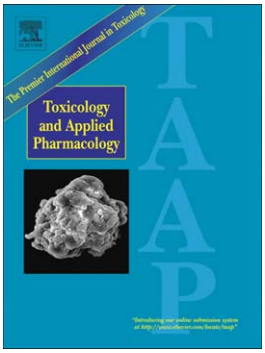

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Use of a combined effect model approach for discriminating between ABCB1- and ABCC1-type efflux activities in native bivalve gill tissue

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1. ABSTRACT

Aquatic organisms, such as bivalves, employ ATP binding cassette (ABC) transporters for efflux of potentially toxic chemicals. Anthropogenic water contaminants can, as chemosensitizers, disrupt efflux transporter function enabling other, putatively toxic compounds to enter the organism. Applying rapid amplification of cDNA ends (RACE) PCR we identified complete cDNAs encoding ABCB1- and ABCC1-type transporter homologs from zebra mussel providing the molecular basis for expression of both transporter types in zebra mussel gills. Further, efflux activities of both transporter types in gills were indicated with dye accumulation assays where efflux of the dye calcein-am was sensitive to both ABCB1- (reversin 205, verapamil) and ABCC1- (MK571) type specific inhibitors. The assumption that different inhibitors targeted different efflux pump types was confirmed when comparing measured effects of binary inhibitor compound mixtures in dye accumulation assays with predictions from mixture effect models. Effects by the MK571 / reversin 205 mixture corresponded better with independent action, whereas reversin 205 / verapamil joint effects were better predicted by the concentration addition model indicating different and equal targets, respectively. The binary mixture approach was further applied to identify the efflux pump type targeted by environmentally relevant chemosensitizing compounds. Pentachlorophenol and musk ketone, which were selected after a pre-screen of twelve compounds that previously had been identified as chemosensitizers, showed mixture effects that corresponded better with concentration addition when combined with reversine 205 but with independent action predictions when combined with MK571 indicating targeting of an ABCB1-type efflux pump by these compounds.

Keywords: environment-tissue barrier; Dreissena polymorpha; multixenobiotic resistance, multidrug resistance associated protein (MRP); P-glycoprotein; mixture effect modeling

2. INTRODUCTION

Aquatic organisms are constantly exposed to potentially toxic natural and anthropogenic chemicals in the water. Certain ATP binding cassette (ABC) transporters constitute a cellular defense eliminating a wide range of chemically diverse compounds from cells. By reducing internal levels of potentially toxic compounds those transporters confer so called multixenobiotic resistance, a phenomenon equivalent to multidrug resistance (MDR) of cancer cells (Kurelec, 1992; Epel, 1998; Bard, 2000). Major MDR proteins are ABCB1[1] (P-glycoprotein), ABCC1 (multidrug resistance associated protein 1, MRP1), ABCC2 (MRP2) and other ABCC subfamily members and ABCG2 (breast cancer resistance protein, BCRP) (Deeley *et al.*, 2006; Sarkadi *et al.*, 2006). In healthy tissues MDR proteins form a biochemical barrier controlling the transition of chemicals across compartment interfaces, such as blood-tissue barriers, and hence are components determining ADME (adsorption, distribution, metabolism, excretion) of pharmacologically or toxicologically active compounds (Leslie et al., 2005).

In gills of bivalves ABC efflux transporters were found to contribute to selective permeability of the tissue for chemicals hindering its permeation by xenobiotics. There is evidence for expression and activity of at least two ABC transporter types in bivalve gills from the Abcb (P-glycoprotein or P-gp type) and Abcc (multidrug resistance associated protein or MRP type) subfamilies (Lüdeking and Köhler, 2002; Lüdeking and Köhler, 2004; Kingtong *et al.*, 2007; Luckenbach *et al.*, 2008; Luckenbach and Epel, 2008; Faria *et al.*, 2011; Navarro *et al.*, 2012).

[1] Our nomenclature for gene/protein names is: *abcc*/Abcc for bivalve and *ABCC*/ABCC for human. Terms "ABCB1-type" and "ABCC1-type" are used in contexts when transporter functions in bivalves are equivalent to mammalian homologs.

Whether ABCG2 homologs, important transporters of xenotoxicants in mammals, are expressed and active in bivalve gills has to our knowledge not been shown so far.

Fluorescent dyes acting as transporter substrates, such as rhodamine dyes or calcein acetoxymethyl ester (calcein-am, Ca-AM), can serve to measure the activity of ABC efflux transporters in bivalve gill tissue. Ca-AM, which was also applied in the present study, is a substrate of both ABCB1- and ABCC-type transporters (Homolya *et al.*, 1993; Holló *et al.*, 1994; Essodaïgui *et al.*, 1998) but not of ABCG2 (Litman *et al.*, 2000; Sarkadi *et al.*, 2006).

The action of inhibitors of mammalian ABCB1, such as the cyclosporin A derivate PSC833, reversin 205 or verapamil, and of ABCC transporters, such as MK571, on dye efflux in bivalve gills indicate ABCB1- and ABCC1-type transporter activities in this tissue (Smital and Kurelec, 1997; Luckenbach *et al.*, 2008; Luckenbach and Epel, 2008; Navarro *et al.*, 2012). Although the considerable phylogenetic distance between bivalves and humans may raise doubts that substrates and inhibitors of mammalian ABC transporters are also specific for respective homologous bivalve subtypes, there is evidence that substrate/inhibitor specificities of transporter subtypes are conserved across taxa. Thus, effect kinetics of inhibitors of mammalian ABCB1 and ABCC transporters in Ca-AM dye accumulation assays are similar across diverse invertebrate species such as sea urchins and bivalves (Hamdoun *et al.*, 2004; Luckenbach and Epel, 2008).

