ageing process.

Our work focused on the effect of commercial ceria nanoparticle (nCeO$_2$) suspensions, towards freshwater micro-algae assessing the effect nCeO$_2$ suspensions with different agglomeration/aggregation state obtained by using an artificial ageing process. Both ageing and non-ageing nCeO$_2$ suspensions were fully characterized using dynamic light scattering (ZetaSizer, Malvern Instruments) or laser diffraction (MasterSizer, Malvern Instruments) and transmission electron microscopy (TEM). In addition, the interaction between NPs and algae were investigated using flow-cytometry and environmental scanning electron microscope technique (E-SEM).

The results obtained showed that the algae growth inhibition was similar after exposure to non-ageing or ageing nCeO$_2$ suspensions. The results obtained from flow-cytometry and E-SEM proved that the ceria NPs are able to tightly entrap the algae cells, which could in part contribute to the effect recorded. Those results also support the fact that aggregation or agglomeration has a few influences when focusing on the standardized algae ecotoxicity test. Moreover by comparison to our previous studies performed with other ceria suspensions, it was shown that the primary particle size and consequently the particle surface area is a relevant parameter in assessing the ecotoxicity of nanoparticles.

Key words: Ceria nanoparticles, Pseudokirchneriella subcapitata, agglomeration state, ageing suspensions
Effect of non-ageing and ageing ceria nanoparticles suspensions toward fresh water micro-algae

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1. Introduction

When assessing the hazards properties of nanomaterials to the environment, the effect of nanomaterials and nanoparticles including both initial form and altered form should be addressed. Thus, the present work aimed at investigating the effects of a commercial ceria nanoparticle composite suspension (nCeO$_2$) toward freshwater algae. The ecotoxic effects were investigated using both non-aging (freshly prepared) or aging (artificially altered) nCeO$_2$ suspensions. The standardized green algae Pseudokirchneriella subcapitata was used for the algae growth inhibition experiments. All the suspensions were fully characterized using dynamic light scattering (ZetaSizer, Malvern Instruments) or laser diffraction (MasterSizer, Malvern Instruments) and transmission electron microscopy (TEM). In addition, the interaction between NPs and algae were investigated using flow-cytometry and environmental scanning electron microscope technique (E-SEM).

2. Nano-ceria suspensions

Commercial nCeO$_2$ were obtained as 248 g/L stable suspension in water. A primary particle size of 10 nm was reported by the supplier. For the ecotoxicity tests, an initial nCeO$_2$ suspension (25 mg/L) was prepared by dilution of the commercial suspension into the algae growth media (OECD 201 growth media). This suspension was artificially aged under light and slow magnetic stirring for 3 days. Another freshly (non-ageing) nCeO$_2$ suspension was prepared 15 minutes prior to the experiment using the same dilution protocol but without ageing process. For the ecotoxicity tests, nCeO$_2$ suspensions with nominal concentrations from 0.195 to 25 mg/l (8 concentrations) were then prepared by dilution of the ageing and non-ageing initial nCeO$_2$ suspensions in the algae growth media. The average specific growth rate of P. subcapitata in each concentration was calculated each day up to 72h for both ageing and non-ageing nCeO$_2$ suspensions.

3. Characterization of non-ageing and ageing suspensions

Investigation of the initial nCeO$_2$ suspensions in algae growth medium showed that ageing and non-ageing suspensions mainly differ in term of agglomeration/aggregation state. As illustrated by figure 1, the non-ageing suspension were mainly composed of small nCeO$_2$ agglomerates or aggregates around 30 nm with some of them up to 500 nm. By comparison, the ageing suspension showed large and loose agglomerated particles up 10 µm (Figure 1).

4. Algae growth inhibition test

The results obtained clearly show that nCeO$_2$ are ecotoxic towards micro algae. Moreover, the effects recorded were similar after both ageing and non-ageing nano-ceria suspension. We have calculated EC$_{50}$ values of 1.4 mg/L and 1.8 mg/L for the non-ageing and the ageing ceria suspensions, respectively. This observation suggested that whatever the agglomeration state, the algae growth inhibition is similar when focusing on the algae growth inhibition test. Moreover, compared with our previous works with other nano-ceria suspensions [1], our data support the view that the primary particle size and consequently the surface area might be an important parameter to take into account, as previously suggested by Van Hoecke et al. [2].
5. Nanoparticles–Algae interaction

The interaction between ceria NPs and algae were investigated by flow-cytometry and using E-SEM technique. The cytogram distributions showed an increase in cell complexity with increasing \( n\text{CeO}_2 \) concentration which suggests a potential adsorption onto the cell wall or a potential internalization into the cell. Without demonstrating the NPs internalization in cells, the results obtained from E-SEM confirmed that the ceria NPs are able to tightly entrap and wrap the algae cells (figure 2). These observations suggest that the algae ecotoxicity could, in part, be due to this close interaction by limiting the transport of metabolites and nutrients across the cell wall, or by inducing cell membrane disruptions and oxidative stress [3].

6. References


Bioavailability of carbon nanotubes to aquatic organisms of different trophic levels and the consequences of CNT-cell interactions to vital functions.

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1. Introduction

There is a distinct lack of analytical methods for the detection of carbon nanotubes (CNT) during ecotoxicological testing and in environmental samples. Hence, quantitative data on the partitioning of this material in aquatic media and distribution to and within biota is scarce. Although some studies suggest that CNT have the capacity to induce toxicity to aquatic organisms, the mechanisms behind observed effects remain largely unknown [1,2].

In this study, radiolabelled CNT were used to quantify bioaccumulation of this nanomaterial in different aquatic organisms. Next to uptake and elimination, transfer of CNT along the food chain, the influence of the presence of sediment or dissolved organic carbon (DOC) on its bioavailability, and distribution of incorporated CNT to different fish tissues were investigated. The results were linked to effect tests with the objective of unravelling possible damage to vital functions in CNT-exposed organisms.

2. Materials and methods

Radiolabelled multiwalled CNT were synthesised by means of catalytic chemical vapour deposition of 14C-benzene. The 14C-CNT product was washed with a 12.5% HCl solution in order to remove excess catalyst (>95% purity) and showed a high specific radioactivity (1.3 MBq/mg). Test suspensions of 1 mg 14C-CNT/L were prepared and ultrasonicated with a microtip (70 W, 2 x 5', 0.2" pulse & 0.8" pause). The homogeneity of dispersions was verified by means of liquid scintillation counting (LSC). Transmission electron microscopy (TEM) analysis showed the presence of single tubes and small agglomerates in the medium.

The uptake of 14C-CNT by algae (Desmodesmus subspicatus), daphnids (Daphnia magna), blackworms (Lumbriculus variegatus) and fish (Danio rerio) from the water phase was determined over time. In a parallel setup, 8 mg DOC/L was additionally supplemented to the medium of daphnids and fish. Furthermore, worms were exposed to the same amount of CNT via both compartments of a sediment-water system. Food chain transfer was investigated by administrating previously exposed algae to daphnids and spiked worms to fish. Fish were dissected after both water and dietary exposure to evaluate distribution of 14C-CNT to different fish tissues. Both liquid (aqueous media and solubilised biota samples) and solid (sediment; up to 1 g in 18 mL cocktail) samples were subjected to LSC. Visualisation of CNT in cells and tissues was performed with TEM.

The influence of the observed interaction of CNT material with gut epithelium (section 3.1) on the digestibility of food by fish was investigated by comparing the condition factor (CF = weight*100/length³) of exposed and control individuals (Table 1). All fish remained unfed during the 4 days the test animals were exposed to 1 mg CNT/L. Then, all fish were transported to clear water and given 10 mg of Artemia nauplii daily for 6 days. After another 24 h of gut purging, the length and weight of the fish were determined.

<table>
<thead>
<tr>
<th>day</th>
<th>start</th>
<th>day 1</th>
<th>day 2</th>
<th>day 3</th>
<th>day 4</th>
<th>day 5</th>
<th>day 6</th>
<th>day 7</th>
<th>day 8</th>
<th>day 9</th>
<th>day 10</th>
<th>day 11</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mg CNT/L (n=10)</td>
<td>suspension renewal</td>
<td>clear water</td>
<td>clear water</td>
<td>feeding (10 mg/fish)</td>
<td>feeding (10 mg/fish)</td>
<td>feeding (10 mg/fish)</td>
<td>feeding (10 mg/fish)</td>
<td>feeding (10 mg/fish)</td>
<td>feeding (10 mg/fish)</td>
<td>feeding (10 mg/fish)</td>
<td>feeding (10 mg/fish)</td>
<td>feeding (10 mg/fish)</td>
</tr>
<tr>
<td>controls (n=10)</td>
<td>clear water renewal</td>
<td>clear water</td>
<td>clear water</td>
<td>clear water</td>
<td>clear water</td>
<td>clear water</td>
<td>clear water</td>
<td>clear water</td>
<td>clear water</td>
<td>clear water</td>
<td>determining L &amp; W</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Setup of the fish effect test (L: length; W: wet weight; n: number of replicates)
Since algae incorporated radioactivity following exposure to $^{14}$C-CNT and single tubes were visualised inside the cells (section 3.1), the cell composition of control and (24, 48, and 72 h) CNT-exposed algae was compared by means of Attenuated Total Reflection Fourier-Transform InfraRed spectroscopy (ATR-FTIR) according to a previously described methodology [3]. The results were interpreted using principal component analysis (PCA) and linear discriminant analysis (LDA).

3. Results and discussion

3.1. Bioavailability experiments

Radioactivity was detected in all test species, which had been exposed to dispersed $^{14}$C-CNT material via the water. Transfer of the organisms to fresh medium led to quick elimination of most but not all radioactivity, except for daphnids that needed the presence of uncontaminated food to clear their guts. Single tubes and small agglomerates were visualised by means of TEM in algal cells, branchial erythrocytes of fish, and gut epithelium of daphnids, worms, and fish. Transfer of CNT from the fish gills to the blood current was confirmed by the presence of radioactivity in blood samples and gonads. However, CNT were not detected in the brain of zebrafish, which indicates that they do not pass the blood-brain barrier. It should be mentioned that practically all incorporated radioactivity was located in the gut of fish and much smaller fractions were present in the gills, the skin, and the filet. Other tissues contained only traces and the data showed large variability among individuals. Waterborne CNT were quickly transported to the sediment. However, sediment spiked CNT (1 mg $^{14}$C-CNT/kg dry weight) were not bioavailable for blackworms ingesting this medium. Similarly, consumption of CNT-containing prey by daphnids (CNT-spiked algae) or by zebrafish (CNT-spiked worms) resulted in lower accumulation in the predator compared to after uptake of equal amounts via the water phase. Although DOC was shown to keep prepared dispersions more stable over time, its presence had no influence on CNT bioavailability.

3.2. Effect tests

The CF of fish that had taken up CNT during four days was significantly lower compared to the one of control organisms. This was only the case at the end of the experiment, i.e. after a subsequent period of six days, in which food was administered to the animals (Table 1). This might indicate that the digestion of food was hindered due to the observed presence of CNT material in gut epithelial cells, causing these fish to have a lower weight compared to unexposed fish. Longer tests with repeated exposure periods are performed at the moment to verify this finding.

ATR-FTIR (PCA-LDA) analysis revealed differences in the composition of exposed compared to control algal cells in biomarker IR-regions that could be aligned to lipids, DNA, and poly-saccharides (PS). In case of PS, the deviation from the control was higher with increasing exposure times. The observed differences for lipids and DNA were the largest in cells of the 48 h CNT treatment, but were not detected after exposing algae for 72 h. These results may indicate subcytotoxic effects of CNT to algae, possibly associated with uptake by and interactions with the cells. However, more research is necessary to investigate the latter.

4. Conclusions

$^{14}$C-tracking allowed quantification of the partitioning of CNT to water, sediment and organisms in aquatic systems. In this way, dispersed $^{14}$C-CNT were shown to be bioavailable for all investigated organisms, which was confirmed with TEM. Sediment functioned as a sink for CNT material, but lowered incorporation in biota. Preliminary effect tests indicate that CNT-cell interactions might involve consequences for organisms.

5. References


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Short-term toxicity of silver nanoparticles on litter-associated fungi and bacteria from streams

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1. Introduction
The risks related to the widespread use of AgNP are closely linked to the antimicrobial property of silver, raising the issue of their harmful effects on aquatic biota and ecosystem functions [1]. A useful system to assess effects of AgNP released in the environment is the complex of microbial decomposers, composed by fungi and bacteria, which degrade large amounts of plant litter in natural ecosystems and play an important role in nutrient recycling and energy transfer through the food chain [2]. The aim of our study was to investigate the short-term toxicity of increasing concentrations of AgNP on leaf-associated microorganisms from streams (i.e. fungi and bacteria), in comparison to the toxicity of AgNO3 (Ag+). This was achieved by assessing the response of various functional endpoints in short-term inhibition tests. The specific questions investigated were: (i) Do AgNP have genuine toxic properties or is toxicity mediated by dissolved Ag+? (ii) Is the toxicity of AgNP and Ag+ time dependent? Do different functional endpoints differ in their sensitivity to AgNP and Ag+?

2. Materials and methods

2.1. Experimental schedule
To allow litter colonisation by microbial decomposers, alder leaves were submerged in a low-order stream in northeastern Germany. After 3 weeks, leaves were retrieved, transported to the laboratory and immediately processed. Leaf discs (12-mm diameter) were cut, placed into 50-mL sterile tubes (132 mm² of total leaf surface/tube), which served as microcosms. A simple nutrient medium (30 mL) was added and supplemented with increasing nominal concentrations of citrate coated AgNP (size 25±13nm; zeta potential -36.6±3.2 mV in nanopure water [3]) or AgNO3 (0, 0.5, 2.5, 5, 25 and 50 µM). All microcosms were agitated at 15°C in the dark. The duration of exposure was 5 and 10 hours.

2.2. Measured functional endpoints
- Fungal endpoints: the short-term toxicity of AgNP and AgNO3 on fungi was monitored by determining the reductin of fungal growth (measured as 14C-acetate incorporation into ergosterol) and sporulation rate of aquatic hyphomycetes (by microscopic counting).
- Bacterial endpoints: the short-term toxicity of AgNP and AgNO3 on bacterial growth was examined by determining 14C-leucine incorporation into proteins.
- General microbial endpoints: The reduction of other functional endpoints targeting microbial activity was evaluated by determining potential extracellular enzyme activity (phosphatase, β-glucosidase, and leucine-aminopeptidase by using fluorescent-linked substrates) and respiration (MicroResp™ method [4]).

2.2. Particle characterization and metal analysis
- AgNPs were characterized in nutrient media in the presence and absence of leaf discs colonised by microorganisms in the field. Measurements were taken after 5 and 10 hours of exposure to AgNP. Size and zeta potential were analysed by Dynamic Light Scattering (DLS) using a Zeta Sizer (Nano ZS, Malvern Instruments) and by Nanoparticle Tracking Analysis (NTA) using a Nanosight (LM10, Nanosight Ltd.).
- The total silver concentration was measured in acidified solution (0.1 M HNO3) by ICP-MS. Silver dissolution after 5 and 10 hours of exposure was examined by centrifugal ultrafiltration through a membrane with a nominal molecular weight limit of 3 kDa. The concentration of Ag+ in the filtrate was related to the total Ag concentration before ultrafiltration as determined by ICP-MS.
3. Results and discussion

The obtained results showed no inhibitory effect of AgNP on microbial respiration, even at the highest concentration and longest exposure time tested. This finding contrasts with the observed reduction in respiration induced by Ag⁺. Furthermore, all potential extracellular enzyme activities measured responded differently to AgNP or Ag⁺ exposure. After 5 hours of exposure, phosphatase activity was stimulated by exposure to the lowest concentrations of AgNP and Ag⁺ (from 0.5 to 5 µM) reaching 150%. However, after 10 hours a significant inhibition of this activity was observed at the highest concentration of AgNP (i.e. 25 and 50 µM) but not following exposure to AgNO₃. Potential leucine amino-peptidase activity was reduced by exposure to both AgNP and Ag⁺. This reduction was more pronounced after 10 hours, reaching a 75% decline indicating that both AgNP and Ag⁺ displayed a time-dependent short-term toxicity. Finally, at all exposure times, β-glucosidase activity was stimulated by AgNP in the same way as phosphatase, but it was inhibited by Ag⁺ even at the lowest concentrations. The results obtained on the three extracellular enzymes examined suggest that AgNP influence nutrient acquisition of litter-associated fungi and/or bacteria in streams. Fungal and bacterial growth and sporulation rate were strongly inhibited by both AgNP and Ag⁺, even at the lowest concentrations. However, the toxicity of AgNP was much higher than that of Ag⁺. These differences were observed even after 5 hours.

Table 1: Summary of the effects of AgNP and Ag⁺ on a range of functional endpoints

<table>
<thead>
<tr>
<th>Respiration</th>
<th>Bacterial growth</th>
<th>Fungal growth</th>
<th>Sporulation</th>
<th>Phosphatase</th>
<th>Leucine-aminopeptidase</th>
<th>β-Glucosidase</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNP</td>
<td>-</td>
<td>+++</td>
<td>++</td>
<td>++</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Ag⁺</td>
<td>+</td>
<td>++</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* No effect detected; + effect detected; * stimulation

4. Conclusion

In conclusion, our hypothesis that toxicity of AgNP may be modulated by dissolved Ag⁺ could not fully explain the results obtained in short-term toxicity tests. AgNP appear to exert a genuine toxicity on fungi and bacteria, independent of the effect of Ag⁺. This study highlights that toxicity effects can vary according to the functional descriptor used, confirming the need to focus on multiple functional endpoints for ecotoxicological investigations into the environmental effects of nano-sized materials.

5. References

The effect of ageing on the bioavailability and ecotoxicity of 
ZnO-NP, bulk ZnO and ZnCl₂ in natural soil

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1. Introduction

Manufactured zinc oxide nanoparticles (ZnO-NP) are used in a wide range of industrial and consumer products and have different uses such as environmental remediation, sunscreen application and waste water treatment. Releases into soils are expected when nanoparticles pass the sewage treatment plant or end up in sewage sludge. Concern has risen about ecotoxicological effects of nanoparticles. Short-term toxicity tests and bioaccumulation studies have already been performed with isopods, earthworms and springtails [1,2,3]. Little information is available on the long-term fate and effects of ZnO-NP in soils. The (bio)availability may change with time due to dissolution, sorption and/or aggregation of the nanoparticles. Long-term processes, called ageing, have shown that zinc availability in the soil decreased with time [4]. It is unknown whether the bioavailability of ZnO-NP in aged soils decreases in a similar manner as the zinc salt. Scheckel et al. (2010) report that ZnO-NP were rapidly converted to Zn²⁺ sorption complexes in a kaolin suspension [5]. However, dissolution of ZnO-NP and the release of the potent Zn²⁺ in soils have not been studied so far.

Here the behaviour of ZnO-NP, bulk ZnO and ZnCl₂ was studied in natural soil to evaluate the influence of aging time on chemical speciation and stability of the nanoparticles. Dissolution and ecotoxicity to the springtail Folsomia candida was determined for the three Zn forms after three, six and twelve months.

2. Materials and methods

ZnO-NP, bulk ZnO and ZnCl₂, were added to Lufa 2.2 natural soil as a suspension in soil extract [6]. Glass jars were stored in a climate room at 20 °C for a one-year period. Twice a month, moisture content of the test soils was checked by weighing the jars, and moisture loss was replenished with Milli-Q. After three, six and twelve months the soil was used to collect pore water and to perform a toxicity test with F. candida.

Toxicity tests were carried out following ISO-guideline 11267 for assessing the effects of chemicals on Collembola [7]. Age-synchronised animals were exposed to aged soil for four weeks and effects on survival and reproduction determined. LC/EC50 was estimated applying a logistic model [8].

After spiking and after one year, soil samples were analyzed for total zinc by digestion in a mixture of Milli-Q, concentrated HCl and concentrated HNO₃ and analysis by flame Atomic Absorption Spectrometry (AAS). Soil pore water was collected by centrifugation soil, after saturation with Milli-Q and one week equilibration. Soils were centrifuged for 50 min. at 2000 g over two paper filters and a membrane filter (0.45 μm), placed inside the tubes [9]. Pore water samples were also analysed by flame AAS.

3. Results and discussion

3.1. Dissolution of ZnO-NP in soil

Recoveries above 80% were found for all test soils after spiking and after one year ageing. Zinc concentrations in the soil pore water ranged from 1.85 to 12.6 mg Zn/l in freshly spiked soil with ZnO-NP and this increase was found to be linear. Porewater concentrations after three, six and twelve months increased in a non-linear manner with increasing soil concentrations for ZnO-NP and bulk ZnO (Fig. 1). Zn concentrations in the pore water increased with time, but peaked at intermediate concentrations. The highest Zn concentrations measured after one year ageing were 67.1 and 66.5 mg Zn/l for ZnO-NP and bulk ZnO, respectively. The amount of dissolved Zn was negligible compared with the total amount of Zn introduced as ZnO-NP, i.e. it was below 2% after one year.

Zn concentrations in pore water, collected from aged soils with ZnCl₂, increased with exposure concentration, but also with time (Figure not shown). The percentage Zn dissolved based on the total amount of Zn introduced at the highest spiking concentration was 8.55% in freshly spiked soil and 16.7%, 17.9% and 14.58% after three, six and twelve months of ageing, respectively.
Fig. 1 Measured zinc concentrations in soil pore water (mg Zn/l) as a function of zinc concentrations in Lufa 2.2 soil (mg Zn/kg) before ultrafiltration in freshly spiked soil with ZnO-NP (T=0), after three months (T=3), six month (T=6) and twelve month (T=12) ageing.

3.2. Ecotoxicity to *F. candida*

ZnO-NP and bulk ZnO were toxic in freshly spiked soil with no significant difference between the two ZnO powders [2]. No effect on Collembolan survival or reproduction was found in three, six or twelve months aged soil with ZnO-NP and bulk ZnO (Table 1). The toxicity of ZnCl₂ decreased with time as shown by the three-fold increase in EC50 value after three months ageing. Table 1 gives an overview of the toxicity data found for the effect on survival and reproduction.

Table 1: LC/EC50 values for the effect on the survival and reproduction of *Folsomia candida* after 28-d exposure to ZnO-NP, bulk ZnO and ZnCl₂ to freshly spiked soil (T=0) and to three (T=3), six (T=6) and twelve months (T=12) aged soil. Values are presented as total concentrations in the soil (mg Zn/kg d.w.) or as soluble Zn (mg Zn/l) in pore water.

<table>
<thead>
<tr>
<th></th>
<th>ZnO-NP (EC50)</th>
<th>Bulk ZnO (EC50)</th>
<th>ZnCl₂ (EC50)</th>
<th>ZnCl₂ (LC50)</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg Zn/kg</td>
<td>mg Zn/l</td>
<td>mg Zn/kg</td>
<td>mg Zn/l</td>
<td>mg Zn/kg</td>
</tr>
<tr>
<td>T=0</td>
<td>1964</td>
<td>10.1</td>
<td>1591</td>
<td>7.94</td>
</tr>
<tr>
<td>T=3</td>
<td>&gt;6400*</td>
<td>&gt;44.5*</td>
<td>&gt;6400*</td>
<td>&gt;42.5*</td>
</tr>
<tr>
<td>T=6</td>
<td>&gt;6400*</td>
<td>&gt;56.4*</td>
<td>&gt;6400*</td>
<td>&gt;46.3*</td>
</tr>
<tr>
<td>T=12</td>
<td>&gt;6400*</td>
<td>&gt;67.1*</td>
<td>&gt;6400*</td>
<td>&gt;66.5*</td>
</tr>
</tbody>
</table>

*No effect on survival or reproduction was observed at the highest test concentration.

- Data did not allow to estimate an EC50 value

4. Conclusions

The release of Zn ions from ZnO-NP was reduced at high spiking concentrations. Sorption and agglomeration may inhibit dissolution and increased sorption may take longer due to the physical and chemical properties of nanoparticles. An increasing solubility throughout one year was observed, although at very low rate when based on total Zn concentrations.

Several studies show that toxicity to soil organisms is related to the free ions released from metal nanoparticles. Our toxicity tests show that an ageing period of three months or longer is able to reduce toxic effects on survival or reproduction to *F. candida*.

5. References

Effects of C$_{60}$ nanoparticles on *Lumbricus rubellus* earthworms: from gene expression to population dynamics

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1. Introduction

Carbon-based nanomaterials, such as carbon nanotubes and C$_{60}$, are among the most commonly available engineered nanomaterials. Production and use are assumed to increase significantly over the next decades (1) and release of these nanoparticles into the environment will be inevitable. Several studies have demonstrated that C$_{60}$ exposure can be toxic to animals [2], including earthworms [3,4].

In the present study the impact of C$_{60}$ exposure to *Lumbricus rubellus* earthworms is assessed at different levels of biological integration. Effects on growth, reproduction and mortality were assessed and used to predict consequences at the population level. In order to relate these observations to potential modes of action, sub-lethal responses (gene expression and histology) were also studied. As an effect on immune response was noted at the molecular level, *in vitro* experiments with immune cells were conducted.

2. Materials and methods

C$_{60}$ was obtained as a dry powder and dissolved in an aqueous soil extract, before addition to the soil [4]. Two experiments were carried out. In the first experiment, adult earthworms were exposed for four weeks and in the second experiment the offspring was exposed lifelong. Earthworms were exposed to nominal C$_{60}$ concentrations of 0, 15 or 154 mg C$_{60}$/kg soil (dry weight).

Growth, reproduction and mortality were assessed in the four week experiment. For the lifelong experiment, the earthworms were checked monthly for survival and growth. These effect markers were used to extrapolate to the population level. The used model was based on a simple Dynamic Energy Budget (DEB) model (4,5). Gene expression analysis was performed, at the end of both experiments, at the National Environmental Research Institute (NERI) in Denmark. Samples were taken from whole earthworms and qPCR was conducted for several selected genes, including heat shock protein 70 (HSP70) and coelomic cytolytic factor 1 (CCF-1). Histological observations were performed at the University of Plymouth on tissue sections from earthworms sacrificed at the end of both experiments.

*In vitro* experiments were performed using primary coelomocytes, extracted from *Lumbricus rubellus* adult earthworms. Cells were exposed for 24 hours to a concentration range from 0.001 to 400 ug C$_{60}$/ml medium, after which survival and phagocytic activity of the cells were tested.

Furthermore, C$_{60}$ was characterized in the exposure media, to elucidate the actual exposure. Samples from C$_{60}$ were studied using ICP-AES, spectroscopy, TEM, DLS and zeta potential analysis.

3. Results and discussion

3.1. Effects on the molecular and cellular level

Changes on gene expression were tested for seven selected genes (HSP70, CCF-1, catalase, GST, SOD, GAPDH and PFK-1), but significant effects were only demonstrated for the general stress effect marker HSP70 and cytokine-like CCF-1. HSP70 showed a significant concentration-dependent down-regulation of expression in both experiments. This down-regulation may be explained by emerging tolerance for C$_{60}$ exposure, up-regulation of other heat shock proteins and/or tissue repair. CCF-1 expression did not
significantly change in the four weeks experiment, but expression was down-regulated significantly in the lifelong exposure experiment. This down-regulation could be caused by mortality of the coelomocytes or immunosuppression [6].

*In vitro* exposure of earthworm immune cells to C₆₀ underline the finding at the molecular level, that C₆₀ exposure can affect the immune response. A decrease of earthworm coelomocyte survival (EC₅₀>400 ug/mL) and phagocytic activity (EC₅₀ of 110 ug/ml) was observed with increasing C₆₀ concentrations.

3.2. Effects on the tissue level

Histological examination demonstrated concentration-dependent effects in both experiments. Both the cuticle and the gut epithelium were affected, suggesting that effects were mediated through dermal exposure as well as via ingestion. Damage to the cuticles was generally accompanied by serious pathology of the underlying ectoderm (e.g. epidermis, circular and longitudinal muscles). Tissue repair (e.g. enlarged cells and more elongated nuclei) was also demonstrated in the earthworms, especially in gut epithelium of lifelong exposed earthworms [6].

3.3. Effects on the individual and population level

Adult earthworms exposed to C₆₀ for four weeks did not show changes of growth or mortality, but the reproductive success (e.g. cocoon production) was significantly reduced. In the lifelong experiment juvenile mortality was significantly increased (up to 40% at the highest exposure concentration) and the growth rate was also affected in the exposed juvenile earthworms [4].

Modelled population growth rate showed a significant decrease with increasing C₆₀ concentrations. The life stage distribution was also affected in C₆₀ exposed populations, with higher percentages of juveniles and lower percentages of individuals in the subadult stage [4].

3.4. Characterization of C₆₀ in exposure media

Heavy metal contamination in the C₆₀ powder was far below threshold levels. Spectrophotometry demonstrated that measured/actual concentrations of C₆₀ in the soil were in the range of the expected/nominal levels. TEM and HRTEM images of soil extract with C₆₀ showed C₆₀ aggregates of 10-15 nm in size, which formed loose structures with each other [4]. Samples of C₆₀ in cell medium will be characterized using DLS and zeta potential.

