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The impact of chemosensitisation on bioaccumulation and sediment toxicity

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Abstract

Cellular multixenobiotic resistance (MXR) transport proteins enhance the efflux of numerous organic pollutants. However, MXR proteins may be blocked or saturated by xenobiotic compounds, acting as inhibitors — also called chemosensitisers. Although effective on a cellular level, the environmental relevance of chemosensitisers has not been conclusively demonstrated. Since sediments are an important source of bioaccumulating compounds in aquatic ecosystems, sediments and sediment–associated hydrophobic pollutants were investigated for their potential to increase exposure and toxicity in the presence of chemosensitisation. In this study, we address this issue by (1) comparing the net uptake of 17 hydrophobic environmental pollutants by zebrafish (*Danio rerio*) embryos in the presence and absence of the model chemosensitiser verapamil and (2) investigating the impact of verapamil on the dose–dependent effect on zebrafish embryos exposed to polluted sediment extracts. None of the 17 pollutants showed a reproducible increase in bioaccumulation upon chemosensitisation with verapamil. Instead, internal concentrations were subject to intra–species variation by a factor of approximately two. However, a significant increase in toxicity was observed upon embryo co–exposure to verapamil for one of three sediment

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extracts. In contrast, another sediment extract exhibited less toxicity when combined with verapamil. In general, the results indicate only a minor impact of verapamil on the uptake of moderately hydrophobic chemicals in zebrafish embryos.

Keywords: `micro--QuEChERS, multi--mode inlet (MMI) GC--MS/MS, large volume injection (LVI), small volume internal concentration, chemosensitisation, bioaccumulation, sediment toxicology, zebrafish embryo toxicity`

1. Introduction

With a steadily increasing number of anthropogenic chemicals introduced to the market, environmental pollution has become as complex, ubiquitous, and diverse as never before (Daughton, 2005). Hence, exposure to xenobiotic compounds is a major stressor for many organisms. Consequently, the toxicokinetic and -dynamic processes, which determine their toxicity in organisms, are among the major concerns in ecotoxicology. While toxicodynamics summarise the modes of actions and effects produced by pollutants, toxicokinetics, or the concept of absorption, distribution, metabolism, and excretion (ADME), encloses transport processes determining an organism's exposure. In addition to passive diffusion, partitioning, and metabolism, active cellular efflux is an important process of the toxicokinetic transport chain (Schwarzenbach et al., 2005; Smital and Kurelec, 1997; Kurelec, 1995). As all those processes interact, resulting in the removal of harmful toxicants from an organism, understanding and predicting the effect of exposure to complex chemical pollution is an inevitable and challenging task. Mixture toxicity research has attracted substantial attention in the last decade (Faust et al., 2001; Backhaus and Faust, 2012; European Commission, 2012). Toxicodynamic models have been developed to predict combined effects in organisms. However, such models can lead to the underestimation of toxicity, especially when biological transport processes are expected to have a major influence (Kurelec, 1997).

This has been hypothesised for the substrates of broadly–binding multixenobiotic resistance (MXR) transport proteins, belonging to the adinosine triphosphate (ATP) binding cassette (ABC) transporter proteins (Kurelec, 1995). Specifically, these proteins are responsible for reducing the concentration of toxicants in the intra–cellular space by translocating them to the extracellular space, where they can be further transported and excreted (Bard, 2000; Aller et al., 2009; Epel, 1998; Kurelec, 1992; Gottesman et al., 2002). However, MXR may be inhibited by environmental pollutants — so–called chemosensitisers —, which either interfere with the required enzyme activity for the supply of energy as ATP or block the system by slow transport kinetics and/or high binding site affinity (Bard, 2000; Choi, 2005; Ford and Hait, 1994; Kurelec, 1997; Sarkadi et al., 2006).

While its importance has been well established on a cellular level, the environmental impact of MXR and its inhibition remains unclear (Kurth et al., 2015). On the one hand, many studies stress the impact of MXR inhibition on bioaccumulation by complex environmental mixtures (Smital and Kurelec, 1997; Shúilleabháin et al., 2005; Zaja et al., 2013; Smital and Sauerborn, 2002). On the other hand, an induction of protein expression overcoming inhibition has been observed in organisms in polluted habitats, for example river sediments (Smital and Kurelec, 1998).

Sediments are especially important in ecotoxicology, as they serve as long–term sinks for environmental pollutants and therefore acquire complex pollution profiles. They have been studied for their MXR inhibitory potential (Zaja et al., 2013), yet less so with respect to the environmental relevance of that potential (Kurth et al., 2015). This relevance depends on both the presence of potent chemosensitisers and the presence of toxic substrates in sediment mixtures.

The zebrafish (*Danio rerio*) embryo is a popular model organism in ecotoxicology and has been used for the assessment of sediment toxicity in the past (Strmac et al., 2002; Kosmehl et al., 2008; Yang et al., 2010; Wu et al., 2010; Kammann et al., 2004). Its advantages include fast development, simple maintenance, high sensitivity, and complexity (i.e. as compared to cellular *in vitro*

tests) (Scholz et al., 2008; Kosmehl et al., 2008). Furthermore, its MXR proteins have recently been characterised and found to be expressed in early life stages (Fischer et al., 2013).