Environmentally relevant anthropogenic chemicals were shown to disrupt ABC efflux transporter activity and thus to affect function of the cellular efflux transporter based toxicant defense (Kurelec, 1997; Smital and Kurelec, 1998; Smital *et al.*, 2004; Epel *et al.*, 2008). The chemosensitizing action of such compounds in bivalve gills was generally associated with effects on ABCB1-type activity (Cornwall *et al.*, 1995; Smital and Kurelec, 1998; Minier *et al.*, 1999; Eufemia and Epel, 2000; Smital *et al.*, 2003; Luckenbach and Epel, 2005; Stevenson *et al.*, 2006; Sandrine and Marc, 2007; Tutundjian and Minier, 2007). However,

the dye-based assays with native tissue that were performed in these studies did not enable to determine ABC transporter subtype-specific inhibitory effects of test compounds. The indicator dye for efflux activity used in the experiments can be substrate of both ABCB1- and ABCC1-type efflux transporters; thus, inhibitory effects of test compounds on efflux transporter activity can be associated with interference of compounds with both ABC transporter subtypes.

In this study, an experimental approach was used that enables distinction between ABCB1- and ABCC1-type transporter efflux activities in native tissue by applying a transporter subtype-specific inhibitor compound in addition to the test compound in a dye accumulation assay. The approach is based on the preconditions that (1) the dye used as indicator for efflux activity is a substrate of both ABCB1- and ABCC1-type transporters, that (2) activities of ABCB1- and ABCC1-type proteins mediating efflux transporter activity are each specifically disrupted by certain inhibitor compounds and that (3) reduced activity of either ABCB1- or ABCC1-type proteins due to the effects of target-specific inhibitors will result in a specific effect on overall efflux transporter activity. The principle of the used approach is that the effect kinetics of binary combinations of inhibitor compounds should allow to conclude whether the compounds in the mixture target the same efflux transporter type, (i.e. act similarly) or target different transporter types (i.e., act dissimilarly). In order to discriminate between similar or dissimilar actions of components in mixtures, experimentally determined combination effects of compound mixtures on efflux activities were compared with predicted combination effects. Combined effect predictions were derived from the individual compounds' activities for inhibiting the respective efflux activities. The model of concentration addition (CA), which is derived from the dilution principle, is thought to work well for predicting a mixture effect where the components affect the same target in the same way (similar action), while the model of independent action (IA) is based on the idea of

statistically independent effects of the components of a mixture, which holds for compounds hitting different targets and independently progressing towards the same outcome (Altenburger *et al.*, 2003).

As an experimental system gill tissue of the bivalve *Dreissena polymorpha* was used. ABCB1- and ABCC1-type efflux activities and expression of transcripts of respective candidate genes in gills of this species were previously reported; coding sequences were however only partially identified (Navarro *et al.*, 2012).

Here we present complete coding sequences for ABCB1- and ABCC1-type transporter proteins with analyses of phylogeny and of protein topologies providing a molecular basis for expression of both putative toxicant transporter types in *D. polymorpha* gill tissue.

Ca-AM served as proxy for ABCB1- and ABCC1-type efflux activities in bivalve gill tissue. ABCB1 and ABCC transporter type - specific inhibitors were reversin 205 or verapamil and MK571, respectively (Yusa and Tsuruo, 1989; Gekeler *et al.*, 1995; Sharom *et al.*, 1999). Further, a range of environmentally relevant compounds were studied, most of which have previously been tested for interference with efflux transporter activity (Cornwall *et al.*, 1995; Galgani *et al.*, 1996; Luckenbach and Epel, 2005; Stevenson *et al.*, 2006); test compounds comprised the artificial musks tonalide, galaxolide, musk ketone and musk xylene; the fragrance alpha-Hexylcinnamaldehyde; the perfluorinated compounds perfluorooctanoic acid and perfluorooctane sulfonic acid; the organochlorine compounds DDT, endosulfan, aroclor 1254 and pentachlorophenol and the pesticide ivermectine.

In a first set of functional experiments, effects of single compounds on efflux activity in *D. polymorpha* gills were quantified. Based on this data compounds were selected for further experimental series with binary combinations of compounds including either the ABCB1 inhibitor REV205 or the ABCC model inhibitor MK571.

3. MATERIAL AND METHODS

3.1 Chemicals

Ca-AM (CAS # 148504-34-1), MK571 (CAS # 115103-85-0), reversin 205 (REV205) (CAS # 174630-05-8), verapamil hydrochloride (VER) (CAS # 152-11-4), aroclor 1260 (CAS # 11096-82-5), dichlorodiphenyltrichloroethane (DDT) (CAS # 50-29-3), endosulfan (alpha+beta = 2+1), ivermectine (CAS # 70288-86-7), pentachlorophenol (PCP) (CAS # 87-86-5), alpha-hexylcinnamaldehyde (H-HEX) (CAS # 101-86-0), galaxolide (CAS # 1222-05-5), musk ketone (KET) (CAS # 81-14-1), musk xylene (XYL) (CAS # 81-15-2), tonalide (CAS # 21145-77-7), perfluorooctanoic acid (PFOA) (CAS # 335-67-1) and perfluorooctane sulfonic acid (PFOS) (CAS # 2795-39-3) were purchased from Sigma-Aldrich (Steinheim, Germany). Ethanol and DMSO (analytical grade) used as solvents were obtained from Merck (Darmstadt, Germany). Stocks of Ca-AM, REV205, aroclor 1260, DDT, endosulfan, H-HEX and PFOA were set up in DMSO; VER, ivermectine, PCP, galaxolide, KET, XYL and tonalide stocks were in ethanol; MK571and PFOS were dissolved in water.

3.2 Experimental animals

Zebra mussels (*Dreissena polymorpha*) were used as experimental organisms. The species is native to the basins of the Black and Caspian Seas and invasive and commonly found in freshwaters across Europe and the North American Great Lakes (Hebert *et al.*, 1989; Karatayev *et al.*, 1998). Adult zebra mussels with valve lengths of 2.0-2.5 cm were collected at water depths of 5-10 m in the Mequinenza reservoir/Ebro River (NE Spain) in the periods May-July 2012 and 2013. Within 3 h of collection, animals were transported in local water in aerated 10 L plastic containers to the lab. The animals were then rinsed and placed in glass aquaria in a density of 0.5 L per individual and maintained in ASTM hard water (ASTM,

1999) at >90% oxygen saturation at 20°C and at a 14 h:10 h/light:dark photoperiod. The water was exchanged three times per week and 2 h before water changes animals were fed at libitum with algae (*Scenedesmus obliquus*).