4. Conclusions

This study shows that C₆₀ exposure affects earthworms. This can be demonstrated at different levels of biological integration, e.g. gene expression, immune system, tissue integrity, individual growth, reproduction and mortality, and population growth rate. Based on the current study, no clear mode of action could be established. Oxidative stress, commonly regarded as an important mode of action for nanoparticle toxicity, could not be confirmed nor ruled out. This study clearly indicates that C₆₀ may be hazardous to earthworms, but further studies are needed to assess their sensitivity.

5. References


Long-term effects of sewage sludge spiked with Ag-NP on soil microorganisms

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1. Introduction

Due to their antibacterial and antimicrobial properties silver nanoparticles (Ag-NP) are widely used, e.g. in textiles, medical applications or cleaning products. This means that Ag-NP will inevitably enter wastewater treatment plants (WWTP). In the literature, research results on the fate and behavior of Ag-NP in WWTPs are reported. Burkhardt et al. (2010) reports that nearly 95% of silver is bound to the sewage sludge whereas around 5% leave the WWTPs with the effluent in ecologically negligible concentrations. In the sewage sludge silver ions are transformed to silver sulphide which precipitates. As in Germany and other countries sewage sludge is used as a fertilizer in agriculture, the goal of the present project is to determine long-term effects of sewage sludge spiked with soluble Ag NO₃ (silver nitrate) and Ag-NP on soil microorganisms over a period of 180 days.

2. Materials and methods

The test soil we used was the loamy sand Refesol 01A which is medium acid and slightly humic. The soil was supplied two weeks before test start, sieved (≤ 2mm) and stored at 4 °C; 3 days before test start the soil was stored at room temperature.

The sludge applied for the test was obtained from a local WWTP in the morning before test start. The activated sludge was spiked with silver nanoparticles, namely NM-300K from the OECD Sponsorship Programme, and silver nitrate and stirred at 300 rpm in two liter beakers. Ag-NP and silver nitrate were added in three steps during one hour to prevent an inhibition of the sludge microorganisms and to ensure a sorption of Ag to the activated sludge. After one hour a polymer was added to the activated sludge to decline the water content in order to reduce the volume before transport to a digestion tank or to the field. The polymer was obtained from the same WWTP that had supplied the sludge to ensure an optimal adjustment of the polymer to the activated sludge. The sludge was centrifuged at 10,500 rpm for 15 minutes. Subsequently the dry matter content of the sludge pellet was determined. According to the German sewage sludge ordinance sludge in a concentration of 5 tons/ha can be applied in three years. This means that we could apply 1.67 g dry sludge/kg soil d.m. (calculation based on a soil depth of 20 cm and a soil density of 1.5 g/cm³). The sludge pellet was suspended in 150 mL deionized water and applied to the test soil. The soil was thoroughly mixed and the maximum water holding capacity was adjusted to 55% with deionized water.

Two replicate batches with 4 kg soil d.m. were used for each concentration. A control without sludge and one with non-spiked sludge served as a reference. The replicate batches were stored in plastic tubes at 20 °C. The pH was determined every sampling day, as described in the guidelines. The water holding capacity was checked every week and adjusted to 55%. Mean silver concentrations of 1.6 and 3.4 mg/kg soil d.m. for NM-300K and 2.1 and 4.0 mg/kg soil d.m. for silver nitrate were measured at five sampling dates with 3 to 9 samples from different positions in the containers. The total content of silver in the sludge/soil-mixture was determined via ICP-OES. The background value of silver in sewage sludge was 0.24 mg/kg soil d.m.

To investigate potential effects on soil microorganisms standardized test systems were used. Ammonium oxidation tests (ISO 15685) and carbon transformation tests (CTT; OECD 217) were performed analysing samples at day 32, 60, 100 and 180 after sewage sludge amendment. For the statistical evaluations the two-sided student t-test and the Wilcoxon, Whitney and Mann u-test were performed.

3. Results and discussion

The aim of the study was to find out whether sewage sludge spiked with silver nitrate/Ag-NP causes adverse effects after degradation in soil within 180 days. The comparison of the controls with and without sludge shows that the sludge stimulates both ammonia oxidation and respiration (Table 1). This indicates that our test design works properly and that the sludge is degraded by the soil microorganisms. After 60 days, minor
effects occurred in the CTT. We found a dose-dependent inhibition which was statistically significant in the ammonia oxidation test and an inhibition in the CTT for silver nitrate and Ag-NP at both concentrations after 100 days. The results from both test systems are presented in detail in section 3.1 and 3.2. Our findings indicate that the sludge is degraded within 100 days and that silver gets bioavailable step by step with ongoing sludge degradation causing adverse effects to the soil microorganisms.

### Table 1: Inhibition [%] of the control in comparison to the control with sludge

<table>
<thead>
<tr>
<th>Test item</th>
<th>D32</th>
<th>D60</th>
<th>D100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ammonia oxidation</td>
<td>-31.1*</td>
<td>-13.6</td>
<td>-31.9</td>
</tr>
<tr>
<td>Carbon transformation</td>
<td>0</td>
<td>-7.9</td>
<td>-44.6</td>
</tr>
</tbody>
</table>

* A negative inhibition expresses a stimulation.

#### 3.1 Ammonia oxidation test (ISO 15685)

The results of the ammonia oxidation test are presented in Table 2. At day 32 and 60 a difference between the sludge control and the tested concentrations could not be observed. After 100 days we obtained a statistically significant dose-dependent inhibition of 33.2% for NM-300K and of 41.3% for silver nitrate at the highest concentration.

### Table 2: Inhibition [%] of the ammonia oxidation

<table>
<thead>
<tr>
<th>Test item; concentration [mg/kg d.m.]</th>
<th>D32</th>
<th>D60</th>
<th>D100</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNO₃ 2.1</td>
<td>-1.9</td>
<td>-4.3</td>
<td>12.8</td>
</tr>
<tr>
<td>AgNO₃ 4.0</td>
<td>0.2</td>
<td>8.9</td>
<td>41.3**</td>
</tr>
<tr>
<td>NM-300K 1.6</td>
<td>2.5</td>
<td>-5.5</td>
<td>11.8</td>
</tr>
<tr>
<td>NM-300K 3.4</td>
<td>-0.6</td>
<td>5.3</td>
<td>33.2*</td>
</tr>
</tbody>
</table>

*0.005 ≥ p ≥ 0.01; **0.01 ≥ p ≥ 0.001

#### 3.2 Carbon transformation test (OECD 217)

The results from the carbon transformation test are presented in Table 3. Effects on the respiration of the microorganisms were not observed after 32 days. Referring to the control with sludge we obtained an inhibition of the microbial respiration activity of 21.7% for the highest concentration of silver nitrate after 60 days, whereas NM-300K had no effect on the respiration. After 100 days both concentrations of silver nitrate resulted in a respiration inhibition of around 31%. For NM-300K a similar respiration inhibition of 28.3% for the lower and 23.3% for the highest concentration was determined at day 100. A statistically significant difference was not obtained.

### Table 3: Inhibition [%] of the carbon transformation

<table>
<thead>
<tr>
<th>Test item; concentration [mg/kg d.m.]</th>
<th>D32</th>
<th>D60</th>
<th>D100</th>
</tr>
</thead>
<tbody>
<tr>
<td>AgNO₃ 2.1</td>
<td>-17.2</td>
<td>7.3</td>
<td>31.2</td>
</tr>
<tr>
<td>AgNO₃ 4.0</td>
<td>-17.2</td>
<td>21.7</td>
<td>30.8</td>
</tr>
<tr>
<td>NM-300K 1.6</td>
<td>-8.6</td>
<td>0.3</td>
<td>28.3</td>
</tr>
<tr>
<td>NM-300K 3.4</td>
<td>-8.6</td>
<td>7.0</td>
<td>23.3</td>
</tr>
</tbody>
</table>

#### 4. Conclusions

Our results indicate that Ag-NP and silver nitrate can become bioavailable in the course of sewage sludge degradation and can cause an inhibition of the soil microorganisms.

#### 5. References


**Acknowledgement** - The study was performed on behalf of the German Federal Ministry of Education and Research and financed by federal funds.
An approach to determine appropriate dose metrics for nanomaterials

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1. Introduction

Traditionally, risk assessors derive safe exposure limits to chemical substances based on information on the toxic potential of the substances obtained from standardized safety studies, in which increasing doses of the substance are administered to groups of experimental animals. For soluble substances, a unique measure of the dose is the total mass of the substance administered or, equivalently, the total number of molecules administered. Consequently, safe exposure limits are generally also based on mass concentrations, such as a maximum tolerable daily intake of X mg of chemical substance Y per kg body weight.

With nanotechnology facilitating the creation of complex spheres, cylinders, pyramids and other forms of different sizes, a whole new array of often insoluble chemical entities has made its appearance. The different characteristics of these nanomaterials (e.g. size, shape, polymorph form of the crystall structure) all may determine their toxic potential. For example, daily intake of X mg of nanomaterial with particle size $d_1$ may be more toxic than X mg of nanomaterial with particle size $d_2$, despite consisting of the same chemical substance Y. In other words, for nanomaterials, information on the administered mass of the chemical substance alone may not be a sufficient description of the dose that determines a particular response in a biological system. As a result, risk assessors are faced with the question of what dose description to use when setting exposure limits for nanomaterials. It has been speculated that exposure limits based on particle numbers, such as used for particulate matter, may be more appropriate, while others suggest that the toxicity of nanomaterials is determined by the administered surface area (Duffin et al. 2007; Monteiller et al. 2007), indicating that safe exposure limits should also be based on surface area.

An adequate dose metric for nanomaterials should describe all relevant characteristics that are necessary to explain differences between responses in experiments. A minimal criterion for an adequate dose metric is that if experiments show a different toxicological or kinetic response, the doses in the experiments, specified according to the dose metric, should be different also. In other words, the dose metric should be able to discriminate doses with different responses (principle of discrimination). In its most complete form, the dose of a nanomaterial can be described by a (distribution) function $P_N$ that specifies the number of particles $N$ with a diameter $d$, surface potential $\zeta$, and crystal structure $\alpha$.

Ideally, a dose metric should be as concise as possible, i.e. should describe the dose with as few dimensions as possible. A reduced dose metric, (for example requiring only information on administered total number of particles, total mass (or volume) or total surface area of a particular nanomaterial consisting of chemical substance Y), would be most pragmatic for risk assessment purposes, since only one exposure limit would have to be derived for various different nanomaterials consisting of chemical substance Y. However, a priori there is no reason why such a reduced dose metric should exist. Therefore, the existence of a reduced dose metric should be established by experimental study.

In this contribution, we present a method to determine whether a reduced dose metric for (a class of) nanomaterials exists. As an illustration, the method is applied to analyse results from experiments with various nanomaterials published recently (Park et al. 2011a; Park et al. 2011b).

2. Materials and methods

Equi-response surfaces

To study properties of a dose metric systematically, one can make use of the requirement that equal doses (specified in a particular metric) should give an equal response in the experimental system. In a comparative study of dose-response relations of nanomaterials, doses should be identified that give rise to the same response. If these doses form a continuous surface (an ‘equi-response’ surface), this indicates the existence of a fixed relation between specific characteristics of nanomaterials and implies that not all parameters are
required to fully characterize the dose. Such a fixed relation between material characteristics, if it exists, can serve as a reduced dose metric.

To illustrate the use of equi-response surfaces, the relative simple (but common) case of solid, spherical nanoparticles is considered. In a plot with the number of particles $N$ and the particle diameter $d$ on the axes every point $(N,d)$ in the plane represents a dose: $N$ nanoparticles of diameter $d$. Equi-response surfaces (curves) can be constructed in this plane by plotting doses that result in equal responses. If, for example, administered surface area $S$, mass $M$ or total number of particles $N$ would be suitable to use as a reduced dose metric, the equi-response curves would have the shapes of the respective curves drawn in figure XX.

**Testing for a reduced dose metric**

Whether the dose of solid spherical nanoparticles can be fully described by a simple reduced dose metric, and whether this dose metric is particle surface area, particle mass, or number of particles, can be systematically tested by comparing the shape of the equi-response curves obtained from experimental data to the curves of constant total particle surface area $S$, constant particle mass $M$ and constant number of particles $N$.

For the total particle surface area the curve follows from:

$$ S = N \times \pi \times d^2 = \text{const} $$

and thus:

$$ N = \frac{c_1}{d^2} $$

with $c_1$ a particular constant. Or, more conveniently, $\log(N) = c_2 - 2 \times \log(d)$

Similarly, for the total volume (or mass) the curve is given by $\log(N) = c_3 - 3 \times \log(d)$

Finally, the curve of constant particle number $N$ is given by $N=\text{const}$. This curve is independent of diameter $d$. In the case that continuous equi-response curves can not be constructed, a reduced metric does not exist. This may be observed when equi-response curves, constructed from experimental dose relations, intersect. Such an intersection is theoretically impossible and may be an indication that points of equal response are in fact not connected. In these cases, there is no fixed relationship between the characteristics of the material, i.e. more characteristics of the material are required to describe doses of equal response.

Thus, the plots of equi-response curves may be used to directly verify whether a reduced dose metric exists and if it does, whether administered number of particles, administered surface area or volume are appropriate dose metrics. If the slope of the equi-response curve in the logarithmic dose-parameter plot equals 0, total number of particles is a complete dose description, if it equals -2, surface area is and if it equals -3, volume is.

If any other curve is found, this still indicates the existence of a reduced dose metric. That is, there is some quantity (a function of $d$) that is the same for doses that lead to equal responses. The interpretation of such a dose metric may not be straightforward, but it may nevertheless be used to extrapolate from one dose to another.

### 3. Results and discussion

#### 3.1. Application of the method to experimental data

The method was applied to data obtained from two different experimental dose-response systems – in vitro cytotoxicity assays in two murine cell lines.

The data obtained from the assays were analyzed using PROAST software version 28.1 (RIVM 2009; Slob 2002) according to methods described previously (Park et al. 2011a; Park et al. 2011b). For data of the silver nanoparticle experiments, the optimal model selected by the Proast software was the exponential model

$$ y = a \exp(b \times). $$

For data of the silica nanoparticle experiments, the optimal exponential model was
\[ y = a \left[ c - (c - 1) \exp(-b x) \right] \]

where the response \( y \) is a function of administered nanoparticle mass concentration \( x \). The dose-response curves obtained were used to quantify doses (referred to as effect concentrations) that give an equal response in the system for a number of response levels, i.e. the concentration of each nanoparticle resulting in a certain % response compared to the solvent control. Since both SiO\(_2\) and Ag particles are spherical, with known diameter and mass density, the mass-based dose, specified in experiment as \( \mu g/l \), can be converted to a number based concentration \( C_N \):

\[ C_N = C_M \times \frac{6}{\pi \rho d^3} \]

The log (base 10) of the number \( N \) of particles administered per liter was plotted against the log of the particle’s diameter for all response levels (see figure XX). To test whether a simple reduced dose metric adequately describes the data, a linear model \( \log(N) = a + b \log(d) \) was fitted to the data by means of a least squares minimization principle. 90% confidence intervals of parameter \( b \) were determined from the 90% confidence intervals in the effect concentrations by bootstrapping.

3.2. Results

The regression lines for both Ag (\( R^2 \) of 0.98) and SiO\(_2\) (\( R^2 \) of 0.97) suggest a linear relation between \( \log(N) \) and \( \log(d) \) for the doses along the equi-response curve, indicating that a reduced dose metric indeed exists (see Figure 1 for an example). The slope of the regression line (parameter \( b \)) gives an indication of what this simple reduced dose metric may be. The analyses of the Ag and SiO\(_2\) dose-response data suggest that the dose response relation of SiO\(_2\) in macrophages is best described by dose expressed in mass (with a \( b \) of -2.9). In contrast, the dose-effect relation of Ag in L929 fibroblasts (\( b = -2.0 \)) is best described by specifying dose in administered surface area \( S \).

![Equi-response curves for Ag in L9:](image)

**Figure 1:** The equi-response curves at various response levels for Ag nanoparticles in L929 fibroblasts.
4. Conclusions

Although additional data are needed to verify the methodology for deriving dose metrics as described above, the results of an array of 4 independent experiments indicate that it indeed is possible to derive a reduced dose metrics for nanoparticles. However, it also appears that there is no unifying dose metrics as amongst others the endpoint of assessment and the nature of the particles used determine the dose metrics derived.

5. References


Acknowledgement - This work was performed within the IRAN project which is part of the strategic research programme of RIVM.
Assessment of environmental risks of nanomaterials throughout the product life cycle

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1. Introduction

Estimation of exposure due to release into the environment represents a key challenge in determining nanomaterials safety. It is therefore important to understand to what extent existing risk assessment approaches and tools can be applied or will require modification to take account of the particular properties of nanomaterials. The present work aims to identify methodologies and tools to assess and manage the environmental risks due to manufactured nanomaterials, identify appropriate risk assessment methods, and develop improved methods for risk assessment. Some currently available nano-specific support tools are evaluated, in order to identify gaps where either risk assessment methods and/or input data are insufficient.

A significant problem is that there still remain many outstanding questions regarding the behaviour of nanomaterials in the environment, which could differ from that of materials of similar composition in bulk form. Reducing the level of these uncertainties is essential to proper risk assessment. There is thus an urgent need for reliable data on the physico-chemical properties, toxicokinetics and degradability of nanomaterials to understand their transport, persistence and fate, and exposure potential in the environment. However, there is still a lack of basic information on the possible release routes for nanomaterials during production, use and final disposal or recycling. For this reason, it is necessary to improve our present knowledge on the release of nanomaterials from products during all phases of their life cycle.

The NanoSustain project [1] is developing innovative solutions for the sustainable use, recycling and final treatment of nanotechnology-based products. Experimental work is carried out to investigate the potential for release of nanomaterials from products during industrial operations, such as sanding and grinding, and at the end of life during disposal by incineration and recycling by melting. Data obtained from such experiments should help reduce the level of uncertainty in the risk assessment.

2. Materials and methods

The materials selected for investigation comprised the following organic and inorganic nanomaterials and associated products: nanocellulose based materials and products; carbon nanotube based composites; nano TiO₂ based products, and nano ZnO based coatings on glass.

Nanocellulose is an innovative material in the forest industry sector with a variety of applications. Although these organic nanomaterials and associated products are generally considered to be environmentally safe, no data or methods to demonstrate their sustainable use, re-use, recycling or final treatment and/or disposal have been established so far.

Because they cannot be melted down, carbon nanotubes cannot be effectively recycled, but instead must be disposed of in landfill or incinerated at the end of their lifecycle. An important but still open question regards their behaviour during incineration. While there is evidence that they will be totally oxidised, no information exists as yet on their final fate.

The nano ZnO in the coatings was synthesized by the sol-gel method. Novel solutions for the sustainable recycling of these nanomaterials are being explored on the laboratory scale by studying the release of nanoparticles during melting of the coated glass, which is a well-established recycling process.

Nano-TiO₂ is increasingly incorporated in paints because of its photocatalytic and antimicrobial properties. The potential release of nanoparticles into the air and resulting exposure to consumers and workers during and after treatment of painted surfaces or paint removal is being studied for nano TiO₂ based materials. Both painted wooden boards and the pure nanomaterial are tested.
3. Risk assessment

Table 1 shows examples of typical hazard sources, pathways and receptors considered for the purposes of environmental risk assessment. The pathway represents the linkage that could potentially allow a particular receptor to be exposed to the source.

<table>
<thead>
<tr>
<th>Sources</th>
<th>Pathways</th>
<th>Receptors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Industrial processes</td>
<td>Air</td>
<td>People</td>
</tr>
<tr>
<td>Recycling</td>
<td>Water</td>
<td>Domestic and commercial property</td>
</tr>
<tr>
<td>Incineration</td>
<td>Soil</td>
<td>Infrastructure</td>
</tr>
<tr>
<td></td>
<td>Food chain</td>
<td>Ecosystems</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Animals and plants</td>
</tr>
</tbody>
</table>

Table 1: Examples of sources, pathways and receptors (adapted from: Royal Society of Chemistry [7])

Two existing nano-specific risk assessment methodologies were evaluated. The Environmental Defense and DuPont framework for the evaluation and management of the potential risks of nanomaterials [2] provides a method for identifying, managing, and reducing the environmental, health, and safety risks of nanomaterials at every stage of the product life-cycle. The precautionary matrix for nanomaterials [3] enables identification of potential risks in the development, production, use and disposal by means of a structured approach that allows identification of the potential risks for existing or new products and processes.

However, at present there are relatively few data concerning the release of nanomaterials from products into the environment, or how they are transported, transformed or accumulate in ecosystems [4]. A published review of the behaviour of nanoparticles in the environment [5] focused mainly on airborne particles. A more recent review [6] covers risk assessment, toxicity, fate and transport of carbon nanotubes and metallic, metal oxide and hydroxide nanoparticles, and quantum dots in terrestrial ecosystems.

4. Conclusions

There is a lack of understanding of the potential risks of nanomaterials in the environment or how they are transported, transformed or accumulate in ecosystems and improved knowledge regarding the behaviour of nanomaterials during use and fate at the end of life is therefore needed. Assessment of the risks requires knowledge of the mobility, reactivity, ecotoxicity and persistence of nanoparticles in the environment, which necessitates extensive characterisation of nanoparticles and their aggregates.

Exposure, environmental fate, and transport will be crucial in determining the overall environmental impact of nanomaterials. However there are a large number of data gaps and little information exists on the material inputs and environmental releases at all stages of the life cycle of nanomaterials. This creates difficulties in carrying out risk analysis because the estimated concentrations in environmental compartments have large variability, due to uncertainties in the production volumes and the life cycles of the products.

5. References


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A Weight of Evidence approach for ranking and prioritization of occupational exposure scenarios for engineered nanomaterials

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1. Introduction

Despite the recognized substantial potential of ENMs to contribute to sustainable innovation, there are extensive gaps in the knowledge regarding both their environmental, health and safety (EHS) profile and the suitable tools to assess their risks for the human health. In the risk assessment framework, the exposure represents a pivotal component, since without exposure there is no risk. Until recently there was no database available to store the existing exposure data for nanomaterials. However, in 2011 the EU-funded FP7 NANEX project (http://nanex-project.eu/) released a public catalogue of 57 (107 contributing) occupational and 5 (24 contributing) consumer exposure scenarios (ES) for CNTs, zinc oxide (ZnO), titanium dioxide (TiO₂), silver (Ag) and carbon black ENMs, derived from the literature, two measurement campaigns (i.e. NanoINNOV and NANOSH) and industry surveys.

Although the NANEX database represents the state of the art in the field of nano exposure assessment, most of the ES are characterized by scarce data and hardly generalizable exposure estimations [1]. A major reason for this is the fact that the studies where information for the database has been collected from, report various types of non-standardized data. This limitation, in combination with the general lack of knowledge on how ENMs compare to bulk materials in terms of behavior in occupational and consumer settings, makes it impossible to generalize information from one situation to another [1] and to use read-across approaches to fill gaps in the current evidence-base. In result, the data in the NANEX ES are not sufficient to use in high-tier exposure models or for quantitative risk assessment.

In this context it is necessary to develop well-validated, easy-to-use approaches for early occupational and consumer safety evaluation [1]. A variety of such tools already exist: e.g. the Swiss Precautionary Matrix for Synthetic Nanomaterials, the Dutch Stoffenmanager Nano, the Danish NANOSAFER, the French Anses system. This is currently a dynamic area of research and several tools are still under development. However, with few exceptions, they all produce qualitative results. The range of tools differs significantly in scope, objectives, and scientific underpinning and they are in need of frequent updating given the rapidly evolving evidence-base. Most of them are not intended to facilitate regulatory decision making, but they are used as preliminary screening and/or research prioritization tools, aimed to help industry in identifying relevant sources of risk in the lifecycle of synthetic nanomaterials and pinpoint areas of knowledge deficits [2]. In this context the development of nano-specific methods for quantitative exposure/risk assessment appears necessary to complement the available toolset.

(max 4):
Engineered Nanomaterials, Occupational Risk Assessment, Weight of Evidence, Multi Criteria Decision Analysis


In this context we propose a quantitative model for relative ranking and prioritization of occupational exposure scenarios (ES), specifically tailored for ENMs. The tool is based on a scoring weight-of-evidence method and it uses expert judgement to estimate exposure potential of nanomaterials in the workplace. The conceptual structure of the approach has been defined on the basis of state-of-the-art exposure models for conventional chemicals, like the Advanced REACH Tool (ART), and for nanomaterials, such as the Stoffenmanager Nano.
The model uses 10 indicators distributed among 4 lines of evidence (LoE) (Figure 1), as their selection was informed by state-of-the-art knowledge and limited by the availability of information provided in the NANEX database. For each indicator a series of rating classes have been identified in the range from 0 to 1, whose values were derived from relevant literature and expert judgement. Then, a score has been assigned to every indicator on the basis of the available data or, in some cases, on the basis of expert deductions or assumptions in order to aggregate them into an index for each LoE. In the next step, the 4 LoE have been combined by means of specific mathematical operators to obtain a final value of potential exposure of workers to ENMs in each studied exposure scenario. Using the model we obtained rankings for 3 groups of inhalation occupational ES obtained from the database of the NANEX project, and, in order to validate our results, we compared them with parallel rankings of the measured exposure concentrations of the materials, reported for the same scenarios.

Since the available NANEX dataset did not allow us to to assign a real datum to each indicator for each scenario, some gaps were filled by information deduced from actual data or, in some cases, by assumptions based on conservative expert judgement. Since this introduces a certain degree of uncertainty related to the reliability of the model performance and output, the error associated with the selection of the input data has been propagated. In addition, probabilistic Monte Carlo sensitivity analysis has been applied in order to test the behaviour of the model in regard to changes in the input parameters.

3. Results and discussion
- The results show high similarity between the rankings of ES based on the WoE model and the parallel ranking of measured exposure concentrations for the same scenarios.
- The model slightly overestimates the exposure in most cases, which proves that it is a conservative approach.
- The error propagation shows that changes of the estimated exposure potential, dependent on the choice of data typology, do not influence considerably the rankings. This behaviour demonstrates both the stability of the model and the reliability of the approach.
- The applied sensitivity analysis shows that the tool always performs in a stable manner in regard to variations in the input data.

4. References

Acknowledgement - The authors thank the FP7-funded ENPRA project.
1. Introduction

Self decontaminating surfaces offer great potential for mitigating biological and chemical agents. Research into these reactive surfaces has included catalysts and enzymatic approaches with the goal to provide a reactive self decontaminating system that does not adversely affect the performance of the system. Metals such as titanium and silver have been incorporated into coatings. Recently advances in materials science have enabled these metals to be used at the nanometer scale. The metal-containing surfaces rely on the surface charge and/or photoactivation mechanism to elicit the biocidal activity. Some of the novel approaches include chemicals added to a polymer coating, such as the quaternary ammonium biocides. These materials have demonstrated biocidal activity when incorporated into polyurethane coatings [1]. Specific material under investigation by Ginsberg and colleagues focuses on nano scale titanium dioxide that, upon photoactivation with ultraviolet light, destroys biological agents. This material has the potential to be incorporated in other matrices such as coatings for non-filter applications.

An approach for providing necessary environmental and human health safety information for nanomaterial use in DoD applications is to conduct a comprehensive environmental assessment (CEA). The CEA approach of Davis [2] enables the evaluation of environmental hazards for technologies through the entire life-cycle (e.g., feedstocks, manufacturing, distribution, storage, use, and disposal). Furthermore, the CEA also takes into account the environmental pathways, fate and transport, exposure – dose, and effects of nanomaterials once they enter the environment. This comprehensive approach provides scientists, risk assessors, and acquisitions personnel to see holistically the potential effects of nanomaterials, as well as data gaps that will help them make better decisions for developing and acquiring materials containing nanomaterials.

The goal of this effort was to evaluate the potential risks of nano scale decontaminating agents through a focused examination following the comprehensive environmental assessment process (CEA). Data gaps identified through the CEA process focused primarily on fate of NP in self decontaminating surfaces and toxicity in relevant biological models.

2. Materials and methods

A CEA conceptual model was developed for the nano TiO$_2$ focusing on implementation of the technology on coated surfaces. Discussions with technology developers indicated best management practices and management controls will be implemented for production of the materials limiting potential releases. However, long-term stability of particles in the surface coating product was identified as a potential concern.

A UV-irradiated titanium dioxide purification based system for air purification similar to that reviewed by Zhao and Yang [3] was being examined for potential release of TiO$_2$ nanoparticles. Stainless steel substrate coupons were coated with anatase titanium dioxide coating having a high surface area and an adsorbed silver ion coating. These coupons were prepared using a sol-gel process to isolate the particles on the stainless steel substrate followed by volatilization. Coupons were evaluated using SEM-EDS to determine...
the surface morphology and elemental composition. Additional analysis included leaching studies, air flow release, and cytotoxicity using alveolar cells in a mixed macrophage culture.

To evaluate a worst case scenario for release of particles, a scrapping of the surface was dry milled by vigorous agitation in a 50 ml polypropylene cylinder containing 5 mm glass beads (4 hours, 300 rpm, horizontal tube orientation). Particles were recovered by dissolution in ultrapure water, separation of the beads, and evaporation of the water (100°C).

3. Results and discussion

Coupons of the reactive surface show a moderate degree of cracking and potential release of particulates (> µm). These fissures show the layers of the coating and silica substrate. Smaller crystals of Ag are observed on the surface and near the edges of fissures. In addition TiO2 on fissure edges demonstrates a potential for the newly exposed surface to be released from the paint coating.

