Information on internal exposure for an improved effect assessment of organic compounds

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Summary

To assess the effect of organic compounds on the aquatic environment, organisms are typically exposed to toxicant solutions and the adverse effects observed are linked to the concentration in the surrounding media. As compounds generally need to be taken up into the organism and distributed to the respective target sites for the induction of effects, the internal exposure is postulated to best represent the observed effects.

The aim of this work is to contribute to an improved effect assessment of organic compounds by describing experimental and modelling methods to obtain information on the internal exposure of contaminants in organisms. **Chapter 2** details a protocol for the determination of bioconcentration parameter for uptake (k_1) and elimination (k_2) of organic compounds in zebrafish (*Danio rerio*) eggs. This enables the simulation of the internal exposure in zebrafish eggs from an ambient exposure concentration over time. The accumulated contaminant amount in zebrafish eggs was also determined, using a biomimetic extraction method. Different bioconcentration estimation models for the determination of internal steady-state concentration of pharmaceutical compounds in fish to an environmental exposure are presented in **Chapter 3**. Bioconcentration factors were estimated from the compounds octanol: water partition coefficient (K_{OW}) to determine the internal exposure to an ambient concentration. To assess the integral bioavailable fraction from the water and sediment phase of environmental contaminants for rooted aquatic plants, the internal exposure in river-living *Myriophyllum aquaticum* plants were determined over time, presented in **Chapter 4**. The plants were collected at different time points, with the accumulated organic contaminants determined using a liquid extraction method. In **Chapter 5** a protocol was established to enable the non-invasive observation of effects in *M. aquaticum* plants exposed to contaminated sediments over time. Since the toxicant effects are a result of all uptake and distribution processes to the target site and the toxico-dynamic process leading to an observed effect during static exposure, information on the internal exposure could thus be gained from the temporal effect expression.

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Ceci n'est pas un poisson.

Chapter 1

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General Introduction and Objectives

General Introduction

The use of single species testing for the prediction of toxic effects of pollutants in ecosystems play an important role for the environmental hazard assessment of organic compounds (Altenburger, 2002). Generally, a chemical is added to an exposure solution in the respective bioassay and after a defined exposure time, the effect on the observed endpoint is correlated to the exposure concentration. The median effect concentration (EC_{50}) can be determined using concentration-response modelling, providing a basic parameter for environmental hazard assessment. However, it is shown that the toxicity of compounds could be underestimated based on nominal exposure concentrations, e.g. when chemicals are not bioavailable for the exposed organism (e.g. Escher and Hermens, 2002, 2004; Hestermann et al., 2000; Riedl and Altenburger, 2007).

It is generally accepted that compounds have to first be taken up into the organism and distributed to the respective target sites for the induction of effects. Thus, usage of target site effect concentrations are assumed to best represent the compounds' potential toxicity to an organism (e.g. Escher and Hermens, 2002, 2004). However, the effect concentration at the target site within an organism is difficult to obtain and the average body concentrations in organisms may be used instead to determine internal EC_{50} (IEC_{50}; Escher and Hermens, 2002). Here, the internal concentration is the result of all uptake (k_1) and elimination (k_2) processes of a contaminant from the surrounding into the organism. This bioconcentration process could reach a dynamic steady-state, where the ratio of the internal to the ambient concentration represents the bioconcentration factor (*BCF*). When the specific bioconcentration parameters (*BCF*, k_1, k_2) of a chemical for an organism is determined, the internal exposure over time can be simulated and correlated to observed effects and an IEC_{50} can be derived (e.g. Ashauer and Escher, 2010).

Assessment of internal exposures in zebrafish eggs

Since microbiotesting became an accepted methodology in the environmental hazard assessment due to its diagnostic power, high-throughput testing ability and cost-effectiveness, the development of microwell-plate bioassays is a fast growing field in aquatic toxicology (Blaise, 1998). Recently a protocol using zebrafish (*Danio rerio*) eggs under short-term exposure has become a substitute for the acute fish assay in the toxicity analysis of wastewater in Germany (DIN, 2001) and it is discussed to become an alternative to the acute fish assay for the testing of chemicals (OECD Draft, 2006). Compared to flow through or tank exposure systems for fish, the stabilisation of exposure concentrations in microwell-plates with its small volume (e.g. 2 mL) may be challenging. It was shown, that the initial concentration

may change dramatically over the exposure time when processes like degradation, sorption or volatilisation occur (e.g. Hestermann et al., 2000; Riedl and Altenburger, 2007; Schreiber et al., 2008). Although, passive dosing approaches were successfully applied to stabilise exposure concentration in microwell-plates (e.g. Brown et al., 2001; Mayer et al., 1999; Smith et al., 2010), the spiking procedure of the passive devices used is sophisticated and thus may not be practical for high-throughput standard testing. In contrast, when bioconcentration parameters of a contaminant for e.g. zebrafish eggs are determined, not only the varying exposure concentration could be considered but also the internal exposure could be simulated. Thus, the correlation of internal exposure with the observed effect may improve the evaluation of compound toxicity.

Environmental hazard prediction from internal effect concentrations

Since the accumulation of pollutants from the ambient environment into fish has been recognised as a substantial environmental hazard, bioconcentration became an important criterion in the environmental risk assessment e.g. in the new European chemical legislation for registration, evaluation, authorization and restriction of chemicals (REACH). To determine bioconcentration parameters for chemicals in fish, standardised protocols like the OECD guideline 305 (OECD, 1996) are used. For a comprehensive overview about *BCF* assessment methods, the reader is referred to the excellent review from Arnot and Gobas (2006).

Considering the hundreds of thousands of compounds in use, experimental determination of fish *BCF*s for every single one is not desirable for ethical reasons but also due to limited test resources (EEC, 1986; IEH, 2001). Bioconcentration in fish has been intensively studied and it is generally agreed that accumulation of non-metabolisable, nonpolar and neutral compounds could be explained by the chemicals' partitioning into fish lipids. As a result, relationships between *BCF*, lipid content and the octanol:water partitioning coefficient (K_{OW}) have been established (e.g. Connell and Hawker, 1988; Mackay, 1982; Veith et al., 1979). Using the *BCF* determination method recommended in the Technical Guidance Document (TGD) for the European Chemical Risk Assessment (Part 2) (TGD model; TGD, 2004), *BCF*s could be estimated based on K_{OW} as descriptor. For chemicals within the models domain (neutral, nonpolar, nonionised and absence of rapid metabolism), the predicted *BCF*s when compared to experimental ones, showed a good accuracy (TGD, 2004).

BCF values estimated from a K_{OW} based regression (e.g. TGD model) have been shown to further be valuable for the prediction of ambient effect concentrations for unknown compounds (e.g. Escher and Hermens, 2002; McCarty, 1986). McCarty (1986) showed that for narcotic acting chemicals, the lethal internal concentration in

fish were similar for all organic chemicals with this biological action. Thus, ambient effect concentration could be predicted based on the lethal internal concentration in combination with the compound's *BCF* (e.g. Escher and Hermens, 2002; McCarty, 1986). For specific acting chemicals like human pharmaceutical compounds (drugs), effect predictions are more complicated, because the internal effect concentrations may vary highly between different drugs, dependent on the compounds biological action. Huggett et al. (2003) proposed an effect prediction model for drugs based on inter-species extrapolations, where human therapeutic plasma concentration (HPC_T) are compared with fish plasma steady-state concentrations (FPC_{ss}). Based on the assumption that human drug target proteins are highly conserved between human and fish, similar or higher FPC_{ss} to HPC_T may lead to receptor mediated responses and thus, potential long-term effects may occur. Also here, FPC_{ss} values were estimated from a bioconcentration model in combination with an ambient drug concentration (Huggett et al., 2003). The usefulness of such ambient effect prediction models is thereby highly dependent on the accuracy of the outcome of the bioconcentration model used.

Internal exposure in wild-living rooted aquatic plants

Origins of current pollutants in aquatic environment include diffuse input such as agricultural runoff, atmospheric deposition and landfills and direct discharge from e.g. municipal or industrial effluents (Doust et al., 2004). Sediments are recognised as major sink for lipophilic organic pollutants, as these compounds generally become associated with sediments and suspended particles when entering the aquatic environment (e.g. Knezovich et al., 1987; Warren et al., 2003). As rooted water plants could not only take up pollutants from the water phase but also via roots, sediment bound pollutants may become a source of contaminants (e.g. Hinman and Klaine, 1992; Turgut and Fomin, 2002). Therefore, to evaluate environmental hazard of pollutants to rooted aquatic plants, both water and sediment exposure have to be considered.

Different monitoring approaches exist for evaluating the environmental contamination of the water phase with organic pollutants, such as spot water sampling or passive sampling. In contrast to spot sampling, where the contamination at a specific time point is determined, passive sampling allows the measurement of time weighted average exposure concentrations (e.g. Stuer-Lauridsen, 2005; Vrana et al., 2005; Zabiegala et al., 2010). As passive sampling is based on the free flow of analytes from the sampling phase (e.g. water) into the sampling device, it is believed that the bioavailable fraction of the compounds could be obtained using passive sampling (e.g. Zabiegala et al., 2010). In contrast, the determination of pollutants exposure from sediments to rooted aquatic plants is more complicated; although analytical

determinations of sediment bound pollutants are well established (e.g. Parkinson and Dust, 2010), the assessment of the bioavailable fraction is still challenging as uptake processes in organisms and desorption processes from sediments are more complex (e.g. Reid et al., 2000). To overcome some of these constraints passive sampling devices have been applied and may become a powerful tool in the future (e.g. Brack et al., 2009; Cornelissen et al., 2008; Ter Laak et al., 2009; Zabiegala et al., 2010). Nevertheless, the most realistic way to evaluate the bioavailable fraction is to determine the local environmental contamination within exposed organisms. Because of their limited mobility, rooted aquatic plants experience the integral local contamination from the water and the sediment phase, thus, they have the potential to function as *in situ* biomonitors for the existing contaminations (e.g. Doust et al., 2006; Gobas et al., 1991; Lytle and Lytle, 2001). Based on the internal exposure of pollutants in rooted aquatic plants, the evaluation of potential environmental hazards could be made on bioavailable fractions of contaminants.

Temporal effect observation provides information about internal exposure and potential toxicity

Many standard test protocols use pre-defined time points to determine effects of an external exposure (e.g. OECD acute tests). However, to evaluate the potential hazards of a toxicant to organisms, uptake and distribution processes have to be considered, as well as the effect translation from the chemical target interaction to the observed effect (e.g. Ashauer and Escher, 2010; Escher and Hermens, 2002, 2004; Jager et al., 2011). These toxico-dynamic processes need time, thus, even at the time point of internal steady-state concentration, effects may not yet fully be expressed. Hence, observing the temporal development of exerted effects includes information about the toxicants uptake and distribution into the organism and the toxico-dynamic processes leading to the adverse effect in exposed organism. A compounds' potential toxicity could thus be assessed during static exposure to a toxicant, from the beginning until the complete effect expression (e.g. Altenburger et al., 2006; Franz et al., 2008). However, typical bioassays, such as the sediment-contact assay with the rooted macrophyte *Myriophyllum aquaticum* (ISO/CD 16191), use invasive observation parameters such as the fresh weight change (FWC) between the beginning and the end of exposure for the effect determination. Thus, observations of temporal effect expressions in individuals to such an endpoint may not be possible.

Common biological actions of pollutants in plants include the inhibition of biological processes such as photosynthesis or mitochondrial electron transport (Babu et al., 2005). It has been shown, that the non-invasive measurement of a plants chlorophyll fluorescence can be used to detect impairments in the photo-

synthetic status compared to a control. This was found to be a reliable method for the identification of the potential hazard of contaminants to plants (e.g. Huang et al., 1997; Krugh and Miles, 1996; Samson and Popovic, 1988). Moreover, the non-invasive observation of the photosynthetic status enables the observation of the effect expression over time (e.g. Franz et al., 2008; Küster and Altenburger, 2007). This enables the modelling of concentration-time-response-relationships for the effect assessment of polluted samples, where contaminants effects could be considered as concentration and time dependent (Altenburger et al., 2006). Further, the determination of toxicant effects from the beginning until complete expression during a static exposure allows the assumption of internal steady-state exposure as well as assessment of the compounds toxic potential to the observed endpoints.

Objectives

The aim of this work is to provide experimental and modelling approaches to gain information on internal exposures in aquatic organism to improve the effect assessment of organic compounds by linking observed adverse effects to exposure concentrations.

In **Chapter 2**, a methodology for the determination of a bioconcentration parameter for lipophilic organic compounds in zebrafish eggs was established. Here, the concentration decrease in a static exposure system is observed when zebrafish eggs are present. Assuming that the only relevant compound loss process is the uptake into zebrafish eggs, bioconcentration parameters could be derived using a two-compartment model.

In **Chapter 3**, the robustness of the fish plasma model (FPM) for indicating possible long-term effects of drugs in fish was studied. In the FPM, the hazard indication based on the comparison of the human therapeutic plasma concentration of a drug to the respective fish steady-state plasma concentration. The dependence of different bioconcentration estimation models for determining the fish steady-state plasma concentration on the FPM outcome was studied.

In **Chapter 4**, the bioavailable fraction of organochlorine pesticides from the water and the sediment phase for the rooted macrophyte *Myriophyllum aquaticum* was studied. The surface water and internal plant exposure of these pollutants in an agricultural and a non-agricultural site of the river Xanaes (province Córdoba, Argentina) was determined at different time points.

In **Chapter 5**, a protocol for a sediment bioassay using *Myriophyllum aquaticum* was established, where the terminal measure of the fresh weight change and the non-invasive measure of the photosynthetic status over time were monitored in parallel. By the observation of effects over time, not only could information about the state of effect development at the photosystem II (PS II) be derived but also information about the internal exposure.

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Chapter 2

A novel in vitro system for the determination of bioconcentration factors and the internal dose in zebrafish (*Danio rerio*) eggs

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A novel in vitro system for the determination of bioconcentration factors and the internal dose in zebrafish (*Danio rerio*) eggs

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Abstract

In this study a novel in vitro approach for the determination of bioconcentration factors (*BCF*) and rate constants of lipophilic substances utilizing zebrafish (*Danio rerio*) eggs is presented. Zebrafish eggs were exposed in a static exposure regime towards a phenanthrene solution and concentration–time profiles of the exposure solutions were analyzed over time. The rate constants and the *BCF* were obtained from the concentration–time profile with the use of a least-square fit to a non-linear model. The determined *BCF* at steady-state (after 72 h of exposure) for phenanthrene was estimated to be only about 1.5 times lower, than the respective *BCF* value reported in the literature. For uptake of solutes in zebrafish embryos, different transport processes are assumed as substances have to pass the chorion first and subsequently the membranes of the embryo. To investigate this, the period to steady-state concentration between zebrafish eggs and the ambient medium for phenanthrene under an agitated and non-agitated static exposure regime were compared. It was found, that this equilibrium was reached within a shorter time frame under agitation, resulting in higher rate constants. In addition to the determination of bioconcentration parameters, the internal phenanthrene dose in zebrafish eggs was determined by utilizing a biomimetic extraction method with water as transfer medium. Approximately 55% of the expected accumulated phenanthrene amount in zebrafish eggs could be re-extracted with a silicone rod extraction method. These results agree very well to what has been observed in abiotic systems. The scope of the proposed in vitro protocol to serve as an alternative for *BCF* determinations using established in vivo animal testing protocols with adult fish is discussed.

Introduction

The new European chemical legislation for registration, evaluation, authorization and restriction of chemicals (REACH) demands that chemicals marketed in quantities of more than 1 tonne per year and manufacturer should be registered, which leads to an assessment load of some 30,000 chemicals (IEH, 2001). Substances of high concern will require additional authorization. One of the concerns beside e.g. to be persistent is the potential of chemicals to bioaccumulate. The bioconcentration factor (*BCF*) describes the concentration of a substance in an aquatic organism (C_F) in relation to the ambient water (C_W) at steady-state and makes up a significant portion of the overall bioaccumulation in aquatic food webs. For *BCF* determinations of chemicals, typically the OECD guideline 305 (OECD, 1996) is used, where fish are exposed in a flow-through system and uptake and clearance of analytes in the fish are studied over time.

The European legislation also calls for the use of non-animal alternative approaches to replace animal testing wherever possible. To this end, de Wolf et al. (2007) proposed an integrative testing strategy to determine *BCF*s of chemicals in fish utilizing a tiered approach. As a first tier, *BCF*s would be estimated by theoretical, computational methods (e.g. via a Quantitative Structure Activity Relationship – QSAR) and the OECD guideline 305 (OECD, 1996) with its 28 d fish assay would be considered as the 'golden standard' last tier. Intermediate tiers consider the use of in vitro methods such as cell-based assays for the *BCF* determination.

Recently, a protocol using fish eggs under short-term exposure has become a substitute for the acute fish assay in the toxicity analysis of wastewater in Germany (DIN, 2001). This is considered to be an animal replacement assay and is currently developed to serve in chemical effect assessment, too (OECD, 2006). One strategy for *BCF* determination of chemicals might therefore be to adapt a protocol using fish eggs as an alternative in vitro testing method.

To measure the internal concentration C_F of chemicals in fish eggs for *BCF* determination, sensitive analytical methods have to be employed, because only a small amount of substance might get accumulated due to the small volume of fish eggs. The *BCF* and rate constants for uptake (k_1) and clearance (k_2) of substances can be determined for fish from the observation of concentration–time profiles of analytes in the exposure solution using an approach developed by Banerjee et al. (1984). In this method, the concentration–time profile of compounds in an aqueous solution may be determined under static conditions whereby the uptake by fish is the only expected substance loss process for the considered solution. This method might also be appropriate for *BCF* determination of substances in fish eggs.

Compared to fish, fish embryos can only take up substances through their membranes as gill ventilation is not yet developed (Petersen and Kristensen, 1998).

As zebrafish embryos are shielded by an outer chorion, solutes have to pass the chorion first and subsequently the membranes of the zebrafish embryo for uptake (Wiegand et al., 2000). There exist evidence, that the major uptake of (lipophilic) substances in zebrafish eggs takes place in the inner zebrafish embryo (e.g. Wiegand et al., 2000). Note, in this work the term zebrafish embryo is used for yolk and developing blastoderm together. To determine reliable rate constants of analytes into zebrafish embryos, the impact of transport processes of solutes through the chorion on rate constants needs therefore to be taken into account.

The aim of the present study was to develop a simple in vitro method to determine rate constants and *BCF*s in zebrafish eggs, which may be used within an integrative testing strategy for *BCF* assessment of chemicals prior to the use of the in vivo OECD test guideline 305 (OECD, 1996). To get insights into uptake processes of solutes into zebrafish eggs, the period to steady-state concentration of a model compound was determined under a non-agitated and an agitated exposure regime. It is assumed, that agitation accelerates the transport of solutes through the chorion into the perivitelline space, while the transport through membranes of the zebrafish embryo remains unchanged. Further, to quantify the internal dose of a chemical in zebrafish eggs a biomimetic extraction method was established. Therefore, previously exposed zebrafish eggs were transferred to clean water, adding a silicone rod (SiRo), and the equilibrium process between the substance concentration in zebrafish eggs, the medium, and the SiRo was used for extraction.

Materials and methods

Chemicals and standard solutions

Acetonitrile (AcN), water (HPLC, ultra gradient grade) and dimethyl sulfoxide (DMSO) were purchased from Merck (Darmstadt, Germany). Phenanthrene (CAS Reg. No. 85-01-8; >98% pure) was purchased from Sigma–Aldrich (Steinheim, Germany). Concentrated solutions were prepared in DMSO and used for HPLC calibration. In exposure solutions the DMSO content was kept at 0.1% (v/v) in standard dilution water (ISO-water) as specified in (ISO, 1996).

HPLC-analysis

Phenanthrene analyses were performed on a Merck-LaChrom (Hitachi, Tokyo, Japan) HPLC-system with a fluorescence (model L-7480) and a diode array (model L-7450) detector and separated on a 5 µm particle size, reversed-phase column (Lichrospher 60 RP select B, Merck). The mobile phase was made up of AcN and water (65:35). Run time was 15 min. The column temperature was set to 25°C, the flow rate was adjusted to 0.5 mL min^{-1}. The fluorescence identification wavelength for

phenanthrene was 252 nm for excitation and 372 nm for emission and the diode array absorbance monitoring wavelength was 251 nm. The detection limit of phenanthrene was 300 ng L^{-1} (fluorescence detector) and the linearity of the calibration function ranged from 0.6 to 1,000 µg L^{-1} (with both detectors).

Fish culture, fish egg collection and incubation conditions

Adult WIK zebrafish (*Danio rerio*, Wild Type Calcutta) were obtained from the Tübingen Zebra Fish Stock Centre (Tübingen, Germany). Fish culture conditions were as described in Küster (2005). Directly after spawning, zebrafish eggs were collected and rinsed with aerated ISO-water to clean them from attached faeces. Zebrafish eggs for all exposure conditions were incubated in a climatic chamber at a temperature of 26.5±1°C and a 12:12 h light:dark regime. Developmental stages were identified according to Kimmel et al. (1995). The volume of one zebrafish embryo was according to Wiegand et al. (2000) assumed to be 0.113 µL at the first developmental day.

Uptake analysis

Two or four fertilized and with aerated ISO-water rinsed zebrafish eggs were placed after 1 h (in the four- to eight-cell stage) in a 4 mL amber vial (VWR International, Darmstadt, Germany) with 2 mL of exposure solution and closed with a commercially available aluminum foil and a polypropylene screw cap. The aluminum foil was used to avoid a concentration decrease due to sorption of phenanthrene to the cap. As a blank control, a 4 mL amber vial without zebrafish eggs was filled with 2 mL exposure solution and sealed similarly in parallel. The amber vial and blank were incubated either with agitation at 75 rpm (horizontal shaker; Edmund Bühler, Hechingen, Germany) or placed without agitation in the climatic chamber under static conditions. The concentration in the exposure solution and the blank were analyzed at defined time points (in general at 0, 1, 4, 7, 24, 32, 55 and 72 h). Only the exposure solution of zebrafish eggs which showed no visible effects were used. Moreover, the concentration in the exposure solution was analyzed even if zebrafish embryos were hatched (e.g. at 72 h). The effect on rate constants and *BCF* was assumed to be negligible, because steady-state conditions were generally reached at this time-point. Each replicate of the 2 mL exposure solution was analyzed three times (i.e. three technical replicates). Thereby, each replicate and blank was utilized for one measurement only and was discarded afterwards. The mean concentration and the standard deviation from three independent replicates at each time-point are presented.

Silicone rod pre-treatment and extraction from zebrafish eggs

The SiRo pre-treatment was done as described in Popp et al. (2004). A silicone elastomer of 1 mm diameter (Goodfellow, Bad Nauheim, Germany) was cut into pieces of 1 cm length with a volume of approximately 8 µL. The SiRo pieces were cleaned by repeated solvent extraction with a methanol:dichlormethane mixture (1:1) followed by heating over night at 250°C in an ultrapure nitrogen stream. After cleaning, the SiRos were stored in vials that were heated before use.

For extraction, zebrafish eggs were examined after 48 h of exposure, using an inverse microscope with a 50× magnification (Olympus IX70-S8F, Hamburg, Germany). After another 30 min, four zebrafish eggs without visible effects were placed without washing in a 4 mL amber vial with 2 mL double-distilled water and a cleaned SiRo. The amber vial was sealed with aluminum foil and a polypropylene screw cap and incubated for 10 min at approximately 70°C to eliminate any enzyme activity and sonicated for another 10 min to homogenize the zebrafish eggs.

For extraction, the amber vial was placed on a horizontal shaker at 200 rpm for 5 h. After extraction the SiRo was removed, rinsed with double-distilled water and dried with a lint-free tissue. Then the SiRo was placed into a HPLC vial with a 500 µL AcN:water mixture (4:1), the vial was closed and sonicated for 10 min. The AcN-water extract was transferred via a 2 mL syringe in another vial for HPLC analysis.

Determination of rate constants

The rate constants for uptake (k_1) and clearance (k_2) were determined according to Banerjee et al. (1984) from the concentration–time profile of the analyte in the exposure solution under static conditions where the uptake by zebrafish eggs was the only expected substance loss process. Under these conditions the instantaneous substance concentration in the exposure solution $[C_W]_t$ can be expressed as in Eq. (2.1), where F represents the proportion of the volume of the zebrafish embryos to the volume of the exposure solution and $[C_W]_0$ is the initial exposure concentration. Please note that the major uptake of phenanthrene in zebrafish eggs was expected to take place in the inner zebrafish embryo and that in the original protocol (Banerjee et al., 1984) F is the proportion of the mass of fish to the mass of the exposure solution.

$$\frac{[C_W]_t}{[C_W]_0} = \frac{1}{k_1 F + k_2}[k_2 + k_1 F e^{-(k_1 F + k_2)t}] \quad (2.1)$$

The rate constants k_1 and k_2 were deduced as fitted parameter utilizing least-square analysis in the software Matlab® (Version 7.4.0.287 R2007a; MathWorks, Natick, MA, USA). A listing of the program is available upon request from the authors.

Results

Uptake into zebrafish eggs under non-agitated exposure

To determine the concentration decrease of phenanthrene in the exposure solution caused by the uptake into zebrafish eggs, two and four zebrafish eggs were exposed. The concentration–time profile of phenanthrene in the exposure solution and a blank were observed (Fig. 2.1). It was found that the concentration of phenanthrene in the exposure solution, which was incubated together with two and four zebrafish eggs, decreased after 72 h by approximately 40% and 60% of the initial value, respectively. In contrast, the phenanthrene concentration in the blank decreased by less than 10%. To estimate the rate constants for uptake (k_1) and clearance (k_2) from the concentration–time profiles, Eq. (2.1) according to Banerjee et al. (1984) was utilized, which assumes that uptake into zebrafish eggs is the only relevant substance loss process. The resulting k_1, k_2, the calculated BCF_{kin} (from k_1/k_2), and the determined BCF_{ss} (steady-state condition; after 72 h exposure) from the measured depletion of phenanthrene from water into zebrafish eggs are shown in Table 2.1.

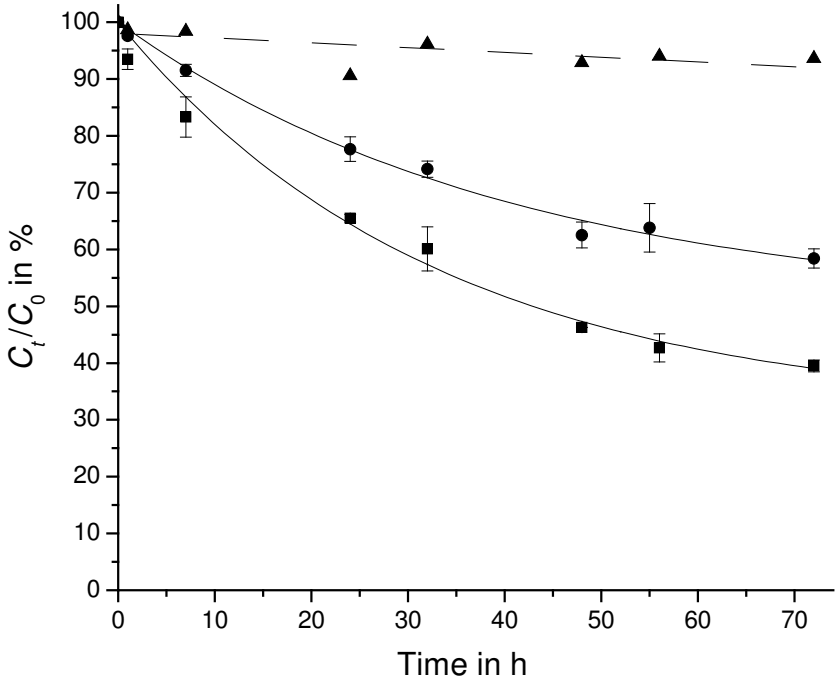

Fig. 2.1. Concentration–time profiles of static and non-agitated phenanthrene solutions over 72 h in closed amber vials, containing two (●) and four (■) zebrafish eggs and a blank (▲) with start concentrations of 138.5, 116.8 and 116.8 µg L^{-1}, respectively. For each data point the concentration from three samples (samples where zebrafish eggs showed no visible effects) was analyzed. The mean concentration and standard deviation is shown.

Uptake into zebrafish eggs under agitated exposure

Simplified, a zebrafish egg consists of an outer chorion, the inner zebrafish embryo and the perivitelline liquid in between. By exposing a zebrafish egg in an exposure solution, for uptake solutes first have to pass the chorion, the perivitelline liquid and subsequently the membranes of the zebrafish embryo where the major uptake of lipophilic compounds is expected. It is hypothesized, that the transport of solutes through the chorion can be accelerated by agitating the exposure system as e.g. the surrounding aqueous boundary layer of the chorion decreases due to a moving exposure medium. To investigate this, again two and four zebrafish eggs were exposed in a static but agitated exposure regime towards a phenanthrene solution. The phenanthrene concentration–time profile in the exposure solution and a blank were analyzed. Again it was found, that the concentration in the exposure solution, which was incubated with two and four zebrafish eggs, decreased after 72 h by approximately 40% and 60% of the starting value, respectively. In contrast, the phenanthrene concentration in the blank decreased by only 3% (Fig. 2.2). As hypothesized, the equilibrium of the phenanthrene concentration between zebrafish eggs and the exposure solution was reached faster, i.e. within 24–32 h of exposure in the static but agitated (Fig. 2.2) compared to 56–72 h in the static and non-agitated exposure regime (Fig. 2.1). To estimate the rate constants k_1 and k_2 from the concentration–time profiles (Eq. 2.1) according to Banerjee et al. (1984) was used. It was found that the rate constants k_1 and k_2 and the calculated BCF_{kin} (from k_1/k_2) differ from the rate constants estimated for the static and non-agitated exposure regime by a factor of 1.5 whereas the determined BCF_{ss} (steady-state condition; after 72 h exposure) remained almost the same (Table 2.1).

Table 2.1. Estimated rate constants (k_1 and k_2) from a concentration–time profile of a phenanthrene solution during an exposure of two and four zebrafish eggs. The BCF_{kin} and the BCF_{ss} of phenanthrene in zebrafish eggs under non-agitated and agitated static exposure conditions were compared. The BCF_{kin} was calculated from the estimated rate constants (k_1/k_2) while the BCF_{ss} was determined from the measured depletion of phenanthrene from water at the steady-state condition (final concentrations at *t* 72 h).

Experimental design	k_1 (per hour)	k_2 (per hour)	BCF_{kin}*	BCF_{ss}
Non-agitated exposure				
Two zebrafish eggs	109.3	0.0121	9,017	6,304
Four zebrafish eggs	92.2	0.0095	9,681	6,777
Agitated exposure				
Two zebrafish eggs	306.7	0.0465	6,599	6,252
Four zebrafish eggs	294.2	0.0443	6,645	6,420

* The parameter *F* in Eq. 2.1 was for two and four zebrafish eggs $1.13 \cdot 10^{-4}$ and $2.26 \cdot 10^{-4}$, respectively.

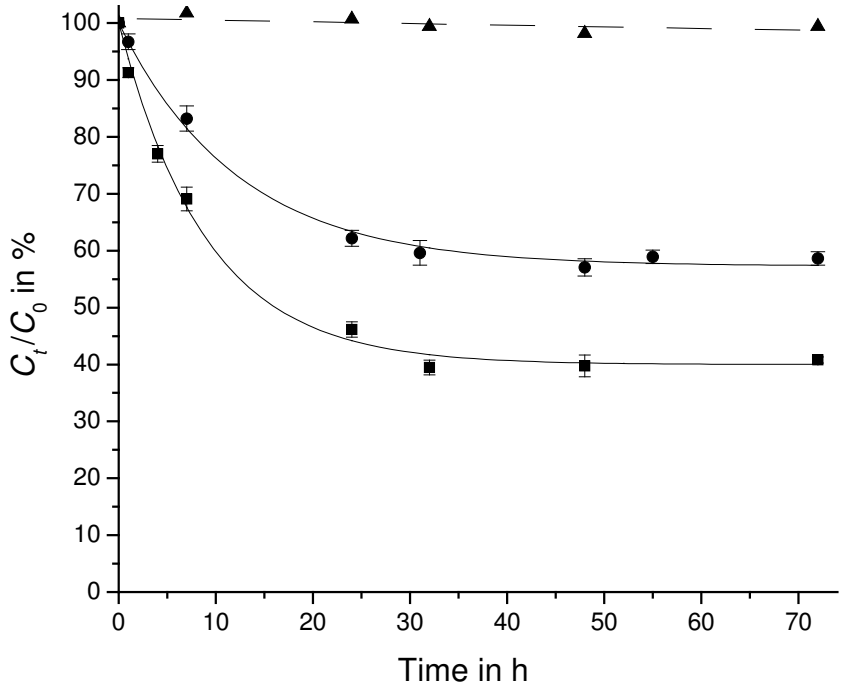

Fig. 2.2. Concentration–time profiles of static but agitated phenanthrene solutions over 72 h in closed amber vials, containing two (●) and four (■) zebrafish eggs and a blank (▲) with start concentrations of 101.8, 204.5 and 204.5 µg L^{-1}, respectively. For each data point the concentration from three samples (samples where zebrafish eggs showed no visible effects) was analyzed. The mean concentration and standard deviation is shown.

Phenanthrene extraction from zebrafish eggs

In the previous sections the uptake of phenanthrene into zebrafish eggs was indirectly determined from the concentration decrease in the exposure solution. In this section we describe the determination of the accumulated phenanthrene amounts by direct extraction from zebrafish eggs utilizing silicone rods (SiRos). For the extraction of phenanthrene from exposed zebrafish eggs with a SiRo, four zebrafish eggs that were previously exposed for 48 h were used. The difference between the amount of phenanthrene (starting minus final concentration multiplied by the volume) in the exposure solution was taken as amount of phenanthrene accumulated by zebrafish eggs. The ratio of the extracted phenanthrene amount from the SiRo in the solvent mixture to the previously indirectly determined amount in zebrafish eggs was defined as the recovery. The mean recovery and the relative standard deviation of four independent experiments was 54.5±1.5%.

Discussion

Uptake and transport processes into zebrafish eggs

The results presented here show a highly reproducible concentration decrease of phenanthrene under static non-agitated as well as agitated exposure conditions invoked by the presence of two or four zebrafish eggs. Further, the steady-state concentration between zebrafish eggs and the ambient medium for phenanthrene was reached within a shorter time frame under agitation, resulting in higher rate constants (Figs. 2.1 and 2.2, Table 2.1).

To interpret these findings the following aspects will be discussed: (a) Is the accumulation volume comparable between different zebrafish eggs and is it constant over time, as is assumed for *BCF* calculations? (b) Is the compartmentalisation relevant for accumulation of (lipophilic) substances in zebrafish eggs? and (c) Is it plausible to assume, that the transport into zebrafish eggs through the chorion is accelerated under the agitated exposure regime? Those three questions are addressed consecutively.

(a) One basic requirement for a reproducible concentration decrease of analytes in water samples is an equivalent accumulation volume of the exposed 'sampler'. As here developing zebrafish eggs instead of an abiotic sampler were used, the finding of this high degree of reproducibility of concentration decreases were therefore quite striking. An explanation for this finding might be, that fish produce large yolky eggs and the egg size is affected/modulated by the nutritional status of the female during ovarian development. In all teleosts studies to date, oocytes show the same basic pattern of growth (Tyler and Sumpter, 1996), so when zebrafish eggs from a highly standardized fish culture are used as it was done in this study, the difference of volume and material of freshly spawned zebrafish eggs should be small at the onset of the uptake experiment. The size of freshly spawned zebrafish eggs are between 0.73 and 0.75 mm in diameter (Selman et al., 1993) when assuming a spherical shape of the chorion the resulting volume is approximately 1.77 µL. In contrast, the volume of a zebrafish embryo has been determined to be only 0.113 µL on the first developmental day (Wiegand et al., 2000). This value has been determined by yolk and embryo diameter measurement, under the assumption of a spherical shape (Wiegand et al., 2000).

During embryogenesis the composition and volume of the chorion is expected to vary only slightly (e.g. Rawson et al., 2000) while the zebrafish embryo undergoes a massive phenotypic change (e.g. Kimmel et al., 1995). Zebrafish embryos are composed of two complex compartments, a large yolk and the developing disclike blastoderm on top of it. Major components of the yolk are vitellogenins, which are multifunctional nutrients sources for the developing zebrafish embryo. In order to

maintain a regulated supply of nutrients and energy during embryonic development, proteolytic degradation of yolk proteins has to be effectively programmed and particularly a regulated proteolysis of vitellogenins was shown by Gündel et al. (2007). Furthermore, developmental stages of zebrafish embryos have been found to be synchronized, for instance the segmentation period (10–24 h), the pharyngula period (24–48 h) and the hatching period (48–72 h) occur within a narrow time frame (Kimmel et al., 1995). This supports the idea of a highly defined developing process of zebrafish embryos during the entire embryogenesis. Together with the assumption of a reproducible volume and material of freshly spawned zebrafish eggs this may suggest a constant accumulation volume between zebrafish eggs from a highly standardized fish culture.

(b) For uptake of substances in zebrafish eggs three possible constitutive compartments were assumed, namely the outer chorion, the zebrafish embryo and the perivitelline liquid between the former. It is generally accepted, that the chorion does not shield the zebrafish embryo against solutes of the exposure solution as it possesses pores of an average diameter of 1.5 µm which occur at distances ranging from 1.5 to 3 µm (e.g. Laale, 1977; Lee et al., 2007; Rawson et al., 2000). Therefore, it is apparently more appropriate to see the chorion as a sieve rather than as a wall. Nevertheless, the chorion has the capability to bind solutes, e.g. Burnison et al. (2006) showed in accumulation studies with ^{109}Ca-labelled Ca^{2+}, that a proportion of 61% of total absorbed Ca^{2+} was bound to the chorion and that only a small proportion of 1% was found in the zebrafish embryo during an exposure of 4 h. The binding of (metal) cations to the chorion was explained by the electrostatic attraction to the anionic glutamic acids as the chorion is rich of them (Rombough, 1985). In contrast, Wiegand et al. (2000) have shown in uptake studies using ^{14}C-labelled trifluoro-acetate (TFA) and ^{14}C-labelled atrazine that these compounds penetrate the chorion very fast and reach the zebrafish embryo within the first 10 s (atrazine) after onset of exposure. The proportion of chorion absorbed amounts to total absorbed amounts of TFA and atrazine have been found to be 30% and 1.8% (calculated from determined values after 48 h of exposure), respectively, while the remainder was found in the zebrafish embryo. In this latter study the perivitelline liquid was basically seen to serve as a transfer medium of the solutes from the chorion to the zebrafish embryo. Furthermore, Wiegand et al. (2000) found that more atrazine compared to TFA was absorbed by the zebrafish egg and that this correlates well with the different octanol water partition coefficient (K_{OW}) of substances (TFA log K_{OW} -4.1, atrazine log K_{OW} 2.58; Wiegand et al., 2000). This leads to the idea, that the uptake of lipophilic substances mainly take place in zebrafish embryos while the perivitteline liquid serves as the transfer medium of the solutes. However, polar (hydrophilic) compounds may also bind in significant amounts to the chorion.