ABCB1- and *ABCC1*-type transporter cDNA sequences were identified from RNA from a *D. polymorpha* individual that was collected in December 2012 in the lake Kulkwitzer See in Leipzig, Germany, from hard substrate at a water depth of about 1 m. It was transferred to the lab in local water, dissected and the gill tissue was transferred to a vial with Trizol (Invitrogen), which was stored at -20°C until RNA extraction.

3.3. Identification of *ABCB1*- and *ABCC1*-type cDNA sequences from *D. polymorpha*

For extension of previously identified partial *D. polymorpha abcb1* (NCBI access. no. JN814910) and *abcc* (NCBI access. no. HM448029) cDNA sequences rapid amplification of cDNA ends (RACE) with the SMARTer RACE cDNA amplification Kit (Clontech) was applied with RACE cDNA made from mRNA according to the manufacturer's instructions. Total RNA was isolated from approximately 100 mg of *D. polymorpha* gill tissue using the Trizol protocol (Invitrogen); mRNA was obtained from total RNA using the Oligotex mRNA Mini Kit (Qiagen). RACE PCR conditions were 94°C for 2 min, followed by 45 cycles with 94°C for 5 sec, 65°C for 10 sec and 72°C for 3 min and a final step with 72°C for 7 min. Primers are listed in Table S1. RACE PCR products were between 2 and 3 kb long. They were gel-purified, cloned and sequenced on an ABI 3100 sequencer and sequences were assembled using Sequencher vs. 5.1. Contigs were confirmed by PCR and cloning of sequences comprising the entire (*abcc*) and major parts (*abcb1*) of the open reading frame.

3.4 Dye accumulation assay for determining chemical effects on ABC efflux transporter activities in *D. polymorpha* gills

The dye accumulation assay is based on the principle that the amount of a fluorescent dye accumulating in *D. polymorpha* gill tissue depends on the cellular efflux transporter activity. With high efflux activity little dye accumulates in the tissue which is indicated by low fluorescence values; with low efflux activity, e.g., due to effects of transporter inhibitors, the amount of accumulating dye is increased. Ca-AM, used as proxy for efflux activity, is a non-fluorescent substrate of ABCB1- and ABCC1-type transporters that is hydrolyzed by intracellular esterases and becomes fluorescent as calcein. Ca-AM freely diffuses across cellular membranes; the more hydrophilic calcein is trapped within the cell. Ca-AM but not calcein is efficiently transported by efflux transporters. The calcein fluorescence quantified in tissue thus reflects the amount of Ca-AM that had entered the cells and is a measure for efflux transporter activity.

Dye accumulation assays were performed as earlier described (Navarro *et al.*, 2012). The animals used in the experiments were collected during the spawning season and the gender of the animals could be determined by visual inspection of the gonads; coloring of gonads is red/orange in males and white in females (Hines *et al.*, 2007). In the tests, gill tissue from freshly dissected mussels was incubated for 90 min in 1 mL of ASTM hard water at 20°C with 0.5 µM Ca-AM and with a given concentration of a test compound or mixture, or in a control with Ca-AM and solvent only. The Ca-AM concentration applied in the tests and the incubation time were evaluated in preliminary experiments with different Ca-AM concentrations (0.25 – 1 µM) and different incubation times (60-120 min). The uptake of Ca-AM by the tissue did not further increase after 90 min incubation with all Ca-AM concentrations. The difference in uptake of Ca-AM between controls and treatments also containing 10 µM of the inhibitor cyclosporine A was most pronounced with 0.5 µM Ca-AM

and 90 min incubation time (data not shown) and we therefore performed subsequent experiments with these conditions. The experiments were set up with DMSO/ethanol/water stock solutions of Ca-AM and test compounds. The overall solvent concentration in all treatments and controls was always adjusted to 0.1 % which does not affect efflux transporter activity in *D. polymorpha* (Faria *et al.*, 2011). Gill pieces from 12 to 38 individuals were used for each experiment comprising a concentration series of a test compound or mixture, with four to eight replicates per treatment. From each mussel two tissue pieces were obtained; one piece was exposed to a test compound or test compound mixture and one piece was used in a DMSO control and served as reference for determining the basal efflux transporter activity in the respective individual. After incubations gill pieces were washed in ASTM hard water to remove medium from the tissue surface, tissue pieces were weighed with a microbalance and sonicated in 0.5 mL ASTM hard water. Homogenates were centrifuged at 3,000 g for 10 min and the fluorescence of the supernatants was measured in a microplate fluorescence reader (Synergy 2, BioTek, Winooski, VT, USA) using excitation/emission wavelengths of 480/530 nm. Arbitrary fluorescence units were standardized to the fresh gill tissue weight (reported as FU mg^{-1}) and are presented as fold changes in fluorescence levels relative to the DMSO control of the respective individual.

3.5 Experimental treatments tested with dye accumulation assays

1) *Effects of single compounds on efflux activity:* Effect characteristics of test compounds on efflux activity in *D. polymorpha* gill tissue were determined in concentration series with model ABCB1 inhibitors REV205 and VER, with ABCC transporter inhibitor MK571 and with twelve environmentally relevant compounds. The applied concentration ranges were: REV205 (0.5-20 µM), VER (1-25 µM), MK571 (2-200 µM), aroclor 1250 (0.01 µM), DDT (0.1,1 µM), endosulfan (20-40 µM), galaxolide (1-10 µM), ivermectin (2, 4 µM),

KET (1-16 µM), PCP (1-20 µM), perfluorooctanoic acid (50 µM), perfluorooctane sulfonic acid (50 µM), tonalide (1-10 µM), XYL (2-25 µM). Concentrations were determined in preliminary range finding experiments. Concentration-response curves were modelled for compounds showing significant effects in dye accumulation assays and regression parameters were determined. Based on these data, binary combinations of test compounds (either model inhibitor/model inhibitor or model inhibitor/environmentally relevant test compound) were composed using a design in which each compound was dosed using a fixed ratio of the total concentration of the mixture. For each studied pairing, fixed ratios of its mixture constituents were selected to maximize the observable response range.