This study aims at providing information on the impact of chemosensitisation at the organism level. Does the bioaccumulation of toxic substrates increase with MXR inhibition? If not, could changes in toxicity still occur, for example in result of an augmented energy demand? In order to answer the first question, zebrafish embryos were exposed to artificial mixtures of a wide range of common hydrophobic pollutants, likely to adhere to the sediment compartment. Embryo net uptake upon co–exposure to verapamil was compared to net uptake in the absence of the model chemosensitiser. To prevent the excessive use of zebrafish embryos, bioaccumulation was determined with a small volume method for the extraction and quantification of internal organism concentration. This requires sensitive chemical analysis, which can be achieved by exposure to artificial mixtures instead of sediment extracts. To address the second question, zebrafish embryos were exposed to sediment extracts in the presence and absence of verapamil by passive dosing. The impact of chemosensitisation upon embryo teratogenicity was quantified by comparing the resulting dose–response curves. Due to their high sensitivity towards chemical pollution, embryos could be passively exposed to sediment extracts, which has the advantage of closely mirroring environmental exposure conditions.

2. Materials and methods

2.1. Chemicals

Chemicals were acquired as analytical standards at the highest purity available from Sigma-Aldrich (Munich, Germany), Honeywell (Seelze, Germany), Merck (Darmstadt, Germany), LGC (Teddington, UK), Toronto Research Chemicals (Toronto, Canada), Dr. Ehrenstorfer (Augsburg, Germany), or ABCR (Karlsruhe, Germany). A mixed standard solution was prepared at 10 µg/mL in acetonitrile and stored at -20 °C.

2.2. Bioaccumulation experiment

Adult zebrafish maintenance and breeding was conducted according to the standard protocol (Westerfield, 1995) and is briefly summarised in the Supplementary Information (SI, Section S1). The collection of eggs and the culturing of embryos was performed as described by Nagel (2001). Briefly, spawning was induced by turning on the light in the morning. Fertilised embryos were collected from glass trays covered with a 3 mm mesh and artificial plants. After rinsing, zebrafish embryos were selected at the 4 to 8–cell stage using a light microscope.

Zebrafish embryos were independently exposed to three compound mixtures of narrow log D ranges (see Table 1) in the presence and absence of the chemosensitiser verapamil. Verapamil is a non–specific inhibitor targeting a variety of MXR proteins (Vehniäinen and Kukkonen, 2015). Experiments were conducted in 10 mL glass vials with aluminum caps containing 2 mL of ISO–water (SI, Section S1) and ten zebrafish embryos. Each experiment consisted of a non–verapamil solvent control, a verapamil–containing solvent control, a non–verapamil compound mixture of each log D group, and a verapamil–containing compound mixture of each log D group. Each group was again subdivided into five exposure replicates per group and five blank replicates, spiked with the respective amount of acetonitrile and methanol. Exposure concentrations amounted to 10 ng/mL (2 µL 10 µg/mL stock solution in acetonitrile), while the verapamil group was additionally spiked with 4 µL 1 mg/mL verapamil (in methanol) to achieve a nominal concentration of 2 µg/mL. Zhu et al. (2014) estimated an LC_{10} of 166 µg/mL for verapamil hydrochloride in *Danio rerio* embryos. In order to account for solvent influence, all exposure replicates in the control and both groups received the same amount of acetonitrile and methanol. All embryos were exposed for 72 hours on a horizontal shaker at 100 rpm and 26 °C. Before exposure termination, embryo lethal, sublethal, and teratogenic effects were recorded (SI, Section S6). Dead embryos were removed from the exposure medium to prevent microbial infestation of healthy embryos and oxygen depletion. Subsequently, embryos were extracted according to a micro–

QuEChERS (Quick, Easy, Cheap, Effective, Rugged, and Safe) method coupled to a gas chromatography tandem mass spectrometer as detailed in Kurth et al. (2017). Briefly, after thorough rinsing with ISO–water and the manual de–chorination of non–hatched embryos, ten embryos were transferred into 200 µL glass inserts. In order to obtain four full exposure replicates, coagulated embryos were replaced by replenishing from the fifth exposure replicate. Water volume (ISO–water from embryo transfer) was reduced to approximately 70 µL, 70 µL of acetonitrile were added, and phase separation was induced by the addition of 28 mg of $MgSO_4$ and 7 mg of NaCl. The acetonitrile phase was used for further analysis. Three independent replicate experiments following the above descriptions were conducted on different days.