(c) Here we discuss, why it is likely, that the transport of phenanthrene into zebrafish eggs through the chorion is accelerated under the agitated exposure regime. As it was discussed before, the relevant uptake of lipophilic substances (phenanthrene log K_{OW} 4.57; Petersen and Kristensen, 1998) can be expected to take place in the zebrafish embryo and therefore we assumed different transport processes for uptake of solutes from the exposure solution. Before solutes reach the zebrafish embryo, they first have to pass the chorion, and subsequently the perivitelline liquid and the membranes of the zebrafish embryo. As one role of the chorion is the protection of the inner sphere (perivitelline liquid and zebrafish embryo) against physical damage (Rawson et al., 2000), a moving exposure medium during agitated exposure may therefore only slightly alter the transport of solutes from the inner chorion surface into the zebrafish embryo. Agitation of the exposure medium also is not expected to alter the passive diffusion of solutes through pores of the chorion into the perivitelline liquid significantly (Lee et al., 2007). Therefore, we believe that agitation should mainly accelerate the transport of solutes from the exposure solution through the outer aqueous boundary layer to the pores of the chorion mainly due to a reduction of the thickness of this layer at the outer chorion surface (Gobas et al., 1986; Kwon et al., 2006).

Bioconcentration parameters in zebrafish eggs

Rate constants and the resulting BCF_{kin} for phenanthrene in zebrafish eggs under non-agitated exposure conditions (Table 2.1) were in excellent agreement with those determined by Petersen and Kristensen (1998) (BCF_{ss} 9,120 [BCF_{kin} 9,300 – this study]; k_1 165–246 h^{-1} [k_1 92–109 h^{-1} – this study]; k_2 0.011–0.025 h^{-1} [k_2 0.010–0.012 h^{-1} – this study]), while the BCF_{kin} from the agitated uptake experiment and BCF_{ss} from non-agitated and agitated experiments (Table 2.1) were estimated to be about 1.5 times lower. The difference between the calculated BCF_{kin} from the estimated rate constants (k_1/k_2) of the non-agitated exposure regime to all other determined *BCF*s may be explained by the assumption of a one-step uptake process in the used pharmacokinetic model (Eq. 2.1). As mentioned above, solutes have first to pass through the chorion of the zebrafish egg to reach the perivitelline liquid and subsequently the substance has to diffuse through the zebrafish embryo membranes. Therefore, this simple pharmacokinetic model might not be appropriate to estimate rate constants when two-steps are rate-determining. Under the agitated exposure regime, the transport through the chorion into the perivitelline liquid is expected to be accelerated, so it could be assumed that the uptake from the perivitelline liquid into the zebrafish embryo should be the rate-determining step during the entire exposure. This would be in accordance with the used pharmacokinetic model. However, the BCF_{ss} determined at steady-state condition in both experimental setups can be

regarded as reliable values, as the phenanthrene concentration in the exposure solutions were constant from 30 to 72 h in the agitated and from 56 to 72 h in the non-agitated exposure regime (Figs. 2.1 and 2.2). Hence the 1.5 times lower BCF_{ss} and the almost similar rate constants compared to the values determined by Petersen and Kristensen (1998), who utilized ^{14}C-labelled phenanthrene for uptake experiments with zebrafish eggs in a semistatic exposure system, indicate that this simple methodology possesses the potential to assess adequate bioconcentration parameters for (lipophilic) substances in zebrafish eggs.

Determination of the internal dose by silicon rod extraction

To determine the internal dose of phenanthrene in zebrafish eggs, the accumulated amounts were extracted from this organisms with a SiRo using water as transfer medium. An equilibrium between the phenanthrene concentration in the medium, zebrafish eggs and the SiRo should be achieved. Compared to zebrafish eggs (median BCF_{ss} 6,438) the SiRo-water partition coefficient ($K_{SiRo:W}$ 23,650; Paschke et al., unpublished report) was approximately 3.7 times higher and the volume of the SiRo was approximately 18 times higher. At equilibrium state therefore the ratio of phenanthrene amounts between SiRo, zebrafish eggs and water were expected to be 97.5%, 1.5% and 1%, respectively. Hence, nearly the entire phenanthrene amount should be extracted by the SiRo from zebrafish eggs and the transfer medium at equilibrium state. Because of this consideration the extraction was thought to provide evidence for the uptake and quantitative dose estimation of phenanthrene in zebrafish eggs. Popp et al. (2004) have developed the SiRo extraction method to extract PAH from water samples and for rapid screening of highly contaminated waste material. For validation they investigated the extraction time and recovery for 14 EPA-PAHs with SiRos from spiked HPLC water. The equilibrium was reached for all PAHs within 3 h of extraction. Recoveries of spiked water samples with PAHs by the SiRo extraction method ranged between 62% and 97% and the total recovery for phenanthrene is 80.2% after the fourth desorption step. At the first desorption step of the SiRo, 66.9% of total phenanthrene have been found in the 100 µL extraction mixture. The slightly lower recovery of phenanthrene from zebrafish eggs (54.5%) in the 500 µL extraction mixture compared to the findings of Popp et al. (2004) from spiked HPLC water (66.9%) might be explained by a non-equilibrium state between the phenanthrene concentration in the medium, zebrafish eggs and the SiRo and by substance loss during extraction treatment like heating and sonication. So in principle, we believe that the indirectly determined dose in zebrafish eggs from the difference between the amount of phenanthrene (starting minus final concentration multiplied by the volume) in the exposure solution is correct. Further, this procedure seems to be a simple method to determine the

internal dose of lipophilic substances in zebrafish eggs, if recoveries in the specific system are known and reproducible.

Scope of the in vitro BCF *assessment procedure*

The European legislation requires that non-animal approaches of testing should be used in the place of experiments with animals wherever possible (EEC, 1986). In the light of the new European chemical legislation REACH, where *BCF*s of some 3,000 substances (de Wolf et al., 2007) will have to be determined, alternative approaches to the 28 d fish assay is an urgent demand (OECD, 1996). To this end, de Wolf et al. (2007) proposed an integrative testing strategy to determine *BCF*s of substances in fish utilizing a tiered approach. Central to the strategy was the question, whether the *BCF* estimate is suitable to make a regulatory decision with sufficient confidence. The first tier proposes the use of estimation models like QSAR (e.g. BCFWIN by Meylan et al., 1999) or biomimetic extraction approaches like semi-permeable membrane devices filled with triolein (Huckins et al., 1990). When determined *BCF*s using the considered methods are not suitable for making a decision then in vitro methods may be used in a second tier. Here, it was suggested to produce or refine estimated *BCF*s with informations of the biotransformation potential of substances in fish using in vitro assays with e.g. slices or cells from liver (Segner, 1998). At the next tier (3) modified in vivo methods like the Banerjee et al. (1984) procedure were suggested to determine a more refined *BCF*. When still this *BCF* measure is not suitable for making regulatoric decisions then the OECD 305 fish assay should be conducted as the ‘golden standard’ last tier (OECD, 1996).

The proposed in vitro zebrafish egg assay may be a method suitable for a tier between purely computational approaches (e.g. QSAR) or cell culture assays and a full animal test. As zebrafish embryos are widely used in toxicology as a fish model for many purposes (Scholz et al., 2008), this organism may also be appropriate for *BCF* determination of substances. Further, it has been described by Banerjee et al. (1984), that bioconcentration parameters of nondegrading lipophilic substances in fish (or here in zebrafish eggs) could easily be determined from the substance concentration–time profile in the exposure solution and the agreement with literature values are satisfactory (de Wolf et al., 2007). In this study, the determined *BCF* value of phenanthrene in zebrafish eggs (median BCF_{ss} 6,438) was in the same order of magnitude as reported *BCF* values for phenanthrene in fathead minnow, which were determined in flow-through systems (*BCF* 1,300–5,100; USEPA, 2007). Therefore, the presented procedure possess the potential to determine suitable *BCF*s of substances in fish to make regulatory decision with sufficient confidence.

A next step forward comprises the understanding of the limitations towards physicochemical properties of investigated substances like lipophilicity, volatilization

and molecular size for the accumulation into zebrafish embryo. This understanding is also needed when regarding processes which may cause an additional concentration decrease of the exposure solution (e.g. adsorption, degradation, volatilization; Schreiber et al., 2008). Assuming that the volume between different zebrafish embryos from a highly standardized fish culture vary only slightly, a standard zebrafish embryo volume (0.113 µL) was utilized. With respect to the effect of substance dilutions by growth of the zebrafish embryo, an additional growth function might also be included in the future. A further step towards an in vitro fish replacement assay for the determination of *BCF* using zebrafish eggs beside the above-mentioned would be to study the correlation between measured *BCF*s of substances in zebrafish eggs and in adult zebrafish. This comprises beside the understanding of limitations towards physicochemical properties also physiological differences, e.g. uptake and elimination processes in fish and zebrafish eggs.

However, in the light of the new European chemical legislation REACH, the proposed method may be useful to replace some of the in vivo fish testings (OECD, 1996). Further this method may be implemented in the proposed integrative testing strategy to determine *BCF*s for fish as an intermediate in vitro assay prior to the use of in vivo fish assays to reduce or replace animal testings (de Wolf et al., 2007).

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Chapter 3

Using the Fish Plasma Model for comparative hazard identification for pharmaceuticals in the environment by extrapolation from human therapeutic data

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Using the Fish Plasma Model for comparative hazard identification for pharmaceuticals in the environment by extrapolation from human therapeutic data

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Abstract

Thousands of drugs are currently in use, but only for a few of them experimental chronic fish data exist. Therefore, Huggett et al. (*Human Ecol Risk Assess* 2003; 9:1789–1799) proposed the fish plasma model (FPM) to extrapolate the potential of unintended long-term effects in fish. The FPM compares human therapeutic plasma concentrations (HPC_T) with estimated fish steady-state concentrations (FPC_{ss}), under the assumption that biological drug targets may be conserved across the species. In this study, the influence of using different input parameters on the model result was characterised for 42 drugs. The existence of structurally and functionally conserved protein targets in zebrafish could not be refuted. Thus, the FPM model application was not in contradiction to its basic assumption. Further, dissociation of drugs was shown to be important in determining the output and model robustness. As the proposed model for FPC_{ss} estimation was considered to predict accurate values for neutral and lipophilic chemicals only, a modified bioconcentration model was used with D_{OW} as predictor. Using reasonable worst case assumptions, a hazard was indicated for one third of the selected drugs. Our results support the notion that this approach might help to prioritise among in use drugs to identify compounds where follow up evidence should be considered.

Introduction

During the last decades, analytical methods to detect pharmaceutical compounds (drugs) in the environment at low concentrations have been improved. Many active drugs have subsequently been detected in various compartments and in different countries at concentrations in the low ng L^{-1} to µg L^{-1} range (Fent et al., 2006; Richardson and Bowron, 1985; Santos et al., 2010). The occurrence of unintended effects in non-target species in the environment like the decline of the Oriental white-backed vulture (*Gyps bengalensis*) population in the Indian subcontinent and in Pakistan due to diclofenac contaminated carcasses (Oaks et al., 2004), the decline of the vulture populations of *Gyps fulvus*, *Aegypius monachus*, and *Neophron percnopterus* in central Spain caused by continuous exposure to antibiotics (Lemus et al., 2008) or the feminisation of male fish related to the exposure to municipal effluents containing endocrine active substances, such as ethinyl estradiol (Purdom et al., 1994; Sumpter and Johnson, 2008), raised concern about the potential hazards of drugs towards wildlife. Given that drugs are designed to elicit a specific biological action and that conservation of drug targets has been shown for a wide range of non-target organisms (Gunnarsson et al., 2008), potentially, long-term and chronic effect information seems relevant for the assessment of unwanted effects and more important than acute data (e.g., Huggett et al., 2003; Länge and Dietrich, 2002; Seiler, 2002). Indeed, available data indicate that determined effect concentrations using established short-term bioassays most often occur at concentrations well above observed environmental concentrations (EnvC), namely in the higher µg L^{-1} to g L^{-1} ranges (Fent et al., 2006; Santos et al., 2010). For most of the drugs currently in use, no systematic environmental risk assessment (ERA) has been performed according to the current European guideline (EMEA, 2006) because they got the granting of marketing authorisation before the guideline came into force. Additionally, there is a noticeable lack of investigation of chronic drug effects on non-target organisms such as fish (e.g., Corcoran et al., 2010; Santos et al., 2010). They have been focus to only a few studies so far, e.g. the effects of the synthetic oestrogen 17α-ethinylestradiol (Länge et al., 2001) or tamoxifen citrate (Williams et al., 2007) on the full-life cycle of fathead minnow. The paucity of data on risks from chronic exposure will probably not change for many years to come.

With respect to animal welfare and the 3R principle (Replacement, Reduction, and Refinement) as defined by Russell and Burch (1959), the extensive conduction of fish experiments to study possible long-term hazards of drugs is further not desirable. Moreover, the European legislation requires, that non-animal approaches should be used to replace animal procedures whenever possible (EEC, 1986). Hence, the prediction of chronic effects by using human therapeutic data in non-

target organisms have been proposed as a way forward to allow a tailor-made risk assessment of drugs and to encourage systematic case studies. Extrapolation approaches, such as the Fish Plasma Model (FPM; Huggett et al., 2003), to model the potential of unintended effects in fish have already been published (e.g., Berninger and Brooks, 2010; Kools et al., 2008; Kostich and Lazorchak, 2008). Based on the assumption of conserved targets in non-target organisms such as fish, these models use pharmacokinetic and pharmacodynamic information produced during drug discovery and development. The FPM in particular compares human therapeutic plasma concentrations (HPC_T) with estimated fish steady-state plasma concentrations (FPC_{ss}), to extrapolate adverse unintended effects in fish for a given drug. Huggett et al. (2003) assume that, when the FPC_{ss} is equal to or higher than the HPC_T, a receptor mediated response in fish and thus potential long-term effects may be possible. Herein the FPM integrates three parts: (i) the use of the HPC_T from available data, (ii) the prediction of a FPC_{ss}, which relates ambient exposure concentrations to internal dose in fish using a lipophilicity-based bioconcentration model and (iii) the assumption that drug target proteins between human and fish based on the primary structure are conserved. For the required decision, on whether or not to consider a target structure as conserved, threshold approaches have been proposed (Christen et al., 2010; Kostich and Lazorchak, 2008).

The first empirical evidence for selected drugs in fish (e.g., Owen et al., 2007) reveal that the model assumptions cannot be dismissed as being completely unrealistic though many details may offer scope for refinement. Nevertheless, systematic investigations of the FPM accuracy are missing. A principle difficulty lies in the fact that systematic experimental validation through e.g., longterm fish studies are not foreseeable due to resource and other constraints. This would be needed to evaluate whether an extrapolation approach produces accurate predictions and thus might be a tool to replace long-term studies such as full life cycle fish tests or might be used as a possible tool in a systematic ERA for drugs currently in use with lacking data according to the current European guideline (EMEA, 2006).

The present work therefore aims to study the likelihood and robustness of the FPM for indicating environmental hazards of drug. We did so by employing the FPM to a broader range of drugs, selected for comprising varying physicochemical and therapeutic properties. Hereby, the different model parts, HPC_T as dependent on the used variables (area under the drugs blood plasma time-concentration curve [*AUC*] or maximum blood plasma concentration after drug administration [C_{max}]), the FPC_{ss} as dependent on the bioconcentration model used as well as on the drugs lipophilicity when regarding dissociation (octanol:water partition coefficients [K_{OW}] vs. apparent K_{OW} at specific pHs) and the basic assumption of the target conservation as dependent on similarity of protein structures have been investigated.

Material and Methods

Compound selection

From the 3000+ drugs that are currently marketed worldwide, we took a sample that was aimed to represent a wide range of physicochemical properties (Table 3.1), comprising major therapeutic application fields and providing known individual and multiple molecular targets (Table 3.2). Thus, we came up with 42 drugs from different therapeutic classes we used within this simulation study. The substances cover a wide range of human therapeutic concentrations (C_{max} values from 54 ng L^{-1} to 2.2 g L^{-1}) and of predicted environmental concentrations (*PEC* values from 150 pg L^{-1} to 22.5 µg L^{-1}). Moreover, the selected drugs span log K_{OW} values from -1.47 to 6.3 and appear in a neutral or dissociated form in the aquatic environment.

Fish plasma model – FPM

Huggett et al. (2003) proposed the FPM to estimate potential hazards of drugs to fish and is based on three key assumptions. Firstly, drug targets are conserved across human and fish species and by this, intended effects in humans may become unintended effects in non-target species. Secondly, if similar targets are present in fish, HPC_T may be compared with respective FPC_{ss} assuming a similar effective internal concentration by calculating an effect ratio (ER; Eq. 3.1).

$$ER = HPC_T / FPC_{ss} \quad (3.1)$$

Thirdly, the FPC_{ss} for a drug can be estimated using a bioconcentration model based on equilibrium partitioning theory. Thereby it uses the drugs blood:water distribution coefficient ($K_{Blood:Water}$) for fish in combination with a *PEC* (Eq. 3.2).

$$FPC_{ss} = PEC \bullet K_{Blood:Water} \quad (3.2)$$

An $ER \leq 1$ indicates that the expected FPC_{ss} is equal or greater than the HPC_T that elicits a therapeutic effect in humans and this is suggested as an indication for receptor mediated responses in fish and thus potential long-term effects.

- HPC$_T$ *Derivation*. For quantifying the HPC_T two different pharmacokinetic (PK) parameters are suggested (Huggett et al., 2003), namely the drugs peak concentration (C_{max}) in blood or the area under the time–concentration curve (*AUC*) after drug administration.
- FPC$_{ss}$ *Derivation*. To estimate FPC_{ss} values, it is proposed to measure or predict EnvC of a respective drug and use this value in conjunction with a predicted $K_{Blood:Water}$ (Eq. 3.2). The partitioning of a drug between the aqueous phase and the arterial blood in rainbow trout (Eq. 3.3) is extrapolated using a correlation function

generated from measured $K_{Blood:Water}$ values of polychlorinated biphenyls, – ethanes and benzene and the respective K_{OW} based on the work of Fitzsimmons et al. (2001):

$$\log K_{Blood:Water} = 0.73 \bullet \log K_{OW} - 0.88 \quad (3.3)$$

Bioconcentration estimation method

Since many drugs do dissociate and/or are not lipophilic, the $K_{Blood:Water}$ (Eq. 3.3) estimate may yield a poor prediction regarding the lipophilic and non-dissociating substances used for the regression. Therefore, the bioconcentration factor (*BCF*) estimation method recommended in the Technical Guidance Document (TGD) for the European Chemical Risk Assessment (Part 2) (TGD, 2004) according to Fu et al. (2009) was used with log D_{OW} values (apparent log K_{OW}; sum of log K_{OW} of neutral and ionic form at a specific pH) as predictors (Eqs. 3.4 and 3.5).

$$\log BCF = 0.85 \bullet \log K_{OW} - 0.70 \quad (3.4)$$

According to the TGD (2004) based on work by Veith et al. (1979), this regression is valid in a log K_{OW} range between 1 and 6. Below log K_{OW} = 1 Fu et al. (2009) set a fixed value of *BCF* = 1.41 (log *BCF* = 0.15). For more lipophilic substances (6≤ log K_{OW} ≤10), a parabolic regression is recommended (TGD, 2004) based on the work of Connell and Hawker (1988):

$$\log BCF = -0.2 \bullet \log K_{OW}^2 + 2.74 \bullet \log K_{OW} - 4.72 \quad (3.5)$$

In the present study, the $K_{Blood:Water}$ (Eq. 3.3) estimation method suggested by Huggett et al. (2003) will be referred to as KBW model throughout, while the *BCF* (Eqs. 3.4 and 3.5) estimation recommended in the TGD (2004) according to Fu et al. (2009) will be referred to as TGD model. Thereby, *BCF* estimates are used for FPC_{ss} predictions as surrogate for $K_{Blood:Water}$ estimates (Eq. 3.2).

Data compilation and methodology for data analysis for ER *estimates*

The compiled data are presented in Table 3.1, in the following paragraphs the origin and treatment of the data is documented. All data were compiled between April and June 2008.

► K_{OW}, pK_a *and* D_{OW} *values*

Several drugs dissociate at environmentally relevant pH values. This can lead to a significant reduction of the compounds lipophilicity and may results in an altered accumulation in fish and thus modifies their FPC_{ss}, respectively. Since experimentally determined apparent K_{OW} (D_{OW}) for specific pH values are scarce in the literature, estimated K_{OW} and D_{OW} values at pH 5, 7, and 10 were used for generating a

consistent data set. The software ACD/LogD Suite (Version 12; Advanced Chemistry Development) was used to estimate pK_a and D_{OW} (K_{OW}) values based on the drugs chemical structure. Structures were created from canonical SMILES (simplified molecular input line entry specification) codes, which were taken from the DrugBank (Wishart et al., 2006) entry of the drug. Hereby drugs were identified using their chemical abstracts service (CAS) registry number.

► C_{max} *and* AUC_{conc} *values*

Pharmacokinetic (PK) parameters like the C_{max} or the *AUC* are highly dependent on the administered therapeutic dose (*TD*) regime of the brand used. Therefore, to gather consistent PK parameter values typically one brand was randomly specified for one active drug. For a worst case hazard ranking, the lowest reported C_{max} and *AUC* values were taken for each drug. Depending on the data availability these data were first looked up in the RxList database (Hatfield et al., 1999), and if not available, in the DailyMed (http://dailymed.nlm.nih.gov/dailymed/about.cfm) database. Finally, for missing cases original communications were retrieved using an internet search on the drug name and the respective PK parameter. The used source of information is provided in Table 3.1. The *AUC* may be interpreted as an 'integral drug exposure over a defined time in blood' rather than a blood concentration. For use in the FPM, it needs to be transformed into a 'therapeutic blood concentration' (AUC_{conc}) before use as surrogate for the HPC_T in Eq. 3.1. To account for the dependence of *AUC* values on systemic uptake and elimination processes of the drug into the blood, *AUC* values were divided by the respective time for the blood concentration to be reduced to 50% of C_{max} (half-life; $t_{1/2}$; Eq. 3.6).

$$AUC_{conc} = AUC / t_{1/2} \tag{3.5}$$

$t_{1/2}$ was chosen as it reflects a relevant biosystem property and is generally available for the drugs. For a worst case hazard ranking, the longest reported $t_{1/2}$ was used.

► PEC *and* TD_{max} *values*

For estimating FPC_{ss}, either measured or predicted EnvCs of the drugs are needed (Eq. 3.2; Huggett et al., 2003). As we did not strive to assess environmental exposure, but rather to provide an uniform data set for comparing the model outputs, the *PEC* values for surface water were calculated using the European legislation Phase I exposure estimation procedure (EMEA, 2006; Eq. 3.7) with default values as used in the European Union.

$$PEC = \frac{TD_{max} \bullet M}{V \bullet D} \tag{3.7}$$

Table 3.1. Identity in means of common name and their chemical abstracts service registry number (CAS-RN) and their respective physicochemical properties (pK_a, K_{OW}, D_{OW} at specific pH values), pharmacokinetic parameters (C_{max}, *AUC*, AUC_{max}, TD_{max}, $t_{1/2}$) and predicted environmental concentration (*PEC*) for the 42 considered pharmaceutical compounds are shown.

Common Name [1]	CAS-RN	pK_a [2]		log K_{OW} [2]	log D_{OW} [2]			C_{max}	*AUC*	$t_{1/2}$	AUC_{conc} [3]	TD_{max}	*PEC* [4]
		1	2		pH 5	pH 7	pH 10	µg L^{-1}	µg•h L^{-1}	h	µg L^{-1}	mg d^{-1}	µg L^{-1}
Aripiprazole	129722-12-9	7.7		3.76	1.59	3.34	3.76	39 [c]	1,994 [c]	94 [a]	21.2	60 [a]	0.3
Atenolol	29122-68-7	9.4	13.9	0.33	-2.75	-2.09	0.21	330 [d]	1,810 [d]	5.7 [d]	320	200 [a]	1
Beclomethasone	4419-39-0	12.2		2.44	2.44	2.44	2.44	0.319 [e]	0.151 [e]	2.7 [e]	0.056	0.336 [a]	0.00168
Betaxolol	63659-18-7	9.4	13.9	2.53	-0.55	0.093	2.43	27.9 [a]	300 [f]	22 [b]	13.6	20 [a]	0.1
Bicalutamide	90357-06-5	11.5		4.13	4.13	4.13	4.11	768 [a]		139 [a]		50 [a]	0.25
Bisoprolol	66722-44-9	9.4	13.9	1.89	-1.2	-0.54	1.76	15.2 [g]	198 [g]	21 [a]	9.45	20 [a]	0.1
Carbamazepine	298-46-4	13.9		1.89	1.89	1.89	1.89	1,900 [a]		40 [a]		1,600 [a]	8
Ciclesonide	141845-82-1			5.3	5.3	5.3	5.3	0.369 [a]	2.18 [a]	7 [a]	0.311	0.64 [a]	0.0032
Citalopram	59729-33-8	9.6		3.47	0.39	1.02	3.34	21.2[h]				60 [a]	0.3
Clofibrate	637-07-0			3.88	3.88	3.88	3.88	5,000 [i]	13,880 [i]	35 [a]	397	2,000 [a]	10
Conjugated Oestrogens	438-67-5			-0.69	-0.69	-0.69	-0.69	0.087 [a]	5.56 [a]	50.7 [a]	0.11	0.3 [a]	0.0015
Diclofenac	15307-86-5	4.2		4.55	3.66	1.77	0.8	583 [j]	682 [j]	2 [a]	341	200 [a]	1
Duloxetine	136434-34-9	10		4.81	1.71	2.05	4.5	12.5 [k]	169 [k]	12 [a]	14.1	120 [a]	0.6
Entacapone	130929-57-6	5.2		2.12	1.96	0.47	-1.04	1,200 [a]		2.4 [a]		1,600 [a]	8
Eplerenone	107724-20-9			0.32	0.32	0.32	0.32	1,500 [l]	7,900 [l]	6 [a]	1,317	50 [a]	0.25
Escitalopram	128196-01-0	9.6		3.47	0.39	1.02	3.34	24.9 [m]	600 [m]	32 [a]	18.7	20 [a]	0.1
Ethinyl Estradiol	57-63-6	10.2		4.11	4.11	4.11	3.91	0.054 [a]	0.28 [a]	24 [a]	0.012	0.03 [a]	0.00015
Fenofibrate	49562-28-9			5.8	5.8	5.8	5.8	985 [n]	45,048 [n]	24 [n]	1,877	130 [a]	0.65
Fluoxetine	54910-89-3	10.1		3.93	0.83	1.15	3.6	190 [o]	4,342 [o]	100 [o]	43.3	80 [a]	0.4
Fluticasone Propionate	80474-14-2	12.5		3.06	3.06	3.06	3.06	0.126 [p]	0.488 [p]	7.8 [p]	0.063	1.76 [a]	0.0088
Furosemide	54-31-9	3		2.3	0.34	-0.79	-1.09	552 [q]	1,203 [q]	2 [a]	602	600 [a]	3
Gefitinib	184475-35-2	7		2.7	0.21	2.41	2.7	2.2E6 [b]	2.93E7 [b]	48 [a]	610,417	500 [a]	2.5

Gemfibrozil	25812-30-0	4.8		4.3	3.86	2.07	0.57	25,790 [r]	86,890 [r]	4 [r]	21,723	1,200 [a]	6
Irbesartan	138402-11-6	2.5	4.2	6.15	5.38	4.23	4.15	942 [s]	5,571 [s]	15 [a]	371	300 [a]	1.5
Lapatinib	388082-78-8	6.3		6.3	4.39	6.21	6.3	1,570 [a]	23,400 [a]	14.2 [a]	1,648	4,500 [a]	22.5
Lenalidomide	191732-72-6	2.8	10.8	-1.47	-1.47	-1.47	-1.53	568 [t]	5,668 [t]	10 [t]	569	10 [a]	0.05
Methylphenidate	113-45-1	9.5		2.31	-0.77	-0.11	2.19	39 [a]	36.3 [u]	4 [a]	9.07	30 [a]	0.15
Metoprolol	37350-58-6	9.4	13.9	1.63	-1.45	-0.81	1.5	125 [v]	421 [v]	7 [a]	60.1	1,000 [a]	5
Milnacipran	92623-85-3	10.4		0.83	-2.27	-2.09	0.28	216 [w]		8 [a]		200 [a]	1
Nadolol	42200-33-9	9.5	13.9	0.56	-2.52	-1.87	0.43	138 [x]	1,828 [x]	24 [a]	76.2	240 [a]	1.2
Norethindrone	68-22-4	13.1		2.86	2.86	2.86	2.86	26.2 [b]	167 [b]	8.51 [b]	19.6	15 [a]	0.075
Paliperidone	144598-75-4	8.1	13	1.41	-1.41	0.26	1.4	40 [y]		23 [a]		12 [a]	0.06
Paroxetine	61869-08-7	9.7		3.7	0.61	1.16	3.53	61.7 [a]	774 [z]	21 [a]	36.9	60 [a]	0.3
Pioglitazone	111025-46-8	5.5	6.4	3.5	2.85	2.76	1.51	524 [aa]	4,590 [aa]	8 [aa]	574	45 [a]	0.225
Pregabalin	148553-50-8	4.2	11.3	1.09	-1.44	-1.41	-1.45	500 [ab]		6.3 [a]		600 [a]	3
Progesterone	57-83-0			3.83	3.83	3.83	3.83	17.3 [a]	43.3 [a]	2.3 [a]	18.8	400 [a]	2
Propranolol	525-66-6	9.5	13.8	2.9	-0.19	0.45	2.77	14.8 [ac]	199 [ac]	12 [a]	16.6	640 [a]	3.2
Sertraline	79617-96-2	9.5		5.08	2	2.7	4.97	123 [a]	2,570 [a]	26 [a]	98.9	200 [a]	1
Spironolactone	52-01-7			3.15	3.15	3.15	3.15	80 [a]	3,401 [ad]	15 [ad]	227	400 [a]	2
Timolol	26839-75-8	9.4	13.4	1.28	-1.79	-0.96	1.2	0.618 [ae]		4 [a]		60 [a]	0.3
Torasemide	56211-40-6	3.4	5.3	1.96	0.92	0.001	-0.04	2,700 [af]		3.5 [a]		200 [a]	1
Valproic Acid	99-66-1	4.8		2.58	2.18	0.4	-1.16	41,500 [ag]	498,300 [ag]	67 [a]	7,437	3,600 [a]	18

[1]DrugBank; [2]ACD/LogD Suite; [3]Calculated value, Eq. 6; [4]Calculated value, Eq. 7; [a]RxList; [b]DailyMed; [c]http://www.aapsj.org/abstracts/AM_2001/252.htm; [d]de Abreu et al., 2003; [e]Daley-Yates et al., 2001; [f]Morselli et al., 1990; [g]www.mhra.gov.uk/home/idcplg?IdcService=GET_FILE&dDocName=CON009658&RevisionSelectionMethod=Latest; [h]de Vane, 2006; [i]Abshagen et al., 1980; [j]http://dgrh.de/fileadmin/media/Die_DGRH/Jahreskongresse/Abstracts_2008_PDFs/K08_AO.01.pdf; [k]Chan et al., 2007; [l]http://www.patentstorm.us/patents/6495165/claims.html; [m]Malling et al., 2005; [n]http://www.gsk-clinicalstudyregister.com/files/pdf/ 25051.pdf; [o]Ouellet et al., 1998; [p]http://us.gsk.com/products/assets/us_flovent_hfa.pdf; [q]Haegeli et al., 2007; [r]www.apotex.com/us/en/products/downloads/bio/gemf_imtb_be.pdf; [s]Vachharajani et al., 1998; [t]Chen et al., 2007; [u]Hungund et al., 1979; [v]http://www4.wiwiss.fu-berlin.de/dailymed/page/drugs/702; [w]http://www.ixel.at/fachinfo.html#5.2; [x]Srinivas et al., 1996; [y]http://ch.oddb.org/ de/gcc/resolve/chapter/kinetic/pointer/:!fachinfo,10164376.; [z]Begg et al., 1999; [aa]Xue et al., 2003; [ab]http://www.kup.at/journals/abbildungen/gross/5005.html#start; [ac]http://www.aapsj.org/abstracts/AM_1998/ 2056.html; [ad]http://www.alopezie.de/diskussion/frauen/uploads/3_aldactone.pdf; [ae]http://www.emea.europa.eu/humandocs/PDFs/EPAR/ganfort/066806en6.pdf; [af]http://ch.oddb.org/de/gcc/resolve/pointer/:!fachinfo,10605141.; [ag]Panesar et al., 1989

Thereby, TD_{max} is the highest recommended daily dose for a drug, *M* is the percentage of the market penetration (default value of 0.01), *V* is the assumed amount of wastewater per inhabitant per day (default value of 200 L inh^{-1} day^{-1}) and *D* is the dilution factor of wastewater by surface water flow (default value of 10). TD_{max} values were file searched in a similar procedure as it was done for the other PK values (Table 3.1).

It has to be noticed, that the European legislation Phase I exposure estimation procedure (EMEA, 2006; Eq. 3.7) calculates *PEC*s of drugs for a reasonable worst case scenario, because for most drugs, the assumption that no metabolism in the patient and no biodegradation or retention in the sewage treatment plant take place, may not be true. Also, no account is taken of aggregate exposure situations, e.g. where the same active ingredient derives from several registered drugs. Thus, the estimated drug *PEC* values may be very different than the actual EnvCs. This translates into over- or underestimation of the FPC_{SS} (Eq. 3.2) and, based on *ER* estimations (Eq. 3.1), this may indicate potential environmental hazard of drugs which may not stand up to refined exposure assessments.

Pharmacodynamic parameter and target conservation analysis

Human drug target protein sequences were taken from Drug-Bank (Wishart et al., 2006, 2008) as identified through the CAS registry number of the drugs in question (Table 3.2). For identification of homologous protein in fish, these were used as 'query sequence' for a non-orthologous one way alignment against NCBI RefSeq zebrafish (*Danio rerio*) protein sequences (Pruitt et al., 2007) applying a BLAST algorithm (Altschul et al., 1990) with the online NCBI BLASTP program version 2.2.21 (http://blast.ncbi.nlm.nih.gov/Blast.cgi) comparing protein sequences. Sequence complexity filtering was set to default, the number of returned alignments to 100 and the EXPECT threshold (statistical significance threshold for reporting matches against database sequences) was 0.01. Results were sorted according to NCBI Bit scoring (normalised raw score see http://www.ncbi.nlm.nih.gov/BLAST/tutorial/Altschul-1.html). The protein with the highest Bit score was considered the protein with the highest homology/conservation to the human drug target and was taken as the fish target homologue. In a second step, for both human drug target and fish homologous protein, general biochemical activity (in Supporting information, Table SI-3.2) and more specific molecular functions (Table SI-3.1) according to Gene Ontology (GO) – criteria (http://www.geneontology.org/GO) were documented. GO criteria were received by searching the UniProt database (http://www.uniprot.org/). For general biochemical activity classification, the following GO criteria were selected: peroxidase (GO0004601), steroid hormone receptor (GO0003707), G-protein coupled receptor (GO0004930), enzyme regulator (e.g., GO0004867),

transporter (GO0006810), ion-transporter (GO0006811), transferase (GO0016740), kinase (GO0004713), oxidoreductase (GO0016491), steroid binding (GO0005496), transcription factor (GO0003700), peptidase (GO0006508), hydrolase (GO0016787), hormone (GO0005179) and cytokine (GO0005125) activity. For information about more specific molecular functions (pathway indication), the GO criteria (number) that described the function most exactly for the fish target homologue was documented. In a subsequent step, these GO criteria were searched for in the UniProt database for the human target proteins.

Homology between human and fish target was investigated in three ways (i) as the relative number of identical or similar amino acids (conservative substitutions) from fish protein compared to whole aligned sequence parts of the human protein target given in percent (Table SI-3.3), (ii) as relative bit scoring values from BLAST analysis (see description below; Table SI-3.3) and (iii) as comparison of GO criteria (Table SI-3.1). For all drugs, all documented targets were included in homology analysis.

As scoring values depend on the total length of the characterised proteins, (Fig. SI-3.1) they were normalised to scoring values received by aligning the human drug target proteins to human sequence data base as best value (see reference score in Table SI-3.1 and Fig. SI-3.3). Firstly, BLAST analysis of human and fish proteins were performed as described above by aligning the human target protein against NCBI RefSeq zebrafish protein sequences and the absolute score ($S[\text{abs}]_{\text{Human:Fish}}$) was documented. Secondly, the same human target protein was aligned against NCBI RefSeq human protein sequences for receiving the reference (optimal) score ($S[\text{ref}]_{\text{Human:Human}}$) when identical proteins are aligned. In a third step, relative scores were calculated by determining the absolute score from the reference score ($S[\text{rel}]_{\text{Human:Fish}}$; Eq. 3.8).

$$S[\text{rel}]_{\text{Human:Fish}} = S[\text{abs}]_{\text{Human:Fish}} / S[\text{ref}]_{\text{Human:Human}} \quad (3.8)$$

Results

In the following sections, the FPM results derived for the 42 selected drugs and the impact of the three main model parts, namely HPC_T, FPC_{ss} and target conservation, on the resulting *ER* estimates are considered. Further, the FPM hazard indication was compared for 7 drugs with reported long-term effects.

Estimates for HPC_T

The HPC_T is a parameter not accessible to direct experimental observation. As proxies, *AUC* or C_{max} values have been proposed by Huggett et al. (2003) for use in the FPM. In the available databases RxList and DailyMed, 15 C_{max} values and 8 *AUC*

Table 3.2. Numbers of targets (No. of Targets) and their protein name of the respective primary (Target 1), secondary (Target 2) and tertiary target (Target 3) described for each of the considered 42 pharmaceutical compounds (drugs) in their respective DrugBank (www.drugbank.ca) entry (DB-No.). The drugs identity is based on the chemical abstracts service registry number (see Table 3.1).