2) *Combined effects of binary mixtures of model inhibitors:* Effect kinetics of binary mixtures of model compounds REV205/VER (ABCB1 inhibitor/ABCB1 inhibitor) and REV205/MK571 (ABCB1 inhibitor/ABCC inhibitor) were compared with model predictions of effects according to the models of CA and IA, respectively. These experiments served to determine whether ABCB1 and ABCC inhibitors target different efflux mechanisms – that may be associated with Abcb1 and Abcc1 activities - in *D. polymorpha* gill tissue.

3) *Combined effects of binary mixtures of model inhibitors and environmentally relevant compounds:* Effect kinetics of binary mixtures of model compounds REV205 (ABCB1 inhibitor) and MK571 (ABCC inhibitor) with environmentally relevant compounds that showed a relatively strong effect and for which an accurate concentration-effect regression could be determined (KET, PCP) were compared with model predictions of effects according to the models of CA and IA, respectively. This data was used as indication whether the test compounds target ABCB1- or ABCC1-type efflux activities in *D. polymorpha* gill tissue.

3.6 Analyses of dye accumulation assay data; curve fitting and mixture analyses

Quantitative prediction of combined effects was performed by adapting previously established approaches (Faust *et al.*, 2003). In principle, it is required to establish concentration-response relationships for the individual components of the mixture of concern (see eq. 1), calculating expected combined effects according to either the model of CA (eq. 2) and/or IA (eq.3), and subsequently compare these predicted with experimentally observed effects. This general procedure had to be specified and adapted as follows: Predicted values of studied individual components were estimated from the obtained concentration – response data, considering fold fluorescence increase relative to control treatments (R) and by fitting observed responses to the modified non-linear HILL model depicted in equation 1.

$$R(c_i) = \min + \frac{(\max - \min)}{1 + \left(\frac{IC50}{c_i}\right)^p} \qquad (1)$$

where

$R(c_i)$ is fold increase at concentration c_i relative to controls

min is fold increase of controls

max is maximal fold increase

c_i is the concentration of inhibitor (i)

p is the HILL number

IC50 is the constant describing the concentration of inhibitor causing 50% of the maximal effect.

Predicted values for mixture combinations considering the CA and IA models can be determined following a previously established procedure (Faust *et al.*, 2003). According to Faust *et al.* (2003) the combined effect (R_{mix}) caused by a mixture with given concentrations of constituents c_i can be estimated by the CA model using equation 2

$$1 = \sum_{i=1}^{n} \frac{c_i}{F_i^{-1}(R_{mix})} \qquad (2)$$

$$\text{with } F_i^{-1} = \frac{IC_{i50}}{\left[\frac{\max_i - \min_i}{R_{mix} - \min_i} - 1\right]^{1/pi}}$$

where F^{-1} is the inverse function of the individual concentration response relationship of the mixture constituents. The value of R_{mix} satisfying this equation has to be solved iteratively using, for example, the Solver–subroutine in the Microsoft(R) EXCEL Analysis Toolpack.

Alternatively, expected responses (R_{mix}) of a n-compound mixture by the IA model can be obtained directly from equation 3 *sensu* Bliss (1939) and many others (Backhaus *et al.*, 2000; Faust *et al.*, 2003)

$$R_{mix} = 1 - \prod_{i=1}^{n} [1 - E(c_i)] \qquad (3)$$

where c_i is again the concentration of the ith component and $E(c_i)$ is the fractional effect of that concentration if the compound is applied alone.

Provided that both inhibitors had different max values, equation 2 could not be solved iteratively for R_{mix} values higher than the smaller max value of the two mixture constituents. To overcome this problem we tested mixture constituent concentrations whose combined effects were expected to not exceed this joint max value. To determine combined effects predicted by the IA model depicted in equation 3 individual fold inhibition changes estimated from equation 1 were first converted to proportional effects dividing them by the larger max

value of the two mixture constituents. Estimated proportional combined effects were then re-converted to fold fluorescence changes. Moreover, to avoid the problem of adding up minimum values of 1, fold change data was re-scaled by subtracting 1 (R = fold change-1).

4. RESULTS

4.1 Identification of *ABCB1*- and *ABCC1*- type cDNA sequences from *D. polymorpha*

Using RACE PCR with cDNA retro-transcribed from mRNA isolated from *D. polymorpha* gill tissue we identified *abcc1* (NCBI accession no. KM892861) and *abcb1* (NCBI accession no. KM892860) cDNAs encompassing the entire open reading frames (ORF). These sequences enabled analyses of respective protein topologies, providing additional useful information for classification of those genes. Our topology and phylogenetic analyses described below confirm previous annotations of initial partial sequences as putatively MDR related Abcc/Mrp and Abcb/P-gp transporters (Tutundjian and Minier, 2007; Faria *et al.*, 2011; Navarro *et al.*, 2012).