2.3. Sediment sampling and extraction

Sediment samples were collected from the Elbe river basin at three sites: Spittelwasser creek, a minor tributary to river Mulde close to the German industrial area of Bitterfeld, characterised by the strong impact of former chemical industry (51°41'41.9"N, 12°17'20.0"E, May 2008), Bilina, an Elbe tributary with marked petro–chemical pollution downstream the city of Most in the Czech Republic (50°30'18.2"N, 13°40'58.5"E, composite sample June 2006/July 2007), and from the River Elbe near Prelouc, a Czech town downstream of a dye and varnish production area (50°02'35.7"N, 15°34'27.6"E, April 2005) (Schwab et al., 2009). After sampling, sediments were freeze-dried and sieved for the silt and clay fraction (<63 µm). Total organic carbon (TOC) was determined by ignition loss (C-230, Leco). Subsequently, sediments were extracted by pressurised liquid extraction (ASE300, Dionex) in 100 mL cells. Hydromatrix was mixed with an appropriate volume of sediment at an approximate relation of 1:6 and filled into the cells, which were capped with a layer of pure hydromatrix and a cellulose filter. Each cell was extracted twice with acetone/ethyl acetate (1:1) with a pressure of 1×10^7 Pa at 120 °C. The removal of elemental sulfur was required to prevent sulfur–related toxicity, which has been reported to dominate toxic effects by organic contaminants in sediments (Svenson et al.,

1998). In order to minimise the effects of elemental sulfur removal on other chemicals, normal phase column chromatography was conducted to separate the sulfur–containing fraction. Sulfur co-elutes with non-polar chemicals, which — in conctrast to compound classes with polar functional groups — are not sensitive to the this treatment.

Prior to column chromatography, silica gel (0.063-0.2 mm, Macherey Nagel) and aluminium oxide (Merck) were activated at 200 °C for six hours and subsequently de–activated with 3% and 6% (w/w) of bi–destilled water, respectively. Additionally, sample–loaded silica gel was prepared by spiking 1 mg of TOC equivalent sediment extract onto 2.5 mg silica gel in a round bottom flask. The solvent was evaporated to dryness in a rotary evaporator. In a next step, normal-phase columns were prepared for extract separation. The bottom of the column consisted of 5 mg de–activated silica gel, overlaid by 3.3 mg de–activated aluminium oxide and a layer of 2.5 mg sample loaded silica gel, capped with approximately 10 mm of hydromatrix. All numbers refer to 1 mg TOC equivalent and were scaled according to column size. In total, four fractions were collected by subsequent elution from the column: n–hexane (1 mL per 27 mg TOC equivalent), 3:1 n-hexane/dichloromethane (DCM) (1 mL per 40 mg TOC equivalent), DCM (1 mL per 20 mg TOC equivalent), and methanol (1 mL per 30 mg TOC equivalent).

Copper clean–up for sulfur removal of the non–polar (n–hexane) fraction was performed using 1 mg of copper per 10 mg of extracted sediment. Clean–up was performed in Erlenmeyer flasks, which guaranteed an optimal surface area to volume ratio. First, copper was activated in 0.01 mol/L hydrochloric acid (HCl) and sonicated for 15 minutes. Afterwards, HCl was replaced by water in multiple washing steps until pH 5-6 was reached. Water was removed by consecutive washing steps with acetone and n–hexane. Finally, the n–hexane column chromatography fraction was added to the activated copper and stored at 4 °C overnight for copper sulphide to precipitate. The next day, the extract was transferred to a fresh vial using a glass Pasteur pipette. The efficiency of sulfur removal was affirmed by GC–MS analysis. As sulfur was still detectable

in Prelouc and Spittelwasser extracts, copper removal was repeated once.

Finally, all column chromatography extracts were re–combined and the solvent was exchanged to methanol. Aliquots of the combined n–hexane/3:1 n–hexane–DCM and the combined DCM–methanol fraction were retained for chemical analysis.

2.4. Dose–response experiment

In order to ensure constant concentrations and plausible exposure patterns, passive dosing was performed for sediment dose–response experiments. Prior to loading, food–grade polydimethylsiloxane (PDMS) O–rings (with an outer diameter of 14.4 mm and a corresponding volume of 171 µL, Altec, UK) were cleaned in excess methanol by shaking twice for 24 hours. Rings were stored in fresh methanol until usage. Loading was conducted in 20 mL screw vials with aluminium covered caps. One ring was inserted into a clean vial and 2 mL of methanol (solvent blank), methanol plus chemosensitiser (verapamil blank), sediment extract (sample), or sediment extract plus chemosensitiser (chemosensitiser sample) was added, respectively. While solvent and verapamil blanks were conducted to monitor background toxicity, sediment specific blanks at sediment concentrations of their previously determined 50% effect concentrations (EC_{50}) were run to compare sediment toxicity in the absence of verapamil, as the experiments were not run on the same day. A volume of 1 mL of bi–distilled water was added every 10 minutes to a final volume of 20 mL to induce compound partitioning into PDMS. Vials were shaken rigorously for 24 hours. Afterwards, rings were removed, blotted dry with lent–free tissue, and transferred into a 10 mL headspace vial with aluminium covered screw cap used for exposure. A volume of 2 mL of ISO–water was added and vials were shaken for another 24 hours. Equilibration time was assessed in a preliminary experiment (SI, Section S7) and considered sufficient for compounds with a log K_{ow} between 3 and 7. After equilibration between ring and water phase was completed, ten zebrafish embryos (approximately 5 hpf) were introduced into each vial and maintained on a shaker at 75 rpm in a climate chamber at 26±1 °C for an exposure period of

96 hours. Lethal, sublethal, and teratogenic effects were recorded on a daily basis. Coagulation, lack of detachment of tail, no somite formation, no heart beat were considered lethal effect. No formation of eyes, no blood circulation, no pigmentation, edema, and hatching without movement were noted as sublethal effects. Malformation, scoliosis, abnormal tail lenght, and behaviour were regarded teratogenic effects (SI, Section S6). Dead embryos were removed from the exposure medium to prevent microbial infestation of healthy embryos and oxygen depletion.