Common name	DB - No.	No. of Targets	Target 1	Target 2	Target 3
Aripiprazole	1238	3	5-hydroxytryptamine 1A receptor	5-hydroxytryptamine 2A receptor	D(2) dopamine receptor
Atenolol	335	1	Beta-1 adrenergic receptor		
Beclometasone	394	2	Corticosteroid-binding globulin	Annexin A1	
Betaxolol	195	1	Beta-1 adrenergic receptor		
Bicalutamide	1128	3	Androgen receptor	Oestrogen receptor beta	Interleukin-6
Bisoprolol	612	2	Beta-1 adrenergic receptor	Beta-2 adrenergic receptor	
Carbamazepine	564	1	Sodium channel protein type 5 subunit alpha		
Ciclesonide	1410	1	Corticosteroid-binding globulin		
Citalopram	215	1	Sodium-dependent serotonin transporter		
Clofibrate	636	2	Peroxisome proliferator-activated receptor alpha	Lipoprotein lipase	
Conj. Oestrogens	286	1	Ostrogen receptor		
Diclofenac	586	3	Prostaglandin G/H synthase 1	Prostaglandin G/H synthase 2	Transthyretin
Duloxetine	476	3	Sodium-dependent noradrenaline transporter	Sodium-dependent dopamine transporter	Sodium-dependent serotonin transporter
Entacapone	494	1	Catechol O-methyltransferase		
Eplerenone	700	1	Mineralocorticoid receptor		
Escitalopram	1175	1	Sodium-dependent serotonin transporter		
Ethinyl Estradiol	977	1	Oestrogen receptor		
Fenofibrate	1039	2	Peroxisome proliferator-activated receptor alpha	Metalloproteinase	
Fluoxetine	472	1	Sodium-dependent serotonin transporter		
Fluticasone Prop.	588	3	Corticosteroid-binding globulin	Cytosolic phospholipase A2	Glucocorticoid receptor
Furosemide	695	2	Solute carrier family 12 member 1	Sodium/potassium-transporting ATPase alpha-1 chain	

Gefitinib	317	1	Epidermal growth factor receptor		
Gemfibrozil	1241	3	Peroxisome proliferator-activated receptor alpha	Lipoprotein lipase	Solute carrier organic anion transporter family member 1B1
Irbesartan	1029	3	Type-1 angiotensin II receptor	Transcription factor AP-1	Angiotensinogen
Lapatinib	1259	2	Receptor tyrosine-protein kinase erbB-2	Epidermal growth factor receptor	
Lenalidomide	480	1	Prostaglandin G/H synthase 2		
Methylphenidate	422	3	Sodium-dependent noradrenaline transporter	Sodium-dependent dopamine transporter	Sodium-dependent serotonin transporter
Metoprolol	264	1	Beta-1 adrenergic receptor		
Milnacipran	4896	2	Sodium-dependent noradrenaline transporter	Sodium-dependent serotonin transporter	
Nadolol	1203	2	Beta-1 adrenergic receptor	Beta-2 adrenergic receptor	
Norethindrone	717	2	Progesterone receptor	Sex hormone-binding globulin	
Paliperidon	1267	2	5-hydroxytryptamine 2A receptor	D(2) dopamine receptor	
Paroxetine	715	3	5-hydroxytryptamine 2A receptor	Sodium-dependent noradrenaline transporter	Sodium-dependent serotonin transporter
Pioglitazone	1132	1	Peroxisome proliferator-activated receptor gamma		
Pregabalin	230	1	Voltage-dependent P/Q-type calcium channel subunit alpha-1A		
Progesterone	396	3	Oestrogen receptor	Cytochrome P450 17A1	Progesterone receptor
Propranolol	571	2	Beta-1 adrenergic receptor	5-hydroxytryptamine 1A receptor	
Sertraline	1104	3	5-hydroxytryptamine 1A receptor	Sodium-dependent serotonin transporter	Cytochrome P450 3A3
Spironolactone	421	2	Type-1 angiotensin II receptor	Mineralocorticoid receptor	
Timolol	373	1	Beta-1 adrenergic receptor		
Torasemide	214	1	Solute carrier family 12 member 1		
Valproic Acid	313	3	4-aminobutyrate amino-transferase, mitochondrial	Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	Histone deacetylase 9

values out of the 42 selected drugs were retrieved. Compared to *AUC* values (33 values), C_{max} data were more frequently reported and could be found for all 42 drugs, when file searching further public sources (Table 3.1).

The dependency of the modelled *ER* on the usage of either C_{max} or AUC_{conc} values was studied. Therefore, FPC_{ss} values (KBW and TGD model) were estimated by using a homogeneous data set of *PEC* and K_{OW} (D_{OW}) values (Table 3.1) and results are presented in Table 3.3. *ER* values for a single drug are within one order of magnitude at maximum, independent of the HPC_T parameter used. The difference between *ER* values based on either C_{max} or AUC_{conc} values was for 75% of the drugs less than a factor of 2.5 and for 95% of the drugs within a factor of 6. Only for clofibrate the difference between *ER*s was greater than 10 (12.6). As the HPC_T is directly compared with FPC_{ss} values for *ER* estimation (Eq. 3.1), the difference between $ER(AUC_{conc})$ and $ER(C_{max})$ values correlated directly with the difference of the respective AUC_{conc} and C_{max} values and was independent of the bioconcentration model used for FPC_{ss} prediction.

Overall, when using the KBW model for FPC_{ss} prediction, *ER* values spread over 8 orders of magnitude from $1.3 \cdot 10^{-2}$ to $1.0 \cdot 10^{6}$ for both parameters. Thus, the difference between substances is far larger than its dependency on the input parameter for therapeutic information as they spread only over one order of magnitude. One of four drugs investigated showed an $ER \leq 1$, independent of the usage of either C_{max} (9 drugs) or AUC_{conc} (11 drugs) values (Table 3.3). Only for 12% of all drugs studied, an $ER > 1{,}000$ was calculated.

The usage of AUC_{conc} compared to the C_{max} typically yielded the more conservative estimation.

Parameters to estimate the FPC_{ss}

The FPC_{ss} represents the concentration of the drug in arterial blood of fish at steady-state after uptake from ambient water (Eq. 3.2). The bioconcentration model for FPC_{ss} estimation in the FPM is based on the drug lipophilicity (K_{OW}) only (Eq. 3.3). Many drugs are polar and possess a low lipophilicity ($K_{OW} < 1$) so that the compounds uptake may not be driven by their lipophilicity only. Further, when their chemical structure shows the potential to dissociate at ambient pH regimes, it can be assumed that FPC_{ss} values are pH-dependent as lipophilicity is influenced by compound dissociation. The pH-dependence and thus the impact of the used FPC_{ss} parameter on *ER* was analysed considering the neutral drug forms (K_{OW}) utilising the KBW and the TGD model or the dissociated (apparent $K_{OW} - D_{OW}$) drug forms with the TGD model only (Fig. 3.1; Table 3.3). D_{OW} values were estimated for different pH values (pH 5, 7, 10). The range of pH 5–10 was taken to cover an environmentally range that might occur in surface waters. HPC_T (based on C_{max} values) and *PEC* values

were kept consistent for the *ER* estimate. Calculated *ER* values for the 42 drugs are presented in Fig. 3.1 and Table 3.3.

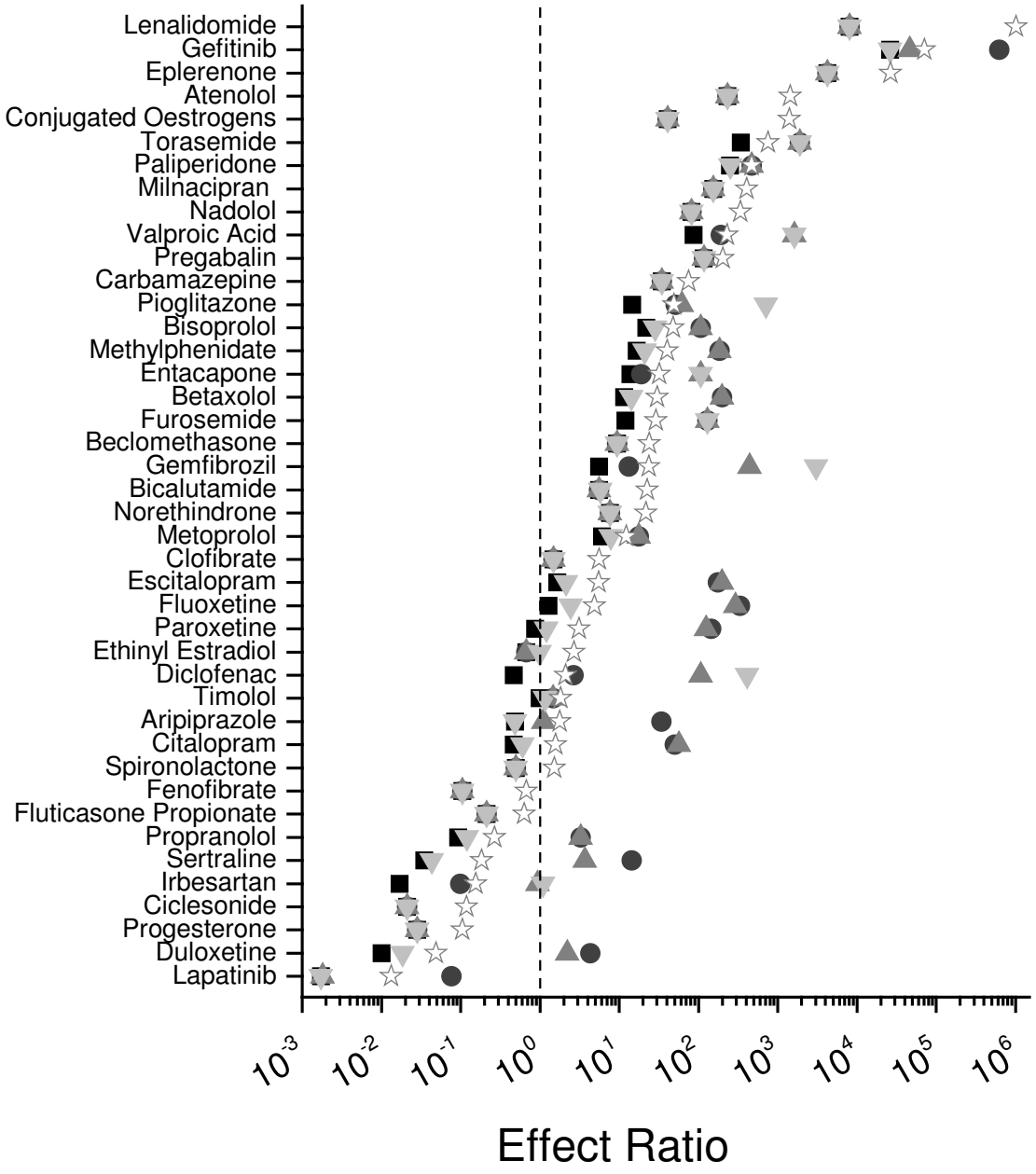

Figure 3.1. Calculated Effect Ratios (*ER*; Eq. 3.1) of 42 pharmaceutical compounds (drugs) dependent on the used bioconcentration model and apparent K_{OW} (D_{OW}) values at different pH's are shown. Hence, the FPC_{ss} parameter (Eq. 3.2) for the *ER* estimate were either based on the KBW model (✯ - Eq. 3.3) or the TGD model (■ – Eq. 3.4 and 3.5) with K_{OW}s as descriptor. For the *ER* dependence on dissociation, the TGD model was used with D_{OW}s at pH 5 (●), pH 7 (▲), and pH 10 (▼). For all FPC_{ss} estimates, the same *PEC* values were used and C_{max} values were used as proxy for HPC_T. Drugs are sorted for *ER* estimate based on the KBW model.

Table 3.3. Estimated effect ratio (*ER*) values for the considered 42 pharmaceutical compounds (drugs) as dependent on usage of different input values for the HPC_T and the FPC_{ss} parameter (Eq. 3.1) are presented. Hereby, C_{max} and AUC_{max} values were used as proxy for the HPC_T parameter while for the FPC_{ss} estimate (Eq. 3.2) two different bioconcentration model were considered ($K_{Blood:Water}$ estimate – KBW [Eq. 3.3] or *BCF* estimate – TGD [Eq. 3.4 and 3.5]). The descriptor for the KBW model was the K_{OW} only while for the TGD model also D_{OW} values at different pH were used. The input data were taken from Table 3.1. For all dark grey indicated *ER* values an $ER \leq 1$ was computed, while light grey indicated drug names signify, that an accurate FPC_{ss} prediction can be assumed when using the TGD model with D_{OW}'s. Furthermore, the homology characterisation as dependent on the methods and the drug target used (Target 1: primary target; Target 2: secondary target; Target 3: tertiary target) for each drug is shown. For each target estimated minimal (min) and maximal (max) homology values are provided, based on the alignment (BLAST) of primary protein structures (AA). Human and fish proteins compared by use of gene ontology criteria (yes: Similar GO criteria for human and fish protein reported).

Common Name	Effect Ratios										Homology								
	C_{max}					AUC_{conc}					Target 1			Target 2			Target 3		
	TGD				KBW	TGD				KBW	AA [%]		GO	AA [%]		GO	AA [%]		GO
	K_{OW}	D_{OW} pH 5	D_{OW} pH 7	D_{OW} pH 10	K_{OW}	K_{OW}	D_{OW} pH 5	D_{OW} pH 7	D_{OW} pH 10	K_{OW}	min	max		min	max		min	max	
Lapatinib	0.002	0.076	0.002	0.002	0.013	0.002	0.080	0.002	0.002	0.014	54	66	yes	59	75	yes			
Duloxetine	0.010	4.32	2.22	0.018	0.049	0.011	4.87	2.50	0.021	0.055	71	83		79	87	yes	69	82	yes
Irbesartan	0.017	0.099	0.939	1.10	0.154	0.007	0.039	0.370	0.433	0.061	48	68	yes	70	83	yes	18	47	yes
Ciclesonide	0.021	0.021	0.021	0.021	0.118	0.018	0.018	0.018	0.018	0.100	31	59	yes						
Progesterone	0.028	0.028	0.028	0.028	0.105	0.031	0.031	0.031	0.031	0.114	40	70	yes	47	68	yes	29	82	yes
Sertraline	0.035	14.5	3.67	0.043	0.183	0.028	11.6	2.95	0.035	0.147	70	81	yes	69	82	yes	55	74	yes
Propranolol	0.093	3.27	3.27	0.120	0.267	0.105	3.68	3.68	0.135	0.301	48	67	yes	75	87	yes			
Fenofibrate	0.105	0.105	0.105	0.105	0.671	0.200	0.200	0.200	0.200	1.28	69	82	yes	50	64				
Fluticasone Prop.	0.211	0.211	0.211	0.211	0.634	0.105	0.105	0.105	0.105	0.315	31	95	yes	71	83	yes	43	64	yes
Diclofenac	0.466	2.66	107	413	2.11	0.272	1.56	62.8	241	1.23	68	81	yes	75	87	yes	48	71	
Citalopram	0.467	50.0	56.5	0.603	1.57						69	82	yes						
Aripiprazole	0.487	34.1	1.11	0.487	1.78	0.265	18.5	0.603	0.265	0.965	70	81	yes	56	83		66	77	yes
Spironolactone	0.495	0.495	0.495	0.495	1.52	1.40	1.40	1.40	1.40	4.32	48	68	yes	44	63	yes			
Ethinyl Estradiol	0.674	0.674	0.674	0.997	2.70	0.147	0.147	0.147	0.218	0.590	40	70	yes						
Paroxetine	0.867	146	125	1.21	3.11	0.518	87.0	74.7	0.723	1.86	56	83		71	83		69	82	yes
Timolol	0.991	1.46	1.46	1.16	1.82						48	67	yes						
Fluoxetine	1.28	336	295	2.44	4.87	0.291	76.6	67.1	0.555	1.11	69	82	yes						
Clofibrate	1.48	1.48	1.48	1.48	5.58	0.118	0.118	0.118	0.118	0.443	69	82	yes	61	79	yes			

Escitalopram	1.65	177	199	2.13	5.54	1.24	133	150	1.60	4.17	69	82	yes							
Bicalutamide	5.58	5.58	5.58	5.81	22.5						38	80	yes	51	71	yes	8	38		
Gemfibrozil	5.60	13.3	440	3,043	23.7	4.72	11.2	371	2,563	19.9	69	82	yes	61	79	yes	43	64		
Metoprolol	6.07	17.7	17.7	7.83	12.3	2.91	8.51	8.51	3.76	5.89	48	67	yes							
Norethindrone	7.62	7.62	7.62	7.62	21.6	5.71	5.71	5.71	5.71	16.2	29	82		18	47	yes				
Beclomethasone	9.43	9.43	9.43	9.43	23.8	1.65	1.65	1.65	1.65	4.18	31	59	yes	59	75	yes				
Betaxolol	11.6	198	198	14.1	30.1	5.68	96.5	96.5	6.91	14.7	40	70	yes							
Furosemide	12.0	130	130	130	29.2	13.1	142	142	142	31.9	56	73	yes	87	94	yes				
Entacapone	13.9	19.1	106	106	32.2						50	75	yes							
Pioglitazone	14.5	51.8	61.8	714	49.2	15.9	56.8	67.7	782	53.9	63	78	yes							
Methylphenidate	16.7	184	184	21.1	40.6	3.87	42.8	42.8	4.90	9.44	71	83		79	87	yes	69	82	yes	
Bisoprolol	22.1	107	107	28.5	48.0	13.8	66.9	66.9	17.8	29.9	48	67	yes	49	71					
Carbamazepine	34.6	34.6	34.6	34.6	75.2						60	75	yes							
Conj. Oestrogens	41.1	41.1	41.1	41.1	1,403	51.7	51.7	51.7	51.7	1,768	40	70	yes							
Nadolol	81.3	81.3	81.3	81.3	340	44.9	44.9	44.9	44.9	188	48	67	yes	49	71					
Valproic Acid	87.1	190	1,632	1,632	229	15.6	34.1	293	293	41.0	62	86	yes	34	63	yes	50	71		
Pregabalin	116	118	118	118	202						68	78								
Milnacipran	153	153	153	153	406						71	83		69	82	yes				
Atenolol	234	234	234	234	1,438	227	227	227	227	1,396	48	67	yes							
Paliperidone	249	472	472	253	473						56	83								
Torasemide	343	1,911	1,911	1,911	760						56	73	yes							
Eplerenone	4,248	4,248	4,248	4,248	26,580	3,729	3,729	3,729	3,729	23,331	44	63	yes							
Lenalidomide	8,042	8,042	8,042	8,042	1.02E6	8,049	8,049	8,049	8,049	1.02E6	75	87	yes							
Gefitinib	26,271	622,992	46,342	26,271	71,364	7,289	172,856	12,858	7,289	19,800	59	74	yes							

● *KBW vs. TGD model.* The results show, that the difference between the *ER* values using the K_{OW} in either the KBW or the TGD model, were less than a factor of 4 for 75% of the drugs and within a factor of 10 for 95% of the drugs. Only for conjugated oestrogens (34.2) and lenalidomide (126.8) the difference between the resultant *ER*s was higher. Using the KBW model, 9 drugs showed an ER ≤1, compared to 16 drugs when the TGD model is used (Table 3.3). Thereby, *ER* values span a range of $2.0 \cdot 10^{-3}$ to $2.6 \cdot 10^{4}$ using the TGD model and of $1.3 \cdot 10^{-2}$ to $1.0 \cdot 10^{6}$ using the KBW model. The bigger difference for conjugated oestrogens and lenalidomide can be explained by the default *BCF* value of 1.41 for substances with log K_{OW} <1 in the TGD model.

Our results indicate that using the TGD model a more conservative *ER* compared to the usage of the KBW model is estimated. For substances, with log K_{OW} values ranging from 1 to 6 the resulting *ER* values differ less than factor 6 while for substances with log K_{OW} <1 and log K_{OW} >6 a larger difference in *ER* values is computed.

● D_{OW} *vs.* K_{OW}. The results show that the *ER* value strongly depends on the dissociation of the drug. Overall, the *ER*s calculated for non-dissociated (K_{OW}) or dissociated drugs (D_{OW}s) were around two orders of magnitude on average different with a maximum of 886 fold for diclofenac (D_{OW} at pH 10). At pH 5, this difference was covered by a factor of 17 and 263 for 75% and 95% of drugs, respectively. At pH 7 a similar situation to that described for pH 5 was found, namely the difference of *ER* values was found to differ within a factor of 19 and 222 for 75% and 95% of the drugs, respectively. At pH 10, the *ER* values varied a factor of 1.5 and 65, for 75% and 95% of the drugs, respectively. Different hazard ranking of drugs depending on the pK_a of the dissociating compounds were computed for the pH values used. Compared to the 16 drugs where with the TGD model an *ER* ≤1 is calculated based on K_{OW} and C_{max} values; 8, 8 and 12 drugs were identified based on D_{OW}s at pH 5, 7 and 10, respectively (Table 3.3).

Regarding the findings of Fu et al. (2009) that the TGD model predicts *BCF* values with a good accuracy only for neutral compounds, weak bases (pK_a <6) and weak acids (pK_a >7.5), drugs were examined for these requirements. Only 16 out of 42 drugs match the required pK_a range, while for the other 26 compounds the *BCF* estimate may be expected to deviate substantially from experimental *BCF* values. For 7 out of these 16 drugs an *ER* ≤1 was estimated using either K_{OW} or D_{OW} at pH 5, 7 and 10 (Table 3.3).

For the drugs considered, the *ER* estimate using the TGD model is more conservative. Further, it was found, that the pH regime with its influence on apparent K_{OW}s (D_{OW}s) of drugs has a larger impact on *ER* values (three orders of magnitude) compared to the usage of either the KBW or the TGD model (one order of

magnitude). Concerning the accuracy of prediction for estimated FPC_{ss} (TGD model), only 16 out of 42 drugs were either non-dissociating substances, weak acids (pK_a >7.5) or weak bases (pK_a <6). For these compounds, a good correlation could be expected.

Selected compounds

Drugs with an *ER* ≤1 were among all tested therapeutic classes (analgetic drugs, cytostatic agents, psychoactive drugs, hormones, broncholytic drugs, β-blockers, lipid-lowering agents, diuretic agents) and no general trend could be derived discriminating therapeutic classes with systematically lower *ER* values.

A multiple regression analysis was done, to investigate the influence of the different model parameters on the model output. This regression resulted in significant influences of all three modelling terms ($K_{Blood:Water}$ or *BCF* from K_{OW} (D_{OW}), HPC_T from C_{max} and *PEC* from TD_{max}). The main driving factor hereby was K_{OW} (D_{OW}) followed by HPC_T and *PEC* terms that had the same impact on the modelling result (data not shown). This result indicate that not one single model parameter alone is responsible for the modelling outcome, regarding the studied drugs.

Target conservation

The following section will concentrate on the aspect of target conservation that is prerequisite for the application of the FPM (Huggett et al., 2003). Only, if a conserved drug target for a defined drug is expected to be present in fish, an effective concentration similar to the HPC_T may be implied in fish. Hence, conservation of the human drug targets for all 42 drugs investigated in the present study in fish was studied to (i) characterise the likelihood of the model applicability, to study (ii) the biochemical background for extrapolating therapeutic data from human to fish and (iii) and to analyse the plausibility of the proposed threshold approach (Christen et al., 2010; Kostich and Lazorchak, 2008). The zebrafish was used as a model organism for fish, as it has a sequenced genome and conservation analysis is thus more reliable compared to any other fish species.

► *Multiple drug targets*

All drug targets for the 42 drugs investigated are proteins and are documented in the DrugBank database. For 18 drugs one specific target, for 12 drugs two targets and for 12 drugs three targets were found (Table 3.2; Fig. 3.2). The second or third target were (i) paralogs of the first target (e.g., Prostaglandin G/H synthase 1 or 2 as first and second target for diclofenac), (ii) from different protein classes such as biotransformation enzymes (e.g., target 2 of progesterone), (iii) binding globulins (e.g., target 2 of norethindrone), or (iv) miscellaneous structures (e.g., target 3 of

furosemide). All human targets or protein binding partners described for one drug could possibly be of interest to characterise a human–fish homology. It has to be noticed, that all data were retrieved between April and June 2008. Hence, the detailed results of the present study are dependent on the retrieval date and might change with increasing knowledge.

To study the dependency of conservation status on different targets from one single drug, human drug target conservation of all targets described for one drug was compared. Results are shown in Fig. 3.2 and Table SI-3.3.

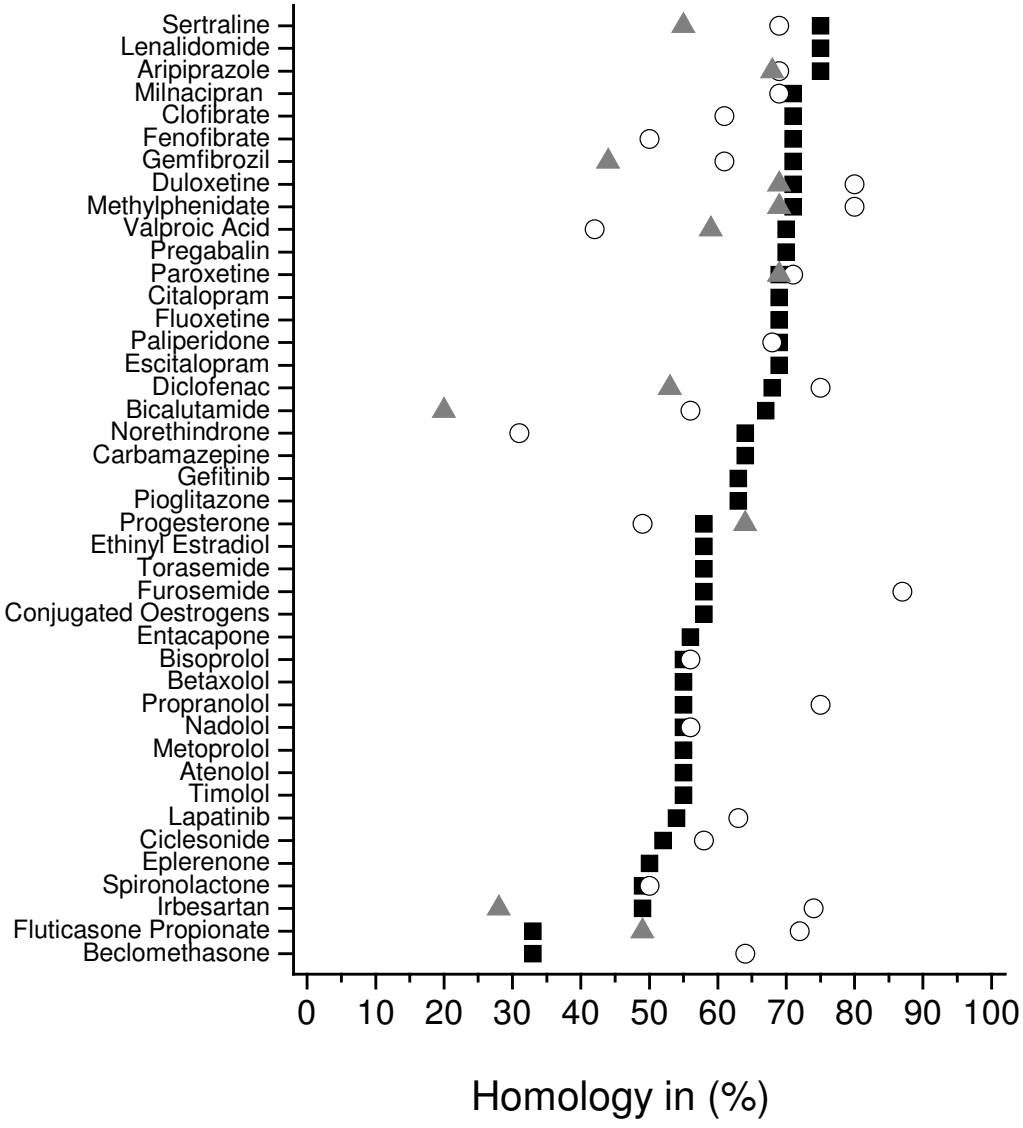

Figure 3.2. Conservation of drug targets in fish as dependent on different targets described for a pharmaceutical compound (drug) are depicted. Up to three drug targets for each drug considered were found in DrugBank: (■) Target 1, (○) Target 2 and (▲) Target 3. Homology was calculated as percentage of identical amino acids in aligned fish and human protein sequences. Drugs are sorted for homology of Target 1.

For some drugs, conservation (fish–human) status of the targets from one single drug were quite different such as for irbesartan target 2 (74%, 2-transcription factor AP-1) and target 3 (28%, angiotensinogen) or bicalumide target 1 (67%, androgen receptor) and target 3 (20%, interleukin). As expected, conservation status of paralogous target proteins were similar such as 75% and 69% for 5-hydroxytryptamine 1A and 5-hydroxytryptamine 2A, respectively; 55% and 56% for beta-1 and beta-2 adrenergic receptors, or 71% and 69% for sodium dependent noradrenaline and serotonin transporter.

The results indicate that the conservation status of several targets from one single drug might be quite different, which is important for the process of decision if a certain human drug has an equivalent drug target in fish or not.

► *Homology criteria*

Regarding the assessment for presence of a potential drug target in fish for all detected 42 human drug targets, conservation in zebrafish was analysed with respect to different approaches: (i) a direct primary structure sequence alignment followed by different homology calculation methods and (ii) based on gene ontology criteria. The results are presented in Fig. 3.3 and the full data retrieved are documented in Table SI-3.3.

In Fig. 3.3, human drug targets are sorted according to relative identity of amino acids to *Danio rerio* proteins. For all analysed 42 human target proteins, a significant match with a fish protein was yielded. Hereby, homology of drug targets was found to range from 20% to 87% with a mean of 59%. Further, 79% of the drug targets in humans showed a homology with *Danio rerio* proteins of greater than 50%. Amino acids with similar functional groups such as glutaminic acid and aspartic acid are likely to perform similar functions within a protein and are thus considered as conservative substitutions. Hence, in addition to identical amino acids, similar amino acids were included in homology characterisation of human and fish proteins. As shown in Fig. 3.3, homology calculated as a relative number of similar in addition to identical amino acids between proteins is higher than only including identical amino acids. This difference amounted to 14% on average. Homology of all characterised 42 human and fish proteins was found to range between 38% and 94% with a mean of around 73% and only three drug targets were seen to have a homology of less than 50% in their fish analogue.

The relative number of identical or similar amino acids between human and fish proteins mainly depends on the length of the compared sequences. In many cases, the full length proteins are aligned by BLAST; however, in some cases the aligned protein sequences cover only parts/domains of the full length proteins. This is the case e.g., for the Progesteron receptor where 383 out of 933 total amino acids for the

full length protein are aligned, or the Androgen receptor where 418 amino acids are aligned although the protein consists of 919 amino acids.

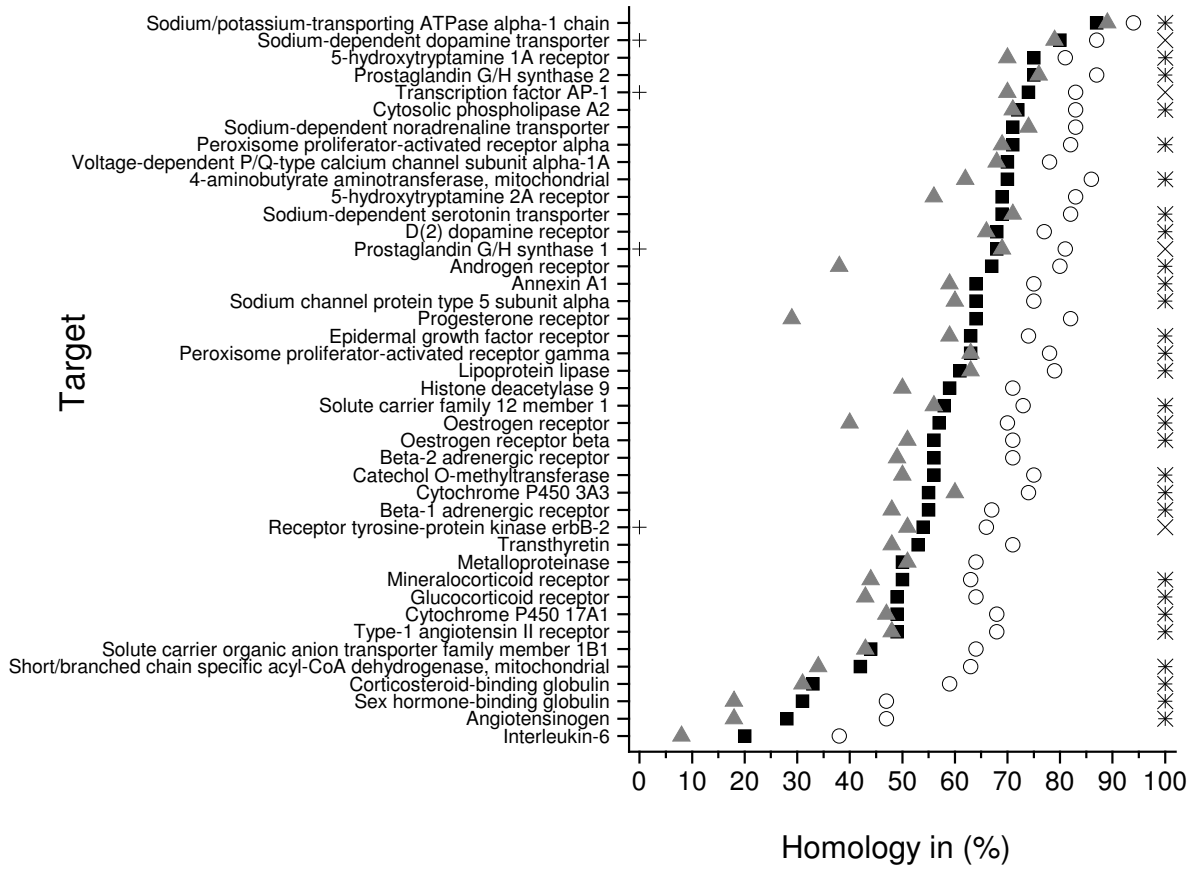

Figure 3.3. Homology of fish analogues to 42 human drug target proteins as dependent on the homology characterisation method are shown. All homology investigations are based on the most similar fish– and human proteins which were identified by use of a BLAST. Human drug targets as Bits were aligned against proteins of the *Danio rerio* RefSeq database. Homology was determined in five different ways. Homology was calculated based on primary protein structure (■) as percentage of identical amino acids in aligned sequences, (○) as percentage of similar amino acids in aligned sequences, (▲) as relative Bit score which was calculated from reference Bit score when human proteins were aligned against proteins of Human RefSeq database (Eq. 3.8) and secondly based on functional criteria referring to GO criteria, (×) as identical biochemical action (100%) or (+) as identical specific molecular function (100%).

Therefore, in a third step, the homology between human drug targets and fish analogues was considered in terms of Bit scores. By applying this method, homology values of all characterised 42 human and fish proteins fell in a range between 8% and 89% with a mean of around 53% (Fig. 3.3). Interestingly, most homology data were similar to the homology data calculated by considering identical amino acids only (Fig. 3.3). As expected, highest differences between the score based method and the one based on identical amino acids was found for the Progesterone and

Androgen receptor, for which alignments were based on only small parts/domains of the protein.

As one additional approach to address the FPM assumption of similar target structures in human and fish, mainly as a tool to help for decision if a target with low conservation status might be still a potential target in fish, the proteins were compared considering their biochemical function by making use of their GO criteria. These were grouped into broad classes of biochemical activity and more specific processes. The limiting factor for the latter, however, was the limited knowledge about fish proteins. GO criteria for both human protein and fish homologue were compared and functional homology was considered as 100% if identical GO numbers could be found. All results are shown in Fig. 3.3. For 73% of the fish analogues, GO criteria have been described (Tables SI-3.1 and SI-3.2). All human drug targets and fish analogues showed 100% homology considering general biochemical activities. Also, for drug targets with a low structural homology such as Interleukin 6 or Angiotensinogen (20% and 28% of identical amino acids, respectively), no difference in general biochemical action between the human and fish proteins was described. Studying the more specific molecular processes, few differences in GO criteria, e.g., for receptor tyrosine-protein kinase erbB-2, Prostaglandin G/H synthase, transcription factor AP-1 and sodium dependent dopamine transporter were found. Moreover, differences for Prostaglandin G/H synthase and sodium dependent dopamine transporter were only due to an inconsistent database entry (see Table SI-3.1). Thus, our results indicate that, considering functional domains derived from protein structure, most target proteins, independent of their sequence homology, seem to be conserved in fish.

Hazard indication

This section will concentrate on the assumption of the FPM that a similar value for FPC_{ss} and for HPC_T may indicate an unintended effect in fish. For this purpose, *ER* values were computed at determined low observed effect concentration (LOEC; instead of the *PEC*, Eq. 3.2) from the few available long-term fish studies of the considered drugs. Hence, if an *ER* value ≤1 is computed, then the FPM indicates the drugs potential to cause long-term effects and thus may be assumed to mirror previous experimental findings.

Only for 7 out of the 42 considered drugs LOECs from long-term fish studies could be retrieved from the literature. Reported LOEC values, with the respective observed effects, the used fish species and the exposure time are presented (Table 3.4). Further, the estimated *ER* value range, modelled in the FPM with different input parameter (K_{OW}, D_{OW}, C_{max}, AUC_{max}) and bioconcentration models (KBW, TGD model) at the LOEC, are shown. The *ER* reasonable worst case (RWC) depicts the

estimated *ER* value at the lowest HPC_T parameter (C_{max} or AUC_{conc}) and the FPC_{ss} estimate for neutral pHs (TGD model; D_{OW} at pH 7) (Table 3.4). This FPC_{ss} estimate was used, because the LOEC values have been determined at neutral pHs in the long-term fish studies used. It was found, that for 3 out of the 7 drugs an *ER* RWC ≤1 was computed, while for 2 drugs an ER RWC >100 was calculated. For the 3 drugs with an ER RWC ≤1, HPC_T values were found to be 14.1, 176, and 9.6 times lower than estimated FPC_{ss} values based on determined LOECs for atenolol, ethinhyl estradiol, and propranolol, respectively (Table 3.4). Thus, based on calculated *ER* RWC values, the FPM has equally either over- or underestimated drugs reported long-term effects.

Discussion

The FPM proposed by Huggett et al. (2003) aims to indicate potential hazards from drugs exposed to fish at EnvC, by making use of human therapeutic data. The usefulness of such a comparative extrapolation approach depends on sufficient discriminatory power and the likelihood of the findings. This work is among the first employing the FPM for a diverse range of drugs undertaking to study the influence of the different model input parameters. The discussion will concentrate on two aspects. Firstly, the model dependencies, as related to the possible input variables will be considered. Secondly, the model plausibility will be discussed with respect to current evidence for long-term drug effects in fish.

Proxies for HPC_T

For the estimation of the HPC_T that is used as an internal dose level indicative for a biological effective concentration, Huggett et al. (2003) proposed to use C_{max} or *AUC* values as dose metrics. These PK parameters mainly depend on the drug administration regime, dosing and the receiving biosystem. The latter is characterised by interindividual differences (e.g., Ginsberg et al., 2002; Renwick and Lazarus, 1998), so that neither a single value nor a homogeneous data quality of the *AUC* and C_{max} values for a given drug can be expected. Moreover, an *AUC* operationalisation using an additional PK parameter ($t_{1/2}$) needs to be performed. Surprisingly, the variability of the retrieved *ER*s when either using C_{max} or AUC_{conc} values was found to lie within one order of magnitude and the ranking of predicted *ER* values for the drugs was similar. Thus, the modelling can be considered as robust considering the different HPC_T input data used. In part, this might be attributed to the employment of the lowest reported C_{max} and *AUC* values during database search, which was done to mirror reasonable worst case scenarios. Direct blood plasma concentrations or even

Table 3.4. Comparison of the FPM derived effect ratios (*ER*; Eq. 3.1) with reported experimental short-term and long-term effect data for fish. The *ER* range depicts the span of all computed *ER* values of the seven considered pharmacological compound (drug) at the lowest effect concentration (LOEC) while the *ER* of the reasonable worst case (RWC) depicts the lowest estimated value at neutral pHs (D_{OW} at pH 7) with their respective HPC_T and FPC_{ss} values. Indicated drug names signify, that an accurate FPC_{ss} prediction can be assumed when using the TGD model with D_{OW}'s (see Table 3.3). For the Experimental Effect Characterisation, from literature retrieved short-term assay data for the standardised 96-h fish assay with lethal endpoint are presented with their respective median lethal concentration (LC_{50}) and the used organism (*Pimephales promelas* - PP, *Oncorhynchus mykiss* – OM, *Oryzias latipes* – OL, *Danio rerio* - DR). Further, literature reported observed effects from a long-term assay are presented with their respective lowest observed effect concentration (LOEC), used exposure time and fish specie.