Dreissena polymorpha abcc1 cDNA/Abcc1 protein: Contigs of RACE PCR product sequences were confirmed by sequences of 4879 bp and 3537 bp PCR products obtained with primers based on RACE PCR product sequences. A contig of available sequences contains a 4698 bp ORF from which a 1565 aa protein of 175 kDa is deduced. Polyphobius analyses (http://phobius.sbc.su.se/poly.html) of the aminoacid sequence reveal protein topology of a "long" ABCC transporter (Table S3, Figure S1), such as human ABCC1, ABCC2, ABCC3, ABCC6, and ABCC7 that in addition to two transmembrane domains (TMD) with 6 transmembrane sections each have an additional TMD at the NH_2 end with five transmembrane helices (Deeley *et al.*, 2006). In addition, the protein has two nucleotide

binding domains (NBD) as indicated by sequence analysis with the scan prosite tool (http://prosite.expasy.org/) (Figure S1). Glycosylation sequons [Asn-X-The/Ser; X can be any amino acid except proline (Kornfeld and Kornfeld, 1985; Gribar *et al.*, 2000)] indicate putative N-glycosylation at five positions in extracellular loops of predicted TMD0 (Asn^{22}-Ser^{23}-Ser^{24}; Asn^{92}- Lys^{93}-Thr^{94}) and TMD2 (Asn^{1016}-Gly^{1017}-Ser^{1018}; Asn^{1033}-Arg^{1034}-Thr^{1035}; Asn^{1038}-Glu^{1039}-Thr^{1040}) (Table S3, Figure S1). *D. polymorpha* Abcc1 thus may be more heavily glycosylated than homologs from other species, such as human ABCC1 with two and the *Mytilus californianus* Abcc homolog with three potential N-glycosylation sites (Hipfner *et al.*, 1997; Luckenbach and Epel, 2008). In a phylogenetic analysis including representative ABCC/Abcc/Mrp sequences the here identified *D. polymorpha* Abcc1 protein falls in a well-supported clade including the human MRPs ABCC1, 2 and 3 and putatively multidrug resistance associated Abcc proteins from other invertebrate taxa represented by *C. elegans* and sea urchin, insect and other bivalve species (Figure 1). Accordingly, the *D. polymorpha* Abcc1 protein sequence is most similar to oyster (*Crassostrea gigas*) Mrp1 with 61 % identity (Table S5); similarities are also high to human ABCC1 and MRP related orthologs from insects *Drosophila melanogaster* and *Cerapachys biroi* with 50 % identities and slightly lower to human ABCC2 and 3 and a range of putative Mrp related orthologs from other species. Sequence identities with the other ABCC/Abcc paralogs are below 40%. Based on its high similarity to the *Crassostrea gigas* MRP1 and human ABCC1 the *D. polymorpha* Abcc protein is called here Abcc1.

Dreissena polymorpha abcb1 cDNA/Abcb1 protein: The complete ORF of 4158 bp was obtained from a contig of 3' and 5' RACE PCR product sequences and a cloned 3568 bp PCR product amplified with primers designed based on 3' and 5' RACE PCR products. Analyses with the scan prosite tool and with polyphobius confirm that the deduced 1385 aa protein of 152 kDa has the typical topology of full ABC transporters with two NBDs and two TMDs

(Table S4, Figure S1). Asn^{176}-Leu^{177}-Ser^{178} and Asn^{204}-Asn^{205}-Thr^{206} mark two putative N-glycosylation sites in the first extracellular loop of predicted TMD1 (Table S4) where N-glycosylation sites are also located in ABCB1/Abcb1 proteins from other species (Chen *et al.*, 1986; Luckenbach and Epel, 2008). In a phylogenetic analysis including ABCB/Abcb/P-gp full transporter sequences from a range of vertebrate and invertebrate species, *D. polymorpha* Abcb1 falls within a cluster with high bootstrap support including the four human full transporter ABCB paralogs (ABCB1, ABCB4, ABCB5, ABCB11) and the respective sequence from one other bivalve, *Azumapecten farreri* (Figure 1). *D. polymorpha* Abcb1 shows highest sequence similarities with the *Azumapecten farreri* P-gp with 58 % and with human ABCB1 and ABCB4 proteins with 55 % identities (Table S5). Percent identities of *D. polymorpha* Abcb1 with respective homologs from other species, including other bivalves as well as sea urchin, crustaceans, insects and *Caenorhabditis elegans* are between 42-50% (Table S5).

4.2 Dye accumulation assay: method evaluation and inter-individual variability

Dye accumulation assays with bivalve gills have previously been performed using the "biopsy punch procedure" with the California mussel *Mytilus californianus*; up to 70 equally sized tissue pieces were obtained per individual enabling experiments with chemical concentration series with tissue from one individual (Luckenbach and Epel, 2005). Morphology and texture of zebra mussel gills do not allow a similar biopsy punch approach and tests with multiple tissue pieces from one individual cannot be performed. Instead, the entire two gill lobes obtained from each individual were used (see Materials and Methods, section 3.4) requiring the use of tissue from multiple individuals in concentration series experiments. To account for data variation resulting from size differences of tested tissue pieces, the calcein fluorescence

data obtained for each tissue piece were related to the wet weight of the respective tissue piece (see also Figure S2); possible inter-individual variations of efflux transporter activities were compensated by relating calcein fluorescence values obtained for each tissue piece to the respective control (tissue piece with Ca-AM and solvent only) that was run for each individual.

When comparing the wet weights of all gill tissue pieces used in the experiments, the coefficient of variation was 36.5 % of the mean [17.0 ± 6.2 mg (mean ± SD), N = 480]; there were no statistically significant differences between wet weights of gill tissue pieces from males and females ($P>0.05$, Student's t test). Wet weight corrected calcein fluorescence values in all control gill tissue pieces, reflecting basal efflux transporter activities, showed a coefficient of variation of 59 % of the mean [122.6 ± 72.0 FU/mg gill tissue (mean ± SD)]; also basal activities in gill tissue pieces were not significantly different between males and females ($P>0.05$, Student's t test).

Fold changes in fluorescence in treatments vs. controls were independent of basal efflux transporter activities. Thus, no significant correlations between maximum fold changes of fluorescence values in gill tissue pieces in treatments with standard ABC transporter inhibitors (20-25 µM REV205, 80-110 µM MK571 or 20-15 µM VER) and fluorescence values in respective controls were found (Pearson coefficients were -0.15, -0.23,and -0.15, respectively; $P>0.05$, N= 8-10). This confirms that standardization of fluorescence values with respective control values was suitable to correct for inter-individual differences in efflux transporter activities.