![SEM-EDS image of a self-decontaminating surface showing the silica substrate, titanium coating, and silver adsorbed coating.](image)

4. Conclusions

Results of our study show the potential for release of particulate titanium coatings as previously described by Reijenders [4]. Coatings that include other adsorbed metals such as silver are likely cause toxicity in in vitro systems however, other evidence suggests particulate and ionic silver to cause inflammation and cell toxicity in vivo [5]. Therefore, long-term use of these coatings may result in release of particles over time. This release may be mitigated through improvement of composites and coating technology. Current studies that will be described include the results of the leaching, air flow release of particles, and cytotoxicity and inflammation responses. These results will provide information about the form, mass release, and toxicity of the particles that may be released from the surface.

5. References


Acknowledgement - Funding for this project was provided in part by the Environmental Quality Research Program – Military Materials in the Environment (Dr. Elizabeth Ferguson, Technical Director), the Center Directed Research Program (Dr. Jeffery Holland, Director) and the Defense Threat Reduction Agency. Permission was granted by the Chief of Engineers to publish this information.
Green Nanotechnology Challenges and Opportunities

Kira JM Matus¹, James E Hutchison², Robert Peoples³, Skip Rung⁴, Robert L Tanguay⁵

Nanotechnology is an emerging field. It is an interdisciplinary science whose potential has been widely touted for well over a decade. Despite significant private and public investment, progress moving nanomaterials from the laboratory to industrial production has been slow and difficult. Two challenges that have slowed development have been the poor understanding of the new hazards introduced by nanotechnology and lack of appropriate policies to manage new risks. Scientists, engineers and entrepreneurs, however, continue to move forward, grappling with challenges that range from the technical to the regulatory and everywhere in between. Just as the concepts of nanoscale invention have required new insights from scientists, they are also demanding new approaches to managing, producing, funding and deploying novel technologies into the larger chemical sector. In this case, there is an unusual opportunity to use science, engineering and policy knowledge to design novel products that are benign as possible to human and environment health. Recognition of this opportunity has led to the development of the “green nanoscience” concept [1,2].

Green nanotechnology has drawn on the field of green chemistry, and the framework of the 12 Principles of Green Chemistry [3] features significantly in work to design new nanotechnologies for joint economic, social, and health/environmental benefit [4]. These efforts have been aided by awareness throughout the nanotech community that they need to address the potential negative impacts of nano from the outset. That has not meant, however, that green nanotechnology has gained widespread and popular acceptance in the scientific and business communities. Awareness is still limited in many sectors, and green nanoscience, along with nanoscience more broadly, still faces significant challenges in transitioning from concept to reality.

In 2010, a summit was held in conjunction with the Safer Nanomaterials and Nanomanufacturing’s (SNNI) Fifth Annual Conference in Portland, Oregon. The authors participated in the conference sessions, in order to develop answers to key questions on four aspects of greener nanoscience:

1. What are the most important technical challenges?
2. What are the challenges to understanding nanotoxicology and the associated informatics challenges?
3. What new policies are necessary to advance greener approaches to nanotechnology? and
4. What are the most pressing industrial deployment challenges?

The summit itself drew approximately 120 participants from academia, industry, NGO’s and government agencies around the United States. The summit presented a wide range of material to participants, and also encouraged debate and discussion of some key issues in the field.

At the conclusion of the conference, the authors came together to identify the key issues and challenges facing green nanotechnology, along with strategies and opportunities for future GCI involvement in order to help move greener nanotechnologies forward, as part of its broader commitment to supporting the development and implementation of green chemistry throughout the chemical enterprise.

The Central Challenge
The challenge of simultaneously developing useful products for the market, advancing the underlying science, and instituting a green nanoscience development and deployment paradigm.

One of the most fundamental challenges particular to green nanotechnology is that the science, the testing, the regulatory strategy, and even the processes needed for commercial production are all being developed and deployed at the same time. From this central challenge flow many early stage challenges. The authors identified six key barriers:

Barriers to the Development and Commercialization of Green Nanotechnology

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1. There are no clear design guidelines for researchers in initial discovery phases of green nanoscience;
2. Many green nanomaterials require new commercial production techniques, which increases the need for basic research, engineering research, and coordination of the two between the industrial and research communities;
3. The lack of a “deep bench” of scientists and engineers with experience developing green nanotechnology;
4. Toxicology and analysis protocols need to be developed and constantly updated to reflect advances in the science;
5. Regulatory uncertainty persists, and green nanotechnologies often face higher regulatory barriers than existing or conventional chemicals;
6. The end-market demand is unclear, especially since there are only a limited number of commercial grade products that can be compared to conventional materials in terms of performance.

Developing an Action Agenda

Green nanotechnology has been making great forward progress, but the challenges presented above point to an agenda of actions where involvement by the scientific research community, industry and government could bring about changes that would be crucial to supporting a more rapid and effective commercialization of green nanotechnology. Such changes have the potential to reestablish competitive leadership in the field, with positive economic implications for the manufacturing and associated job creation.

Specifically, we are proposing that action be taken according the agenda below. The order of the agenda is important. The first, and most pressing need is for more and better analysis and characterization tools. These are a key input which are required to support the rest of the agenda. They are needed for scientists who wish to understand the mechanisms of the reactions that produce nanomaterials in order to develop better synthesis methods. And they will allow for improved and more complete toxicological studies of green nanomaterials, which are required for better and smarter regulation. Similarly, the second item of the agenda, improved mechanistic understanding, is a key part of the foundation for developing green nanomaterial design guidelines. Finally, new regulations, as well as outreach to regulators must be based on the analysis, understanding, and design concepts that are the result of the first three items.

The Agenda
1. Discover, uncover and provide key analysis and characterization tools,
2. Develop, characterize and test precision-engineered nanoparticles for biological and toxicological studies needed to guide greener design,
3. Investigate and understand reaction mechanisms to support more efficient and precise synthesis and production techniques,
4. Develop design guidelines for green nanomaterials,
5. Definition of green criteria for new nanomaterials for fast-track approval by the US EPA, and
6. Education and outreach to regulators to ensure regulatory structures for green nanotechnology reflect accurate knowledge of their intended uses and potential impacts.

Nanotechnology presents an opportunity to develop a revitalized, sustainable chemical and materials manufacturing base. We are at a unique point where we have more understanding of how to go about this than at any time in the past. This new emerging science and associated technologies do not have to follow the typical path of many past innovations in the chemical industry that, despite providing significant benefits, also turned out to have unanticipated costs to human and environment health. The development and commercialization of viable green nanotechnologies is difficult, and the barriers mentioned will require effort from the scientific, research and government communities. But there is a pathway forward, and concrete actions that could construct a solid foundation for a profitable and environmentally sustainable future for nanotechnology.

Rapid in vivo assessment of the nano/bio interface to guide safer nanomaterial design

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ABSTRACT
The rapid rate of discovery and development in the nanotechnology field will undoubtedly increase both human and environmental exposures to engineered nanomaterials. Whether these exposures pose a significant risk remains uncertain. Despite recent collective progress there remain gaps in our understanding of the nanomaterials physiochemical properties that drive or dictate biological responses. The development and implementation of rapid relevant and efficient testing strategies to assess these emerging materials prior to large-scale exposures could help advance this exciting field. I will present a powerful approach that utilizes a dynamic in vivo zebrafish embryonic assay to rapidly define the biological responses to nanomaterial exposures. Early developmental life stages are often uniquely sensitive to environmental insults, due in part to the enormous changes in cellular differentiation, proliferation and migration required to form the required cell types, tissues and organs. Molecular signaling underlies all of these processes. Most toxic responses result from disruption of proper molecular signaling, thus, early developmental life stages are perhaps the ideal life stage to determine if nanomaterials perturb normal biological pathways. Through automation and rapid throughput approaches, a systematic and iterative strategy has been deployed to help elucidate the nanomaterials properties that drive biological responses.

INTRODUCTION
The application of nanotechnology in consumer products is providing numerous exciting new applications. These nano-containing or nano-enabled products in the market will undoubtedly result in increased nanomaterial exposures to both humans and the environment. At present, it is unclear whether these exposures pose a risk to human or environmental health. Although there is an increased research focus on identifying nanomaterial hazard, we are at the early stages in understanding the nanomaterial physico-chemical properties that influences or drive how nanomaterials will behave in complex biological systems. When one considers the limitless diversity of potential NP that are varied in molecular composition, size, and surface functionalization, it is immediately clear that it will not be possible to thoroughly evaluate the toxicity of each nanomaterial in any biological system. We propose instead to take a systematic approach; to generate precisely engineered materials and to isolate the effects of individual nanomaterial features on biological activity. The ideal approach would explore the nanomaterial-biological interface using a sensitive, in vivo, rapid-throughput, and predictive biological assay. We have developed such an approach that exploits the advantages of the zebrafish model.

As a widely accepted model for mechanistic-based toxicological studies, the embryonic zebrafish offers a rapid, high throughput platform to assess the nanomaterial and biological system interactions. Zebrafish have a high degree of homology to the human genome and share many cellular, anatomical, and physiological characteristics with other vertebrates. The embryos are small, develop externally, and are optically clear which allows for non-invasive evaluations. Additionally, a single female zebrafish can produce hundreds of eggs in one spawn, which allows for statistical power, and rapid assessments. The small nanomaterial quantities needed to fully evaluate the toxicity of engineered nanomaterials is also a major advantage for high throughput
assessments. For example, less than one mg is required to fully evaluate the toxicity of a novel nanomaterial, and this includes assessments across a wide range of concentrations in many replicate animals. Finally, early developmental life stages are inherently more susceptible to stressors as this is the most dynamic period in an organism lifespan. The full repertoire of gene products is expressed during embryogenesis because these products are required to successfully accomplish cellular and organ system development. Thus, if there is a unique biological target for a given nanomaterial that must be “hit” to produce a toxicological response, the probability of identifying this interaction is enhanced during the early developmental stage because all targets are expressed.

**Tiered Testing:** Our lab has developed a tiered systematic approach to fully exploit the advantages of the embryonic zebrafish. Tier 1: Rapid 96-well plate screening experiments are conducted to assess the toxicity of a wide range of structurally well-characterized nanomaterials. Nanomaterials found to elicit significant adverse effects proceed to Tier 2 testing. Tier 2: Potential cellular targets and modes of action are defined in vivo using a suite of transgenic fluorescent zebrafish and indicators of cellular oxidative state. Nanomaterials are then grouped according to structural indices and effects. Representative nanomaterials from each group will be selected for Tier 3 testing. Tier 3: Global gene expression profiles are used to define the genomic responses to nanomaterials. Data from these studies are used to define structure-activity relationships. The correlation of structure and the biological response is reported to the Nanomaterial-Biological Interactions (NBI) knowledgebase we have created to collate, organize and analyze data on nanomaterial effects across species and exposure scenarios. To date, over 300 unique nanomaterials have been assessed at least through tier1.

**Assay Details:** In tier 1, morphological and behavioral changes are measured in control and nanomaterial exposed animals. Briefly, six hours post fertilization (hpf) embryos are statically exposed to five concentration of the nanomaterials continuously until 120 hpf. Typically, there are at least 16 replicate animals used for each exposure concentration. At 24 hpf, incidence of mortality and developmental progression are quantified. Embryos are also sampled at this point to quantify nanomaterial uptake. This is routinely done using analytically methods such as inductively coupled plasma mass spectrometry and instrumental neutron activation analysis (INAA) in individual exposed embryos. At 120 hpf, exposed embryos are evaluated for changes in motor activity. Motor behavioral responses are evaluated in commercially available video tracking instruments. Differences in motor responses are then compared across groups to determine if there are interactions between biological targets and specific nanomaterials that lead to disruptions in CNS function. This endpoint strongly highlights the power of this *in vivo* platform to detect subtle responses. This functional systems approach is not possible using cultured cells. In addition to behavioral measures, at 120 hpf each embryo is evaluated for mortality, and for over a dozen morphological malformations including: animal length, body axis angle, yolk sac edema, eye development, snout development, jaw morphology, otic morphology, pericardial edema, brain, caudal fin morphology, pectoral fin development, circulation deficits, and pigmentation pattern). The incidence of mortality and total morbidity are calculated from a mean of three replicates.

In Summary, coupling precision engineering with rapid in vivo testing will reveal the key physicochemical properties that are responsible for distinct biological responses. The identification of these basic nano-bio interaction design rules will ultimately aid in rational safer nanomaterial production.

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Nanomaterials in plant protection products: current state, foreseen applications and research priorities

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1. Introduction

The recent advances in nanotechnology have lead to a corresponding increase in the use of nanomaterials (NM) in a wide range of products. There is already a body of literature that examines the mechanisms of unwanted emission and toxicity of these materials in the environment, e.g. [1, 2]. An ever growing world population is in demand for higher agricultural yields and thus, more effective agricultural practices are needed. Hence, there is an emerging trend for using NM to optimize agricultural practice [3-5]. This could lead to an intentional, and much greater, release of NM to the environment than it has been estimated for other NM applications. Expectations regarding positive aspects of agricultural applications are high: Reduced use and impact of pesticides and nutrients in the environment, reduced costs and improved efficiency of crop production [4, 5], as well as eased remediation and facilitation of soil management [6]. Here, we would like to align the increasingly common but often non-reflected perception of nanomaterial use and benefit for crop protection with actual scientific facts and figures. We will give an integrated overview of the historic development of the “nano-plant protection”-idea, different nano-properties and materials that are envisioned to improve agricultural formulations, and of the current state in the Nano-Agro-Business. To identify research gaps and highlight future research needs, we will also compare the frequency distribution of different NM foreseen for agricultural applications with that in agro-environmental and -toxicological research. The resulting recommendations will be completed by the identification of missing elements of essential NM characterization, that often seem to be overlooked in toxicity studies relevant for agriculture, such as purity of the materials or characterization in the experimental media.

2. Materials and methods

For this compilation, we used the following databases and literature sources: (i) Scientific databases: web of science, Google scholar (ii) Patent databases: WIPO (EU), Free patents Online (EU+US), web of science, and (iii) Grey literature obtained from various sources. The criterion for the literature to be selected was that it should refer specifically to development, testing and application of nano-plant protection, -fertilizer and soil management formulations. For the evaluation of toxicity studies, we used the Card and Magnuson [7] approach. This 2-step method has been developed to assess the quality of studies that examine the toxicity of engineered nanomaterials. In the second step, the completeness of assessing the physicochemical parameters of the NM(s) under investigation is determined. Based on whether information related to these parameters is reported, each parameter contributes 1 point (parameter score) towards a total nanoscore. Step one of the method was excluded, as we focus on NM characteristics.

3. Results and discussion

Publication activity increased exponentially since 2000, both generally in agriculture (Fig. 1, blue squares), but also concerning plant protection and partly also fertilizer and remediation applications (Fig. 1, red dots). Figure 1 also shows that grey literature increase (represented by searches on google) outnumbers that of scientific literature by far: 188,000 hits on google for NM and pesticides in contrast to 16 specific publications and patents in 2011. However, leading companies, such as BASF are contributing to patented technologies in this field, indicating increasing efforts to put agricultural nano-formulations into practice.

Figure 2 provides an overview of the different applications of NM in agriculture that contribute to an intentional input (Fig. 2A). As pesticides constitute the major part of these applications, further information is provided on the type of pesticide (Fig. 2B) and the NM function in the pesticide formulation (Fig.2C and D).
Figure 1: Development of publication activity referring to NM in agriculture since 1990: as a rough search in WoS (Blue squares) and as publications and patents specifically selected in accordance to the criteria mentioned in the methods (red dots) and searches on google (left axis). Maximum values of the respective searches are highlighted with lines.

Figure 2: Purposes of agricultural NM applications (A), types of pesticides containing NM (B), general function of NM in pesticides (C), and task of NM additives in pesticides (D).

Table 1 shows provisional results of the sum of all parameter scores that were determined after [7], as achieved by agriculturally relevant nano-ecotoxicological studies. It is obvious, that important issues and parameters, such as agglomeration of the particles, characterization of the particles in relevant experimental media, purity and surface charge were often not addressed. For example, out of the 34 relevant studies, 30 are indicating the sizes of the used particles, yet only in 9 cases a characterization of the NM was carried out in the experimental media.

<table>
<thead>
<tr>
<th>Particle size / size distribution</th>
<th>Agglomeration / Aggregation</th>
<th>Surface area</th>
<th>Chemical composition</th>
<th>Purity</th>
<th>Crystal structure / crystallinity</th>
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Table 1: Sum of all parameter scores (after [7]) achieved by agriculturally relevant nano-ecotoxicological studies (n=34).

4. Conclusions

Preliminary results already show, that - although heavily discussed in public media and grey literature – the development of nano-plant protection is still at a nascent stage. However, the topic is increasingly attractive to industry. Future development of this field is hard to predict, thus we consider a proactive approach to a risk assessment of these materials necessary. This will be especially useful to aid the development of environmentally safe nano-plant protection products. As results of our survey are still preliminary, final results and recommendations will be presented within the talk.

5. References

5. Ghormade, V., M.V. Deshpande, and K.M. Paknikar, Perspectives for nano-biotechnology enabled protection and nutrition of plants. Biotechnology Advances, 2011(0).
Nanoparticle properties affecting embryotoxicity: toward a design of safer nano-Zinc oxide

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1. Introduction

Metal and metal oxides are globally produced in several thousands tons per year and they are widely distributed in articles of commerce. Thus their impact on human and environmental health is predicted to be increasing. Recently nano ZnO (nZnO) has invaded the market for its UV protective and antibacterial properties, that make it suitable for a wide range of application for functional coating formulations to protect wood, plastics, textiles from UV and microbial degradation. Its effective action as stabilizer agent has promoted the use of nZnO in foods, cosmetics and many other consumer products. To the respect of other metal oxides, nZnO appeared to induce cell responses at lower doses, and its toxic effects are reported in a broad range of biological systems, even the mechanisms are not well understood [1]. The size, shape, surface area and composition of these “new” materials still remain major concerns for toxicological studies. Some authors report soluble metal ions as responsible for the metal oxide NP -induced toxicity, some others indicate that the contact of NP with cell is required for toxicity [1]. This duality needs extra research effort to be clarified, as well as the surface properties driving cell contact and toxicity. The antimicrobial activity can also be photoactivate, not only by UV, but also by visible light irradiation [2], thus introducing new variables to the potential toxic effects of nZnO to cells and organisms.

Previous data already showed that nZnO dysplay potential embryotoxicity on X. laevis [3] and that it was able to mainly affect gut development [4]. These authors clearly demonstrated how nZnO produced severe lesions at the intestinal mucosa and how it can potentially cross the gut barrier reaching the underlying tissues. In this work we used Xenopus laevis embryos to characterize the embryotoxic and teratogenic potential of nZnO according to the modulation of NP size and surface charge, as well as to the irradiation conditions. The purpose was to provide mechanistic data on nZnO ecotoxicology and to suggest criteria to design safer Zinc oxide NPs.

2. Materials and methods

Experimental design - Different sized (<100nm and <50nm) and different charged (positive: +, negative: -) nZnOs were used to investigate the role of NP dimension and surface reactivity on the embryotoxic potential. Mortality, malformation and growth retardation data were obtained. In addition NP biological activity was studied by setting up parallel experiments in the dark and under intense visible light illuminating conditions.

NP characterization – nZnO morphology and size were obtained by transmission electron microscopy (TEM). NPs produced in dry form by chemical or physical processes, has been dispersed in ultrapure water, making a stable suspension (known as nanofluid) by means of ultrasound sonicator (Sonics VCX 750) or high shear mixer (Velp OV5). Once liquid suspensions had been generated, the hydrodynamic behavior was characterized by dynamic light scattering (DLS) equipment. The surface charge was determined by a Zeta potentiometer. The dissolution of zinc ions, that ultimately may contribute to nZnO ecotoxicity, was estimated by atomic absorption spectroscopy (AAS) under the different experimental conditions.

Embryotoxicity test - According to the standardized Frog Embryo Teratogenesis Assay – Xenopus (FETAX), embryos were exposed to increasing NP suspension and after 96h, lethality, malformation rate and growth inhibition were measured. A comparative analyses of the oxidative damage in embryos was determined by TBARS assay.

Microscopical analyses - Embryos were processed for histological and conventional TEM analyses to detect specific lesions at tissue and ultrastructural levels. Whole-mount immunohistochemistry followed by laser
scanning microscopy (LSM) was adopted to visualize specific markers of cell stress, especially targeting cytoskeleton and cell junctions. LSM in the reflection mode and energy-filtering transmission electron microscopy (EFTEM) techniques were used to image and track NPs along the embryo body, focusing on the NP interactions at the level of the intestinal barrier.

**3. Results and discussion**

### 3.1. Embryotoxic effects of nZnO: the role of NP size and surface charge

Firstly, we have standardized the methodology to obtained stable water suspensions of ZnO NPs with dimensions ranging in 50nm and 100nm. These NPs showed a Z-potential of about 25 mV, able to ensure a good resistance to sedimentation. For obtaining high Z-potential it’s sometimes necessary to modify pH of water solution or treat or functionalize NPs by polymers or organic molecules, for increasing steric repulsion in liquid. These preliminary procedures were necessary to then allow realistic comparative results among biological tests.

Very little embryo mortality was observed after exposure to the different-sized, positive-charged, nZnO, while a comparable and significant concentration-dependent growth retardation and induction of specific alterations during gut development were induced. The intestinal mucosa showed diffuse lesions at both histological and ultrastructural level, with epithelial cells showing cytoskeletal disarrangement and alteration at the tight junction level. These lesions were sustained by the induction of oxidative damages in tissues of embryos exposed to nZnO. Both nZnOs were able to cross the intestinal barrier and were mapped along the epithelial paracellular spaces, as well as in the surrounding tissues. Smaller NPs appeared to be more efficient in sneaking through the cells and thus in distributing along secondary target tissues.

Preliminary results indicated that negative-charged nZnO, obtained after NP functionalization (by sodium citrate, PVP or silanes), are less prone to induce embryotoxic effects. Further data are still required to better characterize the mechanisms underlying this difference.

### 3.2. Enhanced nZnO embryotoxicity after NP photoactivation

Visible light irradiation of nZnO was seen to enhance bacteria killing [2]. In our experiments it also caused the severeness embryotoxic effects on *X. laevis*. Such results run parallel with the production of high lipid peroxidation levels, testifying for an increase of light-mediated ROS production at NP surface. We are now verifying if the modification of surface properties may influence this photoactivable nZnO behavior.

### 4. Conclusions

We demonstrated that metal oxide NPs displayed powerful embryotoxicity on *X. laevis*, testifying for a metal-based toxicity, with nZnO-induced toxicity mediated by NPs’ own reactivity rather than ion dissolution.

The ecotoxicological potential of ZnO NPs, is strongly associated with the modality of the biological interactions at the nano-level, which at last depend upon the physical and chemical NP surface properties. These properties are also at the base of the induced oxidative potential by nZNO, which is also very efficiently modulated by light irradiation. Finally NP dimension, and especially surface charge, played a crucial role in determining the embryotoxic potential and the intestinal translocation and lesions of nZnO.

The present results showed how a comprehensive knowledge of the nZnO physical and chemical properties, affecting the interactions at the bio-interface, may contribute to make nanotoxicology a predictive science and may help chemists and material scientists in the design of safer NPs.

### 5. References


Greener nanomanufacturing: Toward low-waste and high-yield synthesis of monodispersed metal oxide nanoparticles

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1. Introduction

Metal oxide nanoparticles have attracted much attention due to their wide variety of applications, such as catalysts, energy storage materials, solar cells, light emitting diodes, sensors, transparent electrodes [1]. In the last decade, there have been many reports of syntheses of metal oxide nanoparticles by using different methods. For practical applications, syntheses should be greener, with low waste and high yields, in order to avoid future environmental health and safety risks and to prevent material resource depletion. In addition to their environmental performance, the nanoparticles must have refined properties that enable high performance applications. The properties of metal oxide nanoparticles offer alternatives to the use of metal elements widely used in industry. If industry plans to use a broad array of metal oxide nanoparticles and employ hundreds of different chemicals to synthesize, we may have to develop a large number of new syntheses and attempt more than hundreds of different toxicological assessments because the specific policies for each environmental impact will be considered including combination of chemicals. To avoid such complicated risks, we require a few generalized synthesis methods of nanoparticles having both technical and environmental performance in order to simplify production and toxicological assessments.

We adopt greener nanomanufacturing for the synthesis of monodispersed metal oxide nanoparticles through a catalytic esterification process. We use only oleyl alcohol and oleic acid, derived from non-toxic natural oils, as solvents, reagents, and surfactants during a one-step synthesis of metal oxide nanoparticles. This greener approach offers precise control of doping concentration as well as monodisperse and high yield of nanoparticles. Also, this approach simplifies the assessment of pollution and toxicity. We will demonstrate syntheses of several different metal oxide nanoparticles.

2. Materials and methods

We have synthesized metal oxide nanoparticles at 230 ºC or less by using an pseudo-first-order catalytic esterification. Mixed solution of metal oleates and oleic acid is injected into preheated oleyl alcohol. The other carboxylic acids, alcohols and carboxylates can be applied as well. Within twenty minutes following injection, the dehydration process from metal hydroxide to metal oxide nanoparticles is complete. This approach offers much lower temperature and shorter reaction time than the other methods to produce monodispersed metal oxide nanoparticles with high yield.

![Figure 1: Reaction of Pseudo-first-order esterification for metal oxide nanoparticles](image)

Since the ingredients are all cis-C18 unsaturated fatty acid and alcohol, solubility of oleic acid and oleates into oleyl alcohol are high enough to be a homogeneous condition for the pseudo-first-order reaction. Also, oleyl oleate which is produced after esterification as a byproduct is known as non-toxic natural oil. Indeed, it seems likely that the synthesis is similar to a "deep-frying" of nanoparticles in olive oil.
3. Results and discussion

3.1. Pseudo-first-order esterification

Since the conventional esterification for nanoparticle synthesis is a second-order reaction in an organic solvent similar to gas-phase reaction (Fig.1a), the reaction temperature is high (over 290 °C) and its reaction rate and yield are low (Fig.1c). Thus, it is difficult to obtain high-yield of nanoparticles to suppress metal/chemical waste. However, our proposed pseudo-first-order esterification which is omitted an organic solvent is much faster (Fig. 1b and 1c) and greener. The esterification between metal oleates/oleic acid and oleyl alcohol completes within five minutes, indicating metal oleates work as catalysts for the esterification. During the reaction, fine bubbles of water are generated. The produced byproducts are water and oleyl oleate. The oleyl oleate could be recycled to oleyl alcohol and oleic acid again by using hydrolysis.

3.2. Synthesis of metal oxide nanoparticles

Figure 2 shows transmission electron microscope (TEM) images of In\(_2\)O\(_3\), 10 % tin-doped ITO, Fe\(_3\)O\(_4\) and SnO\(_2\) nanoparticles. All of metal oxide nanoparticles are monodispersed and show no aggregation. The average diameters of monodispersed In\(_2\)O\(_3\) and ITO nanoparticles are 7.2 nm and 6.3 nm in diameter (with 89 % and 87 % dispersities within ± 1 nm differences from the average diameter), respectively. From selected area electron diffraction (SAED) patterns of In\(_2\)O\(_3\) and ITO nanoparticles, the results indicate that the matrix phase of the nanoparticles is cubic In\(_2\)O\(_3\) without any different phases. The yield of nanoparticles at 230 °C is greater than 90 %.

![Figure 2: TEM images of synthesized metal oxide nanoparticles by pseudo-first-order esterification](image)

This esterification reaction is useful for synthesizing the other metal oxide nanoparticles (Fe\(_3\)O\(_4\) and SnO\(_2\)) shown in Fig. 2 with the almost same uniformity.

4. Conclusions

In conclusion, we have synthesized monodispersed metal oxide nanoparticles using a greener approach that uses a benign solvent (oleyl alcohol) and reagent (oleic acid) in a pseudo-first-order catalytic esterification. The pseudo-first-order esterification offers a number of advantages for metal oxide nanoparticle synthesis because it is rapid, produces high yields, permits precise doping, and minimizes waste.

5. References


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Prioritising Non-Target Identification in Wastewater Effluent: From Picking Peaks to Programs!

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1. Introduction
Non-target analysis of wastewater effluent poses many challenges, including a multitude of peaks and potential interferences of matrix constituents with non-target analysis. However, these samples also offer much valuable information about emerging contaminants in the environment, especially those resulting from urban sources. Here, we detail the results of non-target analysis and prioritisation in several wastewater treatment plant (WWTP) effluents from around Switzerland. The use of several samples allowed us to use various prioritisation strategies to select peaks of relevance for typical wastewater samples and direct subsequent analysis and identification efforts.

2. Materials and methods
The sampling locations around Switzerland are shown in Figure 1. 24 hour flow-proportional composite samples were collected by the respective plant operators in February 2010. The samples were enriched using solid-phase extraction and analysed using high performance liquid chromatography coupled to the high resolution LTQ-Orbitrap mass spectrometer with electrospray ionisation (ESI) for detection.