Common Name	Estimated FPM Effect Prediction				Experimental Effect Characterisation							
					Long-term assays					Short-term assays		
	***ER* range**	***ER*** RWC	HPC_T RWC (µg L^{-1})	FPC_{ss} * at pH 7 (µg L^{-1})	**LOEC** (µg L^{-1})	**Observed Effect**	**Organism**	**Exposure time** (days)	**Literature**	**LC_{50}** (µg L^{-1})	**Organism**	**Literature**
Atenolol	0.071 - 0.450	0.071	320	4,520	3,200	reproduction	PP	21	Winter et al., 2008	>100,000	OL	Kim et al., 2009
Carbama-zepine	277 - 601	277	1,900	6.86	1	histo- and cyto-pathological alterations kidney, liver, gill	OM	28	Triebskorn et al., 2007	45,870	OL	Kim et al., 2009
Diclofenac	0.272 - 413	62.8	341	5.43	1	histo- and cyto-pathological alterations kidney, liver, gill	OM	28	Triebskorn et al., 2004	10,100	OL	Nassef et al., 2009
Ethinyl Estradiol	0.006 - 0.101	0.006	0.012	2.12	$4.0 \cdot 10^{-3}$	lack of sexual differntiation in male	PM	305	Länge et al., 2001	1,700	DR	Versonnen et al., 2003
Fluoxetine	1.16 - 1,345	269	43.3	0.161	0.1	increased plasma estrdiol levels	OL	28	Foran et al., 2004	705 #	PP	Brooks et al., 2003
Metoprolol	14.6 - 88.7	42.5	60.1	1.41	1	histo- and cyto-pathological alterations kidney, liver, gill	OM	28	Triebskorn et al., 2007	>100,000 #	OL	Huggett et al., 2002
Propranolol	0.003 - 0.118	0.118	14.8	141	100	delay in time to hatch, increase in female gonadal somatic index	PP	21	Giltrow et al., 2009	11,400	OL	Kim et al., 2009

* FPC_{ss} estimation based on LOEC values instead of *PEC* (Eq. 3.2); # 48-h acute fish assay

receptor-available doses, which would provide more realistic therapeutic dose estimates were scarcely available and could thus not be used as proxies.

Proxies for FPC_{ss}

The robustness of the model outcome considering the FPC_{ss} term was studied regarding the drugs‘ lipophilicity, as the lipophilicity is assumed to be the driving uptake force into fish. Two approaches were considered, the usage of either the KBW model, which is based on the $K_{Blood:Water}$ estimate according to Fitzsimmons et al. (2001) or the TGD model based on the *BCF* estimate in the TGD (2004) according to Fu et al. (2009) comprising the impact of drug dissociation by using D_{OW} instead of K_{OW} values as variable for the *BCF* estimate.

One central finding was, that the $ER(FPC_{ss})$ values resulting from using either the KBW or the TGD model differed within a factor of 6 for log K_{OW} values between 1 and 6 (Table 3.3). This good correlation is not surprising as the TGD model was elaborated to estimate the *BCF*, which is the substance partitioning between fish and ambient water while the KBW model strives to estimate the compounds partitioning between the fish blood plasma and the ambient water, which can be seen as a ’sub-compartment’ within the fish (e.g., Liao et al., 2005; Nichols et al., 2004). Moreover, using *BCF* values is in good agreement with the FPM principles, as the FPC_{ss} (as well as the HPC_T) is used as surrogate for the concentration of the drugs active form at the respective active site. As the *BCF* is an important criteria in the chemical risk assessment, a large data set of experimental determined *BCF*s are available. Hereby the *BCF* correlation function (Eq. 3.4; TGD, 2004) is based on 267 substances, while the $K_{Blood:Water}$ estimate is based on 11 (Eq. 3.3; Fitzsimmons et al., 2001), still the slopes of the linear regressions are quite similar (0.73, Eq. 3.3 [$K_{Blood:Water}$] and 0.85, Eq. 3.4 [*BCF*]), which reflects the high correlation between the $K_{Blood:Water}$ and the *BCF*. For substances with log K_{OW} <1, the TGD model according to Fu et al. (2009) uses a default value of *BCF* = 1.41. This can be explained by the fact that fish mainly consists of water and when substances partitioning more into water than in a lipophilic phase still an equilibrium between the water phase in fish and ambient water may be reached. For more lipophilic substances (log K_{OW} >6), a polynomial model is used for the *BCF* estimate in the TGD (2004; Eq. 3.5). This correlation is recommended, as measured *BCF*s were found to decrease with increasing K_{OW} (e.g., Connell and Hawker, 1988). Different concepts suggest an explanation for this observation e.g., reduced membrane permeability, reduced lipid solubility or simply analytical artefacts, however, determined *BCF* data were better described by this function (TGD, 2004). Overall it can be assumed, that for neutral, non-polar and non-ionised compounds, the *BCF* estimate recommended in the TGD (TGD, 2004; Eqs.

3.4 and 3.5) according to Fu et al. (2009) may more accurately predict a FPC_{ss} over a wider K_{OW} range compared to the KBW model.

Assuming that using the TGD model with K_{OW} values an accurate *BCF* and therewith a suitable FPC_{ss} could be estimated, ignores that only 7 out of the 42 considered drugs do not dissociate (Table 3.1). Therefore, for more than 80% of the selected drugs the TGD model using K_{OW} values may not be expected to yield accurate *BCF* estimates. However, Fu et al. (2009) compared different *BCF* estimation models for ionisable organic chemicals and achieved good predictions when using the TGD model based on D_{OW} values. By estimating D_{OW} values for relevant ambient environmental pH's using the TGD model, an up to 900 times lower FPC_{ss} value was computed when compared to the respective K_{OW} value. These results clearly demonstrate that dissociation can not be disregarded. Fu et al. (2009) have shown that for monovalent weak organic acids (pK_a >7.5) or bases (pK_a <6) good correlations between *BCF* and D_{OW} (r^2 0.91, r^2 0.80; respectively) exist in fish, while for monovalent strong organic acids (pK_a <6) like valproic acid and bases (pK_a >8) like duloxetine only poor correlations are found (r^2 0.38, r^2 0.34; respectively). This was explained by the fact that at neutral ambient pH's, larger fractions of the non-dissociated form of weak acids and bases are present and therewith $D_{OW} \approx K_{OW}$. Hereby the uptake behaviour is assumed to be similar to those of neutral compounds (Fu et al., 2009). For strong organic acids and bases, the TGD model using D_{OW} values is found to give poor predictions (Fu et al., 2009). Also more sophisticated models, like the dynamic cell model based on the Fick–Nernst–Planck equation or the simpler *BCF* estimation model of Meylan et al. (1999) have been tested by Fu et al. (2009) for their predicting power. Though the cell model is described to show some acceptable results for strong organic bases, none of these models is assumed to estimate *BCF*s with a good accuracy in fish (Fu et al., 2009). Nevertheless, when applying the proposed pH limit, for weak acids (pK_a >7.5) and bases (pK_a <6) for using the TGD model, almost 40% of the selected drugs fulfil this requirement. Thus, a suitable FPC_{ss} estimate could be assumed. For the remaining 60% of the selected drugs, lipophilicity may not be the primary driving uptake force while other processes, e.g., ion trap effects, electrical interaction with proteins or active transport may be dominant. However, as for uptake processes different from lipophilicity driven partitioning up to now no quantitative models exist, the usage of the TGD model with D_{OW} may be seen as a starting-point.

As discussed before, the estimation of an appropriate FPC_{ss} is quite challenging because many drugs are polar and show the potential to dissociate while existing *BCF* prediction models have a good predictive power for neutral, non-ionised and non-polar substances only. Furthermore, in the environment, the uptake is not restricted to bioconcentration alone, but could also occur via dietary and other

ambient sources and thus may alter the FPC_{ss} substantial (e.g., Arnot and Gobas, 2006). A question to be further investigated is whether the FPC_{ss} and HPC_T have the same values at respective active sites for a drug in its active form, where the drug–receptor interaction occurs. However, according to the current European guideline (EMEA, 2006), many currently used drugs have not been evaluated by a systematic ERA. As a result, FPC_{ss} estimates, which use the TGD model with D_{OW} as predictor, may yield *ER* values for a comparative hazard assessment, which will provide reasonable guidance for prioritising drugs for an extended ERA.

Extrapolation of target conservation

Drugs are designed for elucidating effects by specific interaction with defined molecular (mostly protein-) targets. For most drugs, these molecular targets and their intended biological action in humans are well understood (Wishart et al., 2008), although, knowledge on pharmaceutical action is steadily increased. The conservation of human target proteins in fish is one basic requirement for applying models to extrapolate between species, and has been discussed in the literature (e.g., Huggett et al., 2003; Gunnarsson et al., 2008; Kools et al., 2008; Kostich and Lazorchak, 2008). The results presented here confirm the findings of the previous studies (e.g., Gunnarsson et al., 2008; Kostich and Lazorchak, 2008) that many human drug targets may also be present in fish as originally assumed by Huggett et al. (2003). In addition, it has to be recognised as shown for the 42 drugs considered here, that more than one proteinogenic target may exist for a drug and that these may indeed be relevant for understanding unintended effects in fish. As each target showed specific sequence homology to its fish homologue, it might thus be appropriate to assign several values to characterise target homology in interspecies effect extrapolation.

From a qualitative point of view, one might question if proteins having significant matches but only low sequence homology (such as Interleukin, or Angiotensinogen) might still be considered a potential similar target in fish. The inclusion of conservative substitutions in addition to identical amino acids in the calculation of homology might help to further investigate conservation of proteins with low sequence homology. However, the question of the presence of similar targets and thus similar drug functions in fish might primarily be answered by considering also the functional level such as the biochemical action (Länge and Dietrich, 2002). If fish proteins with low sequence homology show similar biochemical actions or are from the same class of proteins (such as iontransporters) like their human analogues, this might hint to the presence of an appropriate target and similar functions in fish. A comparison of criteria according to gene ontology in this work showed that, independent of the ‘degree’ of homology, all fish proteins and their human

homologues belong to the same protein classes and are expected to perform the same biochemical reactions. This can be taken as further indication, that a large number of human drugs might have equivalent targets and provoke similar reaction in fish independent of the degree of homology.

The above analysis was triggered by efforts of defining an overall cut-off value for target and functional similarity between humans and fish as has been proposed by Christen et al. (2010). One suggestion is that human and fish targets may be regarded as sufficiently similar if their primary sequence homology is higher than 50%. Several arguments against using cut-off values can be anticipated from our findings. Firstly, for all investigated proteins, independent of their degree of homology, significant matches in fish and similar proteins were found on a functional base and thus interaction with drugs can not be excluded for the proteins with lower than 50% sequence homology. Secondly, depending on the criteria for homology, such as identical amino acids, conservative substitutions, correction for length of alignment, considering active sites or protein domains, homology values are quite variable for each target. For the progesterone receptor e.g., they ranged from 29% to 82% and for cyclooxigenases they lie between 67% and 100% (Grosser et al., 2002).

Along traditional pharmacological thinking, overall it can be questioned whether a proof of pharmaceutical target interaction can be taken to extrapolate on efficacy and even adverse biological action (e.g., Owen et al., 2007). These subsequent steps are more difficult to assess when only target information are available. For human β1 and β2 adrenergic receptors e.g., it has been shown that while they are 54% identical at the sequence level they are still rather different in terms of there response efficacies upon the same agonists (Owen et al., 2007). This issue may be approached e.g., by the description of affinity constants for the anticipated fish targets. The subsequent comparison of these to affinity constants for the human drug targets, which are determined regularly during drug development, might then help to refine extrapolation of effective doses in fish. The normalisation of the *ER* by accounting for different target sensitivity across species as proposed by Owen et al. (2007) would be an option to refine the FPM. Effective concentrations might also depend on the number of target molecules present in fish and on the involved toxicity pathways and physiological outcome of the target–molecule interactions. These have been shown to be different between human and fish for the case of inhibition or induction of lipolyses caused by β adrenergic receptors (Owen et al., 2007; Vianen et al., 2002). Prediction of effective concentrations thus might be improved through establishing functional assays, which address several endpoints along the drug effect pathway including drug–target interaction and physiological or phenotypic outcome rather than focussing on binding only. A first step in the direction of functional

consideration gene ontology (GO) based pathway analysis may be performed. As demonstrated, this may qualitatively support anticipation of functional similarity based only on sequence information. GO entries, however, are not available for all proteins in fish.

In conclusion, our results can be taken to demonstrate that all investigated human drug targets seem to have homologue structures in fish. Moreover, drugs might have multiple targets that could be important in extrapolating drug action. The definition of cut-off values to discriminate against the existence of similar drug targets in fish seems difficult at the current level of understanding. However, conservation of targets might differ regarding other fish species, which will become accessible with more genomic sequencing in future studies. Further research on the reliability of the FPM, should thus focus on supplementing structural considerations with functional information not only for a qualitative understanding of drug effects in fish but also to quantitatively estimate whether therapeutic concentrations are precise indicators of effects.

What is the FPM good for?

One basic assumption in the FPM is, that similar FPC_{ss} and HPC_T values indicate receptor mediated responses in fish (Huggett et al., 2003). This could be taken to mean that the FPM accurately predicts the kinetics and dynamics of any given drug. Thus, an accurate prediction of single drugs on long-term effects in fish could be made. When considering the uncertainties of the estimated FPC_{ss} values (see Section *Proxies for* FPC_{ss}) and the possible different target sensitivity across species (see Section *Extrapolation of target conservation*), a substantial error in computed *ER* values and thus in long-term effect prediction has to be envisaged. Assuming that with the FPM an accurate prediction for the considered target and the resulting effect could be made, when refining the FPC_{ss} and the HPC_T parameters, still a substantial error in the prediction on long-term effects in fish may be possible. In principle, two crucial scenarios have to be considered, (i) by the interaction with the intended target other biochemical cascades beside the induction of the therapeutically used may be possible and (ii) even an interaction with alternative target could be possible. In both cases, long-term effects may occur at lower concentration than the HPC_T and the respective EnvC.

A further challenge is related to the fact, that even if there are any receptor-mediated responses in fish, these may not necessarily lead to detrimental cellular or even organismic effects. For example, Purdom et al. (1994) found a significant induction of vitellogenin for ethinyl estradiol in rainbow trout at concentrations as low as 0.1 ng L^{-1} during a 10-day exposure, while, during a full-life-cycle exposure, only a 40-times higher concentration caused impairments in sexual differentiation in male

fathead minnow (Länge et al., 2001). Thus, even if there is a receptor-mediated drug response in fish, a subsequent detrimental long-term effect may still not be found (e.g., Corcoran et al., 2010; Sumpter and Johnson, 2008).

Despite the difficulties to make accurate predictions on long-term effects for individual drugs, the FPM with its principal approach may yield *ER* values useful for a comparative hazard ranking. The application of the FPM to 42 drugs, resulted in up to 18 drugs for which a hazard to fish with *ER* values ≤1 was indicated. For most of the indicated drugs, the *PEC* was below 1 µg L^{-1} and these concentrations are well below the typically determined median effect concentrations (EC_{50}) received from the acute effect observations (EC_{50} >100 µg L^{-1}; Fent et al., 2006; Santos et al., 2010; Table 3.4). At the same time, they are in the range of observed EnvC (EnvC $1 \cdot 10^{-4}$ to 10 µg L^{-1}; Fent et al., 2006; Santos et al., 2010). The determined LOECs from the long-term experiments showed (Table 3.4), that at concentrations of about 1 µg L^{-1} several drugs may induce adverse effects. From this it could be concluded that, the FPM does indeed indicate drugs at EnvC, which potentially induce long-term effects.

Fick et al. (2010) derived the EnvC of 500 drugs that is needed to achieve an *ER* value of 1, using the FPM with K_{OW} (KBW model) and C_{max} values as predictors. They found that only for 22% of the drugs the EnvC has to be higher than 1 µg L^{-1} (Fick et al., 2010). For most of these drugs, no ERA has been performed according to the current European guideline (EMEA, 2006). Thus, one may rise the question, how to deal with a situation where for many drugs currently occurring in the environment there will be no chronic fish toxicity information for many years to come. Therefore, the FPM may be a tool for a comparative hazard ranking, that provides guidance on prioritising active drugs from a large pool with no information on environmental fate and effects, for a refined ERA and possible extended fish studies.

Conclusion

Although the FPM model is based only on HPC_T and FPC_{ss}, where HPC_T is using either C_{max} or AUC_{conc} values and FPC_{ss} estimated from EnvC (or *PEC*) and a bioconcentration model based on K_{OW} (D_{OW}), the model shows the potential to (i) indicate potential environmental hazards of drugs at *PEC* (ii) that are far below typically measured EC_{50} values from acute effect observations and (iii) it shows discrimination power against potentially less hazardous drugs. Since most of the drugs have the potential to dissociate, using the *BCF* estimate recommended in the TGD (2004) according to Fu et al. (2009) may yield more accurate FPC_{ss} estimate for neutral drugs, weak acids (pK_a >7.5) and bases (pK_a <6) compared to the KBW model proposed by Huggett et al. 2003. Our results can be taken to demonstrate that all investigated human drug targets have analogues in fish, hereby, one drug might have multiple targets that could be important in extrapolating drug action. The

definition of cut-off values to discriminate against the existence of similar drug target in fish seems impossible at the current level of understanding.

Our results support the notion, that the FPM might be a tool to prioritise drugs according to their predicted environmental hazards based on human therapeutic data. Empirical validation against long-term fish studies is not adequately possible, however, the FPM could be a promising tool to change this shortcoming by identifying priority candidates for testing and thus foster tailor-made risk assessment for drugs currently in use.

Conflict of interest

All authors declare that they have no competing financial interests.

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Supporting Information

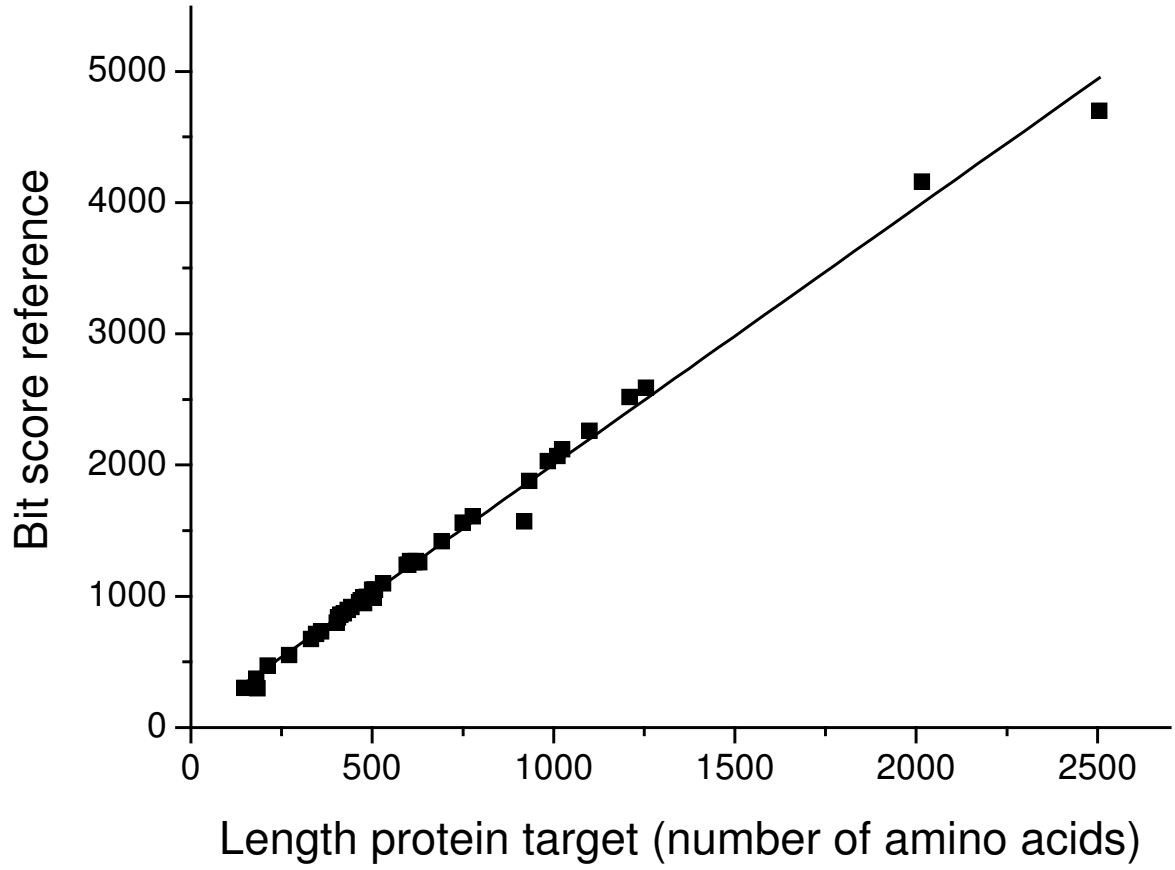

Figure SI-2.1. Correlation of calculated bit scores from Human-Human protein target alignment and length of proteins ([Bit score reference] = 1.96•[Length protein target] + 47.62; r^2 0.998).

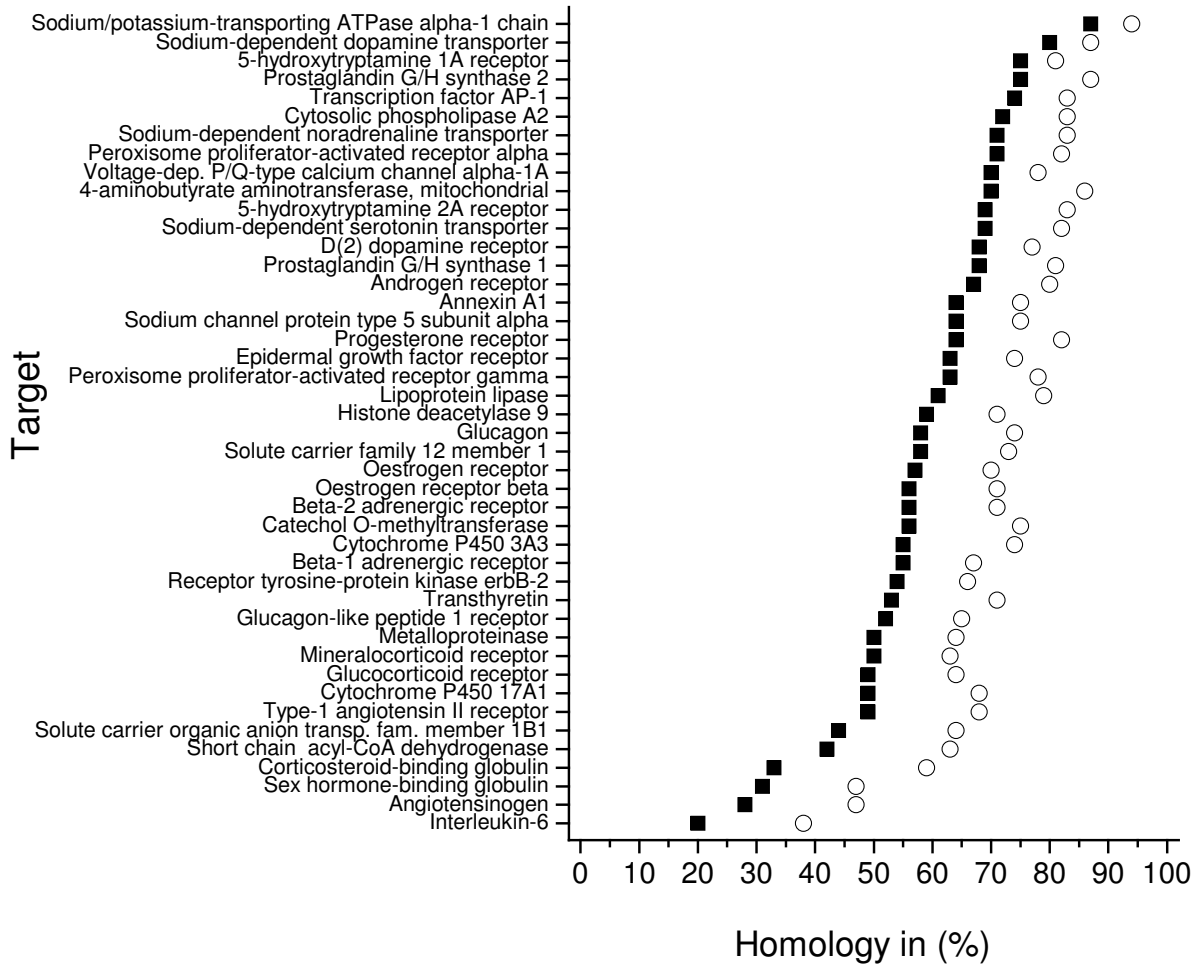

Figure SI-3.2. Comparison of Bit scores derived from alignment of either Human- to Human target proteins (○) or Human- to fish (■) proteins.

Table SI-3.1. Comparison of Human drug target protein functions with functions of *Danio rerio* protein homologs according to GO criteria which were derived from the UniProt (www.uniprot.org) database (n.a.- not available).

Human Drug Target	*Danio rerio* - Analogon	Function of Human Protein	GO-Reference	Function of *Danio rerio* Protein	GO-Reference
Prostaglandin G/H synthase 1	Prostaglandin-endoperoxide synthase 1	Prostaglandin biosynthetic process	GO0001516	Prostglandin-endoperoxide synthase activity	GO0004666
Prostaglandin G/H synthase 2	Prostaglandin-endoperoxide synthase 2b	Response to oxidative stress	GO0006979	Response to oxidative stress	GO0006979
Oestrogen receptor	Oestrogen receptor 1	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Progesterone receptor	Progesterone receptor	Steroid hormone receptor activity	GO0003707	n.a.	n.a.
Beta-1 adrenergic receptor	Beta-1-adrenergic receptor	Adrenoceptor activity	GO0004935	Adrenoceptor activity	GO0004935
Type-1 angiotensin II receptor	Similar to Type-1B angiotensin II receptor (AT1B)	Angiotensin type II receptor activity	GO0004945	Angiotensin type II receptor activity	GO0004945
Mineralocorticoid receptor	Nuclear receptor subfamily 3, group C, member 2	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Peroxisome proliferator-activated receptor alpha	Peroxisome proliferator activated receptor alpha b	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Peroxisome proliferator-activated receptor gamma	Peroxisome proliferator activated receptor gamma	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Glucagon-like peptide 1 receptor	Similar to glucagon-like peptide 1 receptor	Elevation of cytosolic calcium ion concentration	GO0007204	n.a.	n.a.
Corticosteroid-binding globulin	Serine proteinase inhibitor, clade A, member 1	Serine-type endopeptidase inhibitor activity	GO0004867	Serine-type endopeptidase inhibitor activity	GO0004867

Sodium-dependent noradrenaline transporter	Similar to norepinephrine transporter	Monoamine transport	GO0015844	n.a.	n.a.
Sodium-dependent serotonin transporter	Solute carrier family 6 (neurotransmitter transporter, serotonin)	Serotonin transmembrane transporter activity	GO0015222	Serotonin transmembrane transporter activity	GO0015222
5-hydroxytryptamine 2A receptor	5-Hydroxytryptamine (serotonin) receptor 2 A	Serotonin receptor signaling pathway	GO0007210	n.a.	n.a.
5-hydroxytryptamine 1A receptor	5-Hydroxytryptamine (serotonin) receptor 1A a	G-protein coupled receptor activity	GO0004930	G-protein coupled receptor activity	GO0004930
Sodium channel protein type 5 subunit alpha	Sodium channel, voltage gated, type VIII, alpha b	Voltage-gated sodium channel activity	GO0005248	Voltage-gated sodium channel activity	GO0005248
4-aminobutyrate amino-ransferase, mitochondrial	4-Aminobutyrate aminotransferase	4-Aminobutyrate transaminase activity	GO0003867	4-Aminobutyrate transaminase activity	GO0003867
Voltage-dependent P/Q-type calcium channel subunit alpha-1A	Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	Voltage gated calcium channel activity	GO0005245	n.a.	n.a.
Catechol O-methyltransferase	Si:dkey-13a21.15	Catechol O-methyltransferase activity	GO0016206	Catechol O-methyl-transferase activity	GO0016206
Receptor tyrosine-protein kinase erbB-2	v-erb-b2 Erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog	Peripheral nervous system development	GO0007528	Myelination of anterrior lateral line nerve axons	GO0048914
Androgen receptor	Androgen receptor	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Epidermal growth factor receptor	Epidermal growth factor receptor	Transmembrane receptor protein tyrosine kinase activity	GO0004714	Transmembrane receptor protein tyrosine kinase activity	GO0004714

Table SI-3.1. *continued*

Human Drug Target	*Danio rerio* - Analogon	Function of Human Protein	GO-Reference	Function of *Danio rerio* Protein	GO-Reference
Solute carrier family 12 member 1	Solute carrier family 12 (potassium/chloride transporters), member 2	Sodium:potassium:chloride symporter activity	GO0008511	Sodium:potassium: chloride symporter activity	GO0008511
Cytochrome P450 17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1	Monooxygenase activity	GO0004497	Monooxygenase activity	GO0004497
Sex hormone-binding globulin	Sex hormone binding globulin	Androgen binding	GO0005497	Androgen binding	GO0005497
Beta-2 adrenergic receptor	Adrenergic receptor, beta 2	Beta2-adrenergic receptor activity	GO0004941	n.a.	n.a.
Transcription factor AP-1	c-Jun protein	Transforming growth factor beta receptor signaling pathway	GO0007179	Wnt receptor signaling pathway through beta-catenin	GO0060070
Metalloproteinase	Matrix metallopeptidase 17 (membrane-inserted)	Metalloendopeptidase activity	GO0004222	n.a.	n.a.
Lipoprotein lipase	Lipoprotein lipase	Lipoprotein lipase activity	GO0004465	Lipoprotein lipase activity	GO0004465
Glucagon	Novel protein similar to vertebrate glucagon (GCG)	Regulation of insulin secretion	GO0050796	n.a.	n.a.
Annexin A1	Annexin A1a	Phospholipase inhibitor activity	GO0004859	Phospholipase inhibitor activity	GO0004859
Cytosolic phospholipase A2	Cytosolic phospholipase A2, group IVA	Phospholipase A2 activity	GO0004623	Phospholipase A2 activity	GO0004623
Sodium-dependent dopamine transporter	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	Monoamine transmembrane transporter activity	GO0008504	Neurotransmitter:sodium symporter acticity	GO0005887
D(2) dopamine receptor	Dopamine receptor D2b	Dopamine receptor activity	GO0004952	Dopamine receptor activity	GO0004952

Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	Short-chain acyl-CoA dehydrogenase	Acyl-CoA dehydro-enase activity	GO0003995	Acyl-CoA dehydro-genase activity	GO0003995
Oestrogen receptor beta	Oestrogen receptor 2a	Steroid hormone receptor activity	GO0003700	Steroid hormone receptor activity	GO0003700
Sodium/potassium-transorting ATPase alpha-1 chain	ATPase, Na+/K+ transporting, alpha 1a.4 polypeptide	ATPase activity, coupled to transmembrane movement of ions, phosphorylative mechanism	GO0015662	ATPase activity, coupled to transmembrane movement of ions,phosphorylative mechanism	GO0015662
Angiotensinogen	Angiotensinogen	Serine-type endopeptidase inhibitor activity	GO0004867	Serine-type endopeptidase inhibitor activity	GO0004867
Solute carrier organic anion transporter family member 1B1	Similar to solute carrier organic anion transporter family, member 1C1	Sodium-independent organic anion transmembrane transporter activity	GO0015347	n.a.	n.a.
Glucocorticoid receptor	Nuclear receptor subfamily 3, group C, member 1	Glucocorticoid metabolic process	GO0031946	Glucocorticoid receptor activity	GO0004883
Cytochrome P450 3A3	Cytochrome P450, family 3, subfamily A, polypeptide 65	Monooxygenase activity	GO0004497	Monooxygenase activity	GO0004497
Histone deacetylase 9	Similar to histone deacetylase 5	Histone deacetylase binding	GO0042826	n.a.	n.a.
Interleukin-6	Similar to interleukin 6	Interleukin-6 receptor binding	GO0005138	n.a.	n.a.
Transthyretin	Similar to Transthyretin (prealbumin, amyloidosis type I)	Thyroid hormone transmembrane transporter activity	GO0015349	n.a.	n.a.

Table SI-3.2. Comparison of general biochemical activities of Human drug target proteins and *Danio rerio* homologs according to GO criteria which were derived from the UniProt (www.uniprot.org) database (n.a.- not available).

Target Human	*Danio rerio* - Analogon	Biochemical Reaction Human	GO reference	Biochemical Reaction Fish	GO reference
Prostaglandin G/H synthase 1	Prostaglandin-endoperoxide synthase 1	Peroxidase activity	GO0004601	Peroxidase activity	GO0004601
Prostaglandin G/H synthase 2	Prostaglandin-endoperoxide synthase 2b	Peroxidase activity	GO0004601	Peroxidase activity	GO0004601
Oestrogen receptor	Oestrogen receptor 1	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Progesterone receptor	Progesterone receptor	Steroid hormone receptor activity	GO0003707	n.a.	n.a.
Beta-1 adrenergic receptor	Beta-1-adrenergic receptor	G-protein coupled receptor activity	GO0004930	G-protein coupled receptor activity	GO0004930
Type-1 angiotensin II receptor	Similar to Type-1B angiotensin II receptor (AT1B)	G-protein coupled receptor activity	GO0004930	G-protein coupled receptor activity	GO0004930
Mineralocorticoid receptor	Nuclear receptor subfamily 3, group C, member 2	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Peroxisome proliferator-activated receptor alpha	Peroxisome proliferator activated receptor alpha b	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Peroxisome proliferator-activated receptor gamma	Peroxisome proliferator activated receptor gamma	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Glucagon-like peptide 1 receptor	Similar to glucagon-like peptide 1 receptor	G-protein coupled receptor activity	GO0004930	n.a.	n.a.
Corticosteroid-binding globulin	Serine proteinase inhibitor, clade A, member 1	Enzyme regulator activity	n.a.	Enzyme regulator activity	n.a.

Sodium-dependent noradrenaline transporter	Similar to norepinephrine transporter	Transporter activity	GO0006810	n.a.	n.a.
Sodium-dependent serotonin transporter	Solute carrier family 6 (neurotransmitter transporter, serotonin	Transporter activity	GO0006810	Transporter activity	GO0006810
5-hydroxytryptamine 2A receptor	5-Hydroxytryptamine (serotonin) receptor 2 A	G-protein coupled receptor activity	GO0004930	n.a.	n.a.
5-hydroxytryptamine 1A receptor	5-Hydroxytryptamine (serotonin) receptor 1A a	G-protein coupled receptor activity	GO0004930	G-protein coupled receptor activity	GO0004930
Sodium channel protein type 5 subunit alpha	Sodium channel, voltage gated, type VIII, alpha b	Ion transporter activity	GO0006811	Ion transporter activity	GO0006811
4-aminobutyrate aminotransferase, mitochondrial	4-Aminobutyrate aminotransferase	Transferase activity	GO0016740	Transferase activity	GO0016740
Voltage-dependent P/Q-type calcium channel subunit alpha-1A	Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	Ion transporter activity	GO0006811	n.a.	n.a.
Catechol O-methyltransferase	si:dkey-13a21.15	Transferase activity	GO0016740	Transferase activity	GO0016740
Receptor tyrosine-protein kinase erbB-2	v-erb-b2 Erythroblastic leukemia viral oncogene homolog 2, neuro/glioblastoma derived oncogene homolog	Kinase activity	GO0004713	Kinase activity	GO0004713
Androgen receptor	Androgen receptor	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Epidermal growth factor receptor	Epidermal growth factor receptor	Protein tyrosine kinase activity	GO0004713	Protein tyrosine kinase activity	GO0004713

Table SI-3.2. *continued*

Target Human	*Danio rerio* - Analogon	Biochemical Reaction Human	GO reference	Biochemical Reaction Fish	GO reference
Solute carrier family 12 member 1	Solute carrier family 12 (potassium/chloride transporters), member 2	Ion transporter activity	GO0006811	Ion transporter activity	GO0006811
Cytochrome P450 17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1	Oxidoreductase activity	GO0016491	Oxidoreductase activity	GO0016491
Sex hormone-binding globulin	Sex hormone binding globulin	Steroid binding	GO0005496	Steroid binding	GO0005496
Beta-2 adrenergic receptor	Adrenergic receptor, beta 2	G-protein coupled receptor activity	GO0004930	n.a.	n.a.
Transcription factor AP-1	c-Jun protein	Transcription factor activity	GO0003700	Transcription factor activity	GO0003700
Metalloproteinase	Matrix metallopeptidase 17 (membrane-inserted)	Peptidase activity	GO0006508	n.a.	n.a.
Lipoprotein lipase	Lipoprotein lipase	Hydrolase activity	GO0016787	Hydrolase activity	GO0016787
Glucagon	Novel protein similar to vertebrate glucagon (GCG)	Hormone activity	GO0005179	n.a.	n.a.
Annexin A1	Annexin A1a	Enzyme regulator activity		Enzyme regulator activity	
Cytosolic phospholipase A2	Cytosolic phospholipase A2, group IVA	Hydrolase activity	GO0016787	Hydrolase activity	GO0016787
Sodium-dependent dopamine transporter	Solute carrier family 6 (neurotransmitter transporter, dopamine), member 3	Transporter activity	GO0006810	Transporter activity	GO0006810

D(2) dopamine receptor	Dopamine receptor D2b	G-protein coupled receptor activity	GO0004930	G-protein coupled receptor activity	GO0004930
Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	Short-chain acyl-CoA dehydrogenase	Oxidoreductase activity	GO0016491	Oxidoreductase activity	GO0016491
Oestrogen receptor beta	oestrogen receptor 2a	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Sodium/potassium-transporting ATPase alpha-1 chain	ATPase, Na+/K+ transporting, alpha 1a.4 polypeptide	Ion transporter activity	GO0006811	Ion transporter activity	GO0006811
Angiotensinogen	Angiotensinogen	Enzyme regulator activity	n.a.	Enzyme regulator activity	n.a.
Solute carrier organic anion transporter family member 1B1	Similar to solute carrier organic anion transporter family, member 1C1	Transporter activity	GO0006810	n.a.	n.a.
Glucocorticoid receptor	Nuclear receptor subfamily 3, group C, member 1	Steroid hormone receptor activity	GO0003707	Steroid hormone receptor activity	GO0003707
Cytochrome P450 3A3	Cytochrome P450, family 3, subfamily A, polypeptide 65	Oxidoreductase activity	GO0016491	Oxidoreductase activity	GO0016491
Histone deacetylase 9	Similar to histone deacetylase 5	Transcription factor binding	GO0008134	n.a.	n.a.
Interleukin-6	Similar to interleukin 6	Cytokine activity	GO0005125	n.a.	n.a.
Transthyretin	Similar to Transthyretin (prealbumin, amyloidosis type I)	Transporter activity	GO0006810	n.a.	n.a.