4.3 Effects of single compounds on efflux transporter activity in gill tissue

Effects of model transporter inhibitors: For REV205, VER and MK571 concentration-dependent increases of calcein fluorescence in gill tissue showed a bell-shaped profile (Figure

2): At lower inhibitor concentrations fluorescence increased up to a maximum, followed by descending values at higher concentrations. Precipitation of compounds at high concentrations that may have explained decreasing effects was not observed; the cause for reduced calcein fluorescence may be cytotoxicity of test compounds at high concentrations. This may also apply for test compounds KET and PCP (Figure 2, see also below). Ascending values up to the maximum peak were accurately predicted by the concentration-response model (eq. 1) with r^2 values > 0.8 for all three compounds (Figure 2, Table 1). Concentration-effect profiles differed between inhibitors of mammalian ABCB1, REV205 and VER, on the one hand and the ABCC transporter inhibitor MK571 on the other hand (Figure 2, Table 1). Maximum fold fluorescence increases were only 4.2- and 3.3-fold for REV205 and VER, respectively, whereas they were 14.0-fold for MK571 (max values+1, Table 1). IC50 values (concentrations of half-maximal inhibition of efflux transporter activities) were six- and three-times lower for REV205 and VER, respectively, than for MK571; accordingly, lowest observed effect concentrations (LOEC) were five-times lower for REV205 and VER, respectively, than for MK571 (Table 1). The slopes (p) of the regression curves ranged between 1.2 and 2.7; values were particularly low for MK571 and H-Hex (Table 1).

Effects of environmentally relevant test compounds: From twelve tested compounds, eight (aroclor 1254, DDT, endosulfan, galaxolide, ivermectin, perfluorooctanoic acid, perfluorooctane sulfonic acid, tonalide) had no significant effect on Ca-AM efflux in *D. polymorpha* gills; thus, calcein fluorescence levels in tissue exposed to these compounds did not significantly differ from controls ($P > 0.05$; see results in Table S6). These compounds were not considered in further experiments. On the other hand, calcein fluorescence levels showed concentration-dependent increases for KET, XYL, H-HEX and PCP, indicating inhibition of Ca-AM efflux in *D. polymorpha* gill tissue (Figure 2). Maximal changes in fluorescence compared to controls were 2.39-, 1.48-, 2.31- and 1.84-fold for KET, XYL, H-

HEX and PCP, respectively (max. values+1, Table 1). HILL model regression parameters (eq. 1) of fluorescence data obtained for the four compounds had curve fits with r^2 values between 0.65-0.76 (Table 1). The effect characteristics of KET, XYL, H-HEX and PCP regarding efflux transporter inhibition were more similar to Abcb1 inhibitors REV205 and VER than to MK571. Thus, IC50 values of KET, XYL, H-HEX and PCP were in the same range as for REV205 and VER; however maximum fold fluorescence increases were lower for the environmental compounds than for the model inhibitors (Table 1). This may be seen as indication that the environmental compounds are less potent inhibitors of efflux transporter activity than the model inhibitors. For testing effects of mixtures of transporter inhibiting compounds (see below), XYL and H-HEX were not further considered because of relatively low values for maximal fold inhibition and curve fit, respectively.

4.4 Effects of binary mixtures "model inhibitor/model inhibitor" and "model inhibitor/ environmentally relevant test compound" on efflux transporter activity in gills

Six pairings of test compounds REV205, VER, MK571, KET and PCP in binary mixtures with fixed ratios were applied in dye accumulation assays (Table 2). The graphs in Figure 3 show the respective experimentally determined values for the different mixtures with corresponding regression curves (modeled with eq. 1), curves for predicted effects estimated with CA and IA models (eq. 2 and 3, respectively) and the regression curves (eq. 1) based on the experimental data for the single mixture constituents (see Figure 2). The pairings were ABCC1 inhibitor MK571 with REV205, KET and PCP, respectively (Figure 3A, B, C); and of ABCB1 inhibitor REV205 with verapamil, KET and PCP, respectively (Figure 3D, E, F).

"Model inhibitor/model inhibitor" pairings: All experiments showed combined effects clearly discernible from the individual compound activities. For the MK571-REV205 pairing,

expected values based on both IA and CA predictions were at lower concentrations slightly below experimental values. However, at higher concentrations the IA prediction resembled the shape of the regression curve based on experimental values more closely than the CA prediction (Figure 3A) and the IC50 value predicted with the IA model was similar to the IC50 value determined from the experimentally obtained data, whereas the IC50 resulting from the CA model was clearly lower (Table 2). Joint effects in the REV205-VER pairing predicted by IA and CA models were relatively similar, although overall, the CA prediction corresponded better with the regression curve based on the experimental data than the IA prediction (Figure 3D); along these lines, the IC50 value resulting from the CA modeled regression was closer to the IC50 value based on the experimental data than the IC50 value based on the IA-predicted regression (Table 2). These results thus are in concordance with the assumption that MK571 and REV205 target different efflux transporter types in the bivalve gill (ABCC- and ABCB1-type efflux), whereas REV205 and VER act similarly affecting the same efflux transporter type, presumably ABCB1-type efflux.

"Model inhibitor/environmentally relevant test compound" pairings: As in treatments containing pairings of model inhibitors, maximal effects in treatments containing pairings of model inhibitors and other test compounds were generally larger than maximal effects of single compounds. For pairings MK571 with PCP and KET, respectively, IA predictions corresponded better with experimental values and better resembled the regression curves based on respective experimental data (Figure 3B, C). Accordingly, IC50 values based on IA predictions came closer to the experimentally determined median effects than CA predictions and values obtained with IA predictions were close to or within (as for the MK571-REV205 pairing) the 95 % CI for the experimentally obtained IC50 values (Table 2). Thus, for the pairings with MK571 models indicate dissimilar action of mixture constituents.

In contrast, predictions of mixture effects in experiments with pairings REV205-PCP and REV205-KET were better with the CA than with the IA model indicating similar action of mixture constituents in these pairings (Figure 3E, F). Thus, for these pairings the CA modelled curves highly resembled the regression curves based on respective experimental data, whereas the IA modeled curves were more dissimilar (Figure 3E, F). Correspondingly, IC50 values for these pairings were closer to the experimentally determined values for CA than for IA predictions. Further, IC50 values from the CA predictions were for both pairings within the 95 % CI of the experimentally determined IC50 values (Table 2).