![Figure 1: Sampling locations in Switzerland, taken from [1]. The dots indicate the number of residents each WWTP serves (<50,000, <100,000 and >100,000, according to size respectively).](image)

The program enviMass [2] was used to perform both target and non-target identification of all samples. The list of non-target masses from all samples were then compared and prioritised according to intensity. Peaks with intensity above $10^6$ were considered, approximately corresponding 20 ng/L in the environment for well ionisable compounds (e.g. atrazine) and allowing sufficient intensity for MSMS experiments. MSMS experiments were run using the prioritised “non-target” masses as “target” masses in the Orbitrap.
Then, using “the more information the merrier” approach, we gathered as much information as possible from the analysis to hone in on the most likely molecular formulas and thus corresponding candidates. We used MOLGEN-MSMS to calculate the molecular formula on the basis of ppm error, isotope pattern and finally assignment of formulas to MSMS fragments [3]. We then used MetFrag [4] to retrieve candidates from ChemSpider [5] according to exact mass or formula match and fragment these candidates in silico to rank them according to matching MSMS fragments. We also used MetFusion [6] to perform the same search(es), with the addition of a spectral similarity comparison using spectra retrieved from MassBank [7]. Mass Frontier [8] was used to perform rule-based fragmentation prediction to provide additional information about top candidates. Several different calculations for partitioning and retention properties of compounds were used to assist in candidate selection, incorporating values included in MetFrag and ChemSpider.

3. Results and discussion
The enviMass workflow removes sparks (instrument and electronic noise) and peaks in blank/blind samples, as well as identifying target and internal standards, isotope and adduct peaks. Figure 2 shows how we used enviMass to reduce the huge number of peaks in each sample (average ~14,000 peaks, positive only) to isolate the most intense non-targets present in all samples (~2 % of all peaks).

For the intense positive non-target peaks, approximately 3 % contained Cl, 6 % S and 12 % N. In contrast, a much greater percentage of intense negative non-targets had detectable heteroatoms, 31 % contained Cl, 42 % S and 13 % N. The high percentage of S in the negative peak list could be attributed to a large number of sulfonic acid compounds. We will show how the number of database candidates for the peaks, ranging from tens to thousands, could be narrowed considerably using MetFrag and predicted properties.

4. Conclusions
The enviMass workflow allowed a quick and effective selection of peaks of interest and comparison of samples, while the combination of different software streamlines the non-target identification efforts greatly.

5. References

Acknowledgement - The authors thank Philipp Longree and Mark Honti for their assistance.
Screening and toxicological evaluation of organic micro-pollutants in the Rhine and Meuse river basins

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1. Introduction

The rivers Rhine and Meuse serve as drinking water source for millions of people in Europe. In these waters, rapid improvements in chemical and bioanalytical techniques have led to the discovery of all kinds of emerging contaminants at very low concentrations [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. Because thousands of compounds are present in the aquatic environment, it is impossible to detect their presence solely via target analysis in standard monitoring programs. Non-target screening is therefore used as an important additional tool to obtain information on the types of organic contaminants that are present in the aquatic compartment. It functions not only as an intake monitoring tool, but also as a safety net for new or unknown compounds which are not included in the target analysis.

In this study, sensitive GC-MS non-target screening methods were developed (up to LOD 1 ng/L) and applied to different locations in the Dutch part of the Rhine and Meuse river basins in 2010 and 2011. A first goal of the study was to examine which known and unknown compounds are found in the Rhine and Meuse. A second goal of the screening study was to investigate whether there are compounds present that are potentially of concern for the drinking water production. To this aim an integrative ranking system was developed in which the identified compounds were sorted according to aspects as their human toxicological risk, frequency of detection, persistence in drinking water treatment and associated public concern.

2. Materials and methods

2.1 Screening method

Surface water samples were collected at different locations along the Dutch parts of the catchments of the rivers Rhine and Meuse. Every four weeks samples were collected for the standard screening monitoring program of the drinking water companies (Dunea and Waternet) and additionally at Lobith, which is the most important sample point for inflow of water in the Dutch Rhine. This was done in assignment of the Association of River Waterworks (RIWA). A XAD-GC/MS screening method was used to analyze the samples (2L) with a detection limit of 30 ng/L. Every three months, a larger volume (4L) was sampled which enabled a very sensitive measurement of the compounds with a detection limit of 1 ng/L. Samples were passed through a XAD-resin column, which is able to retain organic micropollutants with a relatively large polarity range (log Kow 2-5). The compounds were removed from the column by rinsing the column with dichloromethane and subsequently separated with a gas chromatograph (GC) and measured with a mass selective detector (MS). With the XAD-GC/MS method compounds can be isolated from the water with relatively high efficiencies. The method is semi quantitative. To identify the compounds the obtained mass spectra were compared against the NIST and INFOSPEC mass spectral libraries.

2.2 Data processing

Based on the screening results, an overview was made of known and unknown compounds that are found in the river samples. A selection of relevant compounds was made based on occurrence and concentrations. Compounds that were frequently found (in more than 25% of the samples), compounds that are found in more than one location, or compounds that are found in relatively high concentrations (>0.1 µg/L) were selected. As far as possible the known compounds were assigned to groups based on their application.

A toxicological evaluation was performed for the selected compounds. The results were used for a prioritization of the compounds for their drinking water relevance according to a ranking system addressing...
points to the compounds depending on their toxicological properties, behavior during water treatment (polarity, biodegradability and volatility) and associated public concern.

3. Results and discussion

Up to 400 different compounds were found in the water samples in 2010 and 2011. Around 50% of the compounds were found only once, and around 30% of the compounds had an unknown structure. By mapping the organic pollution in the river basins, and following the trends of pollution over the year, an integrated picture on the presence of organic pollutants in place and time was obtained. The screening results indicated that there are differences in the occurrence of compounds between the Rhine and Meuse, due to differences in activities in their catchments. In the Meuse pesticides and household chemicals dominated, whereas in the Rhine industrial compounds and pharmaceuticals were more prominent. However, some compounds were widespread in both rivers, like tri(chloropropyl)phosphate, Surfynol 104, hexa(methoxymethyl)amine, 5-methyl-1H-benzotriazole and 2,4-di-dimethylpropylphenol. Industrial compounds and flame retardants comprised the largest groups of compounds (as can been seen for the river Rhine in Figure 1).

Figure 1: Groups of compounds that are found in the river Rhine

The screening data resulted in a list of 27 possible relevant compounds for the drinking water. This list includes mostly industrial compounds, but also flame retardants and drugs. Six of the compounds had an unknown structure; one of them was present on all sampling locations. For these compounds it would be recommendable to trace their identity.

A top ten of contaminating compounds that are most relevant for drinking water production from the rivers Rhine and Meuse was compiled. This list will be nominated for political action.

4. Conclusions

- Non target screening is an important tool to identify compounds that are not included in the routine analysis of target compounds.
- An integrated picture on organic pollution in the Dutch river basins of the Rhine and Meuse was obtained, resulting in a list compounds that are potentially relevant for drinking water production.
- Our study showed the transfer of non-target screening to an application outside a strict academic world and the value of non-target screening in combination with human risk assessment.

5. References


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Integrated characterization of a mutagenic waste water treatment plant effluent combining advanced screening techniques and biological assessment

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1. Introduction

Increasing numbers of emerging contaminants have been detected in surface water over the last decade. Waste water treatment plant (WWTP) discharges were identified as main sources of these chemicals and related effects in the environment [1, 2]. For this reason, effluents of WWTPs were assessed to gain knowledge about discharged chemicals, their concentrations and their mutagenic, genotoxic, and estrogenic effects on the environment. Linking the discharged chemicals to effects or identifying the substances causing measured effects is the main challenge for the assessment of WWTP effluents.

To meet this challenge in the present study an integrated biological and chemical approach was applied to identify genotoxic and mutagenic compounds in an effluent of a WWTP that treats both industrial and municipal wastewater as well as contaminated groundwater. The approach combined extensive target and suspect screening with biological effect assessment in vitro and in vivo on the basis of a series of grab samples over several weeks. Effect assessment was based on Ames testing for bacterial mutagenicity applying different diagnostic strains. Thereby, the focus was particularly set on the discrimination of mutagenicity caused by aromatic amines and nitro-aromatic compounds, which were hypothesized to cause mutagenicity in this effluent. This approach was supplemented with in vivo caging experiments of roach in the receiving river Mulde upstream and downstream of the effluent discharge.

2. Materials and methods

2.1. Target and suspect screening

Grab samples were taken after the outlet of the WWTP Bitterfeld-Wolfen, Saxony-Anhalt, Germany weekly for six weeks in a first, every two days in a second sampling campaign. Samples were extracted by solid phase extraction on Chromabond HR-X sorbent (Macherey-Nagel; Düren, Germany). During the first sampling campaign, Blue Rayon (BR), a passive sampler designed for the extraction of planar poly-aromatic compounds, was exposed to the WWTP effluent. BR was cleaned and extracted according to Kummrow et al. [3]. Liquid chromatography - mass spectrometry (LC-MS) analysis was performed on a UPLC system (Agilent Technologies; Waldbronn, Germany) connected to a high resolution, high mass accuracy LTQ Orbitrap MS (Thermo-Fisher Scientific; Bremen, Germany). Target screening was performed for 300 environmentally relevant chemicals. A suspect list of 1800 substances produced and applied at the industrial site and a suspect list of 400 environmentally relevant chemicals from literature was compiled and processed by InstantJChem 5.4 (ChemAxon, Budapest, Hungary). Mass spectrometric data were processed by MZmine, an open source software for MS data processing [4]. Applying a linear solvation energy relationships (LSER) model, retention times of all suspects detected by MZmine were predicted with a 95%-prediction interval of ±2.7 minutes [5].

2.2. Biological characterization of WWTP effluent

Mutagenic effects of WWTP extracts were revealed by the Ames fluctuation assay as described by Perez et al. [6] with a TA98 strain. Further Ames assays were performed with YG1024 strains to get information about the contribution of amino compounds to total mutagenicity. For detection of mutagenicity in vivo, fish, Rutilus rutilus caging experiments were carried out upstream and downstream of the discharge of the WWTP for one week. Exposed fish were examined for micro nuclei.
3. Results and discussion

3.1. Chemical characterization of WWTP effluent

Several target compounds commonly detected in WWTP effluents, have been identified. Concentration trends have been recorded, focusing particularly on indicator compounds for industrial and domestic waste water, as well as for groundwater co-treated in this WWTP. Waste water effluents exhibited relatively constant concentrations of compounds originating from municipal waste water and groundwater, whereas chemicals deriving from industrial waste water differed strongly in concentration. For further chemical characterization and investigation of the influence of certain industries, a suspect screening was performed. Six site specific suspects and two suspects from literature were identified. These suspects include two barbiturates in toxicologically relevant concentrations of up to 190 µg/L. Constant concentrations of prometryn, a no longer registered herbicide, indicates a steadious burden to surface water deriving from contaminated groundwater treated by the WWTP. The combination of high resolution LC-MS/MS with advanced retention time prediction by a LSER model has been proved as a powerful tool for the screening of a large number of targets and suspects.

3.2. Biological characterization of WWTP effluent

Nine of eleven methanol extracts and all BR extracts exhibited mutagenicity in the Ames fluctuation assay after metabolomic activation by a rat liver homogenate. Intensity of mutagenicity varied over time. Time dependent mutagenicity of BR and methanol extracts did not show a good correlation indicating that both approaches sampled different fractions of mutagens. The contribution of aromatic amines was determined by comparison of Ames assay with YG1024 and TA98 strain. In vivo genotoxicity was assessed with caged fish by counting micro nuclei formation.

3.3. Correlation of chemical and biological effects

Correlations between target substances and suspects with mutagenicity of samples will be presented. Revealing potential indicator substances for waste water from particular industries by suspect analysis, these indicators will be set in context with mutagenicity of corresponding samples.

4. Conclusions

The extension of chemical multi-target analysis with a rapid and automated suspect screening exploiting advanced retention prediction allows for an improvement of chemical characterisation of WWTP effluents. The combination with tailored effect monitoring provides additional indications for hazards and risks. Chemical concentrations as well as the measured mutagenic effects for nearly all samples taken, suggest toxicological relevance for the receiving ecosystems and potentially for human health. Further investigations are required to confirm effects on the population and community level. Although, relationships between concentrations of identified chemicals and mutagenic effects provide some assumptions on cause-effect relations. However, further investigations by effect-directed analysis are necessary for the identification of those pollutants causing the measured effects and for the reduction of the environmental burden caused by this WWTP effluent.

5. References


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Identification of biotransformation products (BTPs) formed in freshwater crustaceans

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1. Introduction

Freshwater crustaceans, Daphnia magna and Gammarus pulex are widely distributed in watersheds which are contaminated by anthropogenic compounds. After uptake of organic contaminants the organisms possibly form biotransformation products (BTPs) by means of enzymes involved in detoxification. Little is known on BTPs in crustaceans and their relevance to explain chemical fate and toxicity. There are a number of tools for predicting biotransformation pathways. However, those are originally designed for either mammalian's or microorganisms' BTPs which might be different from BTPs formed in crustaceans. In the present study, D. magna and G. pulex were exposed to selected organic contaminants and then their BTPs were identified through the suspected/non target screening methodology by using high resolution LC-tandem mass spectrometry. The structure elucidation of BTP was performed through MS/MS spectra interpretation with a fragment prediction tool. In addition, the BTP prediction tools currently available were evaluated in terms of the feasibility of application to crustaceans.

2. Materials and methods

- Selected compounds: pharmaceuticals and biocides (e.g., valsartan, clarithromycin, tramadol, venlafaxine, terbutryn, and irgarol) which have been detected in Swiss surface waters.
- Test organisms:
  - D. magna: cultured by OECD method
  - G. pulex: collected from a Swiss stream
- In vivo exposure experiment:
  - Exposed at 100-200µg/L of each compound for 24 h
  - 30 daphnids in 30 mL and 8 gammarids in 500 mL of exposure medium
  - At 20°C (daphnids) and at 13°C (gammarids), 16:8 Light-dark cycle
- Uptake & depuration (toxicokinetic) experiment with/without CYP enzyme inhibitor
- Sampling & Extraction:
  - Homogenization of exposed organisms in MeOH using beads-beating (FastPrep)
  - Filtration with 0.45 µm cellulose filter
  - Injection into 20 mL glass vial filled with deionized water
- Online-SPE-HPLC-Orbitrap-MS/MS:
  - Mixed bed multi layer SPE cartridge [1] (Oasis HLB, Strata-X-AW & -CW, ENV+)
  - Ion source: ESI ±
  - Orbitrap MS: MS range 150-2000, Resolution >60,000, MS accuracy <5 ppm
  - Orbitrap MS/MS: Data dependent acquisition, Resolution >7,500
- Software tools:
  - For BTP prediction: Metworks (Thermo), UM-PPS (Univ. Minnesota), Meteor (Lhasa), Metabolizer (ChemAxon), manual prediction (literature survey, heuristic rules)
  - For MS/MS fragmentation prediction: Mass Frontier 6.0 (Thermo)
  - For non-target screening (for identification of unpredicted masses): SIEVE (Thermo)

3. Results and discussion

Neither abnormal activity nor mortality of exposed organisms was observed during the exposure experiments. Suspected peaks detected through full-scan mode screening of Orbitrap were examined based on exact mass and molecular formula proposed by Xcalibur. The spectral evaluation software, Mass Frontier 6.0 was helpful to interpret the MS/MS spectra of the precursor ion for structure elucidation. Six phase I and one
phase II BTPs were tentatively identified for irgarol (Figure 1). Various reaction mechanisms such as N-dealkylation, O-dealkylation, N-oxidation, hydroxylation, epoxidation and glycine conjugation lead to the BTPs for irgarol, terbutryn, tramadol, or venlafaxine. Glycine conjugation products are not so often reported in literature but have been described for daphnia [2]. Irgarol and terbutryn which both have triazine moiety showed similar biotransformation pathways. No BTPs were identified for valsartan and clarithromycin so far. This may be caused in general by the lower bioaccumulation of the relatively polar and ionized compounds. More BTPs were identified for G.pulex compared to D.magna.

Figure 1: Biotransformation pathway of irgarol suggested for freshwater crustaceans. Biotransformation products are named according to their molecular weights. RT stands for retention time in minutes.

In toxicokinetic experiments, bioaccumulation of irgarol and terbutryn to daphnids reached to equilibrium within 20 h exposure. Glycine conjugation products showed different TK behavior than Phase I BTPs. In the presence of CYP inhibitor, less BTPs were identified, indicating that CYP enzymes were mainly responsible for BTP formation in crustaceans. A number of dealkylation and oxidation products were successfully predicted by the prediction tools. However, the manual prediction based on biochemical knowledge was most successful but it included also the most candidates. Conclusions

Several Phase I and II BTPs were tentatively identified in freshwater crustaceans using LC-HRMS/MS. Oxidation, hydroxylation, dealkylation, and glycine conjugation were possible mechanisms and cytochrome enzymes played a major role in biotransformation of crustaceans. Less BTPs were identified for less bioaccumulative compounds. BTP prediction tools estimated false positive and true negative as well as true positive results.

5. References


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Input and fate of contaminants in surface waters observed by suspect and nontarget screening with LC-Q-TOF-MS

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1. Introduction

The increasing availability and sensitivity of LC-MS systems has widely opened the analytical window for polar analytes, the so-called new emerging contaminants. These are increasing numbers of compounds from pharmaceuticals and personal care products, herbicides, fungicides and industrial chemicals. Several of these compounds show ecotoxicity or human toxicity. Already more than thousand compounds are recognized as relevant for drinking water production. Recent work reveals the importance of transformation products in biotic and abiotic processes in the environment and in water treatment [1]. To cope with the increasing number and variety of contaminants, the identification of unknown compounds is an attractive but also challenging goal.

LC with high resolution mass spectrometry at a high mass resolving power between \(m/\Delta m = 40\,000\) and 100 000 and a high mass accuracy of better than 1 ppm enables to perform screening approaches to detect a considerable number of known and unknown analytes in complex samples ([2, 3]. The identification often is based on matches of the unknown compound in mass spectral libraries with accurate masses and fragmentation data. The availability of LC-MS libraries and the number of entries is however still very limited. Otherwise chemical data bases with a huge number of entries like PubChem or ChemSpider don’t have mass fragmentation data available. Here come software tools from the metabolomics field, like MetFrag [4], into play which allow to predict mass spectral fragmentation from given chemical structures and to match measured with \textit{in silico} fragmentation patterns. This approach is very promising in supporting the workflow of nontarget screening also in environmental analysis.

In this work we will show the application of target and nontarget screening with LC-electrospray ionization-quadrupole-time-of-flight-mass spectrometry (LC-ESI-Q-TOF-MS) combined with computerbased data evaluation based on statistical analysis to reduce the number of relevant compounds and on tools of computational mass spectrometry to elucidate the input and fate of contaminants in surface water.

2. Materials and methods

Surface water samples have been sampled regularly over the period of one year from the river Neckar and the creeks Ammer and Goldersbach near Tübingen. Samples have been preconcentrated 500- to 5000-fold after pH adjustment to 2 and 7 with automated solid-phase extraction on LiChrolut ENV+ and Isolute 101. To be able to use statistical approaches for the selection of relevant compounds three replicates of the samples have been prepared. LC-Q-TOF-MS measurements have been performed on an Agilent 1260 LC coupled to a 6540 QTOF instrument with enhanced mass resolution capability of \(m/\Delta m = 40\,000\). Accurate masses and mass fragmentation patterns have been used to retrieve suitable compounds from a target analyte list and from mass spectral libraries. For the nontarget approach first a deconvolution software was applied to generate mass lists of possible compound candidates. These mass lists were further reduced for example by principal component analysis (PCA) using the statistical software package Mass Profiler Professional to discover relevant masses which should be subjected to further analysis. The high resolution masses and mass fragmentation patterns of the reduced set of unknowns has been further searched in the chemical data bases PubChem and ChemSpider. For each accurate mass a number of candidates between 30 and 2400 have been obtained, which could be ranked by the software tool MetFrag by matching the \textit{in silico} fragmentation patterns of the candidates with the measured mass fragments.

3. Results and discussion

A variety of compounds from pesticides, PPCPs and their metabolites could be found by target and nontarget screening approaches. The number of compounds showed for example an increasing trend from the source of the creek Ammer to the mouth which could be attributed to a large extent to the input of wastewater treatment plant effluents. In the source region the herbicide metabolite desethyl atrazine
indicates an historical background of atrazine pollution of the groundwater. Further intermittent findings of amidotrizoic acid in the source region could be traced back to the influence of storm water overflow basin. Samples taken downstream of a wastewater treatment plant showed more than 10,000 mass peaks retrieved from the chromatogram by the deconvolution software. Typical wastewater indicators have been found like the X-ray contrast media amidotrizoic acid, iomeprol and iopromide, or further pharmaceuticals like carbamazepine and diclofenac, but also the artificial sweeteners acesulfame and sucralose.

Further candidates were retrieved from the huge mass list by PCA which allowed to select relevant candidates for wastewater input or for specific flow conditions of the creek like base flow or high tide. Several compounds could be identified by the application of PubChem search and mass fragmentation match using MetFrag. For example, the search of the exact mass 187.063 retrieved the structures for 726 candidates from PubChem. Matching the measured fragment at m/z 145.0155 against the in silico generated fragments of the 726 proposed structures resulted in only two structures (Figure 1) which made the identification of desethylatrazine straightforward. However, this is an exceptional example, since other masses resulted in much more possible candidates due to the presence of isomers which could not be distinguished by their mass fragmentation. Further background information on possible contaminants or their metabolites was necessary in this case to propose a more plausible candidate and to verify that by an authentical analytical standard.

Figure 1: First two ranks from the MetFrag search of the original compound mass 187.063 and the fragment mass m/z 145.0155 (in the positive mode in PubChem).

4. Conclusions

High resolution MS and different data evaluation approaches for target and nontarget analysis enabled to identify the input of a rather large number of compounds in surface water from agricultural activities and wastewater effluents. To improve the sensitivity and to remove matrix components a preconcentration step is inevitable, but can limit the range of compounds which can be extracted. Statistical analysis to select relevant compounds from the mass list of a sample combined with PubChem search and matching of the fragments with the software tool MetFrag enabled to identify a number of further compounds and their metabolites. However, there is still a large number of candidates to be identified due to inconsistent data base and fragment match and due to a high number of possible isomers. In conclusion, there remains a more urgent call for the availability of mass spectral libraries with sufficient numbers of entries.

5. References


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Can bioanalytical tools help us ensure that our water is safe?

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1. Introduction

Chemical pollution is an increasing threat to our waterways, oceans, and drinking water sources. Its impact will be amplified by population growth and, possibly, by some of the effects of climate change. Many countries have regulations in place for quality assessment of wastewater treatment plant effluent, recycled water and surface waters. For example, the “Standards for Quality of Recycled Water Supplied to Augment a Supply of Drinking Water” cover approximately 360 chemicals. Regular chemical analysis of such a large number of chemicals is not financially viable. Furthermore, conventional chemical monitoring programs have been criticised on the basis that they cannot include the full range of chemical pollutants that could occur in water sources, and they do not account for the combined effects of mixtures of chemicals. Bioanalytical tools may therefore complement chemical analysis for cost-efficient water quality monitoring [1]. They provide measures of the cumulative effects of chemicals that exhibit the same mode of toxic action, for which the selected bioassays are indicative plus they can give a measure of the cytotoxicity of all chemicals acting together in a water sample. Improved detection of the presence of chemicals in water enhances risk assessment and informs water management options, among them water recycling from impaired sources such as sewage or stormwater harvesting and reuse.

2. Materials and methods

Toxicity testing can yield complex information on subtle effects occurring within the body. Cellular events, for instance, can be measured in vitro. These cellular triggers are prerequisites but not sufficient conditions for the manifestation of adverse effect on the organ, organism or population level (Figure 1). While detoxification and defence mechanisms may reduce or eliminate the toxic potency of a single chemical, primary cellular effects can provide a sensitive and thus conservative tool for assessing the potential for effects of contaminant mixtures. The many different chemicals present in a water sample are taken up to varying extent by the human body. After uptake, the xenobiotics are ultimately distributed to organs and cells. The cellular concentration is a measure of the biologically active dose as only the contaminants that reach the cells can undergo interaction with biomolecules in the cells (Figure 1). This initiating event, the interaction of the micropollutant with its biological target, can therefore be applied as a measure of potential effect. Although it is important to keep in mind that repair and defence mechanisms may be able to counteract this initial trigger, the potential to do harm is an important assessment endpoint from a precautionary risk perspective. Reporter gene assays are often used to overcome difficulties measuring the initiating event directly. In reporter cell lines, a receptor or response element in the cellular response pathway is over-expressed and linked to a reporter gene that produces a quantifiable gene product that can be used for easy measurement of the response. Finally, cytotoxicity (cell death or reduced growth) can be quantified as an integrative parameter for all toxicity pathways in a cell. Bioanalytical tools that respond to relevant initiating triggers in a toxicity pathway or to a known mode of toxic action with a defined health outcome are potentially applicable for water quality assessment [1]. Assay robustness and specificity in the presence of matrix components and other chemicals must be characterised prior to implementation as monitoring tools. In vitro assays generally require less space (lower volumes) than direct toxicity testing and are often more practical for assessment of low contaminated environmental samples, for which sample concentration and clean-up are prerequisites.
3. Results and discussion

In this presentation the design of a modular battery of bioassays following the outlined principles will be presented and some illustrative examples from recent applications in Southeast Queensland, Australia. The bioassays were selected from the three main categories of modes of action, namely non-specific, receptor-mediated specific and reactive toxicity. This bioanalytical test battery was used for monitoring organic micropollutants across an indirect potable reuse scheme testing sites across the complete water cycle from sewage to drinking water to assess the efficacy of different treatment barriers, including source control, wastewater treatment plant, microfiltration, reverse osmosis, advanced oxidation, natural environment in a reservoir and drinking water treatment plant. The design of the test battery and all experimental information is published in [1]. Using bioanalytical tools it was possible to follow the removal of groups of micropollutants through the various treatment steps. Treatment efficiency varied between different types of water treatment. Ozonation was very efficient in destroying specific toxicity such as estrogenicity, while baseline toxicant’s effects were better removed by membrane filtration processes, presumably because mild oxidation processes only transform chemicals and do not mineralize them. Herbicides were often recalcitrant towards biodegradation in conventional wastewater treatment plant, while removal of phytotoxicity was much better with tertiary treatment and advanced oxidation processes.

4. Conclusions

Chemical monitoring provides a quantitative assessment of single organic contaminants in a water sample but cannot account for the presence of non-target compounds such as unidentified transformation products and interactions between chemicals. Bioanalytical monitoring is complementary to chemical analysis and provides information on all bioactive micropollutants in a sample according to potency, i.e., chemicals of higher toxicity will be weighted higher than less toxic chemicals. The results of the various studies presented here indicate that bioanalytical tools provide valuable additional information to chemical analysis and should be implemented in the future as a monitoring tool.

5. References


Acknowledgement - This paper is a review of a number of studies, which were mainly financed by the Urban Water Security Research Alliance, Seqwater and Watersecure and additional partner acknowledged in the cited references. We thank the Advanced Water Management Centre of the University of Queensland for collaboration and support.
1. Introduction

Recent studies show that urban areas can be important sources of many semi-volatile organic chemicals (SVOCs) [1], including poly- and perfluorinated alkyl substances (PFASs, e.g. fluorotelomer alcohols (FTOHs) and perfluorooctane sulfonamides (FOSAs)) [2]. PFASs are released in urban areas during use and disposal of polymeric materials, chemicals or consumer products that contain PFAS residuals and directly from manufacturing sites [3]. However, residuals vary considerably among consumer products [4] and it is therefore difficult to estimate diffusive emissions based only on surveys of consumer products. In this work we address this problem by interpreting measurements of four PFASs (8:2 FTOH, 10:2 FTOH, Me-FOSA and Et-FOSA) made in the city of Zurich (Switzerland), during a sampling campaign performed in August 2010 [5] using a multimedia mass balance model. Our estimates are compared with available literature data derived from emission-factor-based methods to obtain more insight into the emission pathways of the four PFASs investigated.

2. Materials and methods

Our multimedia mass balance model tracks the chemical mass balance in environmental compartments including atmosphere, soil, vegetation, water and sediment. Specifically, it has been designed to quantitatively describe the day-night cycling of SVOCs in air considering the atmospheric boundary layer dynamics: after sunset, the land surface cools down by long-wave radiation and thus cools the air close to the surface, which is faster than the cooling of the upper air. Thus, a nocturnal boundary layer (NBL) forms with cold air underlying warmer air. This temperature difference prevents the mixing processes between layers, thus causing enrichment of contaminants within the NBL (see Fig.1, right). When the sun rises, solar radiation heats the ground and the air adjacent to the surface. This heating process causes convectional movement of air parcels and leads to vertical mixing of the contaminants enriched within the NBL with less contaminated upper air (dilution).

![Figure 1: Schematic diagram of the day-night evolution the boundary layer over a city and the enrichment of contaminants within the stable boundary layer at night.](image-url)
3. Results and discussion

3.1. Measured and modelled concentrations of the four PFASs in air in Zurich

Figure 2 shows measured and modeled airborne PFAS concentrations in Zurich. The model describes the observations of all four PFASs within the boundaries of our Monte Carlo uncertainty analysis, with few exceptions. Both measurements and modeled results follow the same diurnal pattern with maxima at night and minima during the daytime. The diurnal boundary layer dynamics was identified as the major factor, leading to enrichment of PFASs at night, when there is no transformation process due to zero OH radical concentration, low inflow and outflow due to low wind speed, and a small volume of air receiving the PFASs.