Table SI-3.3. Protein names, Drugbank source, number of amino acid residues of the Human drug target protein and the respective *Danio rerio* homolog. Based on the primary protein structure, homologies between the homologs were calculated as percentage of either the number of identical amino acids (AA), the number of similar amino acids or the relative Bit score. The relative Bit score was yielded by dividing the reference Bit score derived from a Human- to Human protein alignment by the Bit score derived from the Human- to *Danio rerio* protein alignment (Eq. 3.8).

Human Target Name	**AA Sequence** Source Drugbank	**Number Residues Protein Human**	**Protein Name** ***Danio rerio***	**RefSeq Acession Number** ***Danio rerio***	**Number Residues Protein Fish**	**% Homology** identical AA	**AA Homology** identical AA	**% Homology** similar AA	**AA Homology** similar AA	**Bit score**	**Reference Bit score**	**% Homology Relative Bit score**
Prostaglandin G/H synthase 1	1050	599	Prostaglandin-endoperoxide synthase 1	NP_705942	597	68	398/579	81	474/579	854	1240	69
Prostaglandin G/H synthase 2	480	604	Prostaglandin-endoperoxide synthase 2b	NP_001020675	606	75	441/588	87	512/588	967	1268	76
Oestrogen receptor	1357	595	Oestrogen receptor 1	NP_694491.1	569	57	271/468	70	330/468	496	1243	40
Progesterone receptor	717	933	Progesterone receptor	XP_001923769	617	64	246/383	82	315/383	541	1878	29
Beta-1 adrenergic receptor	195	477	Beta-1-adrenergic receptor	NP_001122161	390	55	237/424	67	288/424	458	949	48
Type-1 angiotensin II receptor	1029	359	Similar to Type-1B angiotensin II receptor (AT1B)	XP_001334570	358	49	168/342	68	234/342	353	731	48
Mineralocorticoid receptor	700	984	Nuclear receptor subfamily 3, group C, member 2	XP_001334570	970	50	513/1014	63	647/1014	887	2025	44
Peroxisome proliferator-activated receptor alpha	1039	468	Peroxisome proliferator activated receptor alpha b	NP_001096037	459	71	337/470	82	389/470	672	970	69
Peroxisome proliferator-activated receptor gamma	1132	505	Peroxisome proliferator activated receptor gamma	NP_571542	527	63	328/513	78	402/513	665	1049	63
Corticosteroid-binding globulin	1410	405	Serine proteinase inhibitor, clade A, member 1	NP_001071226	429	33	128/378	59	224/378	258	838	31

Sodium-dependent noradrenaline transporter	4896	617	Similar to norepinephrine transporter	XP_694138	678	71	443/618	83	517/618	929	1263	74
Sodium-dependent serotonin transporter	215	630	Solute carrier family 6 (neurotransmitter transporter, serotonin	NP_001035061	646	69	437/628	82	515/628	900	1263	71
5-hydroxy-tryptamine 2A receptor	715	471	5-Hydroxytryptamine (serotonin) receptor 2 A	XP_688270	458	69	257/368	83	306/368	541	971	56
5-hydroxy-tryptamine 1A receptor	1104	422	5-Hydroxytryptamine (serotonin) receptor 1A a	NP_001116793	398	75	312/414	81	339/414	613	870	70
Sodium channel protein type 5 subunit alpha	564	2016	Sodium channel, voltage gated, type VIII, alpha b	NP_001038588	1954	64	1308/2020	75	1527/2020	2479	4160	60
4-aminobutyrate aminotransferase, mitochondrial	313	500	4-aminobutyrate aminotransferase	NP_958906	408	70	287/408	86	352/408	645	1046	62
Voltage-dependent P/Q-type calcium channel subunit alpha-1A	230	2505	Calcium channel, voltage-dependent, P/Q type, alpha 1A subunit	XP_690548.3	2349	70	1785/2533	78	1978/2533	3206	4700	68
Catechol O-methyltransferase	494	271	si:dkey-13a21.15	NP_001077312	264	56	128/227	75	172/227	277	553	50
Receptor tyrosine-protein kinase erbB-2	1259	1255	V-erb-b2 erythro-blastic leukemia viral oncogene homolog 2 neuro/glioblastoma derived oncogene homolog	NP_956413.2	1275	54	705/1285	66	859/1285	1307	2585	51
Androgen receptor	1128	919	Androgen receptor	NP_001076592	868	67	282/418	80	337/418	600	1571	38

Table SI-3.3. *continued*

Human Target Name	**AA Sequence** Source Drugbank	**Number Residues Protein Human**	**Protein Name** ***Danio rerio***	**RefSeq Acession Number** ***Danio rerio***	**Number Residues Protein Fish**	**% Homology** identical AA	**AA Homology** identical AA	**% Homology** similar AA	**AA Homology** similar AA	**Bit score**	**Reference Bit score**	**% Homology Relative Bit score**
Epidermal growth factor receptor	317	1210	Epidermal growth factor receptor	NP_919405.1	1191	63	756/1195	74	889/1195	1486	2523	59
Solute carrier family 12 member 1	310	1099	Solute carrier family 12 (potassium/ chloride transporter) member 2	NP_001002080	1136	58	663/1131	73	834/1131	1273	2259	56
Cytochrome P450 17A1	396	508	Cytochrome P450, family 17, subfamily A, polypeptide 1	NP_997971 NP_997884	519	49	239/479	68	327/479	495	1049	47
Sex hormone-binding globulin	783	402	Sex hormone binding globulin	NP_001007152	381	31	107/343	47	162/343	143	799	18
Beta-2 adrenergic receptor	612	413	Adrenergic receptor, beta 2	XP_001919091	456	56	226/400	71	285/400	426	862	49
Transcription factor AP-1	1029	331	c-Jun protein	NP_956281	308	74	246/331	83	278/331	475	675	70
Metalloproteinase	1039	183	Matrix metallopeptidase 17 (membrane-inserted)	XP_698601	588	50	77/151	64	97/151	151	298	51
Lipoprotein lipase	1241	475	Lipoprotein lipase	NP_571202	511	61	284/460	79	367/460	627	994	63
Annexin A1	394	345	Annexin A1a	NP_861423	340	64	221/343	75	258/343	417	712	59
Cytosolic phospholipase A2	588	749	Cytosolic phospholipase A2, group IVA	NP_571370	741	72	537/739	83	617/739	1104	1556	71
Sodium-dependent dopamine transporter	422	620	Solute carrier family 6 (neurotransmitter transporter, do-pamine), member 3	NP_571830	629	80	486/604	87	531/604	999	1266	79

D(2) dopamine receptor	1267	443	Dopamine receptor D2b	NP_922918	452	68	311/452	77	352/452	610	920	66
Short/branched chain specific acyl-CoA dehydrogenase, mitochondrial	313	432	Short-chain acyl-CoA dehydrogenase	NP_001003743	405	42	159/371	63	234/371	308	897	34
Oestrogen receptor beta	1128	530	Oestrogen receptor 2a	NP_851297	553	56	292/521	71	371/521	1437	1101	51
Sodium/potassium-transporting ATPase alpha-1 chain	695	1023	ATPase, Na^+/K^+ transporting, alpha 1a.4 polypeptide	NP_571764	1024	87	895/1024	94	966/1024	4879	2118	89
Angiotensinogen	1029	485	Angiotensinogen	NP_932329	454	28	131/466	47	223/466	446	996	18
Solute carrier organic anion transporter family member 1B1	1241	691	Similar to solute carrier organic anion transporter family, member 1C1	XP_001337850	695	44	291/660	64	427/660	1559	1417	43
Glucocorticoid receptor	588	777	nuclear receptor subfamily 3, group C, member 1	NP_001018547	746	49	384/777	64	505/777	1781	1612	43
Cytochrome P450 3A3	1104	504	Cytochrome P450, family 3, subfamily A, polypeptide 65	NP_001032515 NP_001018633	517	55	278/501	74	371/501	1520	989	60
Histone deacetylase 9	313	1011	Similar to histone deacetylase 5	XP_685659	1115	59	619/1049	71	754/1049	2671	2072	50
Interleukin-6	1128	212	Similar to interleukin 6	XP_001919693	229	20	45/217	38	83/217	87	473	8
Transthyretin	586	147	Similar to Trans-thyretin (prealbumin amyloidosis type I)	XP_001919368	129	53	66/124	71	89/124	363	302	48

Chapter 4

Dynamics of organochlorine contaminants in surface water and in *Myriophyllum aquaticum* plants of the river Xanaes in central Argentina

René Schreiber, Carlos A. Harguinteguy and Martin D. Manetti

In Preparation

Dynamics of organochlorine contaminants in surface water and in *Myriophyllum aquaticum* plants of the river Xanaes in central Argentina

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Abstract

The dynamics of organochlorine pesticides and their major metabolites were studied in surface waters and plants of the river Xanaes (province Córdoba, Argentina). The results of the five months monitoring study showed similar contamination levels in the mountain and in the agricultural area, in both water and plants. The concentrations of compounds detected in the surface water were below 4.5 ng L^{-1}, while concentrations of these substances in *Myriophyllum aquaticum* plants were below 5 µg kg^{-1} (dry weight) with the exception of trans-permethrin (17.6 µg kg^{-1}, dry weight). As no significant differences in the contamination level between samples from the mountain and the agricultural area were observed, it was speculated that these compounds were residues of former use. Further, the concentration-time-trends for organochlorine compounds in the submerged plants showed a generally similar elimination behaviour independent of compound and sampling site, indicating an integral rather then a substance-specific process such as partitioning between the plant and the ambient water. Since rooted macrophytes can take up contaminants via roots, sediments may be the principal source. It is speculated that the concentration decrease over time correlates either with decreased sources of contamination reduced during the almost rainless five-months of sampling or the dependency of compounds desorption rate from sediments on temperature. Furthermore, a concentration increase was found in the plant samples before the concentration decrease was observed for the two detected metabolites p,p'-DDE and endosulfan sulfate. This finding was explained by the possible

metabolisation of the parent compounds. This interpretation was supported by the observation of a similar concentration-time profile of endosulfan sulfate in the surface water. To understand the dynamics of these compounds in the river area more deeply, further research should include the study of the rivers sediment.

Introduction

Organochlorine pesticides (OCPs) such as p,p'-DDT, endrin or lindane were used intensively for decades and e.g. endosulfan is still used in Argentinian agriculture. Because of the toxicity and/or the persistence of OCPs in the environment most of these were prohibited for commercial production and use. Though many of them have not been used for years, OCPs and their metabolites (e.g. p,p'-DDE, endosulfan sulfate) are commonly found in Argentina in different environmental matrixes at low concentrations (e.g. UNEP report, 2002).

OCPs were determined in Argentinian water bodies at concentrations up to µg L^{-1} levels (e.g. UNEP report, 2002). Because of their physicochemical properties such as lipophilicity, they accumulate in aquatic organisms e.g. water plants (e.g. Doust et al., 1994; Gobas et al., 1991). As water plants are food and habitat to many water organisms, they play a key role in aquatic ecosystems. During entry of contaminants by e.g. run-off from agricultural areas, rooted submerged macrophytes with their limited mobility accumulate lipophilic compounds during exposure. Therefore, they can be used as local in-situ biomonitors of waterborne contaminants (e.g. Gobas et al., 1991).

The occurrence of OCPs in environmental matrixes is not just regional, but worldwide due to distributing processes such as atmospheric transport (e.g. Simonich and Hites, 1995). Further processes like spray-drift, or washing-off from plant surfaces during application also have to be considered. Because of their hydrophobic character, these compounds are mainly found associated with organic matter in soil (e.g. Knezovich et al., 1987; Warren et al., 2003). Although, OCPs are associated with sediments, they could be remobilised during e.g. flood events, where they can reach concentrations which are toxic towards exposed wildlife (e.g. Grote et al., 2005). This could, for example, occur annually in areas of intensive agriculture during the first rainfalls after a dry season (e.g. Schulz et al., 2001).

The purpose of this study was to characterise the dynamics of selected OCPs and their main metabolites in the submerged *Myriophyllum aquaticum* plant and surface water at an agricultural and a non-agricultural sampling site of the river Xanaes (province Córdoba, Argentina), directly after the wet season. This was based on the hypothesis that water is the main contamination source. Thus we evaluated (i) the level of contamination of OCPs, (ii) the impact of OCPs run-off from agricultural area and (iii) the remobilisation of OCPs during the wet season.

Material and Methods

Chemicals and standard solutions

Hexane (H), methanol, dichloromethane (DCM) and methyl tert-butyl ether (MTBE) were purchased in pesticide grade from Sintorgan (Sintorgan S.A., Buenos Aires, Argentina). Silica gel and Na_2SO_4 (both analytical grade) were activated for 12 h at 180°C before use. Polychlorinated biphenyl congener 103 (PCB 103; CAS-RN 60145-21-3) was used as an internal standard and was purchased from AccuStandard (AccuStandard Inc., New Haven, CT, USA). Mixtures of standards were used for identification and quantification, purchased from AccuStandard (AccuStandard Inc., USA). One mixture of standards (AS-PBR) consists of α-chlordane, γ-chlordane, chlorobenzilate, chloroneb, chlorothalonil, DCPA, etridiazole, hexachlorobenzene, cis-permethrin, trans-permethrin, propachlor and trifluralin (AccuStandard Inc., USA). The mixture of standards AS-PA consists of aldrin, α-BHC, β-BHC, δ-BHC, γ-BHC, p,p'-DDD, p,p'-DDE, p,p'-DDT, dieldrin, endosulfan I, endosulfan II, endosulfan sulfate, endrin, endrin aldehyde, heptachlor, heptachlor epoxide and methoxychlor (AccuStandard Inc., USA). For details about chemical identities and basic physicochemical properties of the standards see Table SI-4.1 and SI-4.2 (supporting information). Chemicals in standard mixtures were delivered dissolved in MTBE.

Investigation area and period

The source of the river Xanaes (Rio Segundo) is in the Sierras de Córdoba mountains which are located in the centre of the Province Córdoba (Argentina) and it flows further through agricultural areas to lake Mar Chiquita. Thus, one sampling point was in the mountain area (31.7066° S and 64.4892° W) while the other was in an area of intensive agriculture (31.8035° S and 64.3063° W), 40 km downstream. The sampling area is sparsely populated, such that low contribution from waste water are assumed. The sampling was performed between April and August 2010, directly after the wet season. Average precipitation in this period and area is between 9.7 and 52.2 mm (Servicio Meteorológico Nacional, Argentina; http://www.smn.gov.ar/). During the sampling period, light rain occurred occasionally but with no obvious run-off. Surface water and *Myriophyllum aquaticum* were collected at both sides every two weeks during the sampling period (see Table SI-4.3, supporting information).

Sample treatment for water plants

Upper submerged grown *Myriophyllum aquaticum* shoots were collected from the river, enclosed in aluminium foil and transported cooled into the lab. The plants were then freeze-dried, ground finely and stored in amber glass flasks covered with

aluminium until extraction. The method described by Miglioranza et al. (1999) was adapted to prepare the samples. Briefly, 2 g of lyophilised plant powder was mixed with 8 g Na_2SO_4, spiked with 100 µL of PCB 103 (10 mg L^{-1} in MTBE) and transferred into a pre-extracted cellulose extraction thimble. Plants were extracted for 8 h using a Soxhlet apparatus with a mixture of H and DCM (1:1 v/v), 4 extraction cycles per hour. Extracts were concentrated to 3 mL under nitrogen. To separate larger plant compounds such as lipids and chlorophyll from the extract, gel permeation chromatography (GPC) with Bio Beads S-X3 (200-400 mesh; ≤2 kDa) (Bio-Rads Laboratory, Hercules, California, USA) was performed with a successive elution by using a mixture of H and DCM (65:35 v/v). Final clean-up was done using silica gel chromatography using a mixture of H and DCM (50:50 v/v). The extracts were then concentrated to 0.1 mL under nitrogen, diluted with H to 1 mL and stored in amber autosampler vials at –20 °C until GC-ECD analysis.

Sample treatment for surface water

One L of surface water was collected from the river in amber glass flasks covered with aluminium and were transported cooled into the lab. Each 1 L water sample was then spiked with 100 µL of PCB 103 (10 mg L^{-1} in MTBE) and stored for a maximum of 2 days at 3°C until solid phase extraction (SPE). The proposed SPE disk method for quick turnaround aqueous phase EPA extraction (3M Company, St. Paul, MN, USA) was performed to extract OCPs from the surface water (http://www.sigmaaldrich.com/etc/medialib/docs/Supelco/Instructions/1/t709112.Par.0001.File.tmp/t709112.pdf). The extraction apparatus with the Empore™ extraction disk (47 mm, C_{18}; 3M Company, USA) was cleaned with DCM and conditioned with methanol prior to sample extraction. Following extraction of the 1 L water sample, compounds were re-extracted from the Empore™ extraction disk using DCM. The sample flask was rinsed with DCM three times and the wash solution was combined with the extract. Finally, the extract was dried with Na_2SO_4, transferred into a tube and concentrated with nitrogen to 1 mL, diluted with H to 10 mL and concentrated again to 1 mL. The extract was stored in amber autosampler vials at –20°C until GC-ECD analysis.

Analytical method

Gas chromatographic (GC) analysis was performed on a GC Agilent 6890, equipped with a ^{63}Ni µ-electron capture detector (ECD; Agilent Technologies, Santa Clara, Illinois, USA), split-splitless injector in splitless mode, and controlled by ChemStation software (Agilent, Rev. B. 04.01). One µL of the sample was injected and separated on a dimethyl-polysiloxane capillary (model HP-1, Agilent; 30 m • 0.25 mm i.d., 25 µm film thickness) with helium as carrier gas using a column head

pressure of 20 psi. The injector temperature was 300°C. The oven temperature was programmed starting at 80°C and held 1.5 min, followed by increases of 10°C min^{-1} up to 150°C, held for 8 min, then 15°C min^{-1} up to 200°C, held for 6 min, afterwards 20°C min^{-1} up to 230°C, held for 5 min, finally 20°C min^{-1} up to 275°C, and held for 5 min. Each sample extract and standard solution was measured twice.

Peak identification was based on retention time and was performed by the injection of single compounds of the standards AS-PBR and -PA. For peak quantification, various concentrations of AS-PBR and -PA were prepared in MTBE and analysed.

For the determination of the extraction performance from water samples, 1 L of distilled water was spiked with either the AS-PBR or -PA and was treated as a sample e.g. stored one day at 3°C followed by SPE extraction. Similarly the extraction performance from plant samples was determined, with the exception that 10 g Na_2SO_4 were spiked with either the AS-PBR or -PA. Recoveries from water samples ranged from 69.6 to 135.4%, while recoveries from plant samples ranged from 28.6 to 104.4% (Table SI-4.4, supporting information). Depending on the compound, detection limits in surface water ranged from 0.02 to 0.20 ng L^{-1} and in plants from 0.05 to 1 µg kg^{-1} (dry weight) (Table SI-4, supporting information). Contaminations during sample preparation were investigated for water samples by spiking 1 L of distilled water with 100 µL of PCB 103 (10 mg L^{-1} in MTBE) followed by the sample treatment (e.g. stored one day at 3°C, SPE extraction). Similarly the contamination of plant samples was determined, with the exception that 10 g Na_2SO_4 were spiked with 100 µL of PCB 103 (10 mg L^{-1} in MTBE). Depending on the chemical, the concentration of possible laboratory contaminants ranged from n.d. to 0.13 ng L^{-1} in water samples while in plant samples contamination levels ranged from n.d. to 1.16 µg kg^{-1} (Table SI-4.4, supporting information).

The retention time and the likelihood of use were considered when tentatively identifying the chemicals from the water and plant sample extracts. The internal standard PCB 103 and the recovery, determined during extraction performance analysis, were used for quantification. A compound was considered to occur when it's concentration was above the concentration of the possible laboratory contamination during sample preparation and it was detected at least three times at one sampling site.

Mathematical description of the concentration decrease

The first order decay function $C(t)$ was used to describe the decrease in concentration of organochlorine compounds with time in plant samples (Eq. 4.1), where parameter $k_{2,pseudo}$ was estimated using the maximum likelihood in the software Origin (OriginLab Corporation, Northampton, USA).

$$C(t) = (C_{\max} - C_{ss}) \bullet e^{-k_{2,pseudo} \bullet t} \quad (4.1)$$

Here, t represents time, C_{max} the highest observed concentration, C_{ss} the steady-state concentration and $k_{2,pseudo}$ may be interpreted as the pseudo elimination rate. The C_{ss} was calculated using the concentrations from the last four samples.

Results and Discussion

Occurrence of chlorinated pesticides and metabolites

High concentrations of the organochlorine pesticides and their major metabolites were expected in the river and submerged plants for the area of intensive agriculture, while low pesticide level were expected in the forested mountain area. Surprisingly, the concentrations detected in the river and plant samples at both sites were comparable (Table 4.1). Only 10 of the 30 organochlorine compounds considered (AS-PBR, -PA) were found. Of these, 9 and 8 were detected in plants in the agricultural and mountain area, respectively. Seven compounds were found in the agricultural river sample, compared with 6 in the mountain river. The concentration of individual organochlorine compounds in *M. aquaticum* was at both sites below 5 µg kg^{-1} (dry weight) at both sites except for trans-permethrin where concentrations up to 17.55 µg kg^{-1} (dry weight) were found. Concentrations in the surface water were below 4.5 ng L^{-1} at both sampling sites.

Table 4.1. Concentration ranges of organochlorine pesticides and major metabolites in *Myriophyllum aquaticum* and in surface water.

Compound name	***Myriophyllum aquaticum*** in µg kg^{-1} (dry weight)		**Surface water** in ng L^{-1}	
	Agricultural area	**Mountain area**	**Agricultural area**	**Mountain area**
β-BHC	0.59 – 1.76	0.40 – 2.43	0.12 – 0.51	0.10 – 0.35
γ-Chlordane	0.08 – 0.27	–	–	–
Chlorpyrifos	1.58 – 4.91	1.31 – 4.46	0.22 – 0.78	0.17 – 0.48
p,p'-DDE	0.19 – 0.80	0.10 – 0.34	–	0.07 – 0.34
Endosulfan I	0.48 – 2.87	0.43 – 2.90	0.03 – 0.38	0.03 – 0.35
Endosulfan sulfate	0.11 – 4.03	0.19 – 6.31	0.46 – 4.33	0.56 – 2.26
Endrin	0.18 – 2.25	0.22 – 2.41	0.07 – 0.20	–
Heptachlor	1.28 – 4.61	0.74 – 4.26	0.62 – 3.34	0.20 – 1.50
Hexachlorbenzene	–	–	0.08 – 0.25	–
trans-Permethrin	2.93 – 11.22	5.02 – 17.55	–	–

● *Surface water* - No data was found for this river basin during a literature review. Although comparison with levels in different region is not optimal, we will present a few studies here. During an environmental toxicity assessment of the Paraná river delta (Argentina), in December 1996 by Caltaldo et al. (2001), surface water concentrations of up to 0.62 ng L^{-1} for γ-chlordane, 0.63 ng L^{-1} for p,p'-DDE and 1.62 ng L^{-1} for heptachlor were found. We observed a similar concentration range in this study. Baudino et al. (2003) monitored organochlorine contaminant levels in surface and ground water of the two main rivers San Juan and Jáchal in the province San Juan (Argentina), 500 km west of the river Xanaes. Mean concentrations of 6.6 µg L^{-1} for β-HCH, 1.7 µg L^{-1} for p,p'-DDE, 0.4 µg L^{-1} for endosulfan I, 0.48 µg L^{-1} for endrin and 0.97 µg L^{-1} for heptachlor during September 1996 to July 1997. These values are at least 3 orders of magnitude higher (except for heptachlor, 300 times higher) than those observed here in the river Xanaes. Di Marzio et al. (2010) measured higher endosulfan (I+II) levels (up to 25.9 µg L^{-1}) in water samples collected from narrow and shallow streams flowing through cultivated areas in the province Córdoba (Argentina). These samples were taken one day after the application of insecticides and thus, high concentrations of endosulfan were expected (di Marzio et al., 2010). During stream sampling in Pampa Ondulada (Argentina), between October 2002 to March 2004, Marino and Ronco (2005) observed chlorpyrifos concentrations ranging from <0.2 (before application) to 10.8 µg L^{-1} (after application). Only low chlorpyrifos concentrations were determined in this study, compared with Marino and Ronco (2005) (3 orders of magnitude lower). Overall, we conclude that low contamination levels of the 9 detected OCPs with their metabolites are present in the surface water of the river Xanaes.

● *Submerged plants* – Although comparing concentrations in different species is inaccurate due to the different exposure and uptake processes, nonetheless comparison gives an indication of contaminant levels in other areas. Miglioranza et al. (2004) measured the concentration of different organochlorine compounds in the rooted riparian macrophyte *Schoenoplectus californicus* from a creek running into Los Padres lake (province Buenos Aires, Argentina), in an intensive agricultural area. Dry weight concentration of 0.8 µg kg^{-1} for β-HCH, 1.1 µg kg^{-1} for γ-chlordane, 1.3 µg kg^{-1} for p,p'-DDE, 0.1 µg kg^{-1} for endosulfan I, 17.5 µg kg^{-1} for endosulfan sulfate and 0.2 µg kg^{-1} for heptachlor were observed in *S. californicus* stems, which is in the same order of magnitude as the concentrations observed here. Because of the lack of further data, no conclusion about the contamination level in plants could be made.

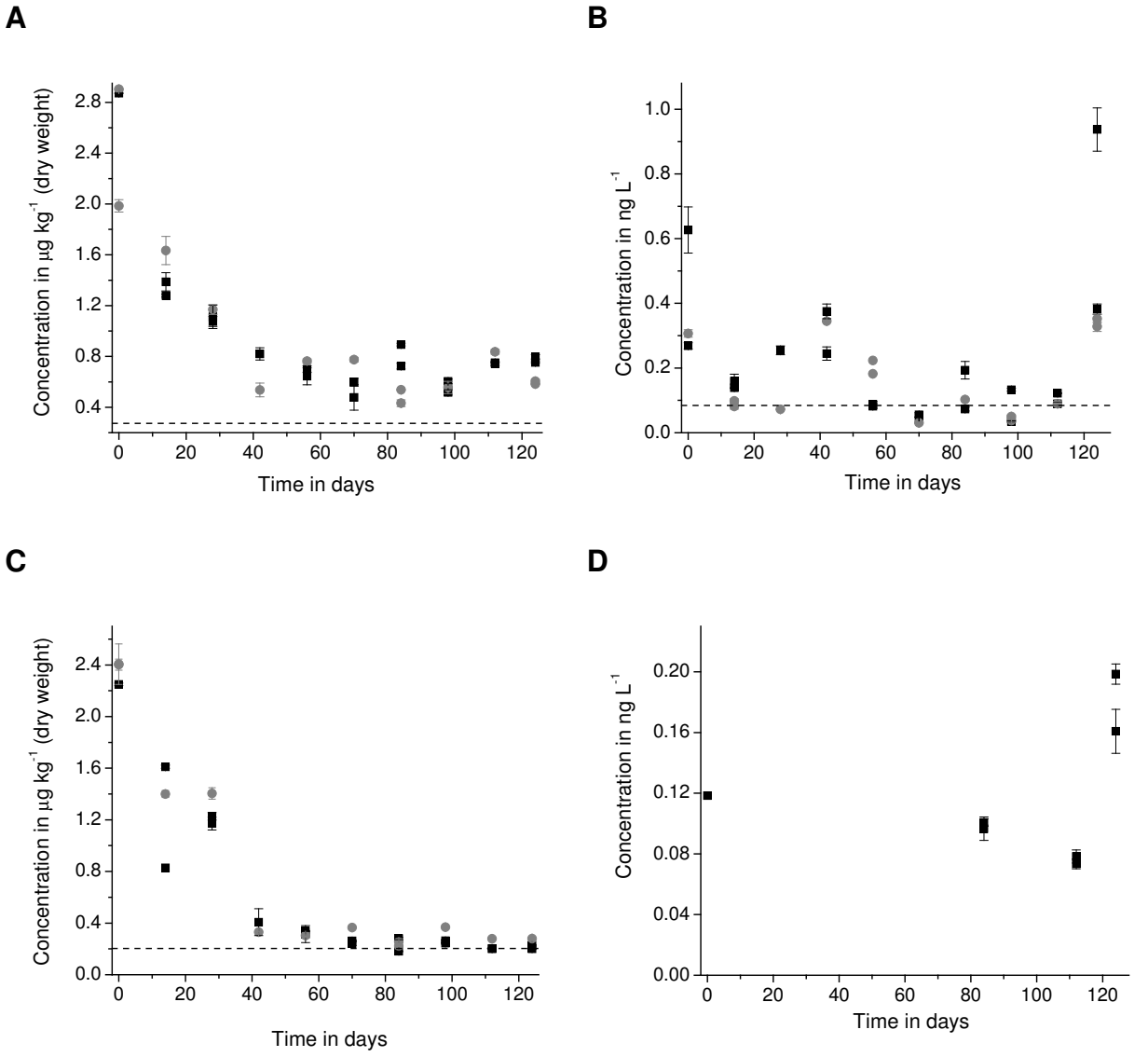

Figure 4.1. Concentration-time-profiles of **endosulfan I** (**A**, **B**) and **endrin** (**C**, **D**) in *Myriophyllum aquaticum* (**A**, **C**) and surface water (**B**, **D**), taken from mountain area (■) and downstream in an agricultural area (●). Every data point represents the mean value of one sample while the standard deviation depicts the difference between technical replicates. The dotted line represents the possible laboratory contamination during sample preparation.

Concentration-time-profiles

The investigated area is characterised by a pronounced dry and wet season. If the major impact of OCPs (and metabolites) in a river is caused through remobilisation and run-off, higher concentrations are expected during and directly after the wet season compared with the dry season. A clear concentration decrease towards a steady concentration was found for plant samples at both sampling sites, for β-BHC, chlorpyrifos, endosulfan I and endrin, while relatively constant concentrations were observed in surface water. This concentration-time behaviour is

shown for endosulfan I and endrin in Figure 4.1. No clear statement about the concentration-time behaviour of γ-chlordane, heptachlor and trans-permethrin could be made, as these compounds were only detected sporadically in both plants and water. A concentration increase was observed in plants for the first sampling days for the two metabolites p,p'-DDE and endosulfan sulfate, followed by a decrease towards a constant concentration (Figure 4.2A and C). A similar concentration-time profile was found for endosulfan sulfate in the water samples taken from the both sampling sites (Figure 4.2B). Only for p,p'-DDE a different concentration-time behaviour was observed in plant samples taken from either the mountain or the agricultural sampling site (Figure 4.2C). The concentration decrease of endrin, endosulfan I, endosulfan sulfate (begin day 28) and p,p'-DDE (begin day 42) in plant samples could be described using a first order decay function (Eq. 4.1; Figure SI-4.1, supporting information), with the respective parameter values for C_{ss} and $k_{2,pseudo}$ presented in Table 4.2. Estimated pseudo elimination rates ($k_{2,pseudo}$) for these four compounds from plants were in the same order of magnitude.

Table 4.2. Pseudo elimination rates ($k_{2,pseudo}$), steady-state concentration (C_{ss}) and log K_{OW} values. C_{ss} was calculated using Eq. 4.1.

Compound name	Agricultural area		Mountain area		log K_{OW}[1]
	C_{ss} (in µg kg^{-1})	$k_{2,pseudo}$ (in days^{-1})	C_{ss} (in µg kg^{-1})	$k_{2,pseudo}$ (in days^{-1})	
p,p'-DDE	0.24	0.076 (±0.009)	0.16	-	6.51
Endosulfan I	0.72	0.081 (±0.009)	0.59	0.048 (±0.006)	3.83
Endosulfan sulfate	0.74	0.087 (±0.030)	0.35	0.076 (±0.004)	3.66
Endrin	0.23	0.042 (±0.004)	0.28	0.042 (±0.006)	5.2

[1]PhysProp Database (http://www.syrres.com/esc/physdemo.htm).

The concentration decreases were well described by a first order decay function. Although $k_{2,pseudo}$ values estimated only result from best fit, these may be interpreted as an overall elimination rate (sum of all uptake and elimination rates) assuming that the uptake and elimination processes follow first order kinetics. Thus, $k_{2,pseudo}$ may represent elimination dynamics for that compound in plants at the respective sampling site. As $k_{2,pseudo}$ values determined differ by less than a factor of 2.5, independent of sampling site and compound considered, the elimination may be dominated by an integral process such as growth dilution. However, growth dilution cannot explain the observed steady-state concentration level (C_{SS}) nor the previous uptake process. Further, Gobas et al. (1991) show, that the elimination rate of lipophilic organochlorine compounds is correlated to the octanol:water partition

coefficient (K_{OW}). Assuming that lipophilic organochlorine compound's elimination rates correlating in *M. aquaticum* with K_{OW} too, differences in $k_{2,pseudo}$ values of detected compounds were expected, but were not observed indicating that partitioning into water may not dominate the observed elimination dynamic. Thus, further compartments and processes needs to be considered.

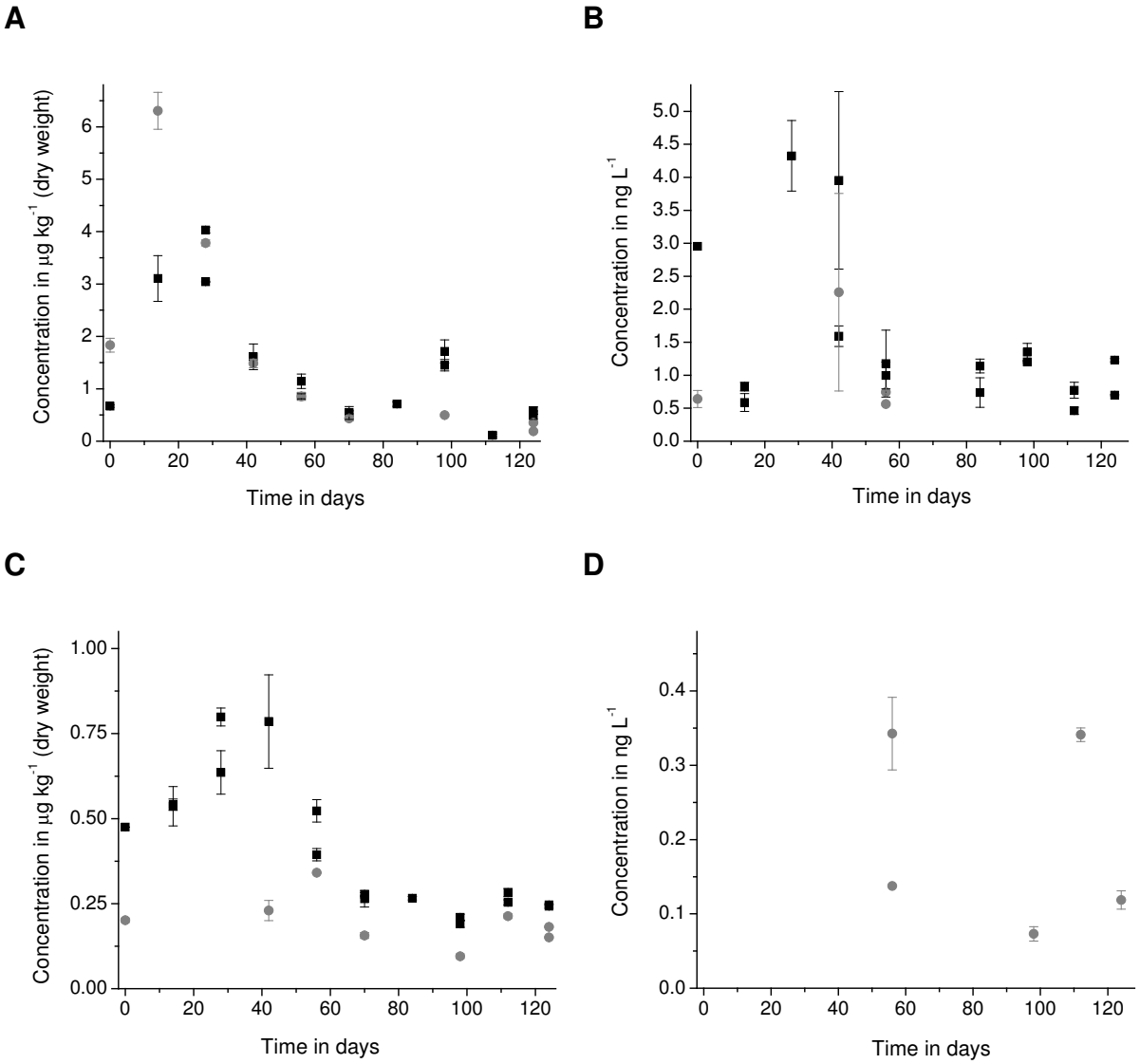

Figure 4.2. Concentration-time-profiles of **endosulfan sulfate** (**A**, **B**) and **p,p'-DDE** (**C**, **D**) in *Myriophyllum aquaticum* (**A**, **C**) and surface water (**B**, **D**), taken from two sampling sites are shown. For further description see Figure 4.1.

Turgut et al. (2002) studied the ability of *M. aquaticum* for root uptake and translocation of pesticides and showed, that all pesticides (trifluralin, triasulfuron, terbutryn, atrazine and cycloxidim) were taken up via the roots and were translocated into shoots. As the contaminantion level of the river sediment was not studied, thus

compound's concentration-time-profiles in sediments and plants could not be compared. If we speculate that OCP concentration levels in sediments and plants correlate, the determined $k_{2,pseudo}$ values describes transport processes in the river sediment. This leads to differnt hypotheses about the sediment contamination: (i) As sampling was done directly after the wet season, it may be speculated that the observed concentration decrease is dominated by the reduced entry of water and suspensed particles. (ii) Another explanation for the broadly similar determined $k_{2,pseudo}$ values from plants takes the temperature dependence of desorption kinetics of organic compounds in sediments into account (e.g. Cornelissen et al., 1997; Enell et al., 2005). Cornelissen et al. (1997) have shown that the slow desorption kinetics are highly dependent on the temperature. During the first 40 days of sampling a decrease in surface waters temperature of about 10 K at both sampling sites was observed, with temperatures remaining approximately steady for the remaining campaign (Figure SI-4.2; supporting information). If the first hypothesis is true, then a decrease in the contamination level in the river sediment during sampling may be assumed. In contrast, if the second hypothesis is true, then during the entire sampling a broadly constant contamination level in the sediment may be assumed.

Both hypotheses may explain compounds similar $k_{2,pseudo}$ values but not the only for p,p'-DDE and endosulfan sulfate previously observed concentration increase. Endosulfan sulfate and p,p'-DDE are major metabolites of endosulfan and p,p'-DDT, respectively. Gao et al. (2000) shown that aquatic plants such as *Myriophyllum aquaticum* could metabolise accumulated p,p'-DDT into p,p'-DDE and it is known, that plants (beside other organisms) could metabolise endosulfan into endosulfan sulfate (Gupta and Gupta, 1979), thus the former determined concentration increase for endosulfan sulfate and p,p'-DDE may be explained by metabolisation of the parent compounds. The partitioning of the synthesised endosulfan sulfate into ambient water may explain the observed concentration peak in the river water.

Conclusion

The results of this study showed relatively similar contamination level in the mountain and in the agricultural area, in both surface water and plants, taken from the river Xanaes. Thus, the observed compounds appear to be residues of former use, otherwise higher concentration in the agricultural area may be observed.