Based on observations made in a range finding experiment, the following TOC concentrations were selected for dose–response experiments: 12.5, 25, 37.5, 50, and 75 mg TOC equivalents for Bilina; 2.5, 5, 10, 15, and 25 mg TOC equivalents for Prelouc; and 5, 10, 20, 30, and 40 mg TOC equivalents for Spittelwasser.

2.5. Chemical analysis

2.5.1. Bioaccumulation experiment

Embryo extracts were analysed applying the programmed temperature vaporisation gas chromatography tandem mass spectrometry (PTV–GC–MS/MS, Agilent 7000 system) method described in Kurth et al. (2017). Briefly, 50 µL acetonitrile QuEChERS extract were injected into an air–cooled liner. There, solvent was vented off and the analytes were transferred to the column. The oven programme started at four minutes after injection with a duration of 36 to 48 min, depending on the log D mixture. The analytes were quantified by multiple reaction monitoring (MRM), using one quantifier and two qualifiers (Table 1). One non–verapamil and one verapamil–containing solvent blank sample was analysed together with each log D exposure group.

2.5.2. Dose–response experiment

To control exposure concentrations, verapamil was analysed in the exposure medium at 0 and 96 h post exposure by liquid chromatography high resolution mass spectromery (LC–HRMS) using a Thermo Ultimate 3000 LC coupled to QExactive Plus HRMS (Thermo) equipped with an electrospray source. For

LC separation, a Kinetex C18 EVO column (Phenomenex, 50 × 2.1 mm, 2.6 µm particle size) and a water:methanol (both with 0.1% v/v formic acid) gradient were used. HRMS data was acquired in postive full scan ion mode at a range of m/z 100-1000 (resolving power 70,000 referenced to m/z 200) combined with data–independent MS/MS scans for the m/z range of 425-475 (resolving power 35,000) at a normalised collision energy of 35 a.u. Exposure media samples were diluted as appropriate, and 50 µL of methanol were added to 1 mL of medium in a 2 ml autosampler vial and quantified against matrix–matched calibration standards prepared in pristine exposure medium spanning a range from 0.5 to 300 ng/mL. Quantification was done using the extracted ion chromatogram of the $[M+H]^+$ ion of verapamil witin a 5 ppm window of the full scan acquisition, while two diagnostic fragments were used for confirmation.

2.6. Data evaluation

2.6.1. Bioaccumulation experiment

GC data was analysed and quantified using MassHunter (Version B.05.01, Agilent). Calibration curves were fit to the required range of quantification using six relevant calibration points in a row. For each experiment, the analyte internal embryo concentration of the verpamil–containing sample was divided by the respective concentration in the non–verapamil sample. Thus, a value of 100% represents equal internal concentrations in both experiments, while a value higher than 100% indicates an increased net uptake in embryos co–exposed to verapamil. Congruently, a value lower than 100% suggests a decreased net uptake in the presence of the model inhibitor. Statistically significant differences between bioaccumulation in non–verapamil and verapamil–containing exposure groups were evaluated with the Welch Two Sample t-test (two–sided with unequal variance) at $\alpha = 0.05$ and $\alpha = 0.01$.

2.6.2. Dose-response experiment

Dose–response curves were plotted using the R package *drc* (R Core Team and others, 2013; Ritz et al., 2005). Equation 1 describes the derived dose–

response function in dependence of minimum (c) and maximum (d) effect, EC_{50} (e), and slope (b).

$$f(x) = c + \frac{d - c}{1 + b^{(log(x) - log(e))}} \quad (1)$$

50% effect levels were determined according to Equation 1. Their uncertainties were evaluated at the 95^{th} confidence interval.

Table 1: List of analysed compounds, their properties, and detection parameters. Partition coefficients (log D and log K_{ow}) were calculated using the calculator plug–ins of JChem (Chemaxon, Budapest, Hungary). TBP: tributylphosphate; TDCPP: tris(1,3-dichloro-2-propyl)phosphate.