Figure 2: modeled (solid lines) and measured (triangles) concentrations of the four PFASs in the air near surface in Zurich; dashed lines indicate the 2.5% and 97.5% confidence interval of the modeled level derived from a Monte Carlo analysis.

3.2. Emission source strength estimates of the four PFASs in Zurich

The good agreement between the measurements and the model results at the Zurich site suggests that the emission rates assumed in the model are a good estimate of the actual emissions of PFASs in the city of Zurich. The estimated yearly emission source strengths of the four PFASs are in the range of 0.4 kg/year to 22.5 kg/year and follow the sequence: 8:2 FTOH > 10:2 FTOH > MeFOSA > EtFOSA.

3.3. Comparison to literature data

To obtain more insight into the emission pathways of the four PFASs, the emission strength estimated from our study were compared to literature data. In general, our estimated emission source strengths of FTOHs are in good agreement with other estimates, which are all based on emission factors along the life cycle of FTOHs. Moreover, our estimates confirm that there is still low but ongoing volatilization of MeFOSA and EtFOSA from consumer products manufactured prior to 2002, when the major producer stopped producing these substances. It may still take years until they disappear from consumer products and the environment.

4. Limitations and outlook

The estimated emission strengths from this study can likely not be extrapolated to the global level, because they represent only diffusive emissions from the city of Zurich, which is a typical urban area but without any manufacturing or processing of PFASs or PFAS-containing products. Therefore, emissions from processes in the PFAS industry are not included in our results. To estimate more comprehensive overall global emission source strengths of these substances, studies using this measurement-and-modelling-combined technique should be carried out in multiple places, including urban areas with fluoropolymer-based industries, especially in new production countries like China.

5. References


Acknowledgement - The Swiss Federal Office for the Environment (FOEN) provided funding for this research at ETH Zurich. We thank Andreas Buser and Bojan Gasic for discussions, and Erol Dedeoglu for IT support.
Uptake of Perfluoroalkyl acids by hydroponically and field grown Lettuce (Lactuca sativa) and Radish (Raphanus sativus)

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1. Introduction

Perfluorinated alkyl acids (PFAAs) are bioaccumulative persistent, organic pollutants (POPs), which can be detected ubiquitously in the environment. PFAAs pose a risk to human health due to accumulation in the food chain. The occurrence of PFAAs in animals, such as fish, birds and mammals including humans is fairly well documented, but little can be found in the literature about crops or plants in general. Also, most studies focus just on the two main compounds perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA). So far only two studies have been published about the uptake of PFAAs by crops [1,2].

Plants grown on contaminated soil are able to take up and accumulate PFAAs, as has become apparent from published data on PFAAs concentrations in crops, e.g. potatoes or cereals. Humans are possibly exposed to PFAAs through consumption of vegetables and other plant-related food items. The objective of this study is to understand the accumulation process of PFAAs in crops.

2. Materials and methods

In a first experiment lettuce (Lactuca sativa, var. attraction) was grown hydroponically in a greenhouse with a spiked nutrient solution to avoid sorption to soil and to make sure the offered PFAAs are completely bioavailable. The lettuces were exposed to a set of 10 perfluorinated carboxylic acids (PFCAs) and 3 perfluorinated sulphonates (PFSAs) in four different concentrations (10 ng/L, 100 ng/L, 1 µg/L and 10 µg/L nominal) to assess the difference in behavior between PFAAs and the influence of concentration.

The greenhouse experiment was set up under the assumption that because of the high water solubility of the compounds the PFAAs will be taken up by the root system of the plants and will be distributed through the plants’ water system. Hence it was assumed that evaporation plays an important role in the uptake, and it was hypothesized that bioaccumulation is especially strong in the leaves of the plants. To test this hypothesis, correlations of the PFAAs uptake with the water uptake were examined.

The plant samples (roots or foliage) were collected after 40 days of exposure. During the growth period the spiked nutrient solution was replaced 3 times. The samples were homogenized with a household blender and then extracted based on the method published by Hansen et al. [3] with modifications proposed by Vestergren et al. [4].

Since the results of the greenhouse experiment led to a new hypothesis that root vegetables or vegetables that form bulbs pose bioaccumulate PFAAs more strongly, a field experiment was set up where lettuce and radish (Raphanus sativus) were grown in 4 lysimeters, each containing different concentrations of spiked soil (initial concentrations: 100 µg/kg, 1 mg/kg, 5 mg/kg and 10 mg/kg DW nominal of the same compounds as used in the greenhouse experiment, except for PFHxS, which was not available) to have a comparison to the greenhouse experiment and a comparison of a leafy vegetable to a root/bulb vegetable. The lysimeters had a surface area of 1 m² and a depth of 0.6 m. About 10 lettuces and 20 radishes were planted in each lysimeter.

Radish and lettuce samples were taken 50 and 70 days after seeding, respectively, when the plants had matured. The samples (roots, bulbs or foliage) from the same lysimeter were pooled and extracted as described above.

The extracts were analysed with HPLC-MS/MS.
3. Results and discussion

3.1. Results of the greenhouse experiment

The results show that except for the short chained PFCAs PFBA and PFPeA the concentrations in the roots were higher than in the foliage. Furthermore the results show that the uptake for the PFAAs with a chain length of more than 6 carbon atoms is not linear with increasing concentration, but follows a Freundlich or Langmuir isotherm instead. This indicates that adsorption to root surfaces might play a more important role in the uptake than the water uptake.

3.2. Results of the field experiment

While no significant difference in growth was observed between the different concentrations in the greenhouse experiment, both the lettuces and the radishes showed a significant decrease in growth in the highest exposure group in the lysimeter study.

The preliminary results of the concentrations in the plant material from the lysimeter study show higher concentrations in the foliage than in the roots or bulbs for both lettuce and radish.

4. Conclusions

The concentrations in the different parts of the lettuces show a different pattern in the field experiment than in the greenhouse experiment, with higher concentrations in the foliage for most of the compounds. One explanation for this could be that the concentration of the compounds in the hydroponic experiment was kept at a constant level, while the soil was only initially spiked. Rain could have washed out the chemicals from the soil, thereby removing chemical that had adsorbed to the root surfaces, while the chemicals that had been translocated to the foliage would have been retained. Another explanation could be the longer growth period in the field experiment. The translocation of the chemicals from roots to the foliage could be time dependent. If you think of the plant as a chromatography system, long chained PFAAs will need more time to reach the foliage than short chained PFAAs.

The hypothesis that root or bulb forming vegetables pose a higher risk than leafy vegetables was not confirmed, since the concentrations in the foliage of the radishes were higher than in the bulbs. This was also observed by Lechner et al. for carrots and potatoes [2].

5. References


Acknowledgement - This study is part of the EU project PERFOOD (KBBE-227525), and the financial support of the European Union is gratefully acknowledged.
Perfluoroalkyl acids in blood serum from first time mothers from Uppsala, Sweden: Temporal trends 1996-2010 and serial samples during pregnancy and nursing

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1. Introduction

Perfluoroalkyl acids (PFAAs) have been detected in human blood samples from several countries around the world [1]. However, little is known about temporal trends of human body burdens of other PFAAs than perfluorooctane sulfonic acid (PFOS) and perfluorooctanoic acid (PFOA). In the present study, concentrations of 13 PFAAs were determined between 1996 and 2010 in pooled blood serum samples from nursing primiparous women living in Uppsala County, Sweden. The aim was to investigate possible effects on human exposure of risk management measures to reduce production, emissions and use of PFOA and PFOS. Moreover, serial maternal serum samples during pregnancy and nursing as well as corresponding cord blood samples were analysed to evaluate if PFAA levels during the nursing period are representative for the fetal development period.

2. Materials and methods

Between 1996 and 2010, 417 primiparous women living in Uppsala County, Sweden, were recruited in a study of temporal trends of body burdens of persistent halogenated organic compounds [2]. The women donated blood samples during pregnancy and/or the nursing period. Additionally, cord blood samples were obtained. The present study consisted of two sub-studies. First, serial maternal blood serum samples during pregnancy and after delivery, as well as cord whole blood samples, were analyzed for PFOS, PFOA and perfluorononanoic acid (PFNA) (n=19). Samples were taken between 1996 and 1999 in the 1st and 3rd trimester of pregnancy, at delivery (cord blood) and 3 weeks and 3 months after delivery to study if PFAA concentrations in the maternal serum were indicative of the body burden of the newborn. Secondly, the blood samples from the homogenous group of young women were used (n=413, one to three pools per year were analysed) to examine temporal changes in human blood levels of 13 PFAAs in Sweden during the last 15 years, including the periods of industrial changes of production and emission patterns of PFAAs.

Samples were spiked with isotope labeled internal standards, extracted with acetonitrile and the extracts were cleaned up on graphitised carbon. Instrumental analysis was performed applying high performance liquid chromatography (HPLC) coupled to tandem mass spectrometry (for the distribution and serial samples studies) or high resolution mass spectrometry (for the temporal trend study).

3. Results and discussion

3.1. Serial blood samples during pregnancy and nursing period

A significant decline in mean PFAA levels in blood serum between the 1st and 3rd trimester of pregnancy was observed for all three investigated compounds. PFNA declined twice as much as PFOA, suggesting compound specific differences in the blood dynamics. Furthermore, after delivery the PFOA levels decreased further, whereas only a non-significant decrease was observed for PFOS. Despite the changes in PFOS and PFOA levels, strong correlations were found between maternal levels at different time points, and between maternal levels and cord blood levels (Table 1). Significant positive, but weaker, correlations were also found for PFNA (not shown). The results suggest that it is possible to use maternal levels as an estimate of fetal exposure. However, the correlations between maternal serum and fetal cord blood PFOS, PFOA and PFNA levels weakened as the time-interval increased between maternal and cord blood sampling (Table 1). This means that PFAA blood levels in maternal serum sampled close to delivery (before and after) give better estimates of the fetal PFAA exposure, as represented by cord blood levels, than maternal PFAA levels in early pregnancy.
Table 1: Statistically significant (p<0.05) Pearson’s correlations between blood levels in serial blood samples.

<table>
<thead>
<tr>
<th></th>
<th>1st trimester</th>
<th>3rd trimester</th>
<th>Cord blood</th>
<th>3 weeks after delivery</th>
</tr>
</thead>
<tbody>
<tr>
<td>PFOS</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>0.79</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cord blood</td>
<td>0.60</td>
<td>0.86</td>
<td></td>
<td></td>
</tr>
<tr>
<td>3 weeks after delivery</td>
<td>0.79</td>
<td>0.92</td>
<td>0.70</td>
<td>0.55 0.82</td>
</tr>
<tr>
<td>3 months after delivery</td>
<td>0.87</td>
<td>0.80</td>
<td>0.55</td>
<td>0.82</td>
</tr>
<tr>
<td>PFOA</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3rd trimester</td>
<td>0.93</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cord blood</td>
<td>0.78</td>
<td>0.89</td>
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<td></td>
</tr>
<tr>
<td>3 weeks after delivery</td>
<td>0.88</td>
<td>0.94</td>
<td>0.82</td>
<td></td>
</tr>
<tr>
<td>3 months after delivery</td>
<td>0.88</td>
<td>0.85</td>
<td>0.70</td>
<td>0.87</td>
</tr>
</tbody>
</table>

3.2. Temporal trends of PFAAs 1996-2010

Diverging trends of the different PFAAs were observed. Increasing levels between 1996 and 2010 were observed for perfluorobutane sulfonic acid (PFBS), perfluorohexane sulfonic acid (PFHxS), PFNA and perfluorodecanoic acid (PFDA), whereas levels for PFOS, perfluorodecane sulfonic acid (PFDS) and PFOA decreased (Figure 1). Other investigated PFAAs were not detected or did not show a significant trend. To our best knowledge, this is the first report of statistically significant upward trends for PFBS and PFHxS in human serum after the year 2000, probably as a reaction to the production change after the PFOS phase-out by the 3M Company. In 2010 the serum levels of PFHxS and PFOS were comparable. In contrast to PFOA, PFNA and PFDA showed increasing temporal trends in blood serum. It is not possible to conclude if the observed upward trend in our study is due to increased exposure to directly emitted PFNA and PFDA, or due to increased exposure to precursor compounds, such as fluorotelomer alcohols.

Figure 1: Temporal trends of PFAAs in pooled blood serum samples 1996-2010. Dots are the geometric means for each year. The regression line is obtained after linear regression analyses of log-normal PFAA levels.

4. Conclusions

The results show that the phase-out of PFOS- and partly PFOA-related production together with emission controls has resulted in decreasing body burdens of the C8 chemicals in young Swedish women. However, exposure to carboxylates with longer carbon chains than PFOA and sulfonates with shorter carbon chains than PFOS (or their respective precursors) is currently increasing.

5. References


Does exposure to 8:2 FTOH affect lung function?

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1. Introduction

Poly and perfluorinated compounds (PFCs) are ubiquitous in our environment. One PFC, 8:2 fluoro telomer alcohol (8:2 FTOH) has been measured as the principal airborne vapour phase PFC in North American and European homes [1-3]. FTOHs are found in PFC treated carpets and textiles as residual unreacted monomers and are unusually volatile for a chemical of such high molar mass leading to strong offgassing [4]. A recent study suggested that inhalation is a major pathway of PFC exposure [3]. Our recent studies have suggested that ingested PFOS and PFOA exposure is capable of modifying lung function in an allergic murine model [5]. As FTOH contributes significantly to human PFC exposure, we similarly hypothesized that 8:2 FTOH exposures could modify pulmonary function and airway responsiveness.

2. Materials and methods

Pregnant BALBc dams (GD16) were exposed to 100mg of 8:2 FTOH that was coated onto a cardboard enrichment which were replaced weekly. Dams continued to be exposed to FTOH in this way and pups were born in the same environment. Upon weaning, 4 female pups were placed into each cage where they continued to be exposed to FTOH in the same way. Control dams and pups received untreated cardboard enrichment huts. One half of the females of each litter received ovalbumin (OVA) IP at days -21 and -4 and intranasal OVA day -4, -3 and -2. Airway resistance (Rw) was measured by flexiVent at 10 weeks of age. Methacholine (MCh) was nebulized and administered in incremental concentrations from 3-50 mg/ml. PC200, the concentration of MCh required to increase the airway resistance 3X from baseline airway resistance was calculated. Data are given as mean±SD.

The concentration of 8:2 FTOH in the air of mouse cage was measured by using a BGI 400 personal air sampling pump and C18 solid-phase extraction (SPE) cartridges and an adopted method [6]. A cardboard enrichment hut was coated with 100mg 8:2 FTOH in methanol, allowed to dry and placed into the mouse cage. After allowing some time for equilibration, air from the cage was drawn through the cartridge using the pump. FTOH was eluted from the cartridge with ethyl acetate and after replacing the solvent with isooctane it was concentrated by a gentle nitrogen stream and analysed by gas chromatography - electron capture detector (GC-ECD).

3. Results and discussion

3.1. Airway resistance of FTOH exposed mice

PC200 MCh was significantly lower for FTOH exposed mice (17±2.1 mg/ml MCh) compared with naive mice (72mg/ml p=0.0016 n=6). While similar to our previous results for PFOA fed mice, these are more significant. Liver weights of PFOA treated animals were much larger when compared to livers of mice exposed to FTOH.

3.2. FTOH concentration in mouse cages

Plotting the concentration versus time showed that the cartridge saturation occurred after about 5 min. The results of the recovery experiments showed a recovery of 80.4% for the analyte. The measured concentration of 8:2 FTOH in the mouse cage as calculated on the linear part of the uptake curve was 51.5ng/L. This concentration is approximately 3 orders of magnitude higher than those found in homes.
4. Conclusions

FTOH exposure significantly increases airway responsiveness in our murine model. The mechanism for this increased responsiveness is unknown, but current studies are assessing airway inflammation and immune responses. From 1970 FTOH production and use paralleled the increase in asthma prevalence and has been widely used in high income countries. We speculate that exposure to this chemical in susceptible individuals may have played some role in the asthma epidemic of the late 20th century.

5. References


Acknowledgement - The authors thank NSERC and CIHR for funding as well as the animal care facilities at the University of Manitoba.
Sorption and desorption of persistent organic pollutants from microplastics in the marine environment

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1. Introduction

Plastic pollution in the marine environment is a growing concern, with research efforts focusing on both the macroplastics (> 5 mm) and the microplastics (< 5 mm) [1-4]. The term “microplastics” was first used in 2004 by Thompson et al. to describe truly microscopic fragments, some of which were around 20 µm in diameter [5]. Since then, the definition has been broadened and the most widely used definition of microplastics are fragments smaller than 5 mm, based on their potential to be ingested by marine organisms [6]. The physical characteristics of most plastics show resistance to ageing and minimal biological degradation. Large and intact items of debris can be ingested by marine organisms and are known to cause adverse health effects [2, 7]. As plastic fragment into smaller pieces they are potentially bioavailable to a wider range of organisms, for example via ingestion. [8-10]. It has also been suggested that the potential for plastics to transport and release chemical contaminants will increase as plastic fragment into smaller pieces [11]. However, studies on sorption/desorption mechanisms of organic contaminants to and from plastics as well as the bioaccumulation of microplastics on marine organisms are limited [12]. This study aims to provide some information on the potential environmental consequences of microplastics in the marine environment and in particular in relation to the sorption, transport and desorption of organic contaminants in seawater and in simulated physiological conditions.

2. Materials and methods

2.1. Sorption of phenanthrene and DDT to PVC and PE

PVC or PE (200-250 µm) were weighed into glass centrifuge tubes and an increasing concentration of the contaminant (14C-phenanthrene or 14C 4-4'-DDT) was added to the walls of the tubes. The concentration of phenanthrene or DDT was determined in the aqueous and solid phase by counting the β decay from the 14C-phenanthrene and 14C 4-4'-DDT by liquid scintillation counting (LSC).

2.3. Desorption of phenanthrene and DDT from PVC and PE

Phenanthrene or DDT was sorbed to plastics as described above. Desorption experiments were conducted both in seawater and in sodium taurocholate. Sodium taurocholate, a vertebrate bile salt, has been shown to be the most accurate mimic of Arenicola marina gut fluids’ solubilisation of individual polycyclic aromatic hydrocarbons (PAHs) [13]. Pseudo-first order rate analyses were used to determine the rate constant for initial desorption.

3. Results and discussion

3.1. Sorption of phenanthrene and DDT to PVC and PE

The $K_d$ values calculated for phenanthrene and DDT sorption onto PVC and PE (Table 1) were in agreement with the $K_d$ values obtained from Teuten et al. (2009).

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Pollutant</th>
<th>$K_d$ (L Kg$^{-1}$)</th>
<th>Teuten et al. (2009)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PVC</td>
<td>Phe</td>
<td>1495 ± 39</td>
<td>1650 ± 200</td>
</tr>
<tr>
<td>PE</td>
<td>DDT</td>
<td>Phe</td>
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<td>-------</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>93745 ± 10271</td>
<td>36994 ± 4263</td>
<td></td>
</tr>
<tr>
<td></td>
<td>52703 ± 9073</td>
<td>38131 ± 5590</td>
<td></td>
</tr>
</tbody>
</table>

Table 1: Distribution coefficient ($K_d$) for sorption of phenanthrene and DDT to PVC and PE ($n = 3$, ± SE). $K_d$ values for phenanthrene sorption onto PVC and PE from Teuten et al. (2009) added for comparison.

3.2. Desorption of phenanthrene and DDT from PVC and PE

Desorption of phenanthrene and DDT from plastic was faster in sodium taurocholate than in seawater with desorption rate enhancements ranging from 1.2 for DDT from PVC to 7.3 for DDE from PE. However the desorption rate of organic contaminants is a much slower process as compared to natural sediments [14]. The results suggest that DDT and phenanthrene will desorb faster under physiological conditions upon digestion by marine organisms than in seawater alone.

4. Conclusions

PVC and PE particles sorbed phenanthrene and DDT from seawater at low concentrations. Binding strength and sorption efficiency was highly polymer and pollutant specific with a higher affinity of phenanthrene for PE and DDT for PVC. Phenanthrene and DDT also showed the capacity to desorb from PVC and PE in both seawater and using a gut surfactant (sodium taurocholate). Desorption in sodium taurocholate was faster with enhancement rates up to 7.3 times the desorption rate in seawater. This indicates that hydrophobic organic pollutants have the potential to desorb faster under physiological conditions experienced in the gut, following uptake of contaminated microplastics by marine organisms. This study outlines the need for further investigation on the sorptive and desorptive capacities of microplastics present in the marine environment.

5. References


Acknowledgement - This study was supported by the Department for Environment, Food and Rural affairs (DEFRA) in the United Kingdom.
Use of immunofluorescence technique in cultured fibroblasts from cetaceans as new “in vitro” tool to investigate effects of microplastic

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1. Introduction
More than 240 million tonnes of plastic each year are used in the world and discarded ‘end-of-life’ plastic accumulates, particularly in marine habitats, where contamination stretches from shorelines to the open-ocean and deep-sea. Degradation into smaller pieces means particles <5 mm, usually defined as microplastic, considered a new priority in marine environment contamination. In fact, ingestion of microplastics, that can be taken up and stored by tissues and cells, provides a potential pathway for the transfer of hydrophobic organic contaminants, monomers, and plastic-additives to organisms with uncertain consequences for their health. Contaminants such as phthalates, bisphenol A (BPA) and polycyclic aromatic hydrocarbons (PAHs) are some of the principal constituents of plastic. Using an indirect immunofluorescence assay, we can detect endogenous proteins induced by these different contaminants. The aim of the present preliminary study is to propose immunofluorescence technique in cultured fibroblasts from cetaceans (Delphinus capensis (n=2), Orcinus orca (n=1), Physeter macrocephalus (n=1), Balaenoptera edeni (n=2)) as a new “in vitro” tool to explore the susceptibility of these marine mammals to different pollutants related to marine microplastic contamination. Here we present the method used for qualitative and quantitative evaluation of Cytochrome P450 1A1 (CYP1A1) and Cytochrome P450 2B (CYP2B) in fibroblast cells after exposure to BPA and PAHs, known inducers of Cytochrome P-450 monooxygenase system (MFO).

2. Materials and methods
All the cetacean samplings have been done with CITES permission (IT02515/EN007). The cell lines were cultured from biopsies of free-ranging cetaceans (D. capensis (n=2), O. orca (n=1), P. macrocephalus (n=1) and B. edeni (n=2)) sampled in the Sea of Cortez (Mexico). Cells were treated for 48 h with contaminants in sterile culture plates with wells having individual sterile covers. The different cell lines were subjected to a PAH mixture containing benzo(a)pyrene (1mM) and beta-naphthoflavone (20mM), solubilized in acetone (0.1%), at three doses: C = (0,5µM BaP + 10µM BnF), B = (2,5µM BaP + 50µM BnF) and A = (12,5µM BaP + 250µM BnF), plus an acetone (0.1%) control and to BPA solubilized in ethanol (0.1%), at four doses: 0,1 µg/ml, 1 µg/ml, 10 µg/ml and 100 µg/ml, plus an ethanol (0.1%) control. After fixing and extraction with methanol and acetone at -20°C, we conducted a first reaction with primary antibodies: anti rabbit cytochrome P450 1A1/1A2 and anti 2B4 cytochrome, diluted 1:500 for 1A1/1A2 and 1:100 for 2B4, for 2 h. Cells were then treated with the respective secondary antibodies (goat anti-rabbit) labelled with a fluorochrome, diluted 1:400, in the dark. Fluorochrome was detected using a solution containing 40% CITIFLUOR and 60% PBS, whereas DAPI was used as marker of chromatin for cell count. The reaction was read using a fluorescence microscope (Olympus mod. BX41). Immunofluorescence was quantified with a specially designed Olympus macro, DetectIntZ [1]. The total fluorescence revealed by the program is divided by number of cells to obtain Arbitrary Unity of Fluorescence (AUF) per cell. Data was processed using Statistica 5.0 (Statsoft).

3. Results and discussion
The main result of these pilot experiments in four cetacean species was the detection of presence of the cytochromes CYP1A1 and CYP2B in fibroblast cells of all species, revealed by immunofluorescence (figure 1A-B); higher basal expression of both proteins was found in B. edeni, the species at the lowest trophic level, while P. macrocephalus showed the lowest levels of these proteins. Table 1 A-B shows the mean values of immunofluorescence of CYP1A1 and CYP2B, revealed in cultured fibroblasts treated with the two experimental mixtures, expressed as index numbers. An increase of CYP1A1 was detected, at least at one dose, in all specimens cultured fibroblasts exposed to PAH mixture, while for the BPA treatment an increase of CYP1A1 expression has been detected only in O. orca (Table 1A). Concerning CYP2B, an increase of expression was found only for D. capensis fibroblasts treated with PAH mixture (Table 1B).
4. Conclusions

In conclusion this methodology, applied to cultured fibroblasts of cetaceans, appears as a powerful “in vitro” technique to assess susceptibility of these marine mammals to different microplastic components.

5. References

Preliminary results on the potential assumption of microplastics by Mediterranean Fin whale: the use of phthalates as a tracer

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1. Introduction

As pointed out by the 2010 scientific expedition undertaken by the program Mediterranean Endangered, a new priority in marine environment contamination are the so-called microplastics (usually defined as plastic particles smaller than 5 mm) (1). Micro debris floating on the Mediterranean Sea have reached 115,000 particles per km² with a maximum of 892,000 particles. Impacts of microplastics on organisms and the environment are largely unknown. More than 180 species have been documented to absorb plastic debris including planktophagous species. Until now no data are reported on the potential assumption and effects of microplastics on baleen whales. One toxicological aspect in the marine environment is the influence that microplastics may have on enhancing the transport and bioavailability of persistent, bioaccumulative and toxic substances. In fact, chemicals with log K(OW) > 5 have the potential to partition >1% to polyethylene, where polyethylene represents a main group of microplastics. Moreover, contaminants such as phthalates and polycyclic aromatic hydrocarbons (PAHs) are some of the principal constituents of plastic.

In this paper we explore for the first time the assumption and potential impact of microplastics in a mysticete species (Fin whale), suggesting the use of phthalates as a potential tracer of microplastics assumption from Mediterranean fin whale through micro litter and plankton ingestion.

The Fin whale (Balaenoptera physalus), the only resident mysticete in the Mediterranean Sea (concentrated during the summer in the Marine Protected Area (MPA) Pelagos Sanctuary), feeds largely on the planktonic euphasiacean species, with each mouthful can trap about 70,000 liters of water (including the surface feeding activities), could potentially undergo to the potential risk of the ingestion and degradation of microplastics. Being characterized by a long average life, chronic exposure to persistent contaminants and several anthropogenic stress may impair population viability.

The dialkyl- or alkyl/aryl esters of 1,2-benzenedicarboxylic acid, commonly known as phthalates, are high-production volume synthetic chemicals and ubiquitous environmental contaminants because of their use in plastics. Di-(2-ethylhexyl) phthalate (DEHP) is the most abundant phthalate in the environment. Public and scientific concern has increased in recent years about the potential human and wildlife health risks associated with exposure to phthalates. The main focus has moved away from the hepatotoxic effects to the endocrine disrupting potency of these chemicals (2).

2. Experimental approaches

The project is implemented through three main steps:

2.1. Phase I: collection and count of microplastics in superficial plankton samples in Pelagos Sanctuary

Mesozooplankton and microplastic particles were collected in two areas of the MPA Pelagos Sanctuary (Fig.1), the Ligurian Sea and Sardinian Sea, in summer 2011, using a WP2 standard net (200 µm mesh size, 57 cm mouth diameter) equipped with a flowmeter for the measurement of the filtered volume. For each sampling, the net was deployed and towed horizontally on the surface at a speed of about 1 knot for 15 minutes. On board, each 2-l sample was split into two separate aliquots of 1 litre each, using a Folsom Splitter. One 1-l aliquot was filtered on a 200 µm mesh sieve and immediately frozen in liquid nitrogen for subsequent analysis of phthalates. The second 1-l aliquot was preserved in 4% formaldehyde-seawater solution buffered with sodium tetraborate, for the subsequent qualitative analyses. A total of 23 frozen and 23 preserved samples were used for this study. The preserved samples were observed under a stereomicroscope Leica Wild M10. The organisms were counted and taxonomically classified, instead the plastic particles were counted and measured. All the data were normalised to the total volume filtered and expressed as individuals and debris per m³.
2.2. Phase II: ecotoxicological investigation of phthalate content in superficial plankton samples of Pelagos Sanctuary

DEHP (di-(2-ethylhexyl) phthalate) and MEHP (mono-(2-ethylhexyl) phthalate) were extracted from the plankton samples (0.5-0.7g) of the two sampling areas (Ligurian Sea and Sardinian Sea), using an ion-pairing extraction procedure and measured by high performance liquid chromatography (HPLC) with electrospray ionization (ESI) tandem mass spectrometry. LOD was 2 ng/g w.w. for MEHP and 10 ng/g w.w. for DEHP. Blanks were analyzed with each set of five samples as a check for possible laboratory contamination and interferences.

2.3. Phase III: ecotoxicological investigation of phthalate content in stranded Fin whale specimens collected on the Italian coasts

DEHP and MEHP were extracted from blubber and liver samples (1 g) of five stranded Fin whale collected along the Italian coasts, using an ion-pairing extraction procedure and measured by high performance liquid chromatography (HPLC) with electrospray ionization (ESI) tandem mass spectrometry.