Since the concentration-time-plots of organochlorine compounds in the submerged *Myriophyllum aquaticum* showed a broadly similar pseudo elimination rate independent of compound and sampling site, partitioning between the plant and the ambient water may not be the dominant elimination process. The concentration decrease may thus correlate either with reduced entry of the contaminant during the

almost dry five months sampling regime, or the dependence of compounds desorption rate from sediments on temperature.

The concentration-time-profiles of the metabolites p,p'-DDE and endosulfan sulfate in the plant samples showed a concentration increase prior to a decrease, which may be explained by metabolisation of the parent compounds. This interpretation is supported by the observation of similar concentration-time behaviour of endosulfan sulfate in the river water.

In contrast to the observed concentration-decrease of the orgaochlorine compounds in plants, a generally constant concentration of these compounds in surface water was found, suggesting that the river water may not be the main source for plant contamination. Since rooted macrophytes can take up contaminants via the roots, sedimends may be the principal source. As the sediment contamination level was not determined here, further research should include the measurement of organochlorine compounds in sediment over time.

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Supporting Information

Table SI-4.1. 13 organochlorine pesticides of the standard mixture AS-PBR. Substance water solubility, vapour pressure and octanol:water distribution coefficient (K_{OW}) were taken from the PhysProp Database (http://www.syrres.com/esc/physdemo.htm).

Common name	**CAS-RN**	**Water-Solubility** (in µg L^{-1})	**log K_{OW}**	**Vapour Pressure** (in mm Hg)
α-Chlordane	5103-71-9	56	6.1	$3.6 \cdot 10^{-5}$
γ-Chlordane	5103-74-2	56	6.22	$5.03 \cdot 10^{-5}$
Chlorbenzilate	510-15-6	13,000	4.74	$2.2 \cdot 10^{-6}$
Chloroneb	2675-77-6	8,000	3.44	$3 \cdot 10^{-3}$
Chlorpyrifos	2921-88-2	1,120	4.96	$2.03 \cdot 10^{-5}$
Chlorothalonil	1897-45-6	810	3.05	$5.7 \cdot 10^{-7}$
DCPA	1861-32-1	500	4.28	$2.5 \cdot 10^{-6}$
Etridiazole	2593-15-9	117,000	3.37	$1 \cdot 10^{-4}$
Hexachlorobenzene	118-74-1	6.2	5.73	$1.8 \cdot 10^{-5}$
cis-Permethrin	61949-76-6	9.75	7.43	$3.6 \cdot 10^{-8}$
trans-Permethrin	61949-77-7	9.75	7.43	$3.6 \cdot 10^{-8}$
Propachlor	1918-16-7	580,000	2.18	$2.3 \cdot 10^{-4}$
Trifluralin	1582-09-8	184	5.34	$4.58 \cdot 10^{-5}$

Table SI-4.2. 12 organochlorine pesticides and 5 major metabolites in standard mixture AS-PA. Substance water solubility, vapour pressure and octanol:water distribution coefficient (K_{OW}) were taken from the PhysProp Database (http://www.syrres.com/esc/physdemo.htm).

Common name	CAS-RN	Water-Solubility (in µg L^{-1})	log K_{OW}	Vapour Pressure (in mm Hg)
Aldrin	309-00-2	17	6.5	$1.2 \cdot 10^{-4}$
α-HCH	319-84-6	2,000	3.8	$4.5 \cdot 10^{-5}$
β-HCH	319-85-7	240	3.78	$3.6 \cdot 10^{-7}$
γ-HCH	58-89-9	7,300	3.72	$4.2 \cdot 10^{-5}$
δ-HCH	319-86-8	31,400	4.14	$3.52 \cdot 10^{-5}$
p,p'-DDD	72-54-8	90	6.02	$1.35 \cdot 10^{-6}$
p,p'-DDE	72-55-9	40	6.51	$6 \cdot 10^{-6}$
p,p'-DDT	50-29-3	5.5	6.91	$1.6 \cdot 10^{-7}$
Dieldrin	60-57-1	195	5.4	$5.89 \cdot 10^{-6}$
Endosulfan I	115-29-7	325	3.83	$1.73 \cdot 10^{-7}$
Endosulfan II	33213-65-9	450	3.83	$6 \cdot 10^{-7}$
Endosulfan sulfate	1031-07-8	480	3.66	$2.8 \cdot 10^{-7}$
Endrin	72-20-8	250	5.2	$3 \cdot 10^{-6}$
Endrin aldehyd	7421-93-4	24	4.8	$3 \cdot 10^{-7}$
Heptachlor	76-44-8	180	6.1	$4 \cdot 10^{-4}$
Heptachlor epoxide (isomer B)	1024-57-3	200	4.98	$1.95 \cdot 10^{-5}$
Methoxychlor	72-43-5	100	5.08	$2.58 \cdot 10^{-6}$

Table SI-4.3. The number of surface water and plant samples collected at the respective sampling site and day.

Sampling date	Agricultural area		Mountain area	
	No. water sample	No. plant sample	No. water sample	No. plant sample
21. April 2010	2	2	1	2
05. May 2010	2	2	2	1
19. May 2010	2	2	1	1
02. June 2010	2	2	1	2
16. June 2010	2	2	2	1
30. June 2010	2	2	1	1
14. July 2010	2	2	1	2
28. July 2010	2	2	2	1
11. August 2010	2	2	1	1
23. August 2010	2	2	2	2

Table SI-4.4. The retention times, recoveries and detection limits of organochlorine compounds detected during GC-ECD analysis. Contamination levels measured in blank samples are shown.

Compound name	RT (in min)	Plant extraction			Water extraction		
		Recovery (in %)	Detection limit (in µg kg^{-1})	Contamination (in µg kg^{-1})	Recovery (in %)	Detection limit (in ng L^{-1})	Contamination (in ng L^{-1})
β-BHC	23.3	104.4	0.10	0.36	135.4	0.10	-
γ-Chlordane	29.8	90.8	0.05	-	96.3	0.10	-
Chlorpyrifos	27.9	37.2	0.05	1.16	132.8	0.10	0.13
p,p' DDE	31.1	40.1	0.10	-	90.5	0.05	-
Endosulfan I	30.4	67.3	0.10	0.27	83.5	0.02	0.09
Endosulfan sulfate	33.4	42.2	0.10	-	69.6	0.20	-
Endrin	32.1	64.9	0.10	0.18	91.7	0.05	-
Heptachlor	25.8	88.2	0.20	-	102.3	0.20	-
Hexachlorbenzene	23.4	60.4	0.05	0.16	80.3	0.05	-
trans-Permethrin	37.6	28.6	1.00	-	-	-	-

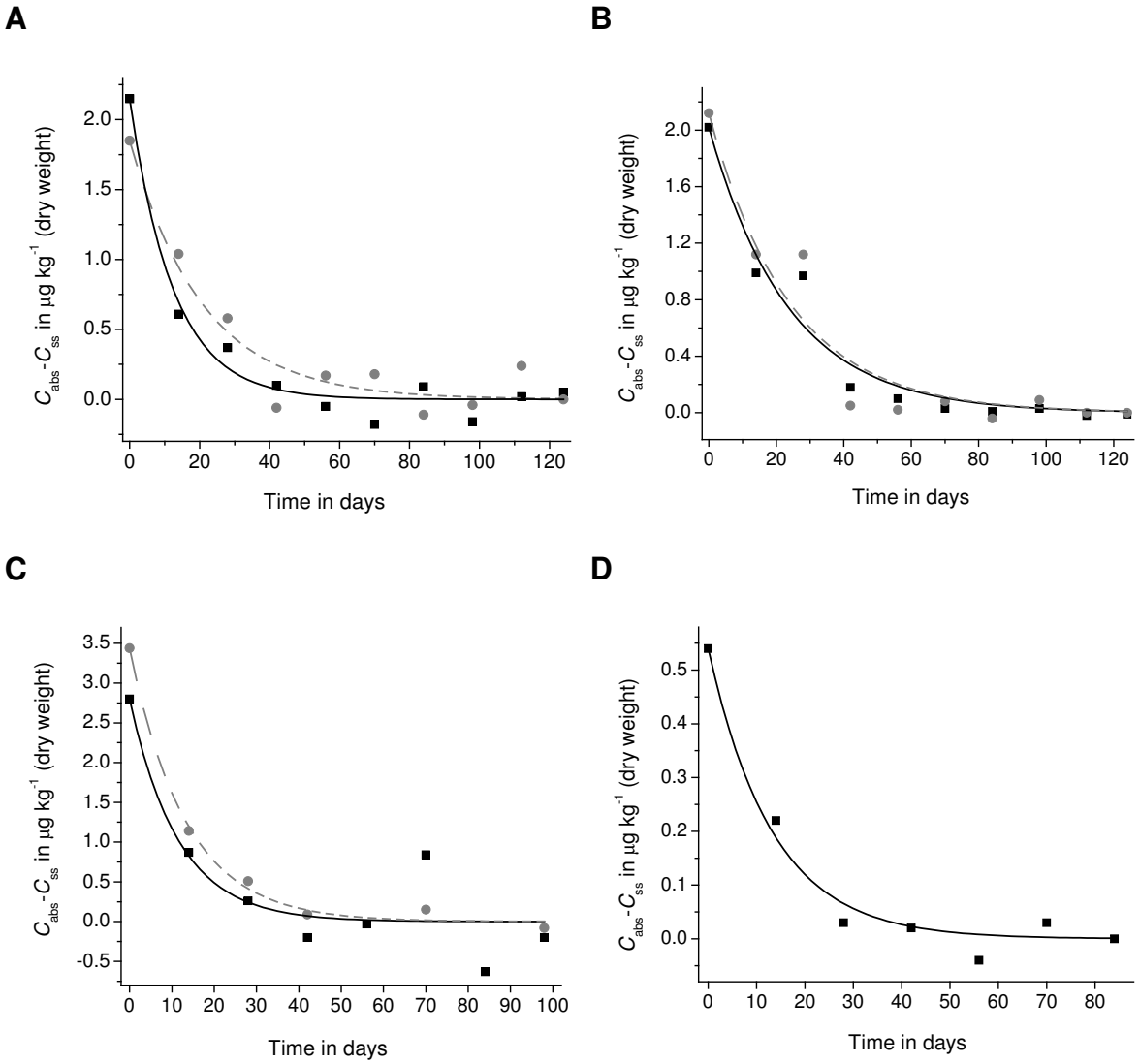

Figure SI-4.1. Relative concentration-decrease-profiles of **endosulfan I** (**A**), **endrin** (**B**), **endosulfan sulfate** (**C**) and **p,p'-DDE** (**D**) in *Myriophyllum aquaticum* taken from two sampling sites are shown. Plant samples were taken from the river Xanaes in a mountain area (●) and downstream in an agricultural area (■). Every data point represents the relative mean concentration, estimated by subtracting the compound's steady-state concentration (C_{ss}) from the mean concentration of all samples taken during one sampling day and site (C_{abs}). For C_{ss} the mean concentration from the last four samplings at one sampling site was used. The dotted and straight line represents the fitted first order decay curve for the relative mean concentrations of plant samples from the mountain and agricultural area respectively by using Eq. 4.1. Note, for **endosulfan sulfate** (**C**) and **p,p'-DDE** (**D**), the elimination was assumed to start at day 28 and day 42 of the sampling campaign, respectively.

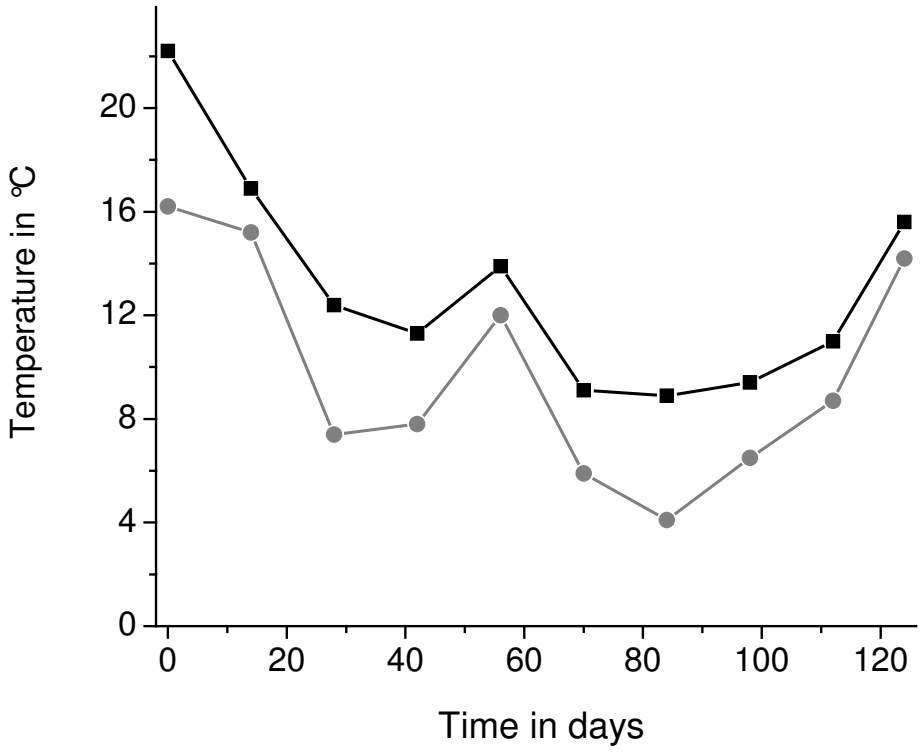

Figure SI-4.2. Surface water temperature during sampling in the river Xanaes at the mountain area (●) and downstream at the agricultural area (■). The temperature was measured 10 cm below the water surface. Generally the sampling started at the agricultural area (around 11 a.m.) and finished at the mountain area (around 1 p.m.). The temperature was measured in the shade at the forested mountain area but in the sun at the woodless agricultural area. The average water depth of the river at the onset of sampling was around 80 cm and 50 cm in the agricultural area and mountain area, respectively. During the five months of sampling the water level decreased by approximately 30 cm at both sampling sites.

Chapter 5

A non-invasive observation parameter to complement sediment bioassays using *Myriophyllum aquaticum*

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A non-invasive observation parameter to complement sediment bioassays using *Myriophyllum aquaticum*

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Abstract

Purpose - The objective of this study was to modify a sediment contact protocol to a multiwell plate exposure system which supplements measurement of the fresh weight change (FWC) using the non-invasive effective quantum yield of energy conversion at photosystem II (PS II) reaction centres (Y(II)) in parallel. Since Y(II) is a functional parameter and FWC represents a whole-plant structural response, the determination of a more pronounced response in one of these parameters may hint at the mode of action of contaminants. By the observation of Y(II) at different time points, extrapolation of effect development over time may be gained from modelling.

Material and methods - An established sediment contact protocol was adapted to an exposure in multiwell plates. During exposure, the Y(II) of exposed *Myriophyllum aquaticum* was measured using an imaging-pulse amplitude-modulated chlorophyll fluorometer. At the end of the 13-day exposure, the FWC was determined and the IC_{50} for FWC and Y(II) was estimated using concentration−response modelling. A concentration−time−response model was used to describe the effect development on Y(II) over time. This protocol was applied to natural sediments and to artificial sediments which were spiked with different contaminants.

Results and discussion - It was shown that for the PS II inhibitor atrazine, the IC_{50} was four times lower on the Y(II) compared to the value for the FWC. In contrast, for the acetolactate-synthase inhibitor metsulfuron methyl, no effect on the Y(II) of exposed *M. aquaticum* could be found, while a 100% inhibition in FWC was detected. For dinitro-*o*-cresol, a decoupler of oxidative phosphorylation, the IC_{50} for the FWC and the Y(II) were of the same order of magnitude. These results are in agreement

with the current mode of action understanding and show the potential of this method to interpret differences in determined *IC* values as mode of action dependent. Moreover, a clear decrease in estimated $I_{Y(II),tx}C_{50}$ values by the end of the 13-day exposure was found for atrazine. This strengthens the hypothesis that effects of contaminants in sediment are not immediately evident but may evolve over time.

Conclusions - With the miniaturised sediment contact assay, mode of action dependent differences in *IC* values on the FWC and the Y(II) could be determined. Based on results from the concentration−time−response modelling, it could be assumed that during the 13-day exposure all contaminants may not fully exert their effects. Since investigated natural sediments did not show inhibitions on Y(II), while pronounced effects on the FWC were found, the FWC may also be worthwhile to be studied over time.

Introduction

Over the last decades, the contamination of river, lake and marine sediments has been increasingly acknowledged. Sediments have been recognised not only as a major sink for persistent anthropogenic chemicals released into the aquatic environment, but also as a source of hazardous substances (Brils, 2002). As sediments are habitat to many organisms and a place for a multitude of biochemical transformations, they play a key role in the assessment of the ecological status of aquatic systems. Numerous bioassays have been developed to simulate exposure of organisms to sediment-bound contaminants to evaluate the hazard of sediments. It has been shown that the bioavailability of contaminants could not be accurately predicted in bioassays that expose organisms to aqueous representations of whole sediments, such as pore-water, aqueous or solvent extracts (e.g. Harkey et al., 1994; Liß and Ahlf, 1997). Thus, whole sediment exposure protocols are assumed to represent the most realistic scenario to simulate the environmental hazard of sediments in the laboratory (Feiler et al., 2005, 2009; Harkey et al., 1994; Liß and Ahlf, 1997; Traunspurger et al., 1997). A recently developed sediment contact assay with *Myriophyllum aquaticum* uses, for example, the growth rate, estimated from the fresh weight change (FWC) over the exposure time, for effect determination of sediments (Feiler et al., 2004) and it is under review to become a standard bioassay for the hazard assessment of sediment contamination (ISO/CD 16191).

Sediments might contain contaminants due to, for example, adsorption from the ambient water that may induce effects in exposed macrophytes. Depending on substance properties, uptake and effects in exposed plants may not occur immediately but evolve over time. Therefore, by restricting the assessment of effects to the measurement at one predefined time point, no information about the temporal

development of exerted effects can be derived. Monitoring the development of effects within organisms may be achieved by the observation of non-invasive parameters. Common modes of toxic action of contaminants in plants include the inhibition of biological processes such as photosynthesis or mitochondrial electron transport (Babu et al., 2005). It has been shown that the detection of an impaired photosynthetic status compared to a control, by measuring the chlorophyll fluorescence, is a reliable method for the identification of the potential hazard of contaminants to plants (e.g. Huang et al., 1997; Krugh and Miles, 1996; Samson and Popovic, 1988).

The monitoring of the photosynthetic status over time for every single exposed plant is time consuming and therefore impractical for high-throughput testing. A new type of pulse-amplitude-modulated chlorophyll fluorometer (PAM) has been developed which enables simultaneous high-resolution fluorescence measurements of whole plants and plant sections in multiwell plates in parallel. Technical applications for the Imaging-PAM (I-PAM) in aquatic biology have been published (e.g. Küster and Altenburger, 2007). The I-PAM was applied for effect detection in short-term bioassays with aquatic macrophyte species (Küster and Altenburger, 2007; Küster et al., 2007). It was shown that with the I-PAM instrumentation, inhibitory effects of contaminants to macrophytes could be detected within 24 h, even if the inhibition of chemicals was not specifically associated with the photosystem II (PS II; Küster and Altenburger, 2007; Küster et al., 2007). Using this observation parameter, inhibitory effects of the PS II could be monitored over time and this enabled the modelling of concentration−time−response relationships for the assessment effect of polluted samples (Altenburger et al., 2006). Information about the state of effect development of contaminants on exposed plants could thus be derived.

The objective of this study was to evaluate whether parallel measurements of different effect parameters could be used in sediment assays and thus be used as a tool in hazard assessment. Therefore, a sediment contact protocol with *M. aquaticum* (Feiler et al., 2004) was adapted to a multiwell plate exposure system, which allows the observation of two effect parameters in parallel. The observation parameters are the terminal measure of the fresh weight change and the non-invasive measure of the photosynthetic status over time of the exposed macrophytes. As a positive control, sediments were spiked with one of the three reference substances with a known mode of action, namely atrazine, dinitro-*o*-cresol (DNOC) or metsulfuron methyl. Subsequently, the developed methodology was applied for characterisation of a series of natural sediments.

Material and methods

Cultivation of M. aquaticum

The stock culture of *M. aquaticum* (parrot feather) was obtained from the Federal Institute of Hydrology (BfG, Koblenz, Germany). The pre-culture was grown non-axenically in artificial sediment (OECD 218) and saturated with modified Steinberg medium as specified in DIN EN ISO 20079. In short, the artificial sediment used consisted of 74% quartz sand (grain size 50 to 200 µm), 20% kaolinite clay, 5% peat (grain size <0.6 mm) and 1% calcium carbonate (OECD 218). The pre-culture was maintained in glass pots at 24±1 °C with a light exposure of 16:8 h light/dark and a light intensity of 80 µE m^{-2} s^{-1}. A new pre-culture was prepared weekly from the head and the three whorls below from 3- to 4-week-old *M. aquaticum* plants.

Spiked sediments

For the preparation of the spiked sediments, the artificial sediment (OECD 218) was saturated with a contaminant solution. Various concentrations of dinitro-*o*-cresol (CAS RN 609-93-8, Sigma-Aldrich, Steinheim, Germany), metsulfuron methyl (CAS RN 74223-64-6, Riedel deHaën, Seelze, Germany) and atrazine (CAS RN 1912-24-9, Riedel deHaën) were prepared in modified Steinberg medium (DIN EN ISO 20079), and were used for spiking. Note that nominal concentrations are presented (see Fig. 5.1) in mass substance per mass sediment dry weight (e.g. mg kg^{-1}). The spiked sediments were conditioned statically in glass pots and sealed with Parafilm® "M“ (American National Can, Chicago, USA) for 1 week under exposure conditions (24±1 °C; 80 µE m^{-2} s^{-1}; continuous light) prior to use in the bioassay. For simplicity, it is considered that all compounds were bound to the sediment after this spiking procedure, even if the contaminants might be present mainly in the water phase or in a non-equilibrium state.

Fluorescence measurements

Chlorophyll fluorescence measurements were carried out as described in Küster and Altenburger (2007). In short, chlorophyll fluorescence measurements were performed in six-multiwell plates using a maxi-imaging pulse-amplitude-modulated chlorophyll fluorometer (Walz, Effeltrich, Germany). The I-PAM employs pulse-modulated measuring light for fluorescence excitation and it continuously monitors fluorescence yield of selected areas of interest. The effective quantum yield of energy conversion at PS II reaction centres (Y(II)) can be measured using the saturation pulse method (for details, see Schreiber et al., 2003). Hereby, under actinic light, the maximum fluorescence yield ($F_{m'}$) is measured during a saturation pulse. The momentary fluorescence yield (F) is determined between the pulses. The

calculation of the fluorescence parameter Y(II) was done according to Genty et al. (1989).

$$\mathrm{Y(II)} = \frac{(F_{m'} - F)}{F_{m'}} \tag{5.1}$$

The measurements were performed during the application of saturation light pulses to samples adapted to an actinic illumination of 80 µE m^{-2} s^{-1} for 2.5 min. A circle that encloses the entire whorl was used as the area of interest. All whorls in slots of the six-multiwell plate were analysed five times in parallel, while only the last four measurements per slot were used for estimating the median Y(II) at each time point.

Natural sediments

Four different sediment samples from German and French rivers or waterways were collected for investigation. One sediment sample, taken from the Spittelwasser River, downstream of Bitterfeld, was collected between March and April 2003 and was previously analysed by Grote et al. (2005a). This sampling site was close to a former chemical production site and the sediment was found to be highly contaminated (for a description of contaminants, see Grote et al., 2005a). The sediment was frozen at –20°C, freeze-dried, sieved (<63 µm) and stored at 3°C. Two sediment samples were taken by the Federal Institute of Hydrology (BfG, Koblenz, Germany) in July 2007; one from the Müritz-Elde waterway (a small tributary of the River Elbe) close to Dömitz (Germany), and the other from an old arm of the River Rhine close to Altrip (Germany). Both sediments were characterised within the joint research project SeKT (Feiler et al., 2009). With regard to priority chemical pollutants, the contamination level was low, apart from moderately enhanced concentrations of heavy metals in the Dömitz sediment. The fourth sediment was sampled from the Borne Creek upstream of Choranche (Isère, France) in June 2008. The latter three sediments were stored unmodified in closed plastic jars at 3°C. The natural sediment samples were conditioned to the incubation temperature (24°C) and thoroughly mixed before use in experiments. The freeze-dried sediment from the Spittelwasser River was saturated with double-distilled water and conditioned at 24°C for 1 week before use.

Sediment contact assay

A sediment contact test with *M. aquaticum* (Feiler et al., 2004) was adapted for its application in multiwell plates. For the miniaturised sediment contact assay, multiwell plates with six slots (Nunc, Roskilde, Denmark) were half-filled (approximately 7 mL) with the respective sediment. Sediment in the slots was water

saturated with modified Steinberg medium if necessary and covered with a non-transparent plastic foil. The stem of the *M. aquaticum* whorl was inserted into the sediment via a hole in the plastic foil (Fig. SI-5.1, supporting information). The plastic foil was used to avoid algae growth at the surface, which would interfere with fluorescence measurements. Before the upper whorls from 3- to 4-week-old *M. aquaticum* were exposed to the sediment, the fresh weight of each whorl was determined. Only whorls with a fresh weight between 10 and 20 mg were used for experiments. After preparation of the multiwell plates, whorls were adapted for 60 min to the exposure condition (24±1°C; 80 µE m^{-2} s^{-1}; continuous light) and afterwards the effective quantum yield of the PS II (Y(II)) of each whorl in the multiwell plate was determined via I-PAM measurement (see Section *Fluorescence measurements*). A circle, which encloses the entire whorl, was used as the area of interest. During the exposure time of 13 days, the Y(II) of each whorl was determined at several stages (day 0, 1, 3, 6, 10 and 13). After 7 days of exposure, 1.5 mL of modified Steinberg medium was added to the sediment in each slot to accommodate for evaporation loss. At the end of the exposure, the fresh weight of each plant (whorl, roots and shoots) was determined.

As a negative control, six slots of a multiwell plate were filled with artificial sediment (OECD 218). For the positive control, mulitwell plates were filled with artificial sediments spiked with different contaminant solutions 1 week before use. All slots were filled with sediment spiked at different concentrations. For each experiment, four parallels were prepared (i.e. each data point represents average values of four independent replicates). To observe the biological effects of natural sediments, five slots of a multiwell plate were filled with the respective sediment and one slot was filled with the artificial (control) sediment (i.e. each presented data point is the average of five replicates). All experiments with natural sediments were carried out in February 2009.

Calculation of inhibitory effects

In the sediment contact assay, two observation parameters were considered; the inhibition of fresh weight change and the inhibition of Y(II) in *M. aquaticum* whorls exposed to natural or spiked sediments in comparison to whorls incubated under control conditions. The FWC (in %) was calculated from the normalised mass gain in fresh weight at day 13 (m_{t13}) of exposed *M. aquaticum* (Eq. 5.2).

$$\mathrm{FWC} = \left(\frac{m_{t13} - m_{t0}}{m_{t0}} \right) \bullet 100 \qquad (5.2)$$

To calculate the inhibitory effects of spiked or natural sediments on the FWC of *M. aquaticum* whorls (I_{FWC} in %), the FWC of exposed whorls (FWC_S) and of the control whorls (FWC_C) were calculated according to Eq. 5.3.

$$I_{FWC} = \left(1 - \frac{FWC_S}{FWC_C}\right) \bullet 100 \tag{5.3}$$

Similarly, the inhibitory effect ($I_{Y(II),tx}$ in %) of spiked or natural sediments to the Y(II) of *M. aquaticum* whorls at a time point (*t*x) was calculated. The Y(II) of exposed whorls at *t*x of the exposure against natural or spiked sediment ($Y(II)_{S,tx}$) and of the control whorls ($Y(II)_{C,tx}$) were calculated using Eq. 5.4.

$$I_{Y(II),tx} = \left(1 - \frac{Y(II)_{S,tx}}{Y(II)_{C,tx}}\right) \bullet 100 \tag{5.4}$$

Concentration–response relationships and concentration–time–effect models

Concentration–response relationships were evaluated using the Hill model where *C* is the concentration, EC_{50} is the median effect concentration, and Θ_1 is the slope (Eq. 5.5). The parameters were estimated using maximum likelihood estimation in the software Origin (OriginLab, Northampton, MA, USA).

$$E(C) = 100 \bullet \frac{C^{\Theta_1}}{EC_{50}^{\Theta_1} + C^{\Theta_1}} \tag{5.5}$$

Estimation of concentration–time–response functions was based on non-linear regression performed in SAS (SAS Institute, 1999) as described in Altenburger et al. (2006). In a modification of the reported approach, the minimum response level was fixed to 0% effect, as we had to deal with inhibition only. However, the slopes of the time functions were clearly better described by an individual parameter (Θ_5) than by the squared time. This is consistent with Haber's rule in its generalised format (Bunce and Remillard, 2003) and leads to the following global concentration–time–effect model (used throughout):

$$E(C,t) = \frac{100}{1 + \left[\frac{C}{\left(\Theta_3 + \frac{\Theta_4}{t^{\Theta_5}}\right)}\right]^{-\Theta_2}} \tag{5.6}$$

where E is the effect, Θ_2–Θ_5 are estimated parameters, C denotes the concentration (in µg kg^{-1} dry weight) and t denotes the time in days. In this form, Θ_3 may be interpreted as the concentration showing 50% effect at infinite time ($E_{t\text{-infinite}}C_{50}$).

Statistical analysis

Experiments were run with at least four replicates and differences in the FWC of control and exposure were analysed for significance using Welch's t test. Normal distribution and heterogeneity of the data variance are assumed.

Results

Positive control using spiked sediments

To investigate the sensitivity of the two observation parameters considered, the non-invasive functional parameter, effective quantum yield of PS II (Y(II)), and the one point measurement for FWC at the end of the 13-day exposure duration, *M. aquaticum* whorls were exposed to control and spiked sediments, using the reference substances atrazine, DNOC and metsulfuron methyl (see Fig. 5.1; Fig. SI-5.2 to SI-5.5, supporting information).

During the exposure of whorls of *M. aquaticum* to control and spiked sediments, the time course of Y(II) was observed six times for each whorl over the 13-day exposure period (see Fig. 5.1a–c). It was found that the Y(II) of *M. aquaticum* whorls in the control sediment show comparable time courses in all of the sequential experiments analysed (see Fig. 5.1a–c). The Y(II) of these whorls varied between 0.55 and 0.65 and showed, more or less, a constant photosynthetic status of PS II of the whorls during the entire duration of the experiment (13 days). In contrast, the Y(II) of *M. aquaticum* exposed to sediments spiked with atrazine showed time and concentration-dependent effects (see Fig. 5.1a). While for the two highest concentrations investigated (10.6 and 3.54 mg kg^{-1}), dramatic and almost immediate effects could be detected, the two intermediate concentrations (1.18 and 0.39 mg kg^{-1}) showed a steady decrease in Y(II) of the exposed whorls over the duration of the experiment. The two lowest concentrations employed (0.13 and 0.04 mg kg^{-1}) did not differ from the controls throughout the experiment. Somewhat similar results were found for the Y(II) of *M. aquaticum* whorls exposed to sediments spiked with DNOC (see Fig. 5.1b). Here, the highest concentration (79.7 mg kg^{-1}) showed a dramatic and fast response within 1 day. The second highest concentration (26.6 mg kg^{-1}) also showed a response after 1 day but subsequently remained around that level (0.45) throughout. The other concentrations did not differ from the control. For

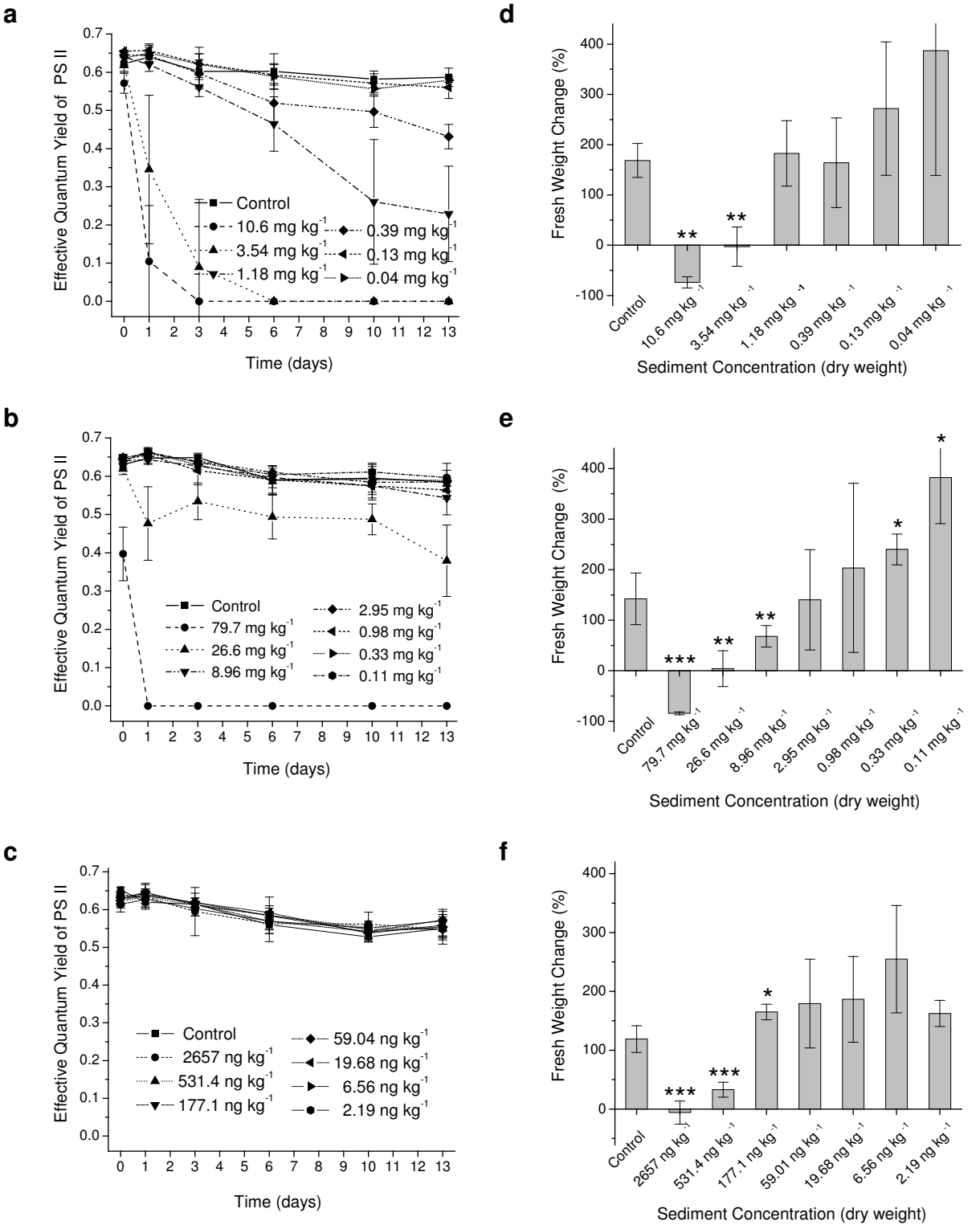

Fig. 5.1. Time course of the effective quantum yield of the PS II (a–c) and the fresh weight change (d–f) of *M. aquaticum* whorls in control and spiked sediments during and after a 13-day exposure. Sediments were spiked either with atrazine (a, d), DNOC (b, e) or metsulfuron methyl (c, f). Data points of spiked sediments represent the mean value of four plants while data points of the control represent the mean value of six plants. Significant differences between the fresh weight change of control and exposure sample were analysed using Welch's *t* test. **P*<0.05; ***P*<0.01; ****P*<0.001.

metsulfuron methyl, no effect on the Y(II) of exposed *M. aquaticum* whorls was found for the concentrations and exposure duration investigated (see Fig. 5.1c).

The second observation parameter, the FWC of *M. aquaticum* whorls in the control sediment, showed a comparable gain of more than double the fresh weight during exposure in all sequential experiments (see Fig. 5.1d–f). The FWC of the whorls within the control varied between 120% and 170% at the end of the 13 days experiment. Thereby, the mean fresh weight of control whorls/plants increased from 12 mg at the onset of the experiment to 29 mg at the end of the exposure. For whorls exposed to atrazine and DNOC, a dramatic effect was observed, with a loss of FWC (whorls were brown and dried-up; Fig. SI-5.3, SI-5.4, supporting information) for the highest concentration. Furthermore, no gain for the second highest concentration was found (see Fig. 5.1d, e). The intermediate concentrations deviated only slightly from the control FWC, while the two lowest concentrations observed demonstrated a gain in FWC that was larger than for the controls. For metsulfuron methyl exposure, the situation was similar to those for atrazine and DNOC exposure (see Fig. 5.1f). The latter findings were in contrast to the observations on photosynthesis function (Fig. 5.1c). For all three contaminants used, the FWC was clearly concentration dependent showing everything from complete FWC inhibition at high concentration to FWC increase at low concentration, when compared with the control.

Concentration relationships and concentration–time–effect models

It is assumed that the difference in Y(II) as well as the FWC of whorls exposed against spiked sediments compared to those values in the control were contaminant induced. Based on the assumption that a reduction in the effective quantum yield of the PS II compared to the value of the control at a similar time point indicates a compromised photosynthesis, and thus an inhibitory effect, Y(II) values of the substance exposed whorls were transformed using Eq. 5.4 into relative inhibition units. Due to this transformation, the resulting numbers are accessible for concentration–effect modelling to determine $I_{Y(II),tx}C_{50}$ values (Eq. 5.5) for specific exposure times. As an example, one concentration–response curve is shown for atrazine at day 13 (Fig. 5.2a). Similarly, a reduced FWC_S change during the 13-day exposure compared to the value of the control was considered to be an inhibitory effect on growth, and the values of exposed whorls were also transformed into relative inhibition units using Eq. 5.3. For the concentration–effect modelling to determine $I_{FWC}C_{50}$ values (Eq. 5.5), only growth inhibition effects (I_{FWC} values between 0% and 100%) were considered. Values higher than 100% were caused probably by dehydration rather than negative growth and, therefore, those values were set to 100%. Negative inhibition values (FWC gain) were not considered because this effect

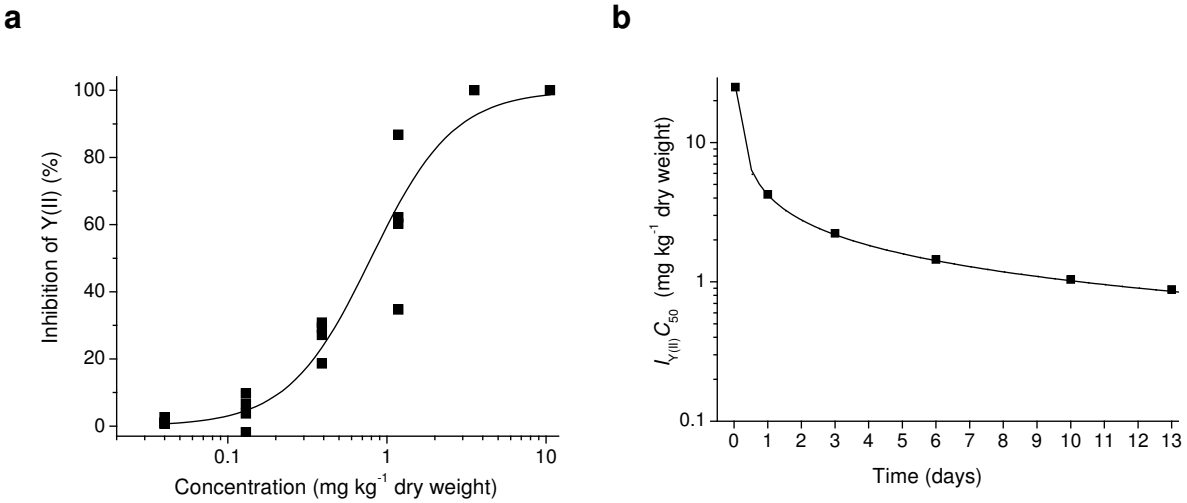

Fig. 5.2. (a) Concentration–response–curve of the inhibition of the effective quantum yield of the PS II (Y(II)) compared with control values of 13-day whorls exposed to sediments spiked with atrazine. The presented concentration–response curve was modelled using Eq. 5.5. (b) Dependence of median effects on cumulative exposure time arising from the concentration–time–response curves for Y(II) of exposed whorls to sediments spiked with atrazine. The global model estimates $I_{Y(II),tx}C_{50}$ values with the concentration–response–effect model according to Eq. 5.6.

was assumed to differ fundamentally in character from the observed inhibitory effect. In Table 5.1, IC_{50} values determined for the two observation parameters considered are shown for the three substances and their respective concentration–response curves are presented in Fig. SI-5.10 (supporting information).