log D	Compound	Molar mass [Da]	log K_{ow}	log D pH=7.5	Retention time [min]	MRM transition 1		MRM transition 2		MRM transition 3	
						m/z	CE	m/z	CE	m/z	CE
4.0-4.3	TBP	266.31	4.09	4.09	11.18	99.0 → 81.0	20	99.0 → 63.0	35	155.0 → 99.0	5
	Piperonyl butoxide	338.21	4.10	4.10	26.65	176.1 → 103.1	25	176.1 → 131.1	15	176.1 → 117.1	20
	Chlorothalonil	265.91	4.10	4.10	15.26	265.9 → 230.9	20	266.1 → 133.0	30	266.1 → 168.0	30
	Prosulfocarb	251.39	4.17	4.17	17.54	128.1 → 43.1	10	128.1 → 86.0	0	91.0 → 65.0	15
	Diazinone	304.35	4.19	4.19	15.12	137.1 → 84.0	15	137.1 → 54.1	15	152.1 → 137.1	0
	TDCPP	427.88	4.28	4.28	24.87	209.1 → 99.0	5	191.0 → 74.9	10	74.9 → 49.0	20
	Pyrene	202.25	4.28	4.28	21.12	202.1 → 201.1	30	202.1 → 200.1	30	100.9 → 100.2	10
	Carfentrazone-ethyl	411.04	4.29	4.29	24.09	326.2 → 177.0	20	325.8 → 121.0	60	330.0 → 310.1	15
4.5-4.8	Flusilazole	315.10	4.58	4.09	22.46	233.0 → 165.1	15	233.0 → 91.0	20	314.7 → 232.9	10
	Bifenox	340.99	4.63	4.63	28.37	189.1 → 126.0	20	189.1 → 133.0	15	340.9 → 309.9	10
	Celestolide	244.37	4.67	4.67	13.96	229.1 → 43.1	25	229.2 → 131.2	15	229.2 → 173.1	5
	Galaxolide	258.40	4.72	4.72	16.03	243.2 → 213.2	15	243.2 → 185.2	15	258.2 → 243.2	10
	Triphenylphosphane oxide	378.09	4.76	4.76	28.61	277.0 → 199.1	35	276.8 → 170.1	60	276.8 → 152.1	50
	2,6-Ditertbutylphenol	206.32	4.76	4.76	10.48	190.8 → 91.0	35	191.1 → 163.1	10	191.2 → 131.1	20
5.5-5.8	Dicofol	370.49	5.56	5.56	18.82	139.0 → 111.0	15	138.9 → 75.0	30	249.9 → 215.1	10
	Permethrin	390.08	5.70	5.70	32.22	183.1 → 168.1	10	183.1 → 165.1	10	183.1 → 153.1	15
	4-Nonylphenol	220.35	5.74	5.74	16.63	107.1 → 77.0	20	107.1 → 51.1	30	219.8 → 107.1	20

3. Results and discussion

3.1. Bioaccumulation experiment

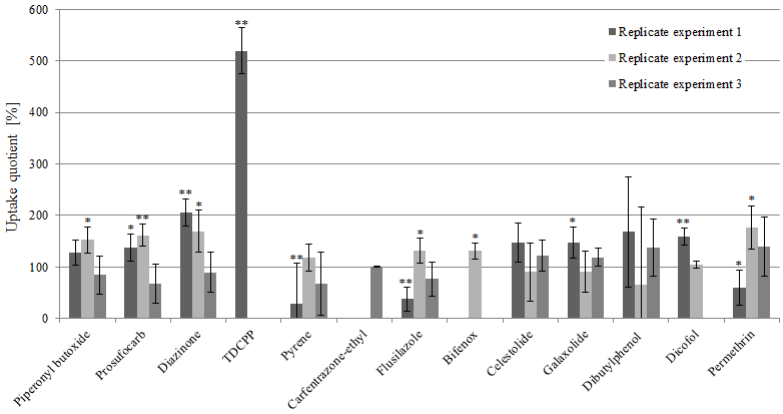

Figure 1: Internal concentrations of organic pollutants in zebrafish embryos exposed to compound mixtures in the presence and absence of the chemosensitiser verapamil. The uptake quotient and its standard deviation in % is given as the internal concentration in zebrafish embryos co–exposed to verapamil (four exposure replicates containing ten embryos, each) divided by the internal concentration in embryos without verapamil (four exposure replicates containing ten embryos, each). Replicate experiments were conducted on different days. Statistical significance was assessed with the Welch Two Sample t–test (two–sided with unequal variance): $^{*}\alpha = 0.05$, $^{**}\alpha = 0.01$.

Internal concentrations were measured in the presence and absence of the model chemosensitiser verapamil after exposing zebrafish embryos for 72 hours to three artificial mixtures (Table 1). Those mixtures consisted of organic pollutants with similar log D ($\delta\,0.3$), which is assumed to result in negligible differences in physico–chemical partitioning within each exposure group. Instead, possible deviations in internal concentration would likely be the result of differences in toxicokinetics. Chemicals were therefore selected according to their log D value. Moreover, the selected compounds are common environmental pollutants associated with the sediment compartment, which has been evaluated less extensively with respect to MXR substrates than the aquatic compartment. TBP, diazinone, TDCPP, pyrene, and triphenylphosphane oxide were further-

more detected in the sediment extracts of Bilina, Spittelwasser, and Prelouc (SI, Section S12). Three replicate experiments were conducted. Figure 1 summarises all results, displaying internal concentrations in verapamil containing normalised to pollutant–only exposure groups. Hence, only compounds detected in both, verapamil–depleted and verapamil co–exposed embryos are illustrated in Figure 1. A result of 100% thus represents no difference in bioaccumulation in the presence of the model chemosensitiser, whereas values higher or lower than 100% account for increased or decreased bioaccumulation, respectively.