3. Results and Discussion

Among the 23 superficial plankton samples, 13 have shown the presence of plastic particles (Fig 1A). The highest "microplastic density" was found in MPM14 sample (9.67 debris/m³), collected close to the Portofino MPA (Ligurian Sea). High concentration of phthalate MEHP and DEHP have been detected in superficial plankton samples collected in the Pelagos Sanctuary areas, with values approximately four time higher in the samples of the Ligurian Sea than the samples of Sardinian Sea.

Regarding chemical harm to Mediterranean Fin whales, related to the potential assumption of plastic derivatives, the preliminary data of this paper underline for the first time the presence in the blubber of four stranded Fin whale relevant concentration of MEHP (Tab 1). This data suggest the potential use of phthalates as a tracer of microplastics assumption by fin whale by micro litter and plankton ingestion.

3. Results and Discussion

<table>
<thead>
<tr>
<th>AREA</th>
<th>DEHP (ng/g)</th>
<th>MEHP (ng/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n.</td>
<td>mean</td>
</tr>
<tr>
<td>Ligurian Sea</td>
<td>8</td>
<td>1.58</td>
</tr>
<tr>
<td>Sardinian Sea</td>
<td>5</td>
<td>0.58</td>
</tr>
</tbody>
</table>

4. Research needs

This is the first evidence of the potential impact of plastic derivatives (phthalates) in a mysticete species and it underlines the importance to develop future research both on the potential toxicological impact of microplastic in cetaceans (particularly mysticete) and in the use of these species as a potential monitoring tool of pelagic environment in the MSFD.

5. References


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Environmental progestin concentrations disrupt oogenesis in amphibians

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1. Introduction
Progestins (synthetic progesterone) are extensively used in human and veterinary medicine in e.g. contraceptives and in other hormonal therapies. Recent research shows that progestins pose a risk to egg laying in wild fish [1, 2]. Information on the susceptibility of frogs to impacts from environmental progestin concentrations is lacking. Levonorgestrel is a progestin found in sewage treatment plant effluents and surface waters at concentrations up to 30 and 7 ng/L, respectively [3,4]. The present study aimed to 1) characterize progestagenic effects on the full cycle of oogenesis (egg development) in frogs, and 2) determine female amphibians’ susceptibility to reproductive impacts from progestins in the environment.

2. Materials and methods
Sexually mature female *Xenopus tropicalis* were exposed to levonorgestrel via the surrounding water for 28 days to 0, 1.3, 18, 160 or 1240 ng/L (measured concentrations). The numbers of individuals in each group were 7, 4, 4, 3, 4, respectively.

The ovaries were analyzed histologically with respect to frequencies of oocytes at various maturation stages. In one section per individual, the oocytes were scored as immature (i.e. in the early stages of meiotic prophase I), previtellogenic, vitellogenic, mature postvitellogenic or atretic oocytes. The percentages of immature and follicular oocytes, i.e. those that have progressed beyond the early diplotene stage of meiotic prophase and are surrounded by follicle cells, were estimated by two analysts. The proportions of various stages of follicular oocytes were calculated as percentages of the total number of follicular oocytes. All histological evaluations were made using coded slides.

3. Results and discussion

3.1. Health status
There was no mortality in any exposure group. Neither was there any weight loss or other signs of general toxicity in the experimental animals.

3.2. Morphometry of reproductive organs and secondary sex characteristics
The females exposed to the highest LNG concentration (1240 ng/L) had significantly decreased gonadosomatic index (GSI) compared with the control females, and their ovaries had transparent regions devoid of mature oocytes, visible to the naked eye. They also had significantly reduced cloacal length (1.93 ± 0.10 mm) (mean, SD) compared to the controls (2.47 ± 0.22 mm), and they all displayed nuptial pads on their forelimbs (male secondary sex characteristic). Exposure to the lower LNG concentrations did not have any significant effects on GSI, oviductal somatic index or cloacal length.

3.3. Oocyte development
The results from the histological evaluation of oogenesis are shown in Table 1. LNG exposure increased the proportions of previtellogenic oocytes and reduced the proportions of vitellogenic and mature oocytes
compared to the controls, although the differences were not always statistically significant. Exposure to the three lowest LNG concentrations reduced the proportion of immature oocytes compared with controls.

Table 1. Frequencies of oocyte stages (mean (SD)) in ovaries of female Xenopus tropicalis after exposure to levonorgestrel (LNG).

<table>
<thead>
<tr>
<th>LNG treatment</th>
<th>Immature oocytes</th>
<th>Follicular oocyte stages</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>*</td>
<td>Previtellogenic oocytes</td>
<td>Vitellogenic oocytes</td>
</tr>
<tr>
<td>Control (n=7)</td>
<td>27 (12)</td>
<td>51 (10)</td>
<td>37 (5)</td>
</tr>
<tr>
<td>1.3 ng/L (n=4)</td>
<td>6 (3)**</td>
<td>76 (7)*</td>
<td>14 (3)**</td>
</tr>
<tr>
<td>18 ng/L (n=4)</td>
<td>2 (3)**</td>
<td>67 (13)</td>
<td>26 (9)*</td>
</tr>
<tr>
<td>160 ng/L (n=3)</td>
<td>5 (5)*</td>
<td>66 (12)</td>
<td>28 (10)</td>
</tr>
<tr>
<td>1240 ng/L (n=4)</td>
<td>30 (6)</td>
<td>92 (3)**</td>
<td>8 (4)**</td>
</tr>
</tbody>
</table>

*aPercentage of oocytes in early meiotic prophase of the estimated total number of oocytes in a histological section of the ovary
*bPercentage of oocytes in various follicular stages of the total number of follicular oocytes in a histological section of the ovary
*cSignificantly different from the control (p < 0.05), Mann-Whitney-test
**Significantly different from the control (p < 0.01), Mann-Whitney-test

4. Conclusions

The present study shows that progestin concentrations found in the aquatic environment impaired oogenesis in adult frogs. To our knowledge, this is the first study to report reproductive effects of adult progestin exposure in amphibians. We have previously reported that LNG is a potent developmental toxicant in amphibians [5]. In the present study the lowest tested concentration, 1.3 ng/L, increased the proportions of previtellogenic oocytes and reduced the proportions of vitellogenic oocytes, indicating inhibited vitellogenesis. Androgenic effects of LNG were observed only at the highest tested concentration. Our results indicate that progestagenic effects on oocyte development include interrupted germ cell progression into meiosis and inhibited vitellogenesis. Considering the crucial role of oogenesis in female fertility our results indicate that progestins in the environment may pose a threat to reproduction in wild amphibian populations at polluted sites.

5. References


Acknowledgement - This work was supported by the Swedish Research Council Formas, the Carl Trygger Foundation and MistraPharma, a research programme supported by the Swedish Foundation for Strategic Environmental Research (Mistra).
Identification of active synthetic steroid compounds in impacted river downstream from pharmaceutical industry

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1. Introduction

The environmental occurrence of emerging pollutants able to disrupt endocrine signalling pathways other than those mediated by estrogen receptors (e.g. corticosteroids, androgens or progestagens – \cite{1}) is of recent concern. In addition recent reviews reported that aquatic organisms could be at risk when exposed to the predicted environmental concentrations of such compounds \cite{2, 3}. Hence, there is a major need to better characterize the occurrence of these compounds and their effects to aquatic wildlife.

Recent evidences have suggested that effluents from pharmaceutical industry release drugs into rivers and trigger adverse effect on wildlife \cite{4}. By using \textit{in vitro} bioassays combined to passive sampling (i.e. Polar organic compound integrative sampler, POCIS), we previously reported the occurrence of estrogenic, glucorticoidic, anti-mineralocorticoidic, progestative compounds and pregnane X receptor (PXR) ligands downstream a pharmaceutical industry release where strong reproductive alteration have been reported in fish \cite{5, 6}.

In this study, we report the use of effect directed approach to identify the compounds responsible for these activities. We first assessed mass balance calculation through chemical analyses directed to toxicity profile. We then fractionated POCIS extracts using RP-HPLC system in order to isolate the active chemicals.

2. Materials and methods

\textbf{In vitro Bioassays}

\begin{table}[h]
\begin{tabular}{|c|c|c|c|}
\hline
Receptors & Cell lines (principle) & Reference ligands (EC50) & Examples of environmental ligands \\
\hline
Estrogen (ER) & MELN (MCF-7, ERE-LUC) & 17\beta-E2 (0.01 nM) & (Xeno)Estrogens, Pharmaceuticals, PCPs \\
\hline
Pregnane (PXR) & HGSLN-hPXR (GAL4RE-Luc/GAL4-hPXR) & SR12813 (70 nM) & Pesticides, Pharmaceuticals, Steroids, Plasticizers \\
\hline
Glucocorticoid (GR) & MDA-kb2 (MDA-MD-453,MMTV-Luc) & Dexamethasone (100 nM) & Steroids, Pharmaceuticals, Vinulolbin \\
\hline
Mineralocorticoid (MR) & HGSLN-hMR (GAL4RE-Luc/GAL4-hMR) & Aldosterone (10 nM) & Steroids? Pharmaceuticals? \\
\hline
Progesterone (PR) & HGSLN-hPR (GAL4RE-Luc/GAL4-hPR) & R5020 (100 nM) & Steroids? Pharmaceuticals? \\
\hline
\end{tabular}
\caption{In vitro bioassays based on reporter cell lines were used in this study}
\end{table}

\textbf{Chemical analyses}

Targeted chemical screening, based on LC-MS/MS system, was directed on natural and synthetic steroids (estrogens, androgens, progestogens and corticosteroids) and pharmaceutical compounds (antibiotics, non-steroidal anti-inflammatory drugs, anti-depressors).

\textbf{RP-HPLC fractionation}

Fractionation was performed using C18 column. POCIS extract was separated at 25°C at a flow rate of 1 mL/min using the following water:acetonitrile (v:v) gradient: 0-10 min (80:20), 60 min (55:45), 100-120 min (0:100), 120.01-125 min (80:20). This fractionation procedure has been calibrated using a mixture of 35 chemicals that represented a broad range of polarity (0 < log Kow < 7) and included the most abundant compounds detected in POCIS extracts.
3. Results and discussion

Chemical analyses in POCIS crude extract showed the occurrence of high concentrations of dexamethasone, spironolactone, canrenone 6-methyl-prednisolone, prednisolone, prednisone, cortisone and levonorgestrel (up to 100 µg/g of sorbent) while 47 antibiotics were also detected.

Mass balance analysis (MBA) showed that GR and anti-MR activities were well explained by natural and synthetic steroids (up to 100%). Dexamethasone, 6methylprednisolone and spironolactone were the main contributors to these activities. Conversely, others biological activities (i.e. ER, PXR) were poorly explained by the detected chemicals.

Finally, sample fractionation allowed the isolation of estrogenic and PXR-like activities from the other ones while many fractions exerted both GR and anti-MR activities in accordance with the physiological function of these receptors (Table 2). It also revealed the occurrence of MR agonists that was masked by the strong anti-MR activity in the crude extract. In addition, HPLC calibration showed a good fitting between retention times of detected chemicals and several active fractions (e.g. 6-methyl-prednisolone in F11, dexamethasone in F12). Nevertheless, some active fractions were not explained by calibration standards suggesting that many active chemicals remain to be identified. Chemical analyses in these fractions are under investigations using LC-HRMS system and results will be presented.

\[
\text{Table 2. Biological activities in RP-HPLC fractions of POCIS extracts}
\]
\[\text{(no activity could be detected between F33 to F40)}\]

4. Conclusions and perspectives

Our study demonstrates the usefulness of MBA approach using pre-directed chemical analyses based on toxicity profile. Such approach allowed the identification of several synthetic steroids as main contributors of GR and anti-MR activities. We also confirmed the strength of the fractionation to unravel complex mixture effect and to isolate the active chemicals. Overall, our results underscore the need to increase knowledge on the effects of corticosteroids and progestogens on aquatic organisms for better risk assessment.

5. References

Mechanism of action of human pharmaceuticals in fish: the 5α-reductase inhibitor dutasteride as case study

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1. Introduction

In recent years, a growing number of human pharmaceuticals have been detected in the aquatic environment, generally at low concentrations (sub-ng/L to low μg/L). These compounds are characterised by highly specific mechanisms of action (MoA), high potency, persistence and prolonged activity in order to minimise dosing requirements and potential toxicity in patients. The evolutionary conservation of drug targets in wildlife species could suggest the possibility that human pharmaceuticals present in the environment may cause toxicological effect acting through the same targets as they do in humans [1, 2].

Our research addressed the question of whether or not dutasteride, a human pharmaceutical mainly used to treat benign prostatic hyperplasia, may cause adverse effects in the teleost fathead minnow (Pimephales promelas) by inhibiting the activity of both isoforms of 5α-reductase (5αR), the enzyme that converts testosterone into dihydrotestosterone (DHT). To our knowledge, this class of pharmaceuticals (5αR-inhibitors) has never before been tested on any fish, or any other aquatic species.

Despite a highly specific and well-known MoA, a significant theoretical issue came to light at the beginning of the study, since a general assumption in fish endocrinology is that DHT is not synthesized in teleosts, or if it is, it has modest or no physiological relevance [3]. Hence, the question arising was: “why study the effects of dutasteride, a 5αR inhibitor, in fathead minnow, if this species seems to lack the biochemical apparatus targeted by the drug?”

In order to answer that question, an integrated testing strategy was adopted, combining the study of the evolutionary degree of conservation of the target and of its functional conservation in our model species, together with a cross-species extrapolation of pharmacological and toxicological information generated during pre-clinical and clinical studies in mammals during the drug development phase. These approaches have been suggested by several authors as key factors that should be taken in account in order to enhance the reliability of environmental risk assessment [1, 2, 4, 5]. The experimental work presented here was divided into two phases: Phase I, focused on the target, and Phase II focused on the effects of the drug in fish.

2. Phase I

2.1 Methods

This preliminary phase was aimed at clarifying if 5αR is present and functional in the fathead minnow. This aim was pursued by using two different approaches:

- Isolation and sequencing of the 5αR genes in the testis of male adult fathead minnow, computational/phylogenetic analysis of the obtained products, and analysis of 5αR gene expression.

- Quantification of the biosynthetic product of 5αR activity, the androgen DHT, circulating in fathead minnow plasma, by Ultrasensitive GC-MS/MS [6].

2.2 Results

Both 5αR1 and 5αR2 genes were expressed in the testis of the fathead minnow. DHT was detected in male fish plasma at concentrations comparable to the human ones (0.6 ± 0.2 ng/mL). On the other hand, DHT concentrations in female fish were approximately between 2 and 15 times higher than the ones measured in women. Furthermore, circulating DHT concentrations in both sexes of fathead minnow were approximately 8% of circulating T concentrations, a value comparable to the typical 10% observed in humans, suggesting that the enzymatic activity of 5αRs in fathead minnow proceeds at similar rates as it does in humans.

These results strongly suggested that DHT has a physiological role in the fathead minnow, and this hypothesis was also sustained by both the results obtained by Margiotta-Casaluci and Sumpter (2011) [7] who exposed fathead minnow juveniles to DHT (20 and 200 ng/L), and by other pieces of evidence, such as
the high binding affinity of DHT to the fathead minnow androgen receptor (AR) and the high potency of DHT to induce the transcription of teleost fish AR.

This set of evidence constituted the rationale for testing the effects of dutasteride, a dual 5αR inhibitor, in the fathead minnow.

3. Phase 2

3.1. Methods

The review of toxicity data and side effects occurring in mammals treated with dutasteride, suggested that exposure to the drug may affect in particular physiological processes such as sex determination, sexual maturation, male fertility, and steroid hormones dynamics. In order to test the hypothesis that similar physiological processes could also be affected if fish were exposed to dutasteride, we performed the following studies:

- Early life stage toxicity test [8] (3.2, 10, 32 and 100 μg/L) followed by recovery period in clean water until sexual maturity, to determine the potential long term effects of an early life stage exposure to dutasteride (e.g. disruption of sex determination, sexual maturation, and sex steroid hormone synthesis);
- 21 days reproduction test to determine the effects of dutasteride (10, 32, 100 μg/L) on reproductive functions (e.g. fertility, fecundity, sperm quality; plasma sex steroid hormone dynamics) [9];
- F1 generation hatchability trials to determine the potential susceptibility of the F1 generation to dutasteride after parental (F0) exposure (e.g. through maternal transfer of dutasteride to embryos).

3.2. Results

Exposure to dutasteride did not cause any effect on embryo hatching rate and hatching time; however, significant effects on growth and survival were recorded in the groups exposed, respectively, to 32 and 100 μg/L. Furthermore, fish exposed to 32 and 100 μg/L for the first 28 days of life presented histologically-evident alterations of gametogenesis in the adult stage (160 dph).

Moreover, exposure to 32 and 100 μg dutasteride/L for 21 days significantly reduced fecundity of fish and affected different aspects of reproductive endocrine function in both males and females. Plasma steroids showed a sex-specific response; spermatogenesis in males was not affected, but there were significant alterations of female ovary histology, characterized by a decreased proportion of vitellogenic oocytes and increased ovarian atresia. Despite the absence of histological alterations in the testis, sperm quality was negatively affected by dutasteride in terms of proportions of motile sperm and non-viable sperm. Alterations of motility parameters were also observed. Finally, F1 generation hatching success and time were not affected by the parental exposure to the drug; however, there was an apparent increase of sensitivity to dutasteride exposure in embryos produced by pairs exposed to the highest dose of dutasteride (100 μg/L).

4. Conclusions

All the results strongly indicate that dutasteride exerts its effect, both in the fathead minnow and in humans, through inhibition of 5αRs activity. None of the observed adverse effects occurred at concentrations of exposure lower than 32 μg/L, with the NOEC at 10 μg/L, indicating that, at present, the potential presence of dutasteride in the environment (PEC=0.03 ng/L) does not represent a risk to wild fish populations.

5. References


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Sub-lethal effects induced by the main cocaine metabolite, the benzoylecgonine, on the freshwater bivalve \textit{Dreissena polymorpha}

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1. Introduction

The increase in global consumption of illicit drugs causes indisputable social, economic and medical problems, but also the onset of a potential new environmental hazard. Considering that global production of major illicit drugs is comparable to that of widely used pharmaceuticals, in analogy with them, residues of many common psychotropic substances also contaminate the aquatic environment of populated areas [1]. It has been established that after human consumption, drugs and/or their metabolites end up in surface waters, after being carried through the sewage system, posing a potential risk for aquatic biocoenosis. Nonetheless, the occurrence of illicit drugs in freshwater was well-documented by many monitoring studies showing concentrations in the high ng/L to low µg/L range worldwide [1, 2, 3], at present any information on their potentially harmful effects on non-target organisms is available. The aim of the present study was the investigation of sub-lethal effects induced by the main metabolite of cocaine, the benzoylecgonine (BE), on a reference freshwater biological model, the zebra mussel (\textit{Dreissena polymorpha}). Our goal was reached by the application of a suite of eight different biomarkers in order to highlight cyto-genotoxic 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 cyto-genotoxic 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 BE.

2. Materials and methods

Several zebra mussel specimens with 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 two concentrations of benzoylecgonine. Mother solution (10 mg/L) for BE was prepared in bi-distilled water. The lowest tested concentration (0.5 µg/L) reflected the mean of the current concentrations measured in surface water worldwide [1, 2]. The highest one (1 µg/L) corresponded to the mean of levels found in the outlet of waste water treatment plants [1, 2]. 14 days \textit{in vivo} exposures were performed in semi-static conditions, the whole water volume was changed daily and the exact volume of BE was added up to the selected concentration. Specimens were fed daily 2 h before water change and contamination. Several bivalves (n=30) were collected every 3 days to measure cyto-genotoxic 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

Each administered concentration of benzoylecgonine was able to induce a significant ($p<0.05$) increase of primary genetic damage (figure 1a), already after 48 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 (figure 1b) and micronucleated (figure 1c) cells. The marked destabilization of the lysosome membranes (NRRA, figure 1d) showed that exposure to BE 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. The analyses of the activity of antioxidant and detoxifying enzymes showed a moderate unbalance of oxidative status of zebra mussel specimens (data not shown). Considering the high potential toxicity of BE on \textit{D. polymorpha}, further analyses should be necessary in order to confirm the role of oxidative stress as induction factor of genotoxicity and to investigate in-depth the mechanism of action of this molecule, enlarging the knowledge on its potential hazard toward the aquatic biocoenosis.
Figure 1: results obtained by the application of cyto-genetic biomarkers on zebra mussel specimens exposed to increasing concentrations of benzoylecgonine. a) LDR values (mean±SEM), b) % of apoptotic cells (mean±SEM), c) ‰ of micronuclei (mean±SEM), d) destabilization of lysosome membrane (mean NRRT±SEM)

4. Conclusions

✓ Benzoylecgonine exposure induced remarkable primary and fixed genetic damage towards *D. polymorpha*;
✓ BE is cytotoxic to zebra mussel specimens and can alter their oxidative status;
✓ in-depth analyses by applying more introspective techniques (e.g. “omic techniques”) should be absolutely necessary to enlarge the knowledge on the BE toxicity towards non-target organisms and to obtain more exhaustive information about its mechanism of action.

5. References

Chronic effects of diclofenac on fish and mussels measured using human diagnostic techniques

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1. Introduction

Human pharmaceuticals are principally introduced into the aquatic environment via treated municipal effluent where they can be found in both effluents and receiving environments at concentrations in the low µg/L to high ng/L range [1]. The non-steroidal anti-inflammatory drug diclofenac is thought to be the most toxic member of this group whose effect is known to occur by damaging renal and gastrointestinal tissue across several vertebrate taxa, and whose accidental exposure has resulted in the near extinction of Asian vulture populations. For this reason diclofenac was highlighted by the European Environment Agency [2] and several authors [3] as being of particular environmental concern. Given their low concentration and persistence in the environment several authors recommend the use of chronic endpoints based on the mode of action of the drug to investigate their potential environmental effect [3]. In the current study we have adapted human diagnostic testing techniques for use with environmental samples (homogenised mussel digestive gland and fish blood plasma). These techniques offer the advantages of being standardised, easy to perform, relatively quick and cost effective. They also allow for a clearer comparison with human responses and allow us to investigate the biochemical effects on non-target organisms, potentially leading to the development of new biomarkers.

2. Materials and methods

Marine blue mussels (Mytilus spp.) were collected from a pristine site (Lettermullan, Co. Galway), sized (4-5 cm) and acclimated in dechlorinated artificial sea water (ASW) for 7 days. Mussels were exposed to 1 µg/L and 1000 µg/L diclofenac dissolved in dimethyl sulfoxide (DMSO) for 14 days under semi-static conditions with the water (ASW) changed and chemical added every 24h. Each treatment, including the control (ASW) and solvent control (DMSO), consisted of 3 replicate tanks. Husbandry conditions were controlled. After 7 and 14 days the visceral mass was dissected and frozen at -80°C. The digestive gland was dissected over ice and 3 animals of each tank, time and treatment were pooled and homogenised in a buffer (10mM Tris-HCl (pH7.2), 0.5M sucrose, 0.15M KCl, 1.0mM EDTA and 1.0mM PMSF). After centrifugation (15,000g for 1h at 4°C) the supernatant (S15) was frozen at -80°C and later defrosted, diluted (1/4 in ddH₂O) and analysed using the general chemistry 13 rotors on the Abaxis Piccolo xpress chemical analyzer.

Juvenile (15-20 g) rainbow trout (Oncorhynchus mykiss) were exposed to diclofenac following the OECD guidelines 203, fish acute toxicity test. Fish were exposed in triplicate to 1 µg/L and 1000 µg/L diclofenac dissolved in charcoal filtered, dechlorinated tap water under semi-static conditions with water changed and chemical added every 24h. Water samples were collected for chemical analysis. Blood samples were taken at time 0 (before exposure) and 96h, centrifuged at 2000g for 10 min and the plasma separated and analysed using the general chemistry 13 rotors on the Abaxis Piccolo xpress chemical analyzer.

3. Results and discussion

3.1. Fish diclofenac exposure

A significant increase in alanine aminotransferase (ALT) occurred between the control and the environmentally relevant concentration of 1 µg/L after 96h exposure. ALT is commonly clinically measured as a part of a diagnostic evaluation of hepatocellular injury to determine liver health. Alkaline phosphatase (ALP) used in humans to indicated liver, bone and intestinal diseases, showed a significant decrease from the control to 1000 µg/L after diclofenac 96h exposure. These endpoints along with aspartate
aminotransferase (AST) and amylase (AMY) used to indicate liver disease and pancreatitis respectively, showed significant differences between the control T0 and the 96h control and exposed fish. This may be due to the stresses induced by the exposure regime.

3.2. Mussel diclofenac exposure

Diclofenac exposure to *Mytilus* showed a significant decrease in digestive gland ALT expression at both 1 and 1000 µg/L after 7 days. At 14 d this effect was only significant against the control. AST also showed a significant decrease in expression after a 7 day 1000 µg/L exposure. Both ALP and AMY showed a reduction after 1 µg/L exposure for 7 and 14 days respectively, but significant only against the control. All molluscs assayed to date have AST and ALT with both being significantly inhibited by metal exposure in the clam *Ruditapes philippinarum*, where it was suggested as a useful biomarker for sublethal stress [4].

4. Conclusions

Other blood chemistry endpoints were also measured. Many of these endpoints have been previously observed in fish and molluscs, but we are proposing their use to evaluate the effects of pharmaceuticals in the environment in a fast, efficient and standardised way using human diagnostic techniques and machines.

5. References


Acknowledgement - The authors thank the Irish EPA for financing this research under the NDP funded STRIVE programme.
Effects of environmental relevant concentration of pharmaceuticals on the immune system of *Lymnaea stagnalis*

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1. Introduction

At present, methods of risk assessment usually focus on the toxicity of single chemicals only. However in the aquatic ecosystems organisms are more frequently exposed to a variety of compounds as a mixture. As municipal effluent is the most important contributor of pharmaceuticals to the environment, these pharmaceuticals do not occur as single contaminants in the real-world, but rather as complex mixtures (Fent et al., 2006). The impact of pollutants on aquatic organism can be assessed at different levels of biological organization. Previous study have shown that the immune system can act as an early warning system of stress. Moreover, several studies have focussed on the effects of pesticides on the immune parameters of *Lymnaea stagnalis*, and proved it usefulness in this area (Russo et al. 2004).

Mixtures of prevailing therapeutic classes were chosen, representing compounds of critical concern (neurological, anti-hypertension, antibiotic, hypolipemic). Their toxicity was tested as a mixture of environmental relevant concentration of each therapeutic class, as a global mixture of all selected pharmaceuticals and compared to the toxicity of water coming from an effluent discharge of Montreal.

2. Materials and methods

*L. stagnalis* were exposed in semistatic conditions to each mixture in triplicates (3 snails per replicate) during 3 days. Hemolymph was collected and immunological parameters measured. Hemocyte count and viability was registered, as well as phagocytosis activity, ROS and thiol production. Gene expression of genes involved the immune response (Allograft Inflammatory Factor, TLR4, Molluscan Defensive Factor, SOD, Catalase, SeGPx, GSR, NOS and NOS bis) was also measured in real-time quantitative PCR.

<table>
<thead>
<tr>
<th>Therapeutic use</th>
<th>Composition</th>
<th>Concentration (ng/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neurological</td>
<td>Venlafaxine</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Diazepam</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>Carbamazepine</td>
<td>200</td>
</tr>
<tr>
<td>Hypertension</td>
<td>Atenolol</td>
<td>500</td>
</tr>
<tr>
<td></td>
<td>Furosemide</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Hydrochlorothiazide</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>Lisinopril</td>
<td>50</td>
</tr>
<tr>
<td>Antibiotic</td>
<td>Novobiocin</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Cirpofloxacin</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Oxytetracycline</td>
<td>200</td>
</tr>
<tr>
<td></td>
<td>Erythromycine</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Trimethoprim</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Sulfamethoxazole</td>
<td>50</td>
</tr>
<tr>
<td>Hypolipemic</td>
<td>Atorvastatin</td>
<td>50</td>
</tr>
<tr>
<td></td>
<td>Gemfibrozil</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>Benzafibrate</td>
<td>100</td>
</tr>
</tbody>
</table>

*Table 1: Mixture composition*

In a second experiment, *L. stagnalis* were exposed in semistatic conditions to water from the St Laurence River, upstream and downstream of the effluent discharge of Montreal. The same immunological parameters were recorded.

3. Results and discussion

Environmental concentration of pharmaceuticals mixtures modulated the immune response at both expression and effect levels.
3.1. Effects of pharmaceutical classes on immune response

All mixtures impaired hemocyte viability and decreased cell count in *L. stagnalis*. Phagocytosis, ROS and thiols production were increased with the antibiotic mixture, as in *in vitro* experiments in *Eliptio complanata*. The neurological mixture was the most potent one, and greatly decreased phagocytosis and ROS production compared to control, while the hypolipemix mixture had no significant effects on these parameters. The global mixture also decreased phagocytosis and ROS production. All mixtures decreased thiols production, suggesting an increase of phase II biotransformation by pharmaceuticals.