Based on the assumption that uptake and distribution processes of contaminants and some buffering and/or detoxification capacities of plants may delay an effect expression, the effect concentrations determined are strongly related to the exposure duration. As Y(II) is a non-invasive parameter, the development of (inhibitory) effects on Y(II) over time could be studied (Fig. 5.1a–c). This opens up the possibility of effect assessment of substances via concentration–time–effect consideration, where effects may not only be regarded as concentration- but also as time-dependent. An example of a concentration–time–effect model is shown for the inhibition of Y(II) of whorls exposed to atrazine using Eq. 5.6 (see Fig. 5.2b). The decrease of the $I_{Y(II),tx}C_{50}$ over time from about 25 mg kg^{-1} after 1 h to a value of 0.79 mg kg^{-1} at day 13 was well described by the model used (Eq. 5.6). The parameter value for Θ_3 was estimated to be –0.22 mg kg^{-1}, which is assumed to represent the $I_{Y(II),t\text{-infinite}}C_{50}$ for atrazine exposure. This indicates that a longer exposure time is needed to derive the steady-state effect level. For DNOC, Θ_3 ($I_{Y(II),t\text{-infinite}}C_{50}$) was estimated to be 3.23 mg kg^{-1} and this is more than half the value compared to the $I_{FWC}C_{50}$ (8.71 mg kg^{-1}) which, similar to the atrazine exposure, indicates that a steady-state effect level has

not yet been reached. In contrast, the modelled concentration–time–effect curve using the estimated parameter values do not convincingly describe the decrease of the $I_{Y(II),tx}C_{50}$ over the time of exposure (Table 5.1; graph not shown). Depending on the contaminant used, one of the two observation parameters considered was found to be more sensitive than the other at the end of the exposure, based on their IC_{50} value. For atrazine, the IC_{50} of Y(II) was found to be almost four times lower than the IC_{50} of the FWC. In contrast, for DNOC, the IC_{50} of the FWC was almost four times lower while for metsulfuron methyl no inhibitory effect on the Y(II) was found for the concentration range investigated (see Table 5.1).

Table 5.1. Estimated IC_{50} values for the two observation parameters considered, the inhibition of the effective quantum yield of the PS II ($I_{Y(II),t13}C_{50}$; day 13) and the inhibition of the fresh weight change compared to the respective control values ($I_{FWC}C_{50}$) of exposed whorls to sediments spiked with either atrazine, DNOC or metsulfuron methyl utilising Eq. 5.5 and the estimated parameters for the global concentration–time–effect model ($I_{Y(II)}$; Eq. 5.6), where Θ_3 may be interpreted as the $I_{Y(II),t\text{-infinite}}C_{50}$.

Substance	Concentration-Response Model Estimated Parameter (Eq. 5.5)				Concentration-Time-Response Model Estimated Parameter (Eq. 5.6)			
	$I_{Y(II),t13}C_{50}$ (mg kg^{-1})	Θ_1	$I_{FWC}C_{50}$ (mg kg^{-1})	Θ_1	$\Theta_3 - E_{t\text{-infinite}}C_{50}$ (mg kg^{-1})	Θ_2	Θ_4	Θ_5
Atrazine	0.79	1.67	2.90	90.6	-0.218	2.67	4.37	0.548
DNOC	30.8	4.04	8.71	3.94	3.23	6.47	$3.3 \cdot 10^{-5}$	4.45
Metsulfuron methyl	-	-	$5.2 \cdot 10^{-4}$	35.4	-	-	-	-

Natural sediments

The applicability of the methodology developed for an effect assessment of *M. aquaticum* whorls exposed to spiked sediments was shown in the previous section. Next, the bioassay developed was applied for effect assessment of natural sediments sampled at sites with expected differences in contamination levels (Fig. 5.3; Fig. SI-5.6 to SI-5.9, supporting information).

The Y(II) of exposed *M. aquaticum* whorls to control and natural sediments, sampled in Altrip, Bitterfeld, Choranche and Dömitz showed a similar constant time course, with values between 0.53 and 0.65 (see Fig. 5.3a). The Y(II) value of whorls exposed to the sediment sampled in Bitterfeld and Dömitz showed at times somewhat lower Y(II) values compared to the control; however, the resulting inhibition was never consistent nor higher than 12% and therewith these effects were assumed to be negligible (see Fig. 5.3a).

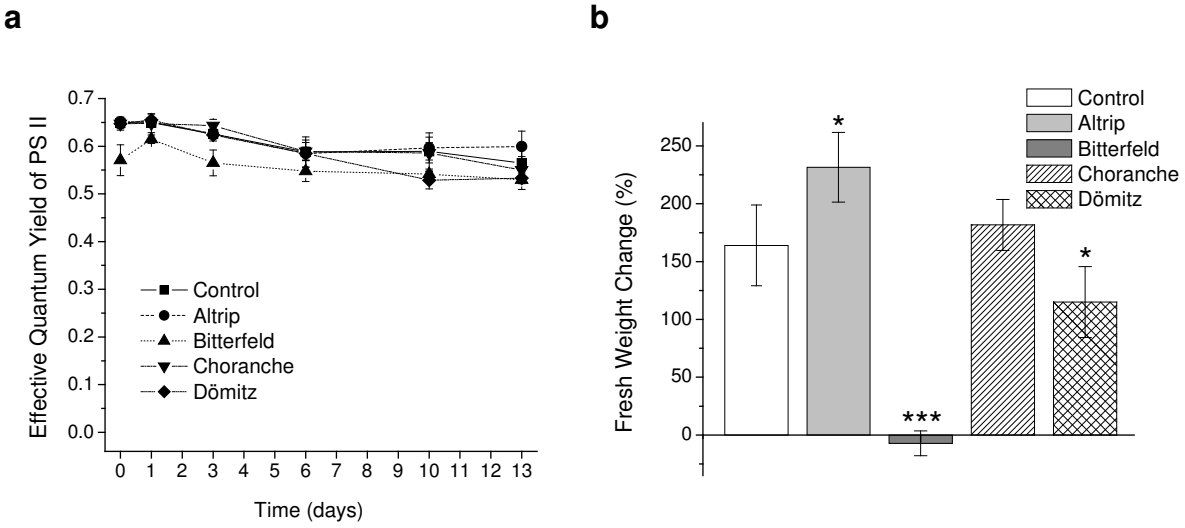

Fig. 5.3. Time course of the effective quantum yield of the PS II (a) and the fresh weight change (b) of *M. aquaticum* whorls in control and natural sediments during a 13-day exposure. Data points of natural sediments represent the mean value of five individual plants while data points of the control represent the mean value of six individual plants. Significant differences between the fresh weight change of control and exposure sample were analysed using Welch's *t* test. *P<0.05; ***P<0.001.

The results of the FWC determination showed higher values for whorls exposed to sediments sampled in Altrip and Choranche (231.6±30.1% and 181.7±22.1%, respectively) compared to whorls in the control sediment (164.0±40.4%). In contrast, whorls exposed to the sediment sampled in Dömitz showed lower (115.0±30.7%) or, for the sediment from Bitterfeld, no FWC increase (–7.1±10.7%) at the end of the exposure. This is equivalent to an inhibition of the FWC increase (I_{FWC}) of 42.6± 16.3% for the sediment from Dömitz and 105.4±6.2% for the sediment from Bitterfeld.

Discussion

The applicability of adapting the sediment contact protocol with *M. aquaticum* to a multiwell plate exposure system will be discussed, considering (1) mode of action dependent differences in responses in one of the observation parameter, (2) the potential of using the photosynthetic status as a bioindicator for a whole plant response, (3) the possibility to extrapolate the effect development over time and (4) the potential to detect contamination of natural sediments.

Positive control for the invasive and non-invasive observation parameter

As the measurement of the effective quantum yield of the PS II is a functional parameter and the fresh weight change is a whole-plant structural response, the determination of a more pronounced response in one of the observation parameters considered may give hints about the mode of action of contaminants in macrophytes.

To investigate this hypothesis, three reference substances with known mode of action have been used.

Atrazine was chosen as a reference substance for the inhibition of Y(II), as it provokes a strong inhibitory effect through the binding to the Q_B site of PS II in the photosynthesis that causes interferences in the electron transport. The Y(II) of *M. aquaticum* whorls exposed to sediments spiked with atrazine show an inhibitory effect at the end of 13 days of exposure, even at a low concentration of 0.13 mg kg^{-1}, while a concentration of 3.54 mg kg^{-1} was needed to cause an inhibition in the FWC. This difference in sensitivity may be attributed to the mode of action of atrazine, as it interferes with the photosynthesis of plants, observed directly via I-PAM measurements (Fuerst and Norman, 1991; Küster and Altenburger, 2007). Because photosynthesis is needed for the energy supply of cells, this leads to growth inhibition due to lack of energy for carbon fixation. However, some compensatory capacity seems to buffer the growth response. In the literature, no information on effect concentrations of atrazine in sediments to *M. aquaticum* could be found. However, Forney and Davis (1981) describe a 60% inhibition of growth of *Myriophyllum spicatum* from 1 mg kg^{-1} atrazine in sediment during 3 weeks of exposure and this compares quite well with the results found in the present study ($I_{FWC}C_{50}$ 2.90 mg kg^{-1}, $I_{Y(II),t13}C_{50}$ 0.79 mg kg^{-1}). Küster and Altenburger (2007) compared the sensitivity of the Y(II) and the growth response of *Lemna minor* upon exposure to atrazine. The EC_{50} for growth of *L. minor* determined by the standardised 7-day growth test (DIN EN ISO 20079) was 97.0 µg L^{-1} while the EC_{50} of the Y(II) for *L. minor* after 24 h of exposure was found to be 28.3 µg L^{-1}. As for this study, the functional observation parameter was found to be almost four times more sensitive compared with the EC_{50} values of the growth response. For these short-term exposures, the sensitivity difference in response between the functional and the growth parameter agrees well with the mode of action of atrazine.

DNOC is a non-systemic insecticide as well as a contact herbicide and is believed to act by decoupling the oxidative phosphorylation in mitochondria. Thereby, DNOC mimics the physiological effects of decouplers by stimulating the respiration of animal and higher plant mitochondria (Vicente et al., 1998). Because of this mechanism, inhibitory effects on Y(II) as well as on growth of exposed *M. aquaticum* whorls were expected as subsequent effects. For DNOC, an inhibition in FWC as well as in the Y(II) of exposed *M. aquaticum* whorls were found for concentrations of 8.96 mg kg^{-1} and higher. Nevertheless, the inhibition of FWC was four times more sensitive compared to the inhibition of the Y(II) based on IC_{50} values. In the literature, information of the effects of DNOC to (aquatic) plants is scarce. Küster et al. (unpublished results) compared the sensitivity of the Y(II) and the growth response of *L. minor* to an exposure of DNOC based on their EC_{50} values. The EC_{50} for the

growth of *L. minor* determined by a 7-day growth test (DIN EN ISO 20079) was estimated as 5.21 mg L^{-1} while the EC_{50} of the Y(II) for *L. minor* after 48 h of exposure was found to be 4.06 mg L^{-1}. Both observation parameters thus showed similar sensitivities to the DNOC exposures, while in the sediment contact test the growth parameter was more sensitive. This might be due to differences in the exposure system (e.g. exposure duration). However, it could be shown that both observation parameters were able to detect effects of a contaminant with a mode of action other than the inhibition of Y(II) to *M. aquaticum* in concentrations of the same order of magnitude.

Metsulfuron methyl was selected due to its known specific binding to the acetolactate-synthase enzyme and the resulting inhibition of the synthesis of the branched amino acids valine, leucine, and isoleucine, leading to a rapid cessation of plant cell division and growth (Obrigawitch et al., 1998). Hence, strong inhibitory effects on the FWC in exposed *M. aquaticum* whorls were expected. A clear growth inhibition of exposed *M. aquaticum* whorls upon metsulfuron methyl exposure was found down to a concentration of 531.4 ng kg^{-1}. An inhibitory effect on Y(II) could be found, only at a concentration of 5,000 ng kg^{-1} (data from screening), which is ten times higher than the concentration compared to the inhibitory concentration for the FWC. Exposed whorls showed a strongly reduced shoot and root growth, which is in accordance with effects described in the literature (e.g. Brown, 1990; Obrigawitch et al., 1998). Specifically, effect concentrations of metsulfuron methyl in sediments to *M. aquaticum* could not be found in the literature. However, Turgut et al. (2003) investigated effects of metsulfuron methyl solutions to different observation parameters in submersed *M. aquaticum* within a 14-day exposure. They found EC_{50} values ranging from 0.61 µg L^{-1} for chlorophyll A content to 6.86 µg L^{-1} (endpoint increase in plant height). Cedergreen et al. (2004) studied the sensitivity of aquatic plants to metsulfuron methyl solutions by detecting the inhibition of the growth rate within a 14-day exposure duration and measured the EC_{50} for *M. spicatum* at 0.29 µg L^{-1} (Cedergreen et al., 2004). Although effective concentrations in sediments and water cannot be compared directly, the high activity of metsulfuron methyl to growth in plants is obvious in both media and this is in accordance with the mode of action.

Although these three compounds were chosen for their modes of action, the IC_{50} values determined were in the range of observed environmental concentrations, which indicates that this sediment contact assay may also be suitable to detect them in natural sediments. For example, Devault et al. (2009) determined atrazine concentrations up to 0.85 mg kg^{-1} (dry weight) in dam sediments from the River Garonne (France). During a monitoring campaign, Degenhardt et al. (2010) measured metsulfuron methyl concentrations of 1.4±1 µg kg^{-1} in wetland sediments, which were sampled from the St. Denis National Wildlife Area (Saskatchewan,

Canada). DNOC concentrations of up to 2.08 mg kg^{-1} (dry weight) were found in sediments from the Grand Calumet River (Indiana Harbor, Indiana, USA) by Hoke et al. (1993).

Photosynthetic status as a bioindicator for a whole-plant response

The experiments with atrazine and DNOC showed that the measurement of chlorophyll fluorescence potentially detects impacts of contaminants that either directly or indirectly inhibit the energy metabolism (Marwood et al., 2001; Küster et al., 2007), while the results for metsulfuron methyl suggest that this parameter is less sensitive to detrimental effects on the synthesis of biomolecules such as amino or fatty acids (Küster et al., 2007). When contaminants inhibit any cellular process downstream of PS II, such as carbon assimilation, respiration, membrane damages or proteins, the associated photosynthetic electron transport will eventually lead to an increased pressure on PS II (Huang et al., 1997). Therefore, the chlorophyll fluorescence was regarded as a possible bioindicator of detrimental effects to plants provoked by several modes of action (e.g. Marwood et al., 2001; Küster and Altenburger, 2007). A fluorescence-based bioassay for aquatic macrophytes using the I-PAM device developed by Küster and Altenburger (2007) was used for effect detection. For the PS II inhibitors atrazine, isoproturon and prometryn, a good correlation was found between EC_{50} values obtained from a standard growth test over 7 days (DIN EN ISO 20079) and the Y(II) after 24 h with *L. minor* and *Chara canescens* (Küster and Altenburger, 2007). For contaminants with a mode of action different from the inhibition of the PS II, a good correlation between EC_{50} values obtained from the standard growth test over 7 days (DIN EN ISO 20079) and the Y(II) after 24 h with *L. minor* were found for paraquat-dichloride (electron acceptor at PS I) and alizarine (impedes photosynthetic electron transport at the cytochrome b6/f complex). In contrast, for triclosan (fatty acid biosynthesis inhibitor) the EC_{50} value for growth was two orders of magnitude lower than the EC_{50} value from the photosynthetic response (Küster et al., 2007). This leads to the hypothesis that substances with modes of action which are not directly linked to energy metabolism, such as metsulfuron methyl here or triclosan in the study of Küster et al. (2007), could be detected with an integral parameter like growth, while the functional parameter of the photosynthetic status may not be a reliable indicator in the exposure time considered. Due to these differences in sensitivity of the two parameters during the defined exposure time, hints about the mode of action of a contaminant could be gained. This hypothesis, however needs some further elaboration on what this linkage between the mode of action and energy metabolism would mean, during the used exposure time.

Effect status

The $I_{Y(II),tx}C_{50}$ of *M. aquaticum* exposed to sediments spiked with atrazine decreased from a value of 25 mg kg^{-1} at day 1 to 0.8 mg kg^{-1} at the end of the exposure. Franz et al. (2008) found an even more drastic decrease of more than three orders of magnitude in the EC_{50} value for the photosynthetic status within the first 6 h of exposure of *Scenedesmus vacuolatus* to triclosan. Küster and Altenburger (2007) also found a decrease in EC_{50} values over time based on photosynthetic status of the atrazine-exposed macrophytes *L. minor* and *C. canescens* by a factor of three and two, respectively (between an exposure time of 1 and 24 h). These examples indicate the strong time dependence of effects and leads to the question of how to adequately assess the effects of the contaminants on macrophytes.

Franz et al. (2008) investigated the dependence of median effects on cumulative exposure time arising from the concentration−response curves using the photosynthetic activity of algae exposed to a triclosan solution. They found that within the first 6 h, the EC_{50} of triclosan for exposed *S. vacuolatus* and *Nitzschia palea* biofilms decreased rapidly to its lowest value, where it remained almost constant until the end of the exposure period (24 and 72 h, respectively). Küster and Altenburger (2007) found a similar behaviour when exposing *L. minor* and *C. canescens* to an atrazine solution. The EC_{50} based on the photosynthetic status of the exposed macrophytes decreased rapidly within the first 5 h and remained more or less at this value until the end of the exposure (24 h). In contrast, the decrease of $I_{Y(II),tx}C_{50}$ values of exposed *M. aquaticum* to atrazine spiked sediments in this study lasted 13 days. This difference in the duration of the effect expression may be explained by the exposure system used. Franz et al. (2008) exposed algae and Küster and Altenburger (2007) exposed macrophytes to a contaminant in solution, while *M. aquaticum* were exposed with the stem/roots contacting the contaminated sediment. The photosynthetic status of the macrophytes exposed is mainly measured on the upper leaves by the I-PAM measuring device, therefore desorption and transport processes from the sediment to the stem/roots as well as uptake and distribution of contaminants to the upper leaves of *M. aquaticum* may delay the response (deCarvalho et al., 2007). In contrast, algae (Franz et al., 2008) and macrophytes (Küster and Altenburger, 2007) were exposed with almost their entire surface contacting the contaminated solution (*L. minor* floating on the surface of the exposure solution), thus uptake into the organisms may occur directly by diffusion through their leaf surface and may induce effects in the whole organism within a shorter time frame.

Because of the various transport processes of compounds from sediments into and inside exposed macrophytes, as well as the possibility for compensatory

capacities to occur, a steady state in effect expression may not be reached for all substances within a 13-day exposure. This may lead to a misjudgement of potential hazards of sediments to macrophytes. As the exposure time of the macrophytes in multiwell plates could not be extended due to space restrictions (plants reach the cover), concentration–time–response modelling may give an appropriate long-term effect estimate. The measurement of the non-invasive observation parameter of the photosynthetic status in exposed macrophytes over time allows the evaluation of the effect development of contaminants regarding this endpoint. Thus, using concentration–time–response modelling means that an $I_{Y(II),t\text{-infinite}}C_{50}$ as well as the time frame for a fully exerted contaminant effect may be estimated. This model, however, has some restrictions. The parameters used (Eq. 5.6, Θ_2–Θ_5) were fitted to the data set and, thus, the models predictive efficiency is basically descriptive. Processes that may alter the subsequent time–response profile are not considered, and thus may lead to a misjudgement, as was seen at the estimated $I_{Y(II),t\text{-infinite}}C_{50}$ value for atrazine (–0.22 mg kg^{-1}). Furthermore, not all contaminants may directly interfere with the energy metabolism and thus the inhibition based on Y(II) may not reflect total sediment hazard alone. A way forward will be to determine the FWC inhibition over time. The two concentration–time–responses in the FWC and the Y(II) may give hints toward a contaminant mode of action and, based on the slope of resulting IC_{50} time curves, a weighing of the effect status could be done to make decisions for further specific in-depth studies.

The occurrence of growth gain

A clear and pronounced FWC gain of macrophytes exposed to low concentrations of atrazine, metsulfuron methyl or DNOC compared to the control (see Fig. 5.2d–f) was observed, where the spiked concentration was one to two orders below the lowest inhibitory concentrations. Although, the natural sediment sampled at Altrip induced a significant gain in FWC in exposed *M. aquaticum* whorls (see Fig. 5.3b). This effect has been described in the literature and is referred to as hormesis (e.g. Calabrese and Baldwin, 2002; Cedergreen et al., 2007; 2008), which is hypothesised to represent an organisms strategy of optimal resource allocation that ensures homeostasis maintenance or simply as chemical stress, which increases growth (e.g. Cedergreen et al., 2009). However, the aim of this study was to investigate the applicability of the sediment contact assay to determine inhibitory rather than evaluate growth gain effects of contaminants to exposed macrophytes.

Natural sediments

The sediment sampled from Bitterfeld completely inhibited the growth in exposed *M. aquaticum*, while Y(II) remained almost unaffected compared with the control. This sediment was sampled after the River Elbe flood in August 2002, when it was expected that large amounts of suspended organic matter were remobilised, transported and settled in downstream affected rivers (Grote et al., 2005a). The hazard assessment of this sediment revealed a strong toxicity, assessed by determining severe effects (effect >70%) in different bioassays, including inhibition of algal reproduction, immobilisation of *Daphnia magna*, development of fish embryos, bacterial luminescence, dioxin-like effects measured by the induction of ethoxyresorufin-*o*-deethylase (EROD) and cytotoxicity (for details, see Grote et al., 2005a). In accordance with previous studies (Brack et al., 1999), the PS II inhibitor prometryn and the phytotoxin *N*-phenyl-β-naphthyl amine were found in concentrations that are expected to induce algae toxicity (6.34 and 15.8 mg kg^{-1}, respectively). It could be shown using cause–effect relationship modelling that these two substances contribute to a high degree of the overall algal toxicity of the sediment (Grote et al., 2005b). Considering this high concentration of the two phytotoxins, it is quite surprising that in exposed *M. aquaticum* only the growth was inhibited, while Y(II) remained almost unaffected compared with the control.

The sediment sampled from Dömitz also induced a significant inhibitory effect in FWC in exposed *M. aquaticum* whorls. Similar observations were described by Feiler et al. (2009), who compared various sediment contact bioassays on natural sediments (e.g. the Dömitz sediment). Adverse effects induced by the Dömitz sediment were observed using a Nematode assay (reproduction), an Arthrobacter assay, a fish-embryo assay and a *Myriophyllum* assay (for details, see Feiler et al., 2009). Interestingly, the sediment sampled at Altrip induced a significant gain in FWC in exposed *M. aquaticum* whorls. The chemical analysis of this sediment showed only a low level of priority pollutants (Feiler et al., 2009).

Overall, it was shown that the miniaturised multiwell plate sediment contact assay presented is able to differentially detect induced effects of natural sediments in exposed plants, which were mirrored by results obtained from different bioassays. No significant inhibition of the Y(II) in plants exposed to these sediments was found. Thus, no effect–time modelling could be performed. Nevertheless, even the finding that the toxic action of a sediment is not connected to the inhibition of Y(II) is valuable. Further, at this moment, a clear judgement about whether the less pronounced differences in the FWC values relative to the control value (Altrip, Choranche, Dömitz) are contaminant induced or simply dependent on differences in sediment properties could not be made.

A way forward to improve our understanding of the bioassay would be to study the dependence of FWC and Y(II) on the geotechnical properties of sediments (e.g. texture, porosity, surface charge and organic matter). Furthermore, natural sediments could be spiked with a substance which is known to induce specific inhibitions on both parameters, during effect assessment, such that the influence of the sediment on the effect expression could be evaluated.

Conclusions

The study shows that the methodology presented for assessing the potential hazards of contaminated sediments to macrophytes enables the detection of detrimental effects of spiked as well as natural sediments in *M. aquaticum* in a multiwell plate exposure system. Depending on the mode of action of the chemicals tested, the measure of the effective quantum yield of the PS II was found to be a useful additional effect observation. The difference in sensitivity between the functional and growth observation may give hints about the mode of action of contaminants in sediments to macrophytes, and offer scope for an advanced hazard assessment. Furthermore, the additional observation of the (non-invasive) photosynthetic status over time allows the evaluation of temporal effect development and this may provide additional information about an appropriate exposure regime. A next step forward will be the study of the dependence of observed effects on sediment properties of natural sediments.

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Supporting Information

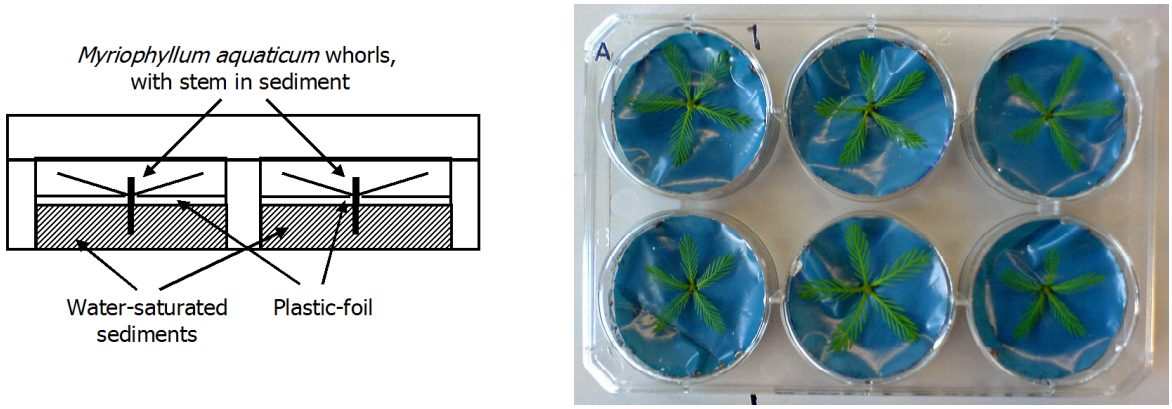

Fig. SI-5.1. On the left side a scheme of the multiwell-plate with 6 slots half-filled with sediment for the sediment contact assay and exposed *Myriophyllum aquaticum* whorls is presented while on the right side an illustration of this exposure system is shown.

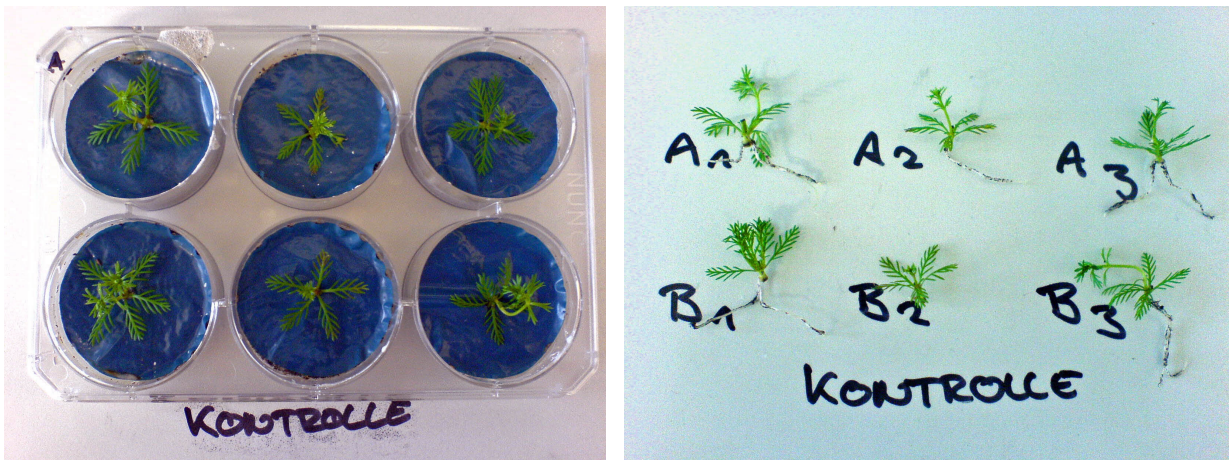

Fig. SI-5.2. *Myriophyllum aquaticum* plants after a 13-day exposure to the artificial sediment (OECD 218) are shown.

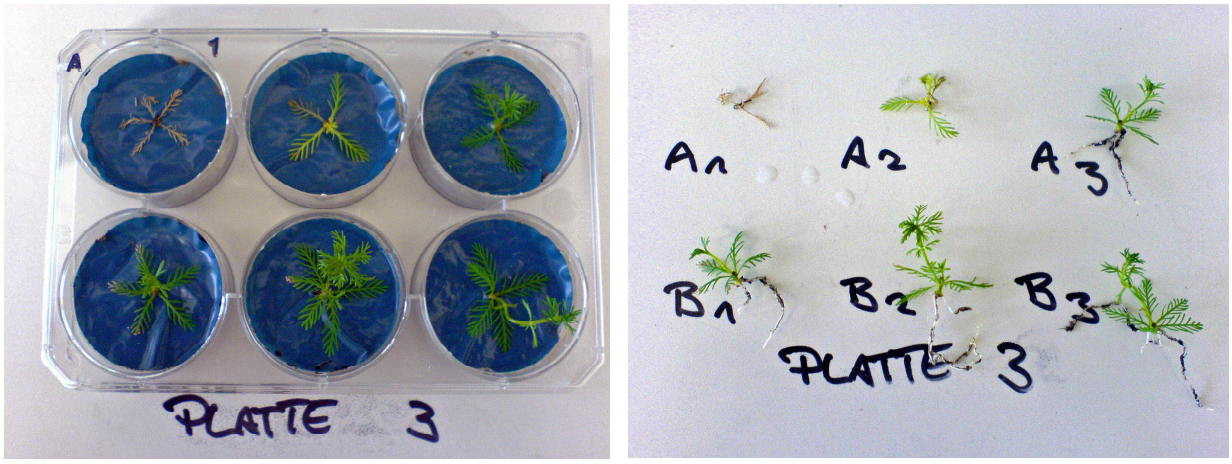

Fig. SI-5.3. *Myriophyllum aquaticum* plants after a 13-day exposure to different concentrations of artificial sediment (OECD 218) spiked with **atrazine**. The **atrazine** concentration was: slot A1 - 10.6 mg kg^{-1}, A2 - 3.54 mg kg^{-1}, A3 - 1.18 mg kg^{-1}, B1 - 0.39 mg kg^{-1}, B2 - 0.13 mg kg^{-1} and B3 - 0.04 mg kg^{-1} (Nominal concentrations are presented as mass toxicant per mass sediment dry weight).

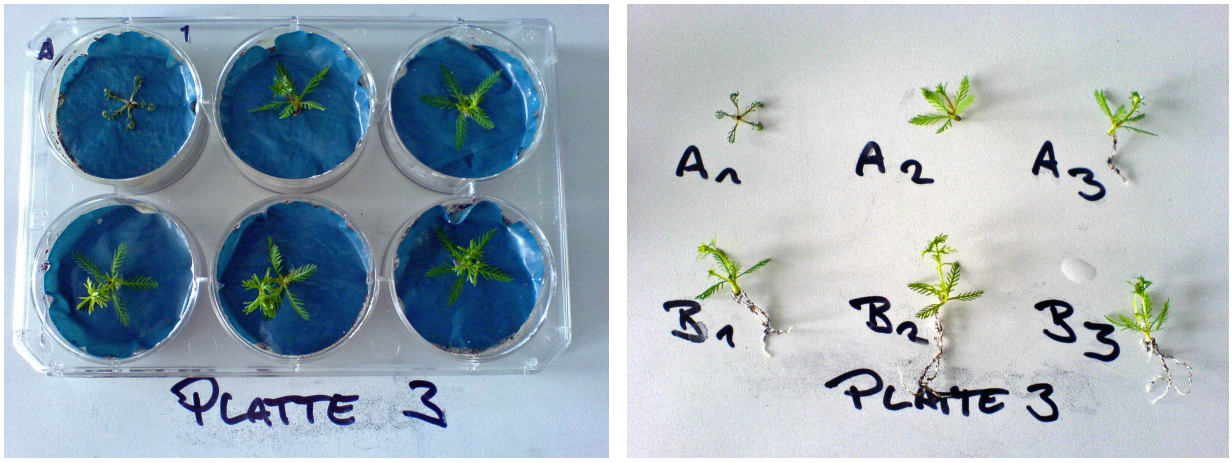

Fig. SI-5.4. *Myriophyllum aquaticum* plants after a 13-day exposure to different concentrations of artificial sediment (OECD 218) spiked with **DNOC**. The **DNOC** concentration was: slot A1 - 26.6 mg kg^{-1}, A2 - 8.96 mg kg^{-1}, A3 - 2.95 mg kg^{-1}, B1 - 0.98 mg kg^{-1}, B2 - 0.33 mg kg^{-1} and B3 - 0.11 mg kg^{-1} (Nominal concentrations are presented as mass toxicant per mass sediment dry weight).

Fig. SI-5.5. *Myriophyllum aquaticum* plants after a 13-day exposure to different concentrations of artificial sediment (OECD 218) spiked with **metsulfuron methyl**. The **metsulfuron methyl** concentration was: slot A1 – 531.4 ng kg^{-1}, A2 - 177.1 ng kg^{-1}, A3 - 59.01 ng kg^{-1}, B1 - 19.68 ng kg^{-1}, B2 - 6.56 ng kg^{-1} and B3 - 2.19 ng kg^{-1} (Nominal concentrations are presented as mass toxicant per mass sediment dry weight).

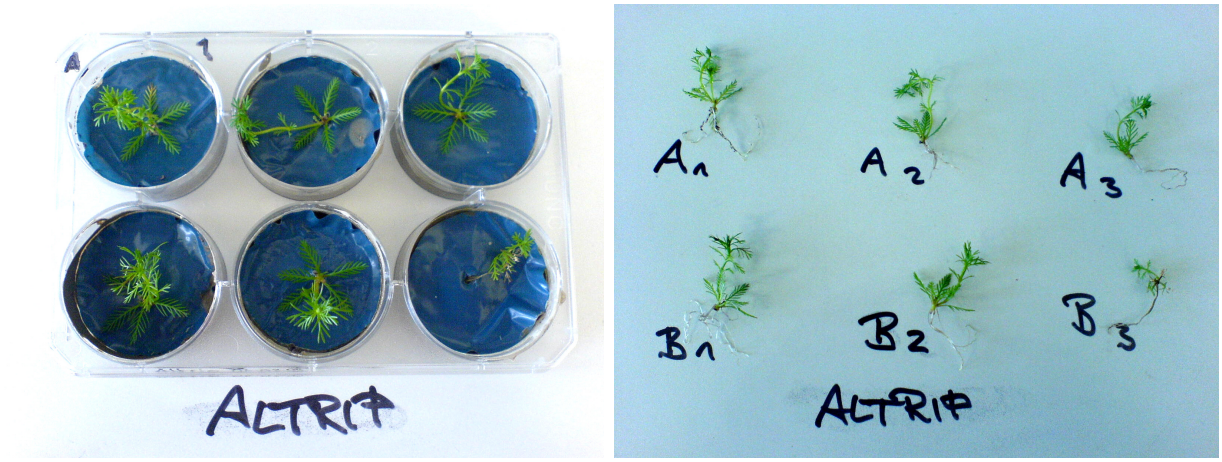

Fig. SI-5.6. *Myriophyllum aquaticum* plants after a 13-day exposure to the artificial sediment (OECD 218, slot A1) and the natural sediment sampled from a river closed to **Altrip** (slot A2 to B3) are shown.

Fig. SI-5.7. *Myriophyllum aquaticum* plants after a 13-day exposure to the artificial sediment (OECD 218, slot A1) and the natural sediment sampled from a river closed to **Bitterfeld** (slot A2 to B3) are shown.

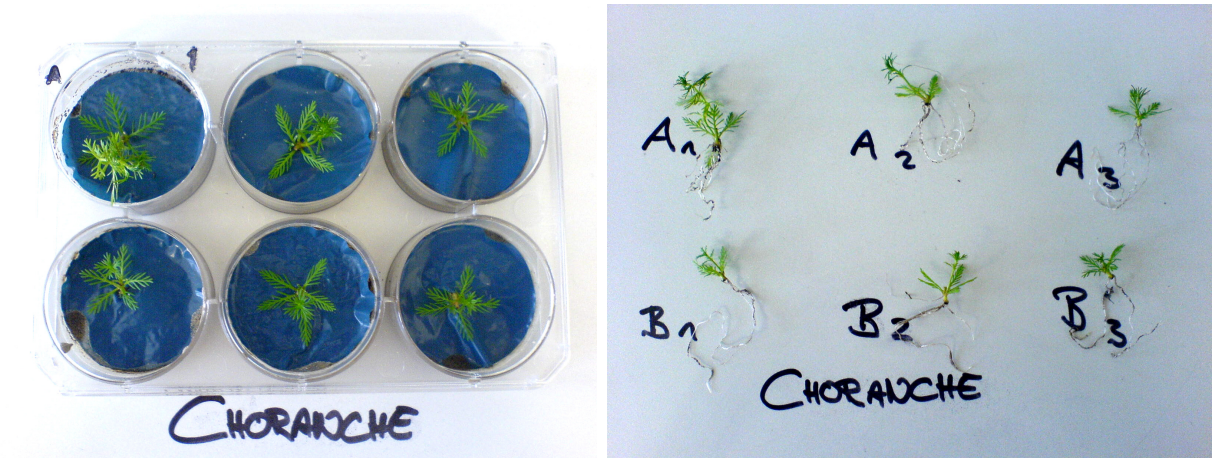

Fig. SI-5.8. *Myriophyllum aquaticum* plants after a 13-day exposure to the artificial sediment (OECD 218, slot A1) and the natural sediment sampled from a river closed to **Choranche** (slot A2 to B3) are shown.