5. DISCUSSION

This study aimed at determining whether an approach involving the application of binary mixtures of inhibitory test compounds and combined effect modeling can be used to (1) discriminate among specific activities of ABCB1- and ABCC1-type transporters and to (2) specify the ABC efflux transporter type targeted by a compound disrupting efflux transporter activity in the Ca-AM dye accumulation assay with native bivalve gill tissue. Expression of *abcb1* and *abcc1* transcripts and sensitivity of Ca-AM efflux to pharmacological ABCB1 and ABCC inhibitors in *D. polymorpha* gill tissue corroborate expression and activities of ABCB1- and ABCC1-type transporters in this tissue.

5.1 Putatively multixenobiotic resistance related *abcb1* and *abcc1* transcripts are expressed in *D. polymorpha* gills

Previous annotations of respective partial sequences from *D. polymorpha* as Abcb and Abcc full transporters (Tutundjian and Minier, 2007; Faria *et al.*, 2011; Navarro *et al.*, 2012) are confirmed based on the here identified complete cDNAs.

Relative abundances of *abcb1* and *abcc1* transcripts have been earlier quantified in *D. polymorpha* gill tissue with qPCR; constitutive *abcb1* and *abcc1* expression levels were in the same order of magnitude with approximately 4.0 and 0.9 mRNA copies (‰S3), respectively (Navarro *et al.*, 2012).

It is not possible to predict specific function of ABC transporters from sequence. However, their phylogenetic relationships and location in gill tissue indicate that *D. polymorpha* Abcb1 and Abcc1 indeed act as toxicant efflux transporters.

Various studies provide evidence for a conserved function of full Abcb and Abcc homologs as toxicant transporters found across invertebrate taxa. Thus, specific functional profiles of respective homologs from sea urchin and fruit fly corresponded to the well-studied mammalian multidrug resistance related transporter homologs (Szeri *et al.*, 2009; Gokirmak *et al.*, 2012). Further, the degree of resistance of cells and organisms against pesticides and other toxicants was linked with expression and activities of respective Abcb and Abcc transporter homologs in nematodes and arthropods [reviewed in Ardelli (2013), Dermauw and Van Leeuwen (2014)].

In the phylogenetic tree, the *D. polymorpha* Abcc1 sequence locates within a distinct cluster with ABCC1, 2 and 3 (Figure 1) indicating that this protein may mediate translocation of conjugated organic anions that are also substrates of mammalian ABCC1-3 but not of ABCB1-type transporters (Cole and Deeley, 1998). The *D. polymorpha* Abcb1 locates in a clade with full ABCB/Abcb transporters that includes toxicant transporters and transporters with other functions, such as mammalian ABCB4 and ABCB11 with highly specific roles in transport of physiological substrates in liver (Figure 1) (van Helvoort *et al.*, 1996; Gerloff *et al.*, 1998). However, since cellular efflux of a wide range of metabolically unmodified xenotoxicants appears to be the primary function of full Abcb transporters this may also be assumed for *D. polymorpha* Abcb1.

Bivalve gills form an interface between water and organism at which respiration, ion regulation and exchange of nutrients and metabolic end products take place. Cellular transporters in this tissue are important regulators of chemical translocation processes. Certain carrier proteins mediate for instance uptake of essential nutritional amino acids by bivalve gill tissue from the water (Wright, 1982). ABC transporters in bivalve gills prevent uptake of toxic compounds from the water and confer disposition of metabolic end products (Luckenbach and Epel, 2008). In this context, it seems evident that Abcb1 and Abcc1 identified here are components of the biochemical barrier against chemical uptake in *D. polymorpha* gills and conferred efflux of Ca-AM from gills in our experiments. It cannot be excluded that also other, unknown Abcb and/or Abcc paralogs expressed in gills contribute to ABCB1- and ABCC1-type efflux activities in this tissue.

5.2 Indications for ABCB1- and ABCC1-type efflux activities in *D. polymorpha* gills by dye accumulation assays with single specific inhibitors and inhibitor combinations

The effects of inhibitors of ABCB1-type efflux, REV205 and VER, on calcein accumulation in *D. polymorpha* gill tissue clearly differed from those of the ABCC1-type efflux inhibitor MK571. Inhibitory effects occurred at higher concentrations of MK571 than of REV205 and VER, while the overall effect amplitude seen with MK571 was substantially larger than with REV205 and VER, respectively (Figure 2, Table 1). The course of the concentration-effect curves included a clear maximum, confirming that concentrations of maximal effects were indeed comprised in the concentration series for all three inhibitor compounds. Likewise, differing effect kinetics of ABCC and ABCB1 inhibitors were found in dye accumulation assays with *D. polymorpha* embryos and larvae (Navarro *et al.*, 2012) and with native cells or tissues from other aquatic invertebrates (Hamdoun *et al.*, 2004; Luckenbach *et al.*, 2008).