The exposure concentration of the model inhibitor verapamil (2 µg/mL) was selected to be high enough to cause inhibitory effects, yet not induce toxicity (SI, Section S4). Luckenbach and Epel (2005) report 50% inhibition (IC_{50}) induced by verapamil at 0.04 µg/mL in mussel gill tissue. At approximately 2 µg/mL, Cunha et al. (2017) observed a significant increase in rhodamine dye accumulation in zebrafish embryos, when exposed to verapamil as compared to a control. Embryos were exposed to target compounds at concentrations of 10 ng/mL, which should not cause lethal effects, while being detectable in the embryos using the analytical method described in Section 2.5.1. During the experiments, no lethal effects were observed. Sublethal effects included yolk sac oedema (all groups), tail malformations (all groups), and seizures (log D 5.5-5.8). Overall, embryo toxicity was not found to be altered by the addition of verapamil (SI, Section S4). Solvent blank embryo internal concentrations were below the limit of detection (<LOD) with rare exceptions. In one case (carfentrazone–ethyl, second replicate experiment), this lead to the exclusion of detected internal concentrations in sample embryos from the evaluation (SI, Section S3).

For the majority of tested compounds, the presence of verapamil has little or no impact on total embryo bioconcentration. Although some compounds are more concentrated in the verapamil exposure groups, this observeration is within a factor of two or not reproducible by replicate experiments. Therefore, they are considered to stem from biological variation. Several compounds display augmented bioconcentration in one replicate experiment, yet not in another, e.g.

diazinone, prosulfocarb, or permethrin. Moreover, flusilazole and permethrin were less concentrated in verapamil co–exposed embryos in the first replicate experiment. Again, the observation was not reproducible. However, 5–fold increased bioconcentration was observed for the flame retardant TDCPP in one replicate experiment. As it could not be detected in the other replicate experiments due to insufficient detection limits (SI, Section S2), this effect could not be confirmed. Tentatively, TDCPP might be a substrate of zebrafish MXR transporters. In the environment, the compound occurs at concentrations of up to 67.0 ng/L, as measured in the German River Scheldt (Bollmann et al., 2012). TDCPP is a known endocrine disruptor in adult zebrafish with impacts on fecundity and reproductive hormone levels (Liu et al., 2013). Moreover, it was reported to produce neurotoxic effects after long–term exposure in zebrafish (Wang et al., 2015). Hence, TDCPP could be a MXR substrate of environmental concern.

While little is known on the increased net uptake of environmental pollutants, studies have been conducted investigating the impact of chemosensitisers on dye or marker compound bioaccumulation. In one of the first papers on chemosensitisation in the environment, Kurelec (1997) describes a 3–fold increase in the net uptake of the radioactive P–gp substrate tritiated vincristine by marine mussels (*M. galloprovincialis*) exposed to diesel–2 oil as compared to non–exposed specimen. Likewise, dos Santos and Martinez (2014) reported a 2–fold increase in rhodamine, a fluorescent P–gp substrate, accumulation in mussel (*C. fluminea*) gills exposed to 10 µg/L Roundup® as compared to clean water. Similar observations were made in zebrafish embryos exposed to 4.5 mg/L of the model inhibitor verapamil. There, rhodamine accumulation increased up to 120% as compared to the water control (100%) (Cunha et al., 2017). The observed effects were non–monotonic, as at a concentration of 7.3 mg/L an increase in rhodamine accumulation of only approximately 105% was measured. Notably, in other cases verapamil induced dye efflux inhibition in the freshwater worm *L. variegatus* in the presence of 4.5 mg/L verapamil, while no difference in dye accumulation was detected in daphnia (Vehniäinen and Kukkonen, 2015).

Although a slight increase in net uptake was observed in the mentioned studies, it should be noted that their experimental conditions were rather worst–case. Verapamil is a model chemosensitiser used in cancer treatment and present in the surface water at up to 20 ng/L (Ozols et al., 1987; Fick et al., 2011). Moreover, in the environment, MXR protein expression was found to vary by a factor of 20 over the year in marine mussels (*M. galloprovincialis*) with highest expression in the warmer and lowest in the colder months (Minier et al., 2000). Similar seasonal variations might occur in other aquatic organisms. Hence, in order to assess the impact of chemosensitisation, knowledge on the quantitative effect of natural processes on MXR protein expression is required.

In addition to the reported inter–species variations (Vehniäinen and Kukkonen, 2015), the data presented in this study also suggest marked intra–species variations, as replicate experiments produce deviating, even contradictory results. For example, while permethrin net update decreased in the presence of verapamil in the first experiment, an increase was observed in the second, and no impact on uptake in the third (Figure 1). Other studies report similarly contradictory findings. For example, Cunha et al. (2017) observed a lack in clear dose–response behaviour in a zebrafish embryo dye accumulation assay. While isoeugenol appeared to inhibit active transport in the lower concentration range, it activated resistance (lowered net uptake) in the medium range. At a higher concentration the compound again displayed inhibiting properties, but no impact on dye accumulation could be observed at its highest concentration.