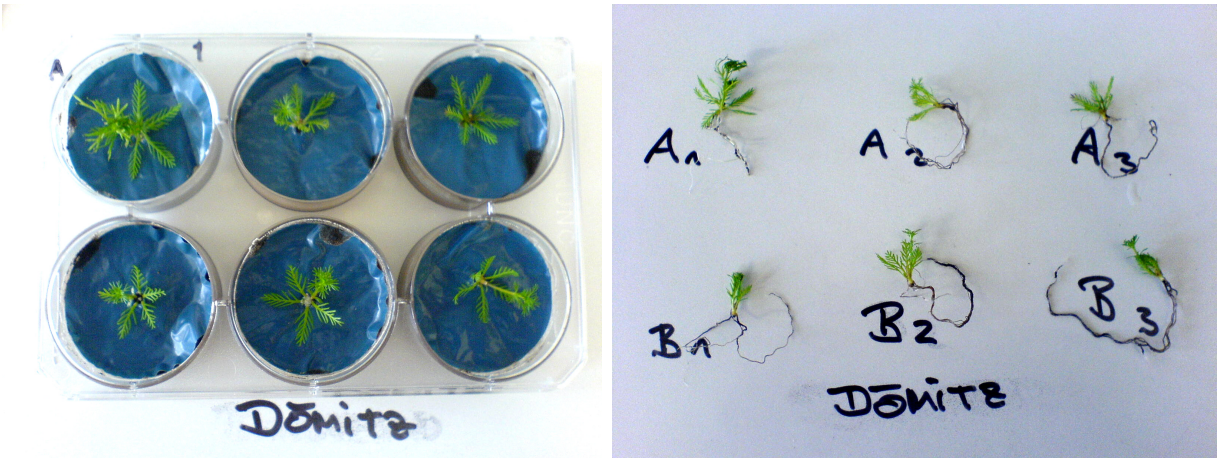

Fig. SI-5.9. *Myriophyllum aquaticum* plants after a 13-day exposure to the artificial sediment (OECD 218, slot A1) and the natural sediment sampled from a waterway closed to **Dömitz** (slot A2 to B3) are shown.

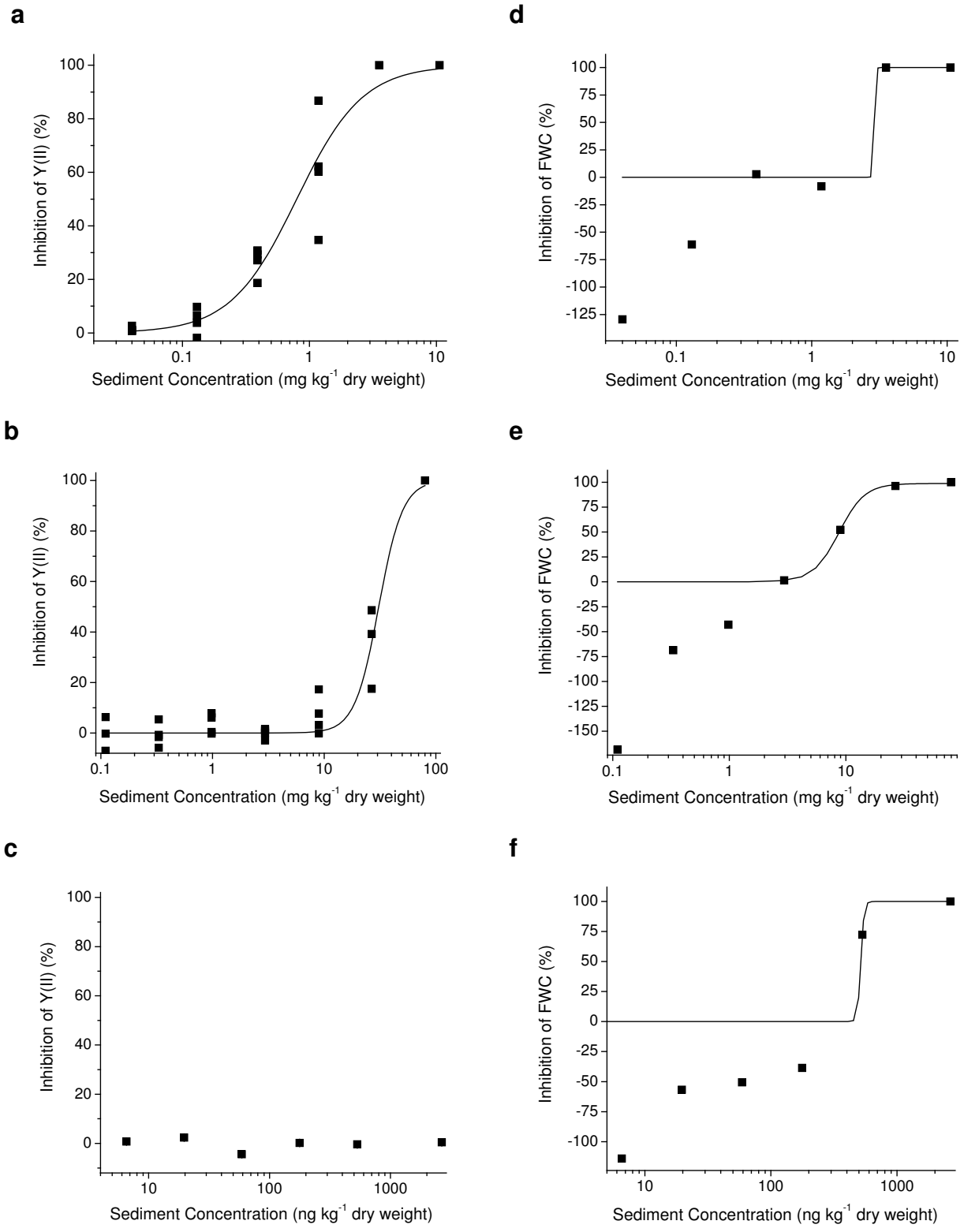

Fig. SI-5.10. Concentration-response-curves of the inhibition of the effective quantum yield of the PS II (Y(II)) (a-c) and the inhibition of the FWC (d-f) compared with control values of 13-day whorls exposed to spiked sediments. Sediments were spiked either with **atrazine** (a; d), **DNOC** (b; e) or **metsulfuron methyl** (c; f). The concentration-response curve was modelled using Eq. 5.5.

Chapter 6

Information on internal exposure for an improved effect assessment of organic compounds

Summary and Future Work

Summary and Future Work

To evaluate the potential hazard of contaminants for the aquatic environment, a battery of single species testing are typically performed (Altenburger, 2002). In general, exposure solutions with different concentrations of a toxicant are prepared and the organisms are exposed. After defined time-points, exposed organisms are observed and the determined effects are linked to the external concentration (e.g. OECD acute testing protocols). As only a fraction of the initial exposure concentration may be bioavailable and induced effects at defined time-points may not fully be expressed, the linkage of determined effects to external exposures may lead to misjudgement of the compound toxicity. The methods presented throughout this thesis will thus contribute to an improved toxicity evaluation of compounds by providing protocols to gain information on internal exposure and by considering the temporal effect of toxicants.

Information on internal exposure for an improved zebrafish egg bioassay

For the evaluation of the potential hazard of chemicals to fish, different fish bioassays have been developed, such as the acute fish toxicity assay (OECD 203, 1992) or the fish early-life stage toxicity assay (OECD 210, 1992). However, with respect to animal welfare and the 3R principle (Replacement, Reduction, and Refinement) defined by Russell and Burch (1959), the extensive use of fish experiments to study a chemical's toxicity is not desirable. Thus, animal replacement assays such as the zebrafish (*Danio rerio*) egg assay are discussed to substitute fish toxicity assays for the testing of chemicals (OECD Draft, 2006). In contrast to the through flow or tank exposure systems used for the acute fish assay (OECD 203, 1992), zebrafish eggs are exposed to a toxicant solution in microwell-plates. It has been shown that the median effect concentrations (EC_{50}) determined from toxicant exposure show a good correlation with respective median lethal concentrations (LC_{50}) determined with the acute fish toxicity assay (OECD 203, 1992). This shows that the zebrafish egg assay may indeed be a suitable surrogate for the testing of chemicals (e.g. Braunbeck et al., 2005; Lammer et al., 2009).

Improved exposure system for the zebrafish egg assay

One validity criteria for the zebrafish egg assay is that the exposure concentrations of tested compounds are maintained satisfactorily (e.g. exposure concentration should at least be 80% of the nominal concentration throughout the exposure; OECD Draft, 2006). It was shown that microwell-plate exposure systems may not be a suitable exposure system for lipophilic and /or volatile compounds, because initial concentrations were found to decrease dramatically during the exposure time (e.g.

Hestermann et al., 2000; Riedl and Altenburger, 2007; Schreiber et al., 2008). For phenanthrene it has been shown, that the concentration decreased by more than 99% under the standard exposure regime for the zebrafish egg assay (microwell-plate, 48 h exposure; Schreiber et al., 2008) and thus, the initial concentration may not adequately represent possible observed effects during an effect assessment. The established protocol for the determination of a compounds bioconcentration parameter in zebrafish eggs presented in Chapter 2, uses amber vials, closed with an aluminium foil. It was shown that with this exposure system, initial phenanthrene concentrations decreased by less than 10% during a 72 h exposure. In contrast to passive dosing approaches, where toxicant spiked dosing devices are used to maintain stable exposure concentrations (e.g. Brown et al., 2001; Mayer et al., 1999; Smith et al., 2010), the exposure concentration could be maintained without using devices, which need sophisticated spiking procedures and further may introduce contamination. Thus, using the amber vial exposure system, the effect assessment with zebrafish eggs could be extended to lipophilic and/ or volatile compounds, where exposure concentration may not be stable in the standard microwell-plate exposure system (Chapter 2). However, further work should include an investigation of the application domain of the exposure system e.g. the range of lipophilicity and/ or volatility of the compounds.

Extraction of accumulated compounds from zebrafish eggs

Assuming that exposure concentrations are stable during effect assessments, the EC_{50} values determined for toxicants with the zebrafish eggs may vary dramatically between different exposures. It could be shown that during a static and non-agitated exposure, the steady-state between the internal and the external phenanthrene concentration was not reached within the standard 48 h exposure time (Chapter 2). Further, it was found that under the static exposure, uptake processes into zebrafish eggs could be accelerated when agitating the exposure solution and the steady-state between the internal and the external phenanthrene concentration was reached within 32 h (Chapter 2). Thus, EC_{50} values determined during toxicant exposure for zebrafish eggs may highly depend on the exposure regime used. Considering that the internal zebrafish egg concentration of a compound may be different at similar external exposure concentrations depending on the exposure regime used, thus, observed toxicant effects during an effect assessment may be better represented by the internal rather than the ambient concentration (e.g. Escher and Hermens, 2002, 2004). To determine the internal concentration of e.g. phenanthrene in zebrafish eggs, accumulated amounts have been extracted from the organism using a biomimetic extraction method, as shown in Chapter 2. Based on the equilibrium between the phenanthrene concentration in the medium, zebrafish

eggs and the silicone rod (SiRo), accumulated amounts could be extracted from the organism reproducibly. For phenanthrene it was shown that approximately 55% of the expected accumulated phenanthrene amount in zebrafish eggs could be re-extracted with the SiRo extraction method. Thus, when compound specific recoveries are determined like for phenanthrene, internal concentrations of exposed zebrafish eggs could be determined using the SiRo-extraction method (Chapter 2). However, further work here should concentrate on the investigation of the application domain of the SiRo extraction method e.g. range of lipophilicity. For compounds outside this application domain, established liquid extraction protocols for fish could be adapted for the extraction of contaminants from zebrafish eggs.

Determination of a compounds bioconcentration parameter for zebrafish eggs

Using, for example the SiRo-extraction method for the experimental determination of internal concentrations from zebrafish eggs, the internal compound concentration in individual zebrafish eggs can only be determined once during exposure and thus, information about the accumulation over time could not be gained. It was found that, similar to fish, the accumulation of phenanthrene by zebrafish eggs could be explained by the compounds partitioning behaviour from the ambient water into the organism (Chapter 2). Thus, a protocol for the determination of a compounds bioconcentration parameter for uptake (k_1) and elimination (k_2) for fish (Banerjee et al., 1984) could be adapted for zebrafish eggs (Chapter 2). Zebrafish eggs were exposed in a static exposure system and the phenanthrene exposure concentration was monitored over time until a steady-state was reached. Under the assumption that partitioning between fish and ambient water is the only concentration loss process, bioconcentration parameters (k_1, k_2, *BCF*) were determined for phenanthrene using a least-square fit to a non-linear model (Chapter 2; Eq. 2.1). The constant rates determined (k_1, k_2) for phenanthrene were found to vary highly depending on the static exposure regime used (e.g. non-agitated vs. agitated), while the resulting *BCF* at steady-state was not influenced (Chapter 2). It was speculated that the phenanthrene transport through the chorion into the perivitelline space was accelerated under agitation compared with the non-agitated exposure (Chapter 2). However, once the specific exposure regime constant rates (k_1, k_2) were determined for a compound, the internal exposure in zebrafish eggs could be simulated over time from the ambient exposure concentration. This enables the linkage of observed effects during an effect assessment of a toxicant with more specific concentration metrics such as the average internal effect concentration (IEC_{mean}), the maximal internal exposure concentration (IEC_{max}) or the area under the internal concentration time curve (*IEAUC*).

Further research should concentrate on the application domain of the presented method for the determination of a compounds bioconcentration parameter e.g. the range of lipophilicity of compounds. It also has to be investigated if the constant rates (k_1, k_2) determined at no observed effect concentrations also be valid for concentration where effects occur. When using internal effect matrices, another question to be solved is at which concentration the observed compounds effect are best represented (e.g. IEC_{mean}, IEC_{max}, $IEAUC$).

The established protocol for the determination of bioconcentration factor and rate constants for lipophilic compounds in zebrafish eggs (Chapter 2) enables the effect assessment with zebrafish eggs for lipophilic and/ or volatile compounds, where exposure concentration may not be stable in the standard microwell-plate exposure system. Further, the internal concentration of lipophilic compounds in exposed zebrafish eggs could be determined with the established SiRo-extraction method. Moreover, when a compounds rate constants have been determined for the specific exposure regime with the protocol presented in Chapter 2, the internal exposure of zebrafish eggs could be simulated over time and this enables the linkage of observed effects with the internal exposure.

Evaluation of the models usability to predict the potential environmental hazard of pharmaceuticals from a comparison of internal exposures

During the last decades, many pharmaceutical compounds (drugs) have been detected in various compartments, including the aquatic environment at low ng L^{-1} to µg L^{-1} (Fent et al., 2006; Richards and Bowron, 1985; Santos et al., 2010). The occurrence of unintended effects in non-target organisms, like the feminisation of male fish from the exposure of municipal effluents containing endocrine active substances, such as ethinyl estradiol (Purdom et al., 1994; Sumpter and Johnson, 2008), raised concern about the potential environmental hazards of pharmaceuticals. Further, Gunnarsson et al. (2008) have shown, that human drug target proteins are highly conserved between human and fish and this was interpreted that receptor mediated responses of drugs could be possible. With respect to animal welfare (Russell and Burch, 1959), the extensive use of long-term fish experiments to study a drugs toxicity is not desirable, thus, the prediction of unintended effects has been proposed to identify drugs where potential environmental hazards may be possible. Huggett et al. (2003) for example, propose an effect prediction model for drugs based on inter-species extrapolations, where the human therapeutic plasma concentration (HPC_T) is compared with the fish plasma steady-state concentration (FPC_{ss}). Based on the assumption that human drug target proteins are highly conserved between

human and fish, similar or higher FPC_{ss} to HPC_T may lead to receptor mediated responses and thus potential long-term effects may occur.

Likelihood of the existence of human drug targets in fish

To investigate the likelihood and robustness of the fish plasma model (FPM; Huggett el al., 2003) for indicating a drugs potential environmental hazard for fish, 42 drugs from different therapeutic classes were used for a simulation study presented in Chapter 3. It could be shown that the existence of structurally and functionally conserved protein targets in zebrafish could not be refuted and thus, the application of the FPM was not in contradiction to the basic assumption of inter-species extrapolation (Chapter 3). The existence of comparable target proteins indicates that the HPC_T may represent an adequate internal effect concentration in fish. Based on the comparison of HPC_T and FPC_{ss}, the detection of possible unintended drug effects in fish seems possible. In further work it may be investigated, if indeed a receptor mediated response in fish occur at a comparable FPC_{ss} with respect to the HPC_T.

Dependence of the FPC_{ss} *estimate on the bioconcentration model*

When assuming that the drugs HPC_T represents an appropriate internal effect concentration in fish, the accurate determination of FPC_{ss} from external concentration is crucial for an adequate long-term effect prediction for drugs. For the estimation of the FPC_{ss}, Huggett et al. (2003) use the drugs partition coefficient between the aqueous phase and the arterial blood of rainbow trout ($K_{Blood:Water}$) in combination with an environmental concentration. When measured drug $K_{Blood:Water}$ values are not available, Huggett et al. (2003) propose to predict this parameter using a correlation function generated from measured $K_{Blood:Water}$ values of polychlorinated biphenyls, -ethanes and benzene and the respective octanol:water partition coefficient (K_{OW}), based on the work of Fitzsimmons et al. (2001). As the $K_{Blood:Water}$ represents the drugs partitioning between the fish blood plasma and the ambient water, a good correlation with the compounds *BCF* (partitioning between the fish and the ambient water) was assumed (see Chapter 3). As *BCF* is an important criteria in the chemical risk assessment, a large data set of experimental determined *BCF*s are available, in contrast to $K_{Blood:Water}$. Hence, established *BCF* estimation methods could thus be assumed to yield more accurate results. When using the *BCF* determination method recommended in the Technical Guidance Document (TGD) for the European Chemical Risk Assessment (Part 2) (TGD model; TGD, 2004), *BCF*s could be estimated based on K_{OW} as descriptor. It was shown that resulting FPC_{ss} values differed by less than a factor 6 (for compounds with a log K_{OW} between 1 and 6) when using either the $K_{Blood:Water}$ or the *BCF* estimation method, thus, the FPC_{ss} estimate seems to be independent of the bioconcentration model used (Chapter 3). It

has been shown that for chemicals within the TGD models domain (neutral, nonpolar, nonionised and absence of rapid metabolism), the predicted *BCF*s compare well with the experimental *BCF*s (TGD, 2004). Hence, for compounds within the TGD model domain, an accurate *BCF* and thus a suitable FPC_{ss} estimate could be expected.

BCF *estimation for compounds outside the TGD model domain*

From the 42 drugs used for the modelling simulation, only 6 compounds did not show the potential to dissociate in water and only 4 of them could be assumed to be nonpolar (log K_{OW} ≥1; Chapter 3), thus, the TGD model could only be used for 4 out of 42 compounds to obtain an accurate *BCF* estimation. Fu et at. (2009) show that for monovalent weak organic acids (pK_a >7.5) or bases (pK_a <6), good correlations between *BCF* and apparent K_{OW} (D_{OW}; sum of log K_{OW} of neutral and ionic form at a specific pH) exist. This was explained by the fact, that at neutral ambient pHs, larger fractions of the non-dissociated form of weak acids and bases are present and therewith, $D_{OW} \approx K_{OW}$. Thus, for these chemicals, the accumulation behaviour is assumed to be similar to compounds within the TGD models domain (Fu et al., 2009). When extending the TGD models domain (neutral, nonpolar, nonionised, absence of rapid metabolism; TGD, 2004) to monovalent weak organic acids (pK_a >7.5) or bases (pK_a <6), then an accurate *BCF* prediction for 13 out of 42 drugs could be assumed. Thus, for 31% of the drugs, the TGD model could be assumed to estimate an accurate *BCF*, while this model may not be applicable for almost 70% of the drugs (e.g. Arnot and Gobas, 2006; Fu et al., 2009; Meylan et al., 1999).

Important processes which substantially alter uptake and elimination rates from the purely lipophilicity-driven partitioning into fish are known, such as active transport, electrical interaction with proteins, ion trapping and metabolisation (e.g. Arnot and Gobas, 2006; Fu et al., 2009; Meylan et al., 1999). For such compounds, different models were developed like the dynamic cell model based on the Fick-Nernst-Planck equation (Fu et al., 2009), the *BCF* estimation model of Meylan et al. (1999) or the usage of D_{OW} values with the TGD model (Fu et al., 2009). However, none of these models showed an overall good performance (Fu et al., 2009). As the majority of the drugs were either ionic, showed potential to dissociate and/ or were not lipophilic, no accurate *BCF* estimation when using the TGD model could be assumed for these compounds and no accurate long-term hazard prediction could be expected based on the estimated FPC_{ss} (Chapter 3).

What to do with drugs out of the BCF *model domain?*

For the estimation of *BCF* for compounds where the bioconcentration process is not driven by the lipophilic partitioning into fish, more complex models have been developed, like the dynamic cell model based on the Fick-Nernst-Planck equation

(Fu et al., 2009) or the advanced fugacity model from Trapp et al. (2010). Such models are highly parameterised, however, and the effort to estimate accurate *BCF* values should be balanced with the final goal to estimate an accurate internal fish concentration for an environmental exposure. In the environment, the uptake is not restricted to bioconcentration alone, but could also occur via dietary and other ambient sources. It was shown that toxicant uptake via e.g. diet contribute substantially to the overall internal concentration for compounds where uptake is not driven by the lipophilic partitioning (e.g. Arnot and Gobas, 2006). Since strong bioaccumulation of pollutants from the ambient environment into fish has been found for lipophilic compounds only (log K_{OW} ≥1; Arnot and Gobas, 2006), thus, using the TGD model with apparent K_{OW} and a fixed *BCF* value for compounds where uptake may not be driven by lipophilicity (apparent log K_{OW} <1; Fu et al., 2009), may seem reasonable, when considering the lack of models for an accurate prediction for the uptake from the environment.

In further analyses, it could be investigated if drugs occur in the environment at concentration levels equivalent to or higher than the HPC_T. If the identified drugs are not within the extended TGD model domain, FPC_{ss} values could may be determined experimentally using established extraction protocols for fish for these target drugs.

To investigate the likelihood and robustness of the FPM for indicating a drugs potential environmental hazard for fish, a set of 42 drugs was used for a simulation study (Chapter 3). It could be shown that the existence of structurally and functionally conserved protein targets in zebrafish could not be refuted and thus, the usage of HPC_T as an internal effect concentration in fish seems possible (Chapter 3). To estimate FPC_{ss} for the effect prediction from an environmental exposure, different bioconcentration models based on correlation functions with K_{OW} were used. As accurate *BCF* estimates could only be expected for drugs within the bioconcentration model domain (neutral, nonpolar, nonionised, absence of rapid metabolism), no accurate FPC_{ss} estimates could be assumed for more than 90% of the investigated compounds (Chapter 3). When extending the TGD models domain to monovalent weak organic acids (pK_a >7.5) or bases (pK_a <6), still no accurate *BCF* prediction could be expected for almost 70% of the drugs. When assuming that the HPC_T is an appropriate internal effect concentration for fish, with the FPM only for a fraction of the existing drugs, accurate long-term effect predictions for an environmental exposure could be assumed. The estimation of accurate FPC_{ss} from environmental exposure for drugs, where uptake may not be driven by lipophilicity, seems challenging at this moment. Considering these, the FPM may provide a reasonable effect prediction for drugs at environmental concentrations, under the assumption that

HPC_T is an appropriate internal effect concentration for fish and drugs are within the bioconcentration models domain.

Internal OCP exposure in river grown *Myriophyllum aquaticum* plant

Organochlorine pesticides (OCPs) such as chlorpyrifos, endosulfan or heptachlor have been used intensively for decades in Argentinian agriculture. As a result, OCPs have not only been found at the site of application, but also in the surrounding environment (e.g. Miglioranza et al., 1999). Because of agricultural runoff and spray-drift, the nearby aquatic environment (e.g. rivers) have been identified as the principal destination for OCPs (e.g. Wauchope, 1978). Because of their physico-chemical properties such as lipophilicity, OCPs generally become associated with sediments and suspended particles when entering the aquatic environment, and thus, sediments are recognised as the major sink (e.g. Knezovich et al., 1987; Warren et al., 2003). Given that OCPs are designed to elicit a specific biological action, potential hazards to the aquatic environment may be possible, especially considering the persistence of these compounds. As rooted water plants could not only take up pollutants from the water phase but also via roots (e.g. Hinman and Klaine, 1992; Turgut and Fomin, 2002), sediment-bound OCPs may become a relevant source. To evaluate the local environmental hazard of OCPs to rooted water plants, the bioavailable fraction from the water and the sediment phase has to be considered. Although the determination of the bioavailable fraction from the water phase is assumed to be adequately assessed, when using e.g. passive sampling techniques (e.g. Stuer-Lauridsen, 2005; Vrana et al., 2005; Zabiegala et al., 2010), the determination of the bioavailable fraction from sediments is still challenging as uptake processes in plants and desorption processes from sediments are more complex (e.g. Reid et al., 2000). Thus, the most realistic way to evaluate the integral bioavailable fraction from the water and the sediment is to determine the local OCP contamination within exposed rooted aquatic plants. Because of the limited mobility and presence all year round, rooted submerged *M. aquaticum* plants have the potential to function as local *in situ* biomonitor for the determination of the bioavailable fraction of OCPs (e.g. Gobas et al., 1991).

Determination of internal OCP levels in M. aquaticum

To investigate the bioavailable OCP fraction for rooted aquatic plants in the river Xanaes (province Córdoba, Argentina), the internal OCP exposure in *M. aquaticum* plants were determined from plants collected in an agricultural and in a mountain area (Chapter 4). Because OCPs, such as chlorpyrifos, endosulfan or heptachlor, have been used intensively in the agricultural area investigated, higher OCP levels in *M. aquaticum* plants sampled from this area were expected compared with the

mountain area. Surprisingly, the results from the five months of monitoring showed similar contamination levels in plants at both sampling sites (Chapter 4). The internal plant concentrations of the detected OCPs β-BHC, γ-chlordane, chlorpyrifos, p,p'-DDE, endosulfan I, endosulfan sulfate, endrin, heptachlor and hexachlorbenzene were below 5 µg kg^{-1} (dry weight), with the exception of trans-permethrin (17.6 µg kg^{-1} dry weight). Based on these results, it is speculated that the OCP contaminations determined appear to be residues from former use, otherwise higher OCP levels in plants from the agricultural area should have been observed.

During the five months of sampling (from April until August 2010), only light rain occurred but with no obvious runoff. As OCP contamination in rivers resulting from agricultural runoff depend on the time period investigated (Schulz et al., 2001), the internal OCP exposure in *M. aquaticum* plants should be determined during the wet summer months in future monitoring campaigns.

Internal OCP concentration-time-profiles in M. aquaticum

To investigate the seasonal variation of internal OCP levels in exposed rooted aquatic water plants, *M. aquaticum* plants were collected at both sampling sites every two weeks beginning in early fall until mid-winter (Chapter 4). Not only were the OCP concentration levels in *M. aquaticum* plants found to be comparable at both sampling sites (agricultural and mountain area), also a clear concentration decrease behaviour towards a steady concentration was found for β-BHC, chlorpyrifos, endosulfan I and endrin at the both sampling sites over time. For the two detected OCP metabolites, p,p'-DDE (agricultural site) and endosulfan sulfate (both sites), a concentration increase was observed over the first sampling days, followed by a decrease towards a constant concentration (Chapter 4). This elimination behaviour with time could be described with a first order decay function (Chapter 4; Eq. 4.1), and resulting pseudo elimination rates ($k_{2,pseudo}$; Eq. 4.1) for the continuously detected OCPs (p,p'-DDE [begin day 28], endosulfan I, endosulfan sulfate [begin day 42] and endrin) were in the same order of magnitude (Chapter 4; Table 4.2). As uptake and elimination processes for lipophilic organic chemicals into water plants are generally compound specific e.g. correlate with compounds K_{OW} (e.g. Gobas et al., 1991), an integral rather than a substance-specific process was assumed. It is speculated that the concentration decrease over time correlates either with reduced OCP entry as a consequence of lessened runoff during the almost rainless sampling period or the dependency of compounds desorption rate from sediments on temperature (Chapter 4). The second hypothesis is based on the assumption that the major uptake of OCPs into plants occurred from sediments, because observed OCP concentration-time-profiles in surface water and in *M. aquaticum* (except for endosulfan sulfate)

showed a poor correlation (Chapter 4). Further, Cornelissen et al. (1997) showed that the slow desorption kinetics from sediments are highly temperature dependent for lipophilic organic compounds. During the first 40 days of sampling, a decrease in surface water temperatures of about 10 K was observed, while resulting temperature levels remained around the same until the end of monitoring. This temperature-time behaviour correlated very well with observed OCP concentration-time-profiles in *M. aquaticum* (Chapter 4). Although these two hypothesis may explain the similar concentration decrease behaviour for the OCPs, they fail to interpret the previously observed concentration increase for p,p'-DDE and endosulfan sulfate (Chapter 4). It is known that plants such as *M. aquaticum* have the potential to metabolise p,p'-DDT into p,p'-DDE (Gao et al., 2000) and endosulfan into endosulfan sulfate (Gupta and Gupta, 1979). Thus, the observed concentration increase for p,p'-DDE and endosulfan sulfate in *M. aquaticum* may be explained by the metabolisation of their parent compounds (Chapter 4).

In further monitoring studies, the OCP concentrations in plants and sediments should be determined in parallel. This may indicate whether sediments are indeed the major source for OCP contaminations in *M. aquaticum*. Moreover, based on determined concentration-time profiles for p,p'-DDE and endosulfan sulfate in sediments and plants, this may uncover the possible contribution of metabolisation to the observed temporal concentration increase in *M. aquaticum*.

To assess the bioavailable OCP fraction for rooted aquatic plants in the river Xanaes (province Córdoba, Argentina), OCP concentrations in the submerged *M. aquaticum* plant and in surface waters were determined over time (Chapter 4). Since the concentration-time-plots of OCPs in surface water and in *M. aquaticum* plants showed a poor correlation, the OCP concentrations in surface water may not adequately represent the exposure of the rooted water plants to the pollutants. Using the submerged *M. aquaticum* plant, the bioavailable fraction from the sediment and water phase could be determined and thus, the local environmental hazard could be realistically evaluated based on the internal OCP exposure rather than external concentrations.

Temporal effect observations to gain information on potential toxicity and internal exposure of contaminants in sediment exposed *M. aquaticum*

Over the last decades, the increased sediment pollution in rivers, lakes, and oceans have raised concern about the potential hazard for the aquatic ecosystem (Brils, 2002). Hence, numerous bioassays such as the sediment contact assay with *M. aquaticum* (ISO/CD 16191) have been developed to evaluate the potential hazard of contaminated sediments for the aquatic environment. For the assessment of the

toxic effect of sediments with the *M. aquaticum* assay, the fresh weight change (FWC) between the beginning and the end of exposure is determined and compared to a control (ISO/CD 16191). Since uptake and effects of contaminants in exposed *M. aquaticum* may not occur immediately but evolve over time, restricting the effect assessment to the measurement at one pre-defined time point may lead to misjudgement of the sediments' potential toxicity. It has been shown that biological functions such as the photosynthesis were commonly affected by toxicants (Babu et al., 2005). Hence, the detection of an impaired photosynthetic status compared to a control by measuring the chlorophyll fluorescence was found to be a reliable method for the identification of the potential hazard of contaminants to plants (e.g. Huang et al., 1997; Krugh and Miles, 1996; Samson and Popovic, 1988). Because the chlorophyll fluorescence in plants could be observed non-invasively, the temporal effect expression of a toxicant could be determined using this end-point.

FWC vs. chlorophyll fluorescence

To determine the toxic effects of sediments on the photosynthetic status in exposed plants, the established sediment contact protocol with *M. aquaticum* (ISO/CD 16191) was adapted to a microwell-plate exposure system (Chapter 5). This enables the non-invasive measurement of the effective quantum yield of energy conversion at photosystem II (PS II) reaction centres [Y(II)] over time in parallel to the terminal measurement of the FWC of exposed *M. aquaticum*. Thus, at the end of a 13-day exposure, the median inhibition concentration of a compound (IC_{50}) on FWC and on Y(II) could be determined at the same time (Chapter 5). To evaluate the dependence of the observation parameter sensitivity on a toxicants biological action, *M. aquaticum* were exposed to sediments spiked with pesticides of different biological action (Chapter 5). For the PS II inhibitor atrazine, it was found that Y(II) was the more sensitive parameter compared to FWC based on the IC_{50} values determined (0.79 mg kg^{-1} and 2.90 mg kg^{-1}, respectively). In contrast, for the acetolactate-synthese inhibitor metsulforon methyl, the IC_{50} value determined for FWC of exposed *M. aquaticum* was 0.52 µg kg^{-1}, while no effects on the Y(II) were observed in the concentration range investigated (Chapter 5). For dinitro-*o*-cresol (DNOC), a oxidative phosphorylation decoupler, IC_{50} values for Y(II) and FWC were of the same order of magnitude. Although the results are in agreement with current mode of action understanding, it shows, that observed effects on FWC and Y(II) at a pre-defined exposure time are highly dependent on the biological action of the contaminant. Thus, hints about the pollutants biological action in *M. aquaticum* plants could be gained based on differences between the inhibitions on the Y(II) and FWC at the end of the 13-day of exposure (Chapter 5).

In future studies, it could be investigated if the sensitivity differences based on IC_{50} values between FWC and Y(II) observed remain valid also during longer exposures. This could reveal whether both observation parameter indeed possess a different sensitivity to toxicants or if this observed sensitivity difference only represents the current effect status of the contaminant for the respective observation parameter.

Information on internal exposure gained from temporal effect observation

To investigate the temporal effect expression of toxicants in *M. aquaticum*, the Y(II) was measured at different time-points. It could be shown that during the exposure of *M. aquaticum* to atrazine spiked sediments, the IC_{50} value on Y(II) steadily decreased from 25 mg kg^{-1} at day one to 0.8 mg kg^{-1} at the end of the exposure (Chapter 5). Further, the inhibition of Y(II) in atrazine exposed *M. aquaticum* could be considered as concentration and time dependent using concentration-time-response modelling. From the results of the concentration-time-response modelling, it was determined, that effects on the Y(II) were not fully expressed within the 13-days of exposure and that a longer exposure time is needed for complete effect expression (Chapter 5). Hence, the decrease in the Y(II) IC_{50} value over time comprises the integral information over all the atrazine uptake and the distribution into and the toxico-dynamic processes leading to the adverse effect.

Küster and Altenburger (2007) investigated the dependence of IC_{50} values on cumulative exposure time using the photosynthetic activity of macrophytes exposed to solutions of different PS II inhibitors. They found that the IC_{50} of atrazine, isoproturon and prometryn for exposed *Lemna minor* and *Chara canescens* decreased rapidly to its lowest value within the first 5 hrs, where it remained almost constant until the end of the exposure period (24 h). If interference to the electron transport in the photosynthesis leads to an observable inhibition on the effective quantum yield of energy conversion at PS II reaction centres [Y(II)] almost immediately after the binding of e.g. atrazine to the Q_B -binding site on the D1 protein, the IC_{50} -time plot for Y(II) represents the uptake and distribution process of atrazine from the exposure media to the target site until steady-state concentration. If this is also true for *M. aquaticum* exposed to atrazine spiked sediments, then the IC_{50} time plot for Y(II) directly correlates with the exposure at the contaminants target site (see Chapter 5).

Assuming that for compounds that directly interfere with the photosynthesis, an inhibition at the Y(II) could be observed immediately after the interaction with their respective target site, the IC_{50} –time plots determined could thus be assumed to correlate with the temporal exposure at the target site. In contrast, for compounds with a different biological action such as DNOC or metsulfuron methyl (Chapter 5), the temporal effect observation may not directly correlate to the internal exposure.

In further studies, it should be investigated which other biological action may lead to an interference in photosynthesis and how long the effect translation from the target site interaction to an observable effect on the Y(II) will take. Moreover, additional non-invasive observation parameters such as the cellular respiration could be included, so that the temporal effect observation may also be possible for compounds with different biological actions.

With the established sediment contact protocol, the inhibition of the Y(II) could be determined in parallel to the whole-structural parameter (FWC) for exposed *M. aquaticum* to contaminated sediments (Chapter 5). It could be shown that, depending on a toxicants biological action, either the FWC or the Y(II) was more sensitive for the exposure time considered based on determined IC_{50} values. As a result, information about the pollutants biological action in *M. aquaticum* plants could be obtained. Since Y(II) could be determined non-invasively, the effect expression on the photosynthetic status could be observed over time (Chapter 5). When the biological action of a toxicant directly interfered with the photosynthesis, the effects on Y(II) could be observed virtually instantly. Thus, the temporal decrease in IC_{50} values determined on Y(II) could be directly related to the temporal exposure level of the toxicant at the organisms target site.

The protocols presented throughout this thesis provide experimental and modelling methods to obtain information on internal exposures in aquatic organisms. This improves effect assessment of organic compounds in aquatic organisms, especially regarding the linkage of observed adverse effects to the exposure concentrations.

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Contributions to the manuscripts

As different authors have contributed to the manuscripts, the following highlights the major contribution by the co-authors:

Chapter 3

"A novel in vitro system for the determination of bioconcentration factors and the internal dose in zebrafish (*Danio rerio*) eggs" by R. Schreiber, R. Altenburger, A. Paschke, G. Schüürmann and E. Küster.

R. Altenburger and E. Küster edited all sections. A. Paschke and G. Schüürmann supervised the work.

Chapter 3

"Using the Fish Plasma Model for comparative hazard identification for pharmaceuticals in the environment by extrapolation from human therapeutic data" by R. Schreiber, U. Gündel, S. Franz, A. Küster, B. Rechenberg and R. Altenburger.

U. Gündel elaborated the target conservation part and she had the main contribution on the introduction. More specifically she prepared in the material and method section the "*Pharmacodynamic parameter and target conservation analysis*", in the result section "*Selected compounds*" and the entire paragraph "*Target Conservation*" and in the discussion section "*Extrapolation of target conservation*". R. Altenburger edited all sections and inserted in material and methods the section "*Compound selection*" and in the discussion section "*Proxies for* HPC_T" and contributed to result section "*Estimates for* HPC_T". S. Franz, A. Küster and B. Rechenberg supervised the work.

Chapter 4

"Dynamics of organochlorine contaminants in surface water and in *Myriophyllum aquaticum* plants of the river Xanaes in central Argentina" by R. Schreiber, C.A. Harguinteguy and M.D. Manetti.

C.A. Harguinteguy and M.D. Manetti supervised the work.

Chapter 5

"Non-invasive observation parameter to complement sediment bioassays using *Myriophyllum aquaticum*" by R. Schreiber, A. Küster, U. Feiler, M. Grote and R. Altenburger.

R. Altenburger edited all sections and elaborated the concentration-time-effect modelling. Herein he contributed more specifically in the material and method section "*Concentration-response relationships and concentration-time-effect models*" and in the result section "*Concentration-relationships and concentration-time-effect models*". A. Küster, U. Feiler and M. Grote supervised the work.

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Publications

Schreiber, R., Altenburger, R., Paschke, A., Küster, E., 2007. How to deal with lipophilic and volatile organic substances in microtiter plate assays. *Environmental Toxicology and Chemistry* 27, 1676-1682.

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Conferences

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Poster at the SETAC Latin America, Mar del Plata, Argentina.

Hiermit erkläre ich an Eides statt, dass
ich die vorliegende Arbeit selbstständig
durchgeführt und keine anderen als die
angegebenen Hilfsmittel verwendet habe.

Leipzig, den 3. Oktober 2011