Specificity of inhibition of ABCB1- and ABCC1-type efflux activities in native bivalve tissues by certain inhibitory compounds was indicated earlier when it was shown that the combination of the two inhibitors PSC833 and MK571 resulted in a larger effect amplitude of calcein accumulation in tissue than with single inhibitor compounds. Moreover, the concentration-effect relationship of the combination of the inhibitory compounds was better predicted by the IA than by the CA effect model (Luckenbach *et al.*, 2008). The concentration-dependent effects of the combination of MK571 and REV205 on calcein accumulation in *D. polymorpha* gills found in this study were along these lines, showing better prediction of combined effects by this mixture by IA, whereas combined effects by the REV205/VER mixture followed the CA based prediction (Figure 3, Table 2). This also indicates that efflux activities in *D. polymorpha* gill tissue are mediated by two different transporter types, which apparently are specifically targeted by different inhibitory compounds. We here provide evidence of expression of at least one candidate paralog for each ABCB1-type and ABCC1-type efflux activities, however, as also mentioned above, it is conceivable that other paralogs mediating efflux activities of either type are also expressed and active in the gills. In particular, this may be assumed for Abcc subfamily paralogs as different paralogs of this subfamily are known to mediate MRP-type multidrug resistance (Cole and Deeley, 1998). The different effect kinetics of the inhibitors indicate differences in functional properties of ABCB1- and ABCC1-type efflux transporters. ABCB1-type efflux is based on one or several transporters with high affinity to substrates but with comparatively low capacity, whereas ABCC1-type efflux is mediated by a low affinity/high capacity transporter system. These differences between transporter types may be seen as adaptation to differences in abundances of respective specific substrates. Further, when considering that substrate spectra of ABCB1- and ABCC1-type transporters overlap (Litman *et al.*, 2001), combinations of efflux activities of the different transporter types with different substrate

affinities and transport capacities may enable efficient efflux of putative xenotoxicants within a wider concentration range.

5.3 Environmentally relevant compounds acting as chemosensitizers disrupt ABCB1-type efflux activity in *D. polymorpha* gills

The inhibitory effect profiles of environmentally relevant test chemicals KET, XYL, H-HEX and PCP resembled more those of REV205 and VER than of MK571 (Figure 2) suggesting inhibition of ABCB1-type efflux by these compounds. For KET and PCP this was further confirmed in mixture effect experiments where concentration-effect profiles of compound mixtures with REV205 followed the CA model indicating corresponding target sites of each mixture constituent, i.e., ABCB1-type efflux. In the contrary, mixtures with MK571 followed the IA prediction in all cases indicating different target sites of constituents, i.e., ABCB1- and ABCC1-type efflux activities, respectively (Figure 3, Table 2).

How do effect concentrations in our experiments relate to environmental levels of the test compounds? Inhibition of efflux activity in *D. polymorpha* gills by KET, XYL, H-HEX and PCP occurred in the low µM range (≙ high µg/L to low mg/L range); concentrations of the musk compounds and of PCP reported for water samples from the environment (environmental data for H-HEX are not available) are generally several orders of magnitude lower (low ng/L ≙ nM range) (Yamagishi *et al.*, 1983; Rimkus *et al.*, 1999; Zheng *et al.*, 2012). It can thus be excluded that short-term exposures to single compounds at environmental concentrations result in significant transporter activity disruption. However, log Kow values of 3.9 (musks) and 4.8 (PCP) [log Kow values from (ChemSpider)] indicate a comparatively high degree of lipophilicity of the compounds; accordingly tissue concentrations of exposed organisms were found to be three to four orders of magnitude above levels in the water (Gatermann *et al.*, 2002; Kondo *et al.*, 2005). Thus, tissue burdens

of those compounds resulting from long-term exposure may be at levels that could lead to inhibition of efflux transporter function. Even if environmental concentrations of single compounds are below effect levels it further needs to be considered that man-made chemicals generally occur as complex mixtures in the environment; significant effects on cellular efflux transporter function could result from the combination of different compounds with transporter inhibition potency (Kurth *et al.*, 2015).

In contrast to other studies we did not find effects on efflux activity by aroclor 1254, DDT, endosulfan, galaxolide, ivermectin, perfluorooctanoic acid, perfluorooctane sulfonic acid and tonalide (Table 3). However, results of the here considered studies are in some cases also not consistent (Table 3). For instance, aroclor 1254 and DDT were reported to act as efflux inhibitors by Galgani *et al.* (1996); in contrast, Cornwall *et al.* (1995) did not find effects by those compounds (Table 3). Assay procedures were similar in both studies, but experiments were performed with different bivalve species. In some cases, differences in results across studies may thus mirror species-specific differences of compound – transporter protein interactions.

Further, the use of different indicator dyes may account for differences in results among the studies. Instead of Ca-AM rhodamine B was used in dye accumulation assays with bivalve gill tissue in the other studies considered (Cornwall *et al.*, 1995; Galgani *et al.*, 1996; Smital *et al.*, 2004; Luckenbach and Epel, 2005; Stevenson *et al.*, 2006). Ca-AM and rhodamine B act as substrates of both ABCB1- and ABCC1-type efflux transporters, but there appears to be a bias of ABCB1-type efflux of rhodamine B and of ABCC1-type efflux of Ca-AM, respectively. This is indicated by effect kinetics of efflux transporter inhibiting compounds that clearly differ depending on the indicator dye used. In a previous study with *D. polymorpha* larvae, both indicator dyes were used in dye accumulation experiments (Faria *et al.*, 2011). Changes in dye accumulation in cells by the ABCB1 inhibitor REV205 were

greater in treatments with rhodamine B compared to Ca-AM. The opposite was seen with the ABCC inhibitor MK571 that caused more pronounced effects in treatments with Ca-AM than in ones with rhodamine B (Faria *et al.*, 2011). Consequently, dye accumulation assays with rhodamine B may overall be more sensitive in indicating ABCB1-type efflux inhibition by test compounds. The absence of effects by some of the tested compounds in this study may thus be related to lower sensitivity of Ca-AM efflux to ABCB1-type inhibitors.

5.4 Summary and conclusions

Our study supports previous findings of expression and activities of at least two distinct types of ABC efflux transporters, ABCB1- and ABCC1-type, in gills of the bivalve *D. polymorpha*. Using pharmacologic inhibitors of human ABCB1 and ABCC efflux transporters, activities of these transporter types were specifically disrupted as indicated by distinct effect kinetics of the specific inhibitors when applied singly and which followed the IA prediction derived from the assumption of different targets when applied in binary combinations. Although more specific cellular *in vitro* assay systems based on overexpression of a particular transporter will be necessary to explore specific functional properties of the identified transporters, the dye accumulation assays with native tissue, as applied here, appear to be suitable for identifying the efflux transporter type targeted by a chemosensitizer compound. Native tissue as experimental system enables determining effect concentrations of