Another important factor influencing substrate bioaccumulation by chemosensitisation is the increase of MXR protein activity upon constant exposure to pollution, which enables organisms to counteract the process of competitive inhibition. Several experiments compare the MXR activity of organisms collected from polluted and unpolluted environments. They found that organisms from polluted sites accumulate less P–gp substrate than those from unpolluted sites (Kurelec, 1997; Kurelec et al., 2000). The authors conclude that in face of constant exposure organisms induce MXR as a means of protection. This induction has been shown to occur within only a few days. Smital and Kurelec (1998)

transplanted snails and mussels from unpolluted to polluted sites for 2-3 days, afterwards measuring their MXR activity. Transplanted organisms accumulated the model substrate tritiated vincristine at a comparable level to organisms originally collected from the respectively polluted site. In turn, snails previously exposed to pollution were found to reduce MXR activity when transferred to clean water for seven days (Assef et al., 2014). Similar findings were also made by Stevenson et al. (2006) and Fernández-Sanjuan et al. (2013) in marine mussels upon verapamil and perfluorinated chemical (PFC) exposure. Notably, such resilience was reported to arise at the cost of increased oxygen consumption and thus higher metabolic cost to the organism (Fernández-Sanjuan et al., 2013; Stevenson et al., 2006). Hence, although organisms successfully mitigate their exposure to xenobiotic pollution, toxicological effects might result from the increased energy consumption associated with MXR upregulation.

3.2. Dose–response experiment

Since competitive inhibition and MXR upregulation may simultaneously influence xenobiotic net uptake, it is difficult to conclude on the relevance of chemosensitisation for an organism. Even though internal concentration was found to have a limited impact on the bioaccumulation of moderately hydrophobic chemicals, toxic effects might still be expected in more complex mixtures. Thus, in a next step, we investigated the toxicity of zebrafish embryos co–exposed to polluted sediments and verapamil by observing lethal and sub–lethal effects in a dose–dependent manner. The sulfur–depleted sediment extracts of three polluted river sites were passively dosed using silicone O–rings. Passive dosing aims at maintaining constant exposure levels throughout the experiment and mirroring environmental partition to establish realistic exposure scenarios. Three dose–response experiments were conducted with each sediment extract: no, low ($0.015\pm0.006\,\mu g/mL$), and high ($0.364\pm0.220\,\mu g/mL$) verapamil exposure. Verpamil concentrations were determined at the beginning of the experiments. Additionally, verapamil was measured at the end of the low verpamil experiment after 96 h of exposure. The concentration was found to have in-

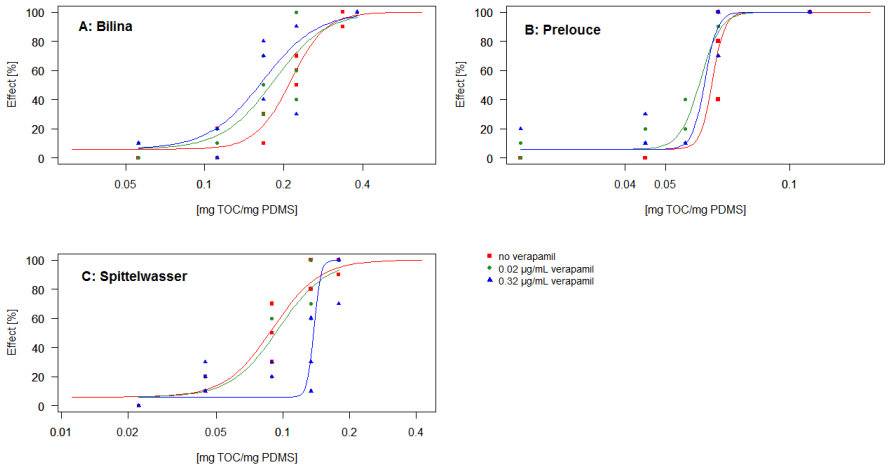

Figure 2: Zebrafish embryo toxicity in the presence and absence of the chemosensitiser verapamil. Embryos were passively dosed to sediment extracts from Bilina (A), Prelouc (B), and Spittelwasser (C) at different sediment extract and verapamil exposure levels. Verapamil exposure was selected to represent the highest attainable concentration by passive dosing (blue) and an environmentally relevant concentration (green). Three exposure replicates were conducted for each sediment dose level, which in turn contained ten zebrafish embryos. The effect depicted on the y–axis includes lethal, sublethal, and teratogenic effects (SI, Section S11).

creased to 0.024±0.014 µg/mL, which is thought to result from water having evaporated to the cap of the vial. Hence, exposure might have been slightly higher than measured at the onset of exposure. Verapamil is not thought to have induced observable toxic effects in the mixture, as supported by the observations made in the bioaccumulation experiment (SI, Section S4).

Results are illustrated in Figure 2. The amount of sediment is reported in relation to its TOC content and was normalised to mg PDMS. While no significant difference in embryo toxicity in the presence and absence of verapamil can be observed upon co–exposure to the Prelouc sediment extract, a slight increase in toxicity is noted in the presence of verapamil with Bilina sediment exposure. There, EC_{50} decrease from 0.213±0.004 mg TOC/mg PDMS without verapamil to 0.184±0.009 mg TOC/mg PDMS (low verapamil) and

0.167±0.012 mg TOC/mg PDMS (high verapamil). Based on the 95% confidence interval, both the low and high verapamil EC_{50} are significantly different from sediment EC_{50} in the absence of verapamil, while no significant difference is observed between the low and high verapamil dose.

No significant change in effect of the low verapamil concentration (EC_{50} = 0.096±0.006 mg TOC/mg PDMS) is revealed for Spittelwasser. However, upon verapamil exposure, EC_{50} significantly increases from 0.090±0.005 mg TOC/mg PDMS (no verapamil) to 0.146±0.008 mg TOC/mg PDMS (high verapamil).

The three different sediments induced no, increased or lower effect on embryos when co–exposed with verapamil. The already discussed interacting mechanisms of MXR induction and chemosensitisation could be responsible for the diverging outcomes. First, after four days of exposure, MXR activity could have increased to counteract the augmented stress on transporter proteins induced by verapamil, leading to a decrease in toxicity as possibly depicted in the Spittelwasser graph. However, as stated by Fernández-Sanjuan et al. (2013) and Stevenson et al. (2006), an impact on sublethal and teratogenic embryo effects might still have been expected from metabolic stress. Second, sediment extracts contain substrates and inhibitors of MXR proteins, themselves. The presence of substrates could lead to an increase in toxicity upon verapamil co–exposure which would result in observations similar to those in the Bilina experiment, while the presence of potent inhibitors might mask verapamil–induced chemosensitisation. The latter would be illustrated by a lack of impact as seen in the Prelouc experiment. All three sediments stem from heavily polluted sites and contain a complex mixture of xenobiotic compounds (Brack et al., 1999; Jacobs et al., 2015; Schwab et al., 2009). Considering the unspecificity of MXR protein binding, it is likely that many substrates and competitive inhibitors are present in each sediment extract.

Similarly contradictory observations were made by Campos et al. (2014), who exposed *Daphnia magna* juveniles to pair–wise combinations of the toxic substrates mitoxantrone and chlorambucil and the inhibitors reversin 205, MK571, and cyclosporin A. In substrate/inhibitor combinations after 48 hours of ex-

posure, two of six displayed antagonistic (less than additive) while four of six showed synergistic effects (more than additive) when compared to a concentration addition model. In a study with *Dreissena polymorpha* larvae, Faria et al. (2011) assessed the toxicity of the substrate vinblastine in binary combinations with each of the three inhibitors verapamil, MK571, and celestolide. Whereas the first two combinations resulted in synergistic effects, celestolide and vinblastine generated concentration additive toxicity.

The experiments conducted in this study together with the results by Campos et al. (2014) and Faria et al. (2011) seem to illustrate the toxicological impact of the complex molecular responses to MXR modulation. Evidently, chemosensitisation and the resulting MXR induction can lead to both the increase and the decrease of toxicity in organisms exposed to chemosensitisers.

4. Conclusion

This study aimed at investigating whether the bioaccumulation of common hydrophobic toxicants and their effects on the model organism zebrafish embryo could be influenced by the suppression of biological active efflux. This process, termed chemosensitisation, has been found to increase substrate net uptake in freshwater mussels, worms, daphnia, and zebrafish embryos (Kurelec, 1997; dos Santos and Martinez, 2014; Cunha et al., 2017).

Active efflux was inhibited in zebrafish embryos with the potent model inhibitor verapamil. In response, no reproducible increase in bioaccumulation was observed for the 17 hydrophobic compounds detected in zebrafish embryos. Furthermore, the variability of internal concentrations by a factor of approximately two indicated that biological variability should be considered when concluding on relevantly increased net uptake in consequence of chemosensitisation. When zebrafish embryos were co–exposed to sediments and verapamil, one of three sediments showed a slight but significant increase in toxicity as compared to exposure without the model chemosensitiser.

Although the results indicate that the bioaccumulation of moderately hy-

drophobic chemicals and the toxicity of contaminated sediments in zebrafish embryos might be slightly enhanced by verapamil, the effects were rather moderate and not observed in all tested mixtures. Possibly, MXR transporter induction could have counteracted chemosensitisation. Perspectively, both processes should therefore be considered, when assessing the environmental relevance of chemosensitisation, which could be achieved by additional Western blotting or real time quantitative polymerase chain reaction (RT–qPCR) analysis. Similar information as derived in this study is also required on other organisms and environmental compartments. For example, the complimentary testing of water samples and hydrophilic substances could present a next step towards characterising the environmental relevance of chemosensitisation.

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- Zebrafish embryos were exposed to sediment extracts by passive dosing
- Upon chemosensitisation, embryo toxicity increased for one sediment extract
- Toxicity remained unaltered or decreased for two other sediment extracts