<table>
<thead>
<tr>
<th>Neurological</th>
<th>Hypolipemic</th>
<th>Hypertension</th>
<th>Antibiotic</th>
<th>Mixture</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability ↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Cell count  ↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Phagocytosis ↓↓</td>
<td>↓</td>
<td></td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>ROS ↓↓</td>
<td>↓</td>
<td></td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Thiols      ↓</td>
<td></td>
<td></td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Genes expression ↑ SeGPx, ↓ AIF, CAT, ↑↑ TLR</td>
<td>↑ SOD</td>
<td>↑↑ MDM, TLR</td>
<td>↑↑ CAT, TLR</td>
<td>↑↑ SeGPx</td>
</tr>
</tbody>
</table>

Table 2: Immune effects of different therapeutic class mixtures compared to control

The TLR4 gene, coding for the lipopolysaccharide receptor, was the most modulated by the exposure, particularly increased by the hypolipemic mixture. However, it was decreased by the neurological mixture. Drugs interaction with the expression of this receptor has often been described in vertebrates. Gene expression was generally decreased by the mixtures, except the hypolipemic one.

3.2. Comparison of the effect of the mixtures and the effluent of Montreal

The effluent also modulates the immune response. However, the effects of the bacteria cannot be excluded as the bacterial load was very different between sites and the further the downstream, the higher. Globally, phagocytosis, ROS production were increased downstream of the release, while thiol production was decreased. Gene expression was also modulated, and not surprisingly particularly the TLR4 expression.

<table>
<thead>
<tr>
<th>Immediate downstream</th>
<th>Downstream 1km</th>
<th>Downstream 5km</th>
</tr>
</thead>
<tbody>
<tr>
<td>Viability ↓↓</td>
<td>↓</td>
<td>↓</td>
</tr>
<tr>
<td>Cell count ↓</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Phagocytosis ↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>ROS ↑</td>
<td>↑</td>
<td>↑</td>
</tr>
<tr>
<td>Thiols ↓↓</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Genes expression</td>
<td>↑ CAT, SeGPx, TLR</td>
<td>↑ CAT, TLR</td>
</tr>
<tr>
<td>Bacterial load (10^6 UFC/g) 14</td>
<td>44</td>
<td>86</td>
</tr>
</tbody>
</table>

Table 3: Immune effects of the Montreal effluent compared to upstream

The effect of the mixtures of pharmaceuticals on the immune system of *L. stagnalis* are different from those of the effluent. Indeed, pharmaceuticals tend to decrease the cell count and phagocytosis, while the effluent increases them. However, the immediate effluent, which has the lowest bacterial load decreases cell count and greatly decreased thiols production, as the mixtures. Thus, the effects can partly be explained by the pharmaceutical, as well as other factors as the bacteria present.

4. Conclusions

Environmental relevant concentrations of pharmaceutical mixtures modulate the immune response of *L. stagnalis*, the neurological mixture being the most potent, and the antibiotic one having opposite effects compared to the global mixture, but similar effects to the effluent. The effluent also modulate the immune response, but the effects of the bacteria seem very important.

5. References


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Does the environmental risk assessment within the marketing authorization procedure ensure the environmental safety of Human Pharmaceuticals?

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1. Introduction

The European council directives and regulations on medicinal products (2001/81/EC, 2001/82/EC and 726/2004/EC) were established to ensure the safety of human beings, animals and the public health by ensuring a high quality, safety and efficacy of medicinal products. The authorization of medicinal products consists of two phases: a pre-market and a post-market surveillance. An application for marketing authorization for a medicinal product has to include all the administrative information and scientific documentation necessary to demonstrate that potential risks arising from the use of the medicinal product are outweighed by the therapeutic efficacy of the product. Due to the fact, that not all side effects of a drug may be anticipated based on preapproval studies a comprehensive system of post market surveillance, the pharmacovigilance system, was established. The competent authorities use the information obtained within the post market surveillance to update drug labeling, and, if necessary, to reevaluate the approval or marketing decision.

For all new marketing applications of human medicinal products the European legislation requires an assessment of potential risks to the environment in the pre-approval phase. The respective European guideline on environmental risk assessment of human medicinal products came into effect in 2006 (EMEA/CHMP/SWP/4447/00) and an amending question and answers document in 2010. The marketing approval of a medicinal product may be linked with risk management measures to reduce identified risks for the environment. However, for human medicinal products (HMPs) the environment is not part of the benefit-risk balance. Consequently the marketing authorization of a HMP cannot be refused because of environmental concerns. Furthermore, there is no obligation to monitor environmental risks in the post-market surveillance.

The environmental risk assessment according to the EMA-guideline is a tiered process in which Phase I is an action limit approach only considering environmental exposure. If the predicted environmental concentration in surface waters (PECsw) exceeds the action limit of 0.01 µg/L, an in depth ERA based on studies on environmental fate and effects has to be performed in a Phase II. For estimating a potential environmental risk, the predicted environmental concentration (PEC) is compared to the predicted no effect concentration (PNEC). A PEC/PNEC ratio ≥ 1 indicate an environmental risk.

In Germany about 3000 active substances are used in more than 9500 medicinal products. Since 2006 the Federal Environment Agency (UBA) assessed more than 700 marketing authorization applications. The major therapeutic groups assessed so far are antiinfectives, analgesics, psychotropics, cytostatics and hormones. Based on predicted and measured environmental concentrations a potential risk can be identified e.g. for some hormonal and psychotropic substances.

2. Results and discussion

In the light of a worldwide harmonization of the assessment of pharmaceuticals it was a political decision to structure the EMA guideline similar to the corresponding US Guideline (FDA, 1998): The first phase (Phase I) estimates the exposure of the environment to the drug substance and a PEC action limit triggers the in depth ERA based on experimental study results (Phase II). The PEC action limit of 0.01 µg/l was derived from available toxicity data for aquatic organisms. However, from a scientific point of view there are arguments against a PEC-trigger value for substances of "no concern".

- The use of a tiered system including trigger values requires that conservative input values are used in the first step. Otherwise it would not be possible to ensure that substances with a potential environmental risk will be identified in this first screening phase. However, there are some assumptions,
i.e. the uniform spatial and temporal distribution of the drug use and the dilution factor, in the PEC estimation according to EMA (2006) which are probably not protective.

- There is another fact which does not make much sense from an environmental perspective: the product related authorization: Active ingredients are often used in dozens and even hundreds of different medicinal products. The same active ingredient may also be used in quite different medicinal indications. This could lead to an underestimation of the predicted environmental concentration of the single medicinal product in phase I resulting in the fact that a Phase II ERA based on experimental fate and effects data may not be required.

- More than 150 drug substances were already detected in Germany in various environmental compartments, mainly in surface water. However, for most of these substances which are often used in large quantities hardly any data on fate and effects in the environment are available. The environmental risk of these drug substances may not be evaluated. The reason for that is simple: The respective medicinal products were approved before the requirement for an ERA was introduced into the legislation in 2006.

- Furthermore, for most generic human medicinal products no fate and effects data are provided within the authorization procedure.

The above described problems of the functioning of the ERA could be overcome with the establishment of a monograph system on environmental safety of the active ingredients of HMPs. Such a monograph system on environmental fate and effects data would also avoid duplicate testing and repetition of studies. The establishment of a monograph system for drug substances should first of all focus on substances which are of high environmental concern. There are already several projects dealing with prioritization of drug substances based on risk indices to identify substances of high environmental concern available.

3. Conclusions

To ensure a high standard for the environmental safety of medicinal products for human use a monograph system on active drug substances should be established. Only a pre-marketing monograph system on fate and effects data of drug substances in conjuction with an effective monitoring e.g. of the occurrence of active substances in the environment within the post-market surveillance will be able to ensure the environmental safety of human and veterinary medicinal products in use.

The aims of the presentation are:

- To illustrate the assumptions and uncertainties of the environmental risk assessment according to EMA (2006)
- To discuss and compare the legal provisions as well as the environmental needs
- To discuss the benefit of a monograph system on environmental fate and effects data of drug substances
- To present ideas on an targeted monitoring within the post-market surveillance

4. References

Prioritizing cytotoxic drugs present in aquatic systems and their occurrence in the environment

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1. Introduction

There is growing concern regarding the presence of cytotoxic drugs (used in chemotherapy), in the wider aquatic environment following breakthrough and release from wastewater treatment plants (WWTP). By their nature, cytotoxic drugs have high pharmacological potency and can be considered to have fetotoxic, genotoxic and teratogenic properties, possibly posing a risk to aquatic biota and serving as low level contaminants to downstream water supplies. There are a large number of cytotoxic drugs currently used in anti-cancer therapies and their presence in wastewater is through human excretion of non-metabolised and metabolised drugs during and following cancer therapy. Due to the nature of chemotherapy administration, the likely route of environmental exposure is through municipal wastewater, although hospital wastewater also contributes to the overall load. Most of these compounds are polar, non-volatile and poorly biodegradable, and consequently leach through WWTPs into receiving waters [1], a large number of these chemicals are likely to be efficiently removed from waste effluent during sewage treatment. To date, there is a paucity of measurement data for cytotoxic drugs in the aquatic environment. In this study we examine the specific use/consumption of cytotoxic drugs based on data from a number of hospitals located in NW England and shortlist those chemicals which are likely to be prevalent in receiving waters, based on their use/consumption, physical-chemical properties and persistence. In addition, we report measured concentrations from WWTP influent, effluent and receiving waters from the River Ribble catchment in NW England.

2. Experimental approach and initial results

2.1. Prioritization of cytotoxic drugs

Table 1 presents a list of commonly used cytotoxic drugs and their likely fate and persistence in aquatic systems as well as their reported presence in environmental wastewater. The table serves to demonstrate the likely occurrence of key cytotoxic drugs in receiving waters.

<table>
<thead>
<tr>
<th>Drug name</th>
<th>Consumption at NW England Cancer Unit (g/year)</th>
<th>Urinary excretion rate as a percentage of the unchanged parent drug</th>
<th>Predicted environmental persistence</th>
<th>Hydrolysis/photolysis/biodegradation as a likely removal pathway in receiving waters</th>
<th>Measured in the environment</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyclophosphamide</td>
<td>614.64</td>
<td>21</td>
<td>HIGH</td>
<td>NO (~0%)/HIGH</td>
<td>YES</td>
</tr>
<tr>
<td>Ifosfamide</td>
<td>0.00</td>
<td>26</td>
<td>HIGH</td>
<td>NO (~0%)/HIGH</td>
<td>YES</td>
</tr>
<tr>
<td>Treosulfan</td>
<td>857.40</td>
<td>22</td>
<td>HIGH</td>
<td>PARTIAL (~30%)/HIGH</td>
<td>YES</td>
</tr>
<tr>
<td>Methotrexate</td>
<td>8.00</td>
<td>87</td>
<td>HIGH</td>
<td>PARTIAL (~10%)/HIGH</td>
<td>YES</td>
</tr>
<tr>
<td>Pemetrexed</td>
<td>151.20</td>
<td>80</td>
<td>HIGH</td>
<td>HIGH</td>
<td>YES</td>
</tr>
<tr>
<td>5-fluorouracil</td>
<td>3113.40</td>
<td>25</td>
<td>HIGH</td>
<td>YES/NO (0-100%)/HIGH</td>
<td>YES</td>
</tr>
<tr>
<td>Gemcitabine</td>
<td>2286.84</td>
<td>7</td>
<td>HIGH</td>
<td>PARTIAL (~50%)/HIGH</td>
<td>YES</td>
</tr>
<tr>
<td>Capecitabine</td>
<td>63546.00</td>
<td>3</td>
<td>HIGH</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>Etoposide</td>
<td>89.76</td>
<td>44</td>
<td>HIGH</td>
<td>PARTIAL/NO/YES</td>
<td>YES</td>
</tr>
<tr>
<td>Epirubicin</td>
<td>94.70</td>
<td>11</td>
<td>HIGH</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>Cisplatin</td>
<td>189.46</td>
<td>33</td>
<td>HIGH</td>
<td>NO</td>
<td>YES</td>
</tr>
<tr>
<td>Carboplatin</td>
<td>948.36</td>
<td>43</td>
<td>HIGH</td>
<td>PARTIAL (4-54%)/HIGH</td>
<td>YES</td>
</tr>
<tr>
<td>Oxaliplatin</td>
<td>134.04</td>
<td>36</td>
<td>HIGH</td>
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<td>YES</td>
</tr>
<tr>
<td>Irinotecan</td>
<td>80.40</td>
<td>22</td>
<td>LOW</td>
<td>PARTIAL (4-54%)/LOW</td>
<td>YES</td>
</tr>
</tbody>
</table>

Table 1: commonly used cytotoxic drugs and their likely fate and persistence in aquatic systems
The environmental load of each cytotoxic drug is calculated using highly accurate consumption data from a survey of hospitals with non-specialised oncology units and combined with urinary excretion rates from clinical studies.

Profiling of each chemical to determine the fate of the cytostatic entering/leaving a WWTP: The fate of the chemical entering a WWTP depends on both the nature of the chemical and the treatment process; cytotoxic drugs may sorb to sludge, undergo volatilisation, or be discharged in the aqueous effluent. In general cytotoxic drugs are highly water soluble, with low \( k_{OW} \), vapour pressure and Henry’s Law coefficients – indicating that it is unlikely that the compounds will volatilise to air under ambient conditions. Knowledge on the partitioning tendencies allows us to assess which chemicals are rapidly lost during wastewater treatment, for example, paclitaxel, vinorelbine and irinotecan are lost to sludge.

Biological and chemical degradation processes will serve to remove chemicals in both the WWTP and receiving waters. Biodegradation is significant in the WWTP for gemcitabine and 5-fluorouracil, whereas hydrolysis is significant for irinotecan. SPARC and EPI-SUITE are useful chemical fate models used to estimate degradation under environmental conditions.

### 2.2. Quantification of environmental concentrations in WWTP influent, effluent and receiving waters

Collection of influent (n=10) and effluent (n=10) samples was undertaken at a number of WWTPs across England and again at WWTPs and downstream river water along the Rivers Calder and Ribble; a largely urban catchment in NW England. Samples were filtered and extracted using a combination of Strata-X and Florisil SPE cartridges in an analytical method developed by Llewellyn et al [2]. Analysis was performed by LC-MS/MS with chromatographic separation on a Thermo Scientific Hypersil GOLD C18 column (1.9µm 50 X 2.1mm) using a CHOOF-buffered H₂O/MeOH mobile phase. The mass spectrometer was operated in highly selective reaction monitoring mode and heated electrospray ionisation (HESI) by Quantification by internal standardisation was achieved using custom synthesised d4-cyclophosphamide. An initial set of samples were screened and optimised for cyclophosphamide and ifosfamide only. The limits of detection were between 0.03-0.12ng/L and 0.05-0.09ng/L for cyclophosphamide and ifosfamide respectively. Cyclophosphamide was detected in most samples, corresponding to its relatively high consumption and persistence (i.e. low removal rates from WWTPs). The range of concentrations measured in nine WWTPs was 0.0-22.7ng/L, interestingly cyclophosphamide concentrations were greater in the final effluent than the raw influent, suggesting the ‘release’ of cyclophosphamide, possibly from a conjugated moiety (e.g. metabolite complex), during the wastewater treatment process.

Pending results: Samples have also been collected at different stages of the waste water treatment process (i.e. primary, secondary and tertiary treatments) to examine the influence of these treatment processes on drug concentrations. River water samples taken in the vicinity of, and downstream from, a series of WWTWs that serve large populations with hospitals in their catchment areas are currently being screened for cytotoxic drugs.

### 3. Conclusions and further research

The specific use and consumption of cytotoxic drugs and factors which affect the occurrence of cytotoxic drugs in the environment have been examined allowing the development of a ‘hit list’ of key chemicals for water screening programmes. Field data is to be reconciled with the consumed cytostatics within a given region. Ongoing work will involve the development of a multi-compound analytical method for the shortlist of chemicals based on the cyclophosphamide/ifosfamide method. Additional analyses include 5-fluorouracil, methotrexate, etopside and doxorubicin. The analytical method can then be used to test new sorbents for the development of a novel passive water sampler and to screen environmental samples.

### 4. References


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Ecotoxicity from antimicrobials and analgesics during an influenza pandemic

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1. Introduction

During the course of a pandemic, large quantities of drugs might be used to treat severe cases of influenza and influenza-associated complications, mitigate the epidemic spread, and reduce the burden on the health care system. This necessarily varies depending on the transmission potential of the new virus, its pathogenicity and the rate of occurrence of mild to severe illness and complications. The majority of the ingested antibiotics and antivirals are excreted from the human body in the faeces and urine in a biologically-active form, constituting a potential risk to the environment. In order to quantify this environmental risk, we integrated a spatially structured global epidemic model to a wastewater and river flow model and to toxicity models to produce ab initio estimates of drug usage patterns, their release in the sewerage system, their impact on the WWTP functioning and the potential contamination of receiving rivers.

2. Materials and methods

We used the GLobal Epidemic and Mobility (GLEaM) model to map 6 billion individuals worldwide and integrate mobility data at the worldwide scale including air travel and commuting patterns. GLEaM was used to generate in silico epidemics simulating the number of cases at each stage of disease progression and the quantities of drugs used for each geographical census area, with projections down to the spatial resolution scale of ¼° and with a time resolution of one day. Given the large uncertainty associated with an emerging influenza pandemic, we explored different situations ranging from a mild transmission potential with a basic reproductive number $R_0 = 1.65$, through a moderate situation with $R_0 = 1.9$, to a severe scenario, with $R_0 = 2.3$, where $R_0$ indicates the average number of infections generated by an infectious individual in a fully susceptible population. The explored $R_0$ ranges from values consistent with the recent estimates of the current H1N1 pandemic, to values up to available estimates for the 1918 pandemic. As such, this study analyses a wide range of ecotoxicologic risks corresponding to differing pandemic severity and associated pharmaceutical mitigation strategies. Pharmaceutical interventions considered in GLEaM are assumed to differ according to the selected transmission scenarios. For more severe pandemic scenarios but not for the mild scenario, we consider the implementation of antiviral treatment (AVT) with Tamiflu in all countries with available stockpiles, assuming a 1 day reduction of the infectious period, a reduced transmissibility of the infection and a reduced complication rate. In addition, in all scenarios we explore a set of interventions based on antiviral prophylaxis (AVP) assumed for short durations (2 or 4 wks) from the start of the outbreak in a country. The case with no prophylaxis was also considered. Antibiotics treatment for influenza-associated complications was based on the empirical guidelines of the British Thoracic Society. Treatment with analgesics (mainly paracetamol and ibuprofen) was also included.

A major English river catchment, the Thames, was used as a case study, because it is one of the most populous and production dense catchments in the world. The coupling of GLEaM with a wastewater and river flow model, Low Flows 2000 – Water Quality Extension (LF2000-WQX), allowed the accurate description of the evolution of the pandemic and of its ecotoxicologic impact.

Ecotoxicity modelling was utilised to examine the potential toxicity of antibiotics to microorganisms endemic to WWTP and rivers during each pandemic scenario. Similarly, the potential toxicity of ibuprofen and paracetamol to aquatic organisms was investigated. Toxicity is measured in terms of the ‘potentially affected fraction’ (PAF), i.e. a measure of the fraction of species within a WWTP or a river, projected to be growth inhibited by antibiotics exposure or acutely or chronically affected by analgesics. The PAF was calculated by use of bacterial species sensitivity distributions (SSD) of antibiotic toxicity constructed from compilations of minimum inhibitory concentrations (MIC). In the absence of sensitivity data for WWTP microbial consortia, MICs of human pathogens were used as a surrogate. We accounted for the presence of multiple antibiotics
through mixture toxicity models. For analgesics, SSD were constructed by use of acute and chronic toxicity of ibuprofen and paracetamol in aquatic species.

3. Results and discussion

The quantity of antibiotics estimated to reach WWTPs at the peak of a mild pandemic corresponds to a negligible 1% (95% reference range (RR), 0.4% to 23%) increase in inter-pandemic usage of the same antibiotics in England in 2007-8. Antibiotic usage (as total mass excreted) was projected to increase by 13% (95% RR, 1% to 83%) and 252% (95% RR, 158% to 279%), for moderate and severe transmission scenarios mitigated by massive antiviral treatment intervention, respectively, as compared to the inter-pandemic baseline. The RR is obtained from the RR of the drugs usage pattern predicted by the stochastic epidemic model.

Simulated ecotoxicity for each WWTP for the different transmission scenarios and pharmaceutical interventions show that the entire RR of toxicity in the mild pandemic is below the 5% PAF threshold for all WWTPs, while the upper bound of the 95% RR in the moderate scenario ranges from 1.4 to 14% PAF. In the severe pandemic, the 95% RR of the WWTP toxicity varies from approximately 8% in the WWTPs experiencing the lowest toxicity to about 32% in the WWTPs predicted to reach the maximum toxicity.

Results obtained for the ecotoxicity in rivers indicate that a mild and moderate pandemic are unlikely to pose a significant environmental risk. The maximum antibiotic toxicity reached by a river stretch in a moderate pandemic was projected to be lower than 15%. On the other hand, a severe pandemic would exceed the 5% PAF threshold in about half the river stretches under all prophylaxis interventions considering the upper bound of the 95%RR, equating to approximately 35 to 40% of the total river length within the basin. River stretches above the threshold could reach up to 30% toxicity. With respect to the effect of analgesics on aquatic organisms, acute effects are not likely to occur even during a severe pandemic. Concentrations of ibuprofen high enough to raise concerns about chronic effects possibly occur during both a moderate and severe pandemic, with the constraint of limited availability of experimental data on chronic effects.

The systematic use of antiviral drugs to mitigate the impact of a pandemic might be viewed as a mechanism for reducing antibiotic usage and thus ecotoxicologic risk. Furthermore, existing ecotoxicologic data on Tamiflu suggests little cause for concern for organisms within WWTPs, rivers and coastal marine habitats. However, high concentrations of antivirals in rivers can potentially spread antiviral resistance in wildfowl, as previously discussed. In addition, WWTPs performance could also be compromised by high antiviral use.

4. Conclusions

While analgesic use during an influenza pandemic was not projected to result in acute effects to aquatic biota, chronic effects on sensitive species cannot currently be excluded. Also, effects of antibiotics on WWTP functioning might occur especially during a severe pandemic. Still, models of microbial growth-inhibition resulting from exposure to projected antibiotic concentrations in WWTP during an influenza pandemic have a high degree of uncertainty, because the sensitivity of WWTP consortia as compared to pathogens is unknown. Widespread WWTP failures were not reported during the current H1N1 pandemic, as was projected by this study for a mild transmission scenario. However, future pandemics, depending on their severity, might test the resilience of WWTPs due to increased pharmaceutical use to control the pandemic—such a response could have serious and widespread ecotoxicologic implications.
Emission of human antibiotics and anti-neoplastics into the environment: identification of high risk exposure scenarios in Europe

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1. Introduction

It is generally accepted that most human pharmaceuticals do not pose an ecological threat at the concentrations present in the environment, with only a few known exceptions.¹,² Furthermore, human pharmaceuticals are unlikely to pose a significant threat to human health via environmental exposure.³ However, it must be noted that this insight is mainly based on data from healthy adults. Therefore, potentially more vulnerable groups, such as (unborn) children, elderly and infirm people, are not taken into account.⁴,⁵ Next to this, up till now studies have mainly focused on the assessment of single pharmaceuticals separately, leaving out possible mixture effects. It is shown that a considerable mixture effect can result, even if all components of a mixture of pharmaceuticals are present in low, individually non-toxic concentrations.⁶,⁷ From these points, it can be concluded that the conviction that small amounts of pharmaceuticals in (drinking) water are harmless is not yet without doubt. Here thus lies a need for a significant improvement of the science, so that the risks to the environment and public health can be more accurately defined. The current research focuses on antineoplastic drugs and antibiotics.³ Antineoplastic drugs can be cytotoxic, genotoxic, mutagenic and teratogenic, and it is generally accepted that no safe threshold value can be given for some of them. Next to that, it is a group of pharmaceuticals that has yet been studied very little. For the second group of substances, the antibiotics, their presence in the environment has been studied more often⁸, but the extent to which they could be responsible for the induction of genetic resistance is still poorly understood.⁹ Because of the sheer impracticality to study and assess the wide range of possible exposure scenarios in detail, with an exposure scenario defined as the description of a situation in which one or more receptors are exposed to one or more stressors under specific spatial and temporal conditions, we developed a modeling tool to identify high risk exposure scenarios for humans and aquatic ecosystems. It was used for the ranking of exposure scenarios in Europe with national consumption data as input. In its development, the main focus of the tool was on the risk via surface water (aquatic ecosystem) and drinking water (human health).

2. Materials and methods

To make a comparison between exposure scenarios on a European scale possible, we used a spatially explicit approach. Country-specific characteristics such as the inpatient and outpatient use of the pharmaceuticals, compliance, the practice of disposal of unused medicines and the possible application of sewage sludge on agricultural land, and substance-specific characteristics such as human excretion rates and degradation and removal processes in sewage treatment plants and the environment were gathered. They were combined with spatial data on the distribution of European agglomerations and sewage treatment plants, and, subsequently nation-specific emissions were estimated.

The emissions to surface water and agricultural soil on the scale of the individual STPs and agglomerations were used as input for the environmental distribution module from the multimedia fate model SimpleBox, which consists of a set of eight well-mixed homogeneous compartments on local, regional, continental and global scales.¹⁰,¹¹ We used data on the spatial characteristics of Europe¹²,¹³ to estimate environmental concentrations per substance. Furthermore, we estimated concentrations of the substances in foodstuffs and drinking water with the use of bioconcentration (BCFs), root concentration (RCFs), and biotransfer factors (BTFs), as well as data on the removal during purification processes in drinking water plants.

Four age-based target groups were defined, i.e. infants, children, adults and elderly. Behavioral data per target group were linked with the concentrations derived from the fate calculations. The human exposure routes that we included are food and drinking water consumption, swimming and diving in surface water (recreational as well as occupational), ingestion of soil particles and air inhalation. Furthermore, we included specific consumption and activity patterns that could potentially cause higher risks, e.g. the consumption of locally grown crops.
Acute toxicity data from literature and publicly available databases, i.e. EC50s, LC50s, and IC50s were used for the ranking of the exposure scenarios with respect to their risks for the aquatic environment. Spearman rank correlation tests showed that aquatic toxicity of the substances does not significantly correlate between taxonomic groups. Therefore, the ranking of exposure scenarios for the aquatic environment was based on the most vulnerable taxonomic groups. LD50s for rats and mice and human maximum recommended therapeutic dose (MRTD) values did correlate significantly for our set of substances. Because of this, and because of limited data availability, we used the combined oral HD50 values for the ranking of the human exposure scenarios.

3. Results and discussion
This study will result in a set of rankings of aquatic and human exposure scenarios on a European scale and the identification of scenarios most likely to cause risks for the aquatic environment or human health. The rankings will be incorporated in a set of GIS-maps to visualize spatial variations in risks. Necessary assumptions were made within a worst-case approach, causing scenarios for which data are partially absent to be assigned a higher rank.

4. Conclusions
The new scenario selection tool can be used to identify high risk exposure scenarios and to pinpoint those situations that should be given priority in further, more indepth, risk assessment studies. Because the rankings incorporate spatial and interindividual variations, the tool can be used to identify those locations and target groups in Europe that have the highest risks. Furthermore, the tool is also suitable for the identification of current gaps of knowledge for the risk assessment of antibiotics and antineoplastics. Its limited input requirements make this method potentially useful for policy makers as a screening tool for the prioritisation of pharmaceuticals for further assessment.

5. References
1. Introduction

Environmental pressures, such as physical factors, diet and contaminants may affect interactions between microbial symbionts and their multicellular hosts. In virtually all species, these host-symbiont relations are important for the host nutrition, immune responses and reproduction [1]. Therefore, selective elimination of specific microbial symbionts by antibiotic contaminants released into the aquatic environment via wastewater effluent and agricultural runoff, would ultimately cause indirect effects on their hosts. However, despite obvious relevance, effects of antimicrobial contaminants on host-symbiont interactions in non-target aquatic organisms are largely unknown [2].

To directly explore effects of antimicrobials on bacterial diversity and development of a copepod host, the harpacticoid copepod *Nitocra spinipes* was used as a model system. Commercially used antibiotics were used to perturb composition and structure of symbiotic bacterial communities. We hypothesized that this exposure would alter composition of bacterial assemblages associated with the copepods, and that the disrupted copepod-bacteria interactions would translate into retarded copepod growth and development. To examine changes in the copepod-associated bacteria following the exposure to the antibiotics, we used a 16S rRNA gene clone library approach. To the best of our knowledge, this is the first report on how antibiotics affect both the copepod-associated bacterial community and the development of the host.

2. Materials and methods

Newly hatched nauplii were subjected to three antibiotic treatments (ciprofloxacin, sulfamethoxazole and trimethoprim) at low concentrations (~5 mg/L), and three respective solvent controls (synthetic sea water, aceton, and DMSO). The exposure period was about 2 weeks, with regular addition of food and partial change of the test solutions.

Upon the termination of the experiment, survivorship, percentage of metamorphosed individuals and the development index that integrates survivorship and development rate were calculated for each experimental unit. For each treatment/control, a pooled sample of ~25 individuals was used for molecular identification of copepod associated bacteria. The rRNA gene clone libraries were created from PCR products that were amplified from *N. spinipes* DNA extracts using bacteria specific primers. Bacterial diversity indices were derived based on the ribotype frequencies and related to the copepod survival and development.

3. Results and discussion

3.1. Alterations in symbiotic bacterial communities following the antibiotic treatments

The exposure of *Nitocra spinipes* nauplii to antibiotics altered taxonomic composition of copepod-associated bacterial communities and reduced their diversity and evenness (Figure 1). The most intriguing finding was,
however, identification of bacteria closely related to the genus *Cardinium*. This endosymbiotic bacterium has been shown to cause reproductive manipulations in various terrestrial arthropods, but never in any aquatic crustaceans, including copepods [3]. As evidenced by ribotype distribution in the bacterial clone libraries, the exposure to antibiotics caused a shift in dominance from Betaproteobacteria to *Cardinium* bacteria (Figure 1), which indicates a weakened immune status in the antibiotic treated copepods.

### 3.2. Alterations in copepod development and survival

Development in ciprofloxacin and trimethoprim treated copepods was significantly retarded, with a complete arrest in trimethoprim, coinciding with significantly poorer survivorship (Figure 2). Although, the developmental alterations were not apparent in sulfamethoxazole treatment and varied significantly among the controls, the overall positive correlations between different measures of bacterial diversity and copepod development were significant (copepod development index vs. bacterial diversity indices: Spearman rank $r > 0.88$; $p < 0.05$). Based on these findings, we suggest that compromised juvenile development was, at least partially, related to changes in structure and, possibly, abundance of bacterial communities living in symbiosis with *N. spinipes*.

![Figure 2: Survival and development of antibiotic-treated and control groups of *Nitocra spinipes*.](image)

(A) Survivorship, %; (B) percentage of copepodites (%Copepodites); and (C) copepod developmental index. All data are shown as median and range observed at the termination of experiment. Treatments: ciprofloxacin (Cipro), sulfamethoxazole (Sulf), and trimethoprim (Trim); statistical comparisons are based on the Box Cox transformed data; * ($p<0.05$) and *** ($p<0.001$) denote significant differences from the respective controls.

### 4. Conclusions

In addition to providing evidence that the antibiotic-induced perturbation of the microbial community associates with reductions in growth-related traits of the host, this study is the first record of a copepod serving as a host for endosymbiotic *Cardinium*. Taken together, our results suggest that

1. bacteria-mediated traits in copepods may be altered by antimicrobials, a recently recognized type of environmental contaminants, which can result in both short- and long-term consequences for the host fitness,
2. development rate, survival and immune status are the traits responding within days to weeks of the exposure, and
3. similarly to other arthropod species, reproductive biology of copepods may be affected by *Cardinium* bacteria.

### 5. References


Acknowledgement - This research was supported by grants from The Swedish Research Council for Environment, Agricultural Sciences and Spatial Planning (FORMAS) to EG, Swedish Foundation for Strategic Environmental Research (MISTRA) through the MistraPharma programme (MB), and the Department of Systems Ecology, Stockholm University (AE).
1. Introduction

Though over 25% of the United States population employs decentralized on-site technologies for wastewater treatment, a comparative understanding of treatment efficacies of these systems remain less understood than those from centralized municipal wastewater treatment plants (Garcia et al., in review). In fact, it is estimated that 10 to 20 percent of these systems malfunction annually, releasing untreated wastewater to groundwater and surface water, which can degrade water quality and result in impacts on surface waters in regions experiencing high groundwater – surface water exchange (Jantrania and Gross, 2006). Even less understood is a comparative understanding of contaminants of emerging concern (CECs) associated with these systems and subsequent loadings to the environment.

The primary objective of this study was to assess the occurrence and removal efficiencies of select CECs among several different wastewater treatment systems. Our secondary objective was to evaluate the influences of seasons and influent dosing scenarios on-site aerobic treatment systems. The current study further examined the occurrence of CECs, including drug of abuse metabolites, between weekend and weekday sampling events.

2. Materials and methods

The study site was located at the Baylor Wastewater Research Program (BWRP) adjacent to the City of Waco Metropolitan Area Regional Sewerage System (WMARSS), Texas, USA. This highly novel facility allows experiments to be performed with various on-site technologies, which receive influent wastewater from WMARSS. Thus, influent domestic wastewater from WMARSS was introduced to the centralized municipal treatment plant (MTP/WMARSS), on-site aerobic septic treatment systems (ATS), an on-site septic treatment system (STS), and a constructed subsurface wetland treatment system (WTS) that received effluent from the STS. (See Figure 1)

WMARSS is an activated sludge treatment plant (MTP) with a design capacity of approximately 40 million gallons per day (MGD) and an average load of about 25 MGD. The ATS systems were 1,500-gallon multi-chambered units with a pretreatment tank, an aeration chamber and a final clarifier (HOOT Systems, Lake Charles, Louisiana, USA). The STS was a typical two-chambered 750-gallon tank with no filter on the effluent discharge. STS effluent was introduced to a subsurface flow WTS, which is described elsewhere (Garcia et al in review).
One STS and four identical ATS systems were studied, but each received different influent loading scenarios. One ATS and the STS received a standard 40 design loading of influent, while three other ATS systems received continuous influent at three different volumes per day (influent equalization; IE). 4L of effluent from each treatment system and WMARSS influent were collected on four sampling dates, which accounted for differences in hydrologic retention times among the treatment types, to evaluate CECs on weekends and weekdays. The studied CECs covered a variety of common pharmaceutical classes, including analgesic, anti-hypertension, antibiotic, psychostimulant metabolites, anti-histamine, anti-seizure, benzodiazepine, anticoagulant, anti-inflammatory, antilipemic, caffeine, and the artificial sweetener sucralose, which appears to represent a robust tracer of anthropogenic activities (Soh et al., 2011). All the samples were extracted and analyzed using an analytical protocol that was described elsewhere (Vanderford et al., 2006). ANOVA was performed to test differences of treatment type and season on CEC concentrations and other routine water quality parameters.

3. Results and Discussion
An isotope dilution liquid chromatography tandem mass spectrometry (LC-MS/MS) method (Du et al., in review) that employed independent isotopically-labeled standards for quantitation of each compound was applied to quantitate target analytes for all samples. By using this method, inherent matrix effects were minimized. Method detection limits (MDLs) of target analytes are typically below and/or at low ng/L. Quality of data was checked by evaluating the matrix spike recoveries in analysis of each batch of samples, resulting in the criterion typically within 80-120%.

![Figure 2: Representative LC-MS/MS total ion chromatogram resulting from standards of target compounds and isotopically labeled analogs.](image-url)

Results showed that concentrations of CECs are typically in the range of <1-100 ng/L in the effluent discharges, which are generally consistent with peer-reviewed literature reports for these CECs. CEC concentrations from advanced on-site systems were reduced relative to septic systems. The present study, which included CECs with varied physicochemical properties, provides an initial understanding of the range of environmental loadings from common on-site wastewater treatment systems.

4. References

Acknowledgement – This research was supported by Baylor University Department of Environmental Science, the City of Waco Water Utilities, and the Texas Commission on Environmental Quality. The Institute of Ecological, Earth, Environmental Sciences and the Graduate School at Baylor University provided additional travel support.
Sorption and leaching behaviour of four emerging pollutants pharmaceuticals in agricultural soils

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1. Introduction

Pharmaceuticals are introduced in the soil by wastewater irrigation, that find increasing interest nowadays. Traditional approach to study the mobility of organic pollutants taking into account the Kow parameter would not be appropriated for ionic or polar pharmaceuticals [1]. For those substances ionised at wastewater and environmental pH, an ionic interaction mechanism could be responsible for their retention by soils. Soil factors such as cationic exchange capacity (CEC), pH, or organic matter content (OM) modified by the wastewater irrigation could influence the mobility of pharmaceuticals in soil [2]. The aim of this study was to investigate the mobility of some frequently detected wastewater derived pharmaceuticals in soils under laboratory conditions. Four pharmaceuticals found in wastewater at 1 µg/L level were chosen: carbamazepine(CBZ), venlafaxine (VEN) and their respective major human metabolites, 10,11-dihydro-trans-10,11-dihydroxyl carbamazepine (DIOL), and O-Desmethyl Venlafaxine (ODV). Two experimental approaches were carried out in order to: 1) study the sorption/desorption behaviour of the selected compounds on two agricultural soils; and 2) investigate the leaching behaviour of the compounds through soils columns under influence of ionic strength and the presence of cationic or anionic surfactants.

2. Materials and methods

Selected compounds: Among selected compounds (Figure-1), CBZ is neutral and VEN is positively charged at environmental pH. D parameter that include pH influence was used for VEN. DIOL has a smaller Kow value than CBZ, and ODV has a similar pKa value than VEN.

<table>
<thead>
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<th>Pharmaceutical</th>
<th>pKa</th>
<th>log Kow</th>
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<td>Carbamazepine</td>
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</tr>
<tr>
<td>Diol-Carbamazepine</td>
<td>n.i.</td>
<td>0.13</td>
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Agricultural soils: Two agricultural soils from Salamanca (NW-Spain) with low organic matter (OM) (<1.5%) which differ from their CEC values, (4.83 cmol/Kg for soil Al and 9.75 cmol/Kg for soil Mu) were used. Sorption/Desorption experiments: Batch sorption/desorption study was largely based on OECD 106 guidelines. Influence of pH (2, 4, 6, 8, 10, and 12) was studied in sorption experiment. Influence of ionic strength (0, 10, 100, and 500 mM CaCl₂ solution) was studied in desorption experiment. Leaching study: Leaching experiment was carried out in soil columns under saturation washing with: pure water, CaCl₂ solutions (10 and 100 mM), cationic surfactant hexadecyltrimethylammonium (HDTMA) 0.5% solution and anionic surfactant sodium dodecyl sulfate (SDS) 0.2% solution. Chloride was used as ion tracer. Sample preparation and analytical method: All liquid samples were centrifugated at 3000 rpm for 20 minutes and filtered through 0.45 µm syringe filter before analysis. Solid soil samples were extracted by a mixture of acetonitrile and 100 mM CaCl₂ solution (v:v 70:30). Pharmaceuticals were analyzed by HPLC-UV/DAD method with C8 column (Zorbax Eclipse XDB-C8 3.0*150 mm, 3.5µm, Agilent).

Figure 1: Four wastewater derived pharmaceuticals

Agricultural soils: Two agricultural soils from Salamanca (NW-Spain) with low organic matter (OM) (<1.5%) which differ from their CEC values, (4.83 cmol/Kg for soil Al and 9.75 cmol/Kg for soil Mu) were used. Sorption/Desorption experiments: Batch sorption/desorption study was largely based on OECD 106 guidelines. Influence of pH (2, 4, 6, 8, 10, and 12) was studied in sorption experiment. Influence of ionic strength (0, 10, 100, and 500 mM CaCl₂ solution) was studied in desorption experiment. Leaching study: Leaching experiment was carried out in soil columns under saturation washing with: pure water, CaCl₂ solutions (10 and 100 mM), cationic surfactant hexadecyltrimethylammonium (HDTMA) 0.5% solution and anionic surfactant sodium dodecyl sulfate (SDS) 0.2% solution. Chloride was used as ion tracer. Sample preparation and analytical method: All liquid samples were centrifugated at 3000 rpm for 20 minutes and filtered through 0.45 µm syringe filter before analysis. Solid soil samples were extracted by a mixture of acetonitrile and 100 mM CaCl₂ solution (v:v 70:30). Pharmaceuticals were analyzed by HPLC-UV/DAD method with C8 column (Zorbax Eclipse XDB-C8 3.0*150 mm, 3.5µm, Agilent).
3. Results and discussion

3.1. Sorption/Desorption experiment
Sorption capacities were higher in Mu than in Al soil, in agreement with their CEC. There was very little influence of pH on sorption except for the extreme values (>10) probably due to change of ionisation equilibrium of the compounds (see pKa values). CBZ and DIOL were poorly sorbed (CBZ more sorbed than DIOL) with partition coefficients (Kd) under 3 ml/g. VEN and ODV showed Kd values higher than 90 ml/g, and higher for VEN than for ODV (Table 1). The ionic strength clearly influence desorption, specially for the more sorbed VEN and ODV. More concentrated CaCl2 solutions have leached more sorbed compounds. Desorption of CBZ and DIOL showed also somehow influence of ionic strength, but this was in minor extent.

<table>
<thead>
<tr>
<th></th>
<th>ODV</th>
<th>DIOL</th>
<th>VEN</th>
<th>CBZ</th>
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<tr>
<td>Mu</td>
<td>229.9±8.2</td>
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</table>

*Table 1: Kd values (ml/g) of compounds on two agricultural soils (0.5 g soil, 5 mg/L pharmaceuticals, 10 ml solution)*

Mechanism of sorption of VEN and ODV was probably due to cationic exchange, whereas the sorption mechanism of CBZ and DIOL seems not dominated by cationic exchange.

3.2. Leaching study
As expected, CBZ and DIOL were neither influenced by leaching conditions (leaching breakthrough curves (BTCs) not statistically different) nor soil characteristics. CBZ showed a retardation in its leaching, while DIOL was leached at the same time than the ion tracer Cl- (see Figure-2a,2b). VEN and ODV were leached much later than CBZ and DIOL, and their BTCs showed a dependence with ionic strength of the leaching solution (see Figure-2a,2b). They only leached with 100 mM Ca2+ solution. VEN leached after ODV. In accordance with its higher CEC, Mu soil had a higher retention capacity than Al soil for both VEN and ODV: ODV was leached much later from Mu soil than from Al soil and VEN was only leached from Al soil.

![Figure 2: Breakthrough curves for the leaching of selected pharmaceuticals in soil Al columns](image)

4. Conclusions
This work showed a clear influence of soil CEC in the sorption/desorption mechanism of ionic pharmaceuticals VEN and ODV. CBZ and DIOL weakly sorbed on organic matter poor soils and leached easily in all tested conditions. Other parameters related with CEC should be taken into account.

5. References

Acknowledgement - The authors thank French national research center CNRS, Spanish national research council CSIC, Regional Council of Languedoc-Roussillon and Montpellier 1 University.
Uptake of Pharmaceuticals in the Terrestrial Environment

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1. Introduction

Increasing amounts of pharmaceutical are being detected in soils. This is primarily due to sewage sludge being applied to fields for use as a soil amendment as well as the use of reclaimed wastewater for irrigation. Both sewage sludge and reclaimed wastewater contain high levels of pharmaceuticals which are then transferred to the soil.

Previous studies have already highlighted the effects of pharmaceuticals on terrestrial organisms such as the anti-inflammatory drug diclofenac causing declines in vulture populations in Asia [1] and antibiotics which have effected plant growth, soil microbial and enzymatic activities [2]. Once in soil, pharmaceuticals can be taken up by soil dwelling organisms. Accumulated pharmaceuticals may then affect the health of the organisms or be transferred up through food chains potentially creating wider ecosystem effects. However, to date research into the uptake of pharmaceuticals in terrestrial organisms still remains an area which is relatively unexplored.

This study therefore investigated the uptake of pharmaceuticals into earthworms (Eisenia fetida) with the ultimate aim of developing models to improve the assessment of the risks posed by pharmaceuticals in the terrestrial environment.

2. Materials and methods

The uptake of fluoxetine and diclofenac into earthworms (Eisenia fetida) was investigated. Diclofenac is an acidic pharmaceutical whereas fluoxetine is a basic compound (Table 1). The studies were based on the OECD Guideline 317 and involved a 21 day uptake phase followed by a 21 depuration phase. Earthworms were incubated in a controlled growth chamber in glass jars with 50 g of soil containing spiked test material. Studies were performed using radiolabelled compounds to allow for a lower limit of detection thus enabling environmentally relevant concentrations of both compounds to be used in the study. For fluoxetine, soil was spiked at 0.108 mg/kg whilst the diclofenac test concentration was 0.05 mg/kg. Blank controls and solvent controls were used to account for any solvent or pharmaceutical effects. Organisms were sampled throughout the uptake and depuration phase. Earthworms were removed from their respective beaker and allowed to purge their guts for 24 hours.

<table>
<thead>
<tr>
<th></th>
<th>Usage</th>
<th>pKa</th>
<th>Log Kow</th>
<th>Soil Kd (L/kg)¹</th>
<th>Structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diclofenac (Acidic)</td>
<td>Anti-inflammatory</td>
<td>4.00</td>
<td>4.15</td>
<td>170</td>
<td><img src="image" alt="Diclofenac" /></td>
</tr>
<tr>
<td>Fluoxetine (Basic)</td>
<td>Anti-depressant</td>
<td>10.05</td>
<td>4.05</td>
<td>360</td>
<td><img src="image" alt="Fluoxetine" /></td>
</tr>
</tbody>
</table>

Table 1: Pharmaceutical properties

Earthworms were then extracted and analysed by liquid scintillation counting. Assessment of the analytical method showed that recoveries of > 90 percent were achieved for both compounds. Selected samples were also analysed by radio HPLC to assess whether any metabolism had occurred. Soil samples were also analysed using validated methods to explore the persistence of the compounds in soil.
The results from the uptake and depuration study were modelled using OpenModel software (v 1.2 Nottingham University). The model was a one compartment toxicokinetic model. The modelling assumed that uptake was from soil pore water. The modelling results were used to evaluate the suitability of existing quantitative structure-activity relationships that are used in environmental risks assessment, for use in pharmaceutical assessment.

3. Results and discussion

3.1. Results of uptake and depuration experiment

Experimental data on the uptake and depuration of fluoxetine and diclofenac are shown along with the best model fit in Figures 1 and 2. For both compounds there was uptake within the first 6 hours of the experiment, this initial uptake was especially rapid for fluoxetine. After which, the uptake of diclofenac and fluoxetine slowed. The uptake rate constants from the model (Kin) also show that fluoxetine has a higher overall uptake rate than diclofenac at 0.7428 L/kg ww d\(^{-1}\) and 0.1484 L/kg ww d\(^{-1}\) respectfully. When *E. fetida* were transferred to clean soil for the depuration phase both compounds were seen to be immediately eliminated from the organism. By the end of the depuration phase there were still traces of both pharmaceuticals in the earthworms. The depuration phase was considerably slower than uptake for both pharmaceuticals with rates (Kout) of 0.0012 d\(^{-1}\) and 0.01 d\(^{-1}\) for diclofenac and fluoxetine respectfully.

![Figure 1: Uptake and depuration of fluoxetine](image1.png)

![Figure 2: Uptake and depuration of diclofenac](image2.png)

Bioconcentration factors were estimated from the uptake and depuration rates. The diclofenac BCF\(_{pw}\) was 167 and the BCF for fluoxetine was 133. Both of these values are comparable to BCF\(_{pw}\) values estimated from equations used in regulatory risk assessment, such as that found in the Technical Guidance Document.

4. Conclusions

This research shows earthworms, *E. fetida*, can accumulate pharmaceuticals if they are present in soils at environmentally relevant concentrations. Work is currently ongoing to assess the uptake of additional pharmaceuticals into earthworms, as well as to investigate how uptake can be influenced by soil parameters such as change in pH.

5. References

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The anti-arrhythmic drug flecainide: environmental detection and conserved mode of action in fish

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1. Introduction

Flecainide is sodium channel blocker used to treat cardiac arrhythmic disorders in humans. It has a narrow therapeutic index and even slight overdoses can be life threatening. Flecainide is widely used in Sweden, and based on sales statistics, we calculated the predicted environmental concentration (PEC) in surface water to be 47 ng/L. Together with a logP of 4.0 and a presumably conserved mode of action in fish, we therefore hypothesized that flecainide could cause adverse environmental effects. However, according to the US EPA, concerted environmental surveys have not been performed for any anti-arrhythmic drug, and the literature revealed no measurements of flecainide in environmental samples. Nor have we found studies on the effects of flecainide in fish. We therefore developed methods for quantifying levels of flecainide in environmental samples. In addition we also studied the effects of flecainide in rainbow trout (Oncorhynchus mykiss), both in vivo and in vitro, using endpoints reflecting its known mode of action in humans.

2. Materials and methods

Field sampling: Water samples were collected directly into 1 litre polyethene (PE) bottles, surface water was taken as grab samples and sewage water was taken as flow proportional samples. Fish were caught using fishing net, muscle was dissected from the dorsal muscle, and a composite sample from around ten individuals from each site was prepared.

Analytical determinations: A triple stage quadrupole MS/MS TSQ Quantum Ultra EMR, coupled with an Accela LC pump, and a PAL HTC autosampler were used as analytical system. Setting of key parameters, SRM transitions, absolute recoveries, etc is stated in [1]. Possible memory effects were excluded by blank injections of Milli-Q water after standard samples and field and laboratory blank samples were included in each batch.

In vivo and in vitro studies: Commercially farmed rainbow trout (n=7) were cannulated via the buccal cavity in accordance with Axelsson and Fritsche [2] and left to recover for 24 hours before tests were started. Once a stable baseline was attained, stepwise increments (10x at each step) of flecainide was injected via the cannula, where the max dose was based on the defined daily dose (DDD) for flecainide in humans (2 mg/kg). We recorded blood pressure (BP) and heart rate (HR) for 2 hours after each injection. Ventricular and gastric strip preparation from rainbow trout was used to evaluate the effects of flecainide in vitro. Force and rate of contraction was recorded, measured according to Shiels and Farrell [3].

3. Results and discussion

Flecainide was found in effluent water from 5 separate sewage treatment plants (STPs) in Sweden, with a mean concentration of 123 ± 17 ng/L, and in surface water at 6 different sites, either upstream (2.7 ± 1.2 ng/L) or downstream (36.8 ± 14.4 ng/L) from STPs. Concentrations decreased with increasing distance from the STPs. Flecainide was also detected in muscle issue of wild fish (0.16 ± 0.03 µg/kg). Ongoing analyses of plasma concentrations of flecainide in wild fish allow a better estimation of the risks for pharmacological effects [4].

Intraarterial injections of flecainide affected HR and possibly also BP, Fig. 1. The effects were seen at 2 mg/kg, which is equivalent to the human DDD. Heart rate decreased significantly after 5 min; this was reversed after 25 min. Trends towards an elevated BP were found at 10 and 15 minutes following an injection with 2 mg/kg (p=0.055).
Mean flecainide levels in plasma from these individuals were 0.9µg/ml, which is twice the therapeutic plasma concentration in humans (0.4µg/ml). Together, this indicates an at least partly conserved mode of action between human and fish as well as a roughly similar potency. We will continue by monitoring effects on HR and BP in future exposure studies with waterborn flecainide. Bioconcentration studies will be conducted in order to assess uptake from water.

Preliminary (n=4) results from measurements of twitch force and contraction speed in gastric strip preparation show decreases in both end points at levels equal to human therapeutic plasma concentration (0.4µg/mL) and, with regard to force, also at 0.04µg/ml; Fig. 2. Ventricular strips also show trends towards a decreasing twitch force and rate of contraction with increasing flecainide concentrations, though these results were not significant (not shown).

4. Conclusions
- Flecainide is found in the environment and is taken up in wild fish.
- Injection with flecainide equivalent to human doses acutely affects heart rate in vivo in fish, suggesting an at least partly conserved mode of action and roughly similar potency in fish.
- In vitro studies indicate the possibility of effects on non-target organs, i.e. the gastrointestinal tract, in exposed fish.
- Waterborn dose-response studies, controlled bioconcentration studies and analyses of plasma levels in wild fish will allow for further assessment of environmental risks with flecainide.

5. References

Acknowledgement - The authors thank Mistra and Vetenskapsrådet for funding. We would also like to thank Emma Forsmark, Zohreh Tajik, Isabell Dahlgren, Nina Sjölin and Isabel Runneberger for technical assistance.
Accumulated antidepressants in wild-caught fish - a relaxed wildlife?

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1 Introduction

Residues of pharmaceuticals and their metabolites have been found globally in the influent and effluent of waste water treatment plants [1] [2] or in waste water treatment plants themselves [3]. In addition numerous pollutants (e.g. propranolol, carbamazepine or diclofenac) have been detected in surface water and downstream of waste water treatment plants, to some extent in relatively high concentrations [4]. In Switzerland, three drugs in particular ibuprofen, diclofenac and clofibric acid have been identified in surface water from lakes and rivers [5] [6], whereas more recently monitoring studies of cytostatic pharmaceuticals have been conducted [7]. In contrast to former studies the current project analysed residues of polar to lipophilic pharmaceuticals from different trophic levels. The aim was to determine the fate and behaviour of pharmaceuticals within the aquatic food web. A national study in US has already demonstrated an accumulation potential for norfluoxetine, sertraline, diphenhydramine, diltiazem, and carbamazepine in fillet of wild fish species [8].

In addition, tissue-specific concentrations of pharmaceuticals can be used in the tissue residue approach to deduce mechanisms of toxic action, evaluate the toxicity of mixtures, and interpret field data on bioaccumulated toxicants [9].

2 Study design and measurements

2.1 Biota samples

Sampling of fish eating birds, fish and macrobenthon from various Swiss rivers and one lake was performed twice a year (summer and autumn) in 2006 and 2007 and once in 2011. About 500 samples were collected originating from four small to medium-sized rivers situated near Basle, Zurich, in the canton of Thurgau and from Lake Thun. All sampled water bodies were to some extent exposed to treated waste water effluents.

The caught fish species were barb, trout and chub. The sampled macrobenthic organisms were crustacean species and mussels (zebra and swan mussel) and the fish eating birds were cormorants.

2.2 Sample preparation

An identical extraction procedure was applied independent of the sampled species (fish eating bird, fish or macrobenthon) or organ (e.g. liver or muscle tissue).

One gram of tissue (muscle, liver or whole organism in case of macrobenthic species) was homogenized and subjected to liquid-solid extraction using methanol and acetonitrile as solvent.

After extraction the homogenate was centrifuged and the supernatant was reduced in volume, resuspended in methanol and cleaned up by a syringe filter. Subsequently samples were analysed by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS).

2.3 HPLC-MS Analysis of pharmaceuticals

In a first campaign determined pharmaceuticals were frequently detected pharmaceuticals including beta blocker atenolol, antihistamine diphenhydramine, calcium channel blocker diltiazem, anticonvulsant carbamazepine, antidepressant fluoxetine and its main metabolite norfluoxetine, and analgetics ibuprofen and diclofenac. Mefenamic acid, a non-steroidal anti-inflammatory drug and sulfamethoxazole which is a sulphonamide bacteriostatic antibiotic were also analysed. Deuterated pharmaceuticals were spiked to all biota samples to monitor extraction efficiency.
Most of these pharmaceuticals are degradation resistant in waste water treatment plants and therefore often found in surface water, or as in the case of diltiazem, the substance has been already extracted from fish tissue. The selected compounds comprise additionally a wide range of different physico-chemical properties. From these determined pharmaceutical residues it can be expected to achieve a better understanding of general uptake behaviour in aquatic organisms.

Since our preliminary results have revealed exclusively residues of norfluoxetine, fluoxetine, diphenhydramine and carbamazepine in fish and fish prey the list of analytes were extended by other antidepressants (sertraline, its main metabolite N-desmethyleraline and citalopram) but also by more lipophilic pharmaceuticals like gemfibrozil, fenofibrate and naproxen and lipophilic metabolites of diclofenac (2-[2-(chlorophenyl)amino]benzaldehyde) and ibuprofen (2-[4-(2-hydroxy-2-methylpropyl)phenyl]propionic acid), which have been in parts detected in surface water or fish muscle and liver (e.g. sertraline and gemfibrozil [8]).

As valuable tools for studying pharmaceuticals in environmental matrices an HPLC-MS multi-residue method was used. The LC–MS/MS system consisting of an Agilent (Walldbronn, Germany) 1100 HPLC system (binary pump, degasser, autosampler and UV–vis detector) was coupled to an ‘LC/MSD Trap XCT plus’ equipped with an orthogonal ESI interface (Agilent, Waldbronn, Germany). The chromatographic separation was achieved by using a Zorbax SB-C18 column (150mm×3.0mm, 3.5μm particle size) and a C-18 guard column, both from Agilent Technologies (Wilmington, DE, USA) at a column temperature of 30°C. Identification and quantification of pharmaceuticals in biota were arranged in a different elution time window to increase measurement sensitivity.

3 Results and Discussion

To calculate recovery for biota extraction, biota samples were spiked with each pharmaceutical standard which was measured within the present method. Recovery of pharmaceuticals varied from 77% for ibuprofen to 97% for diltiazem. Recoveries consistently higher than 70 % for all pharmaceuticals documented a proper extraction efficiency to educe traces of pharmaceuticals out of various biota tissues.

Preliminary fish and macrobenthon analysis confirmed four substances (norfluoxetine most important active metabolite of widely used antidepressant fluoxetine, fluoxetine itself, diphenhydramine, and carbamazepine) to be present in fish prey and fish. The identified concentrations of analytes were higher in liver than in muscle tissue. Furthermore analysis indicated different patterns of pollutants depending on species, trophic level or feeding type whereby mussels for example accumulated in parts other substances than fish.

Residue data has additionally highlighted that accumulation is not only depending on lipophilicity of compounds since carbamazepine (log P = 1.9) as a pseudopersistent pharmaceutical has also proven the potential to accumulate in the body of wildlife species.

The residue data of fluoxetine and norfluoxetine will contribute to a more comprehensive risk assessment due to the fact that there is a lack of fate data for norfluoxetine and the fate of fluoxetine (cationic substance at environmental pH values) cannot be predicted using partition coefficients established for nonionic compounds [10].

4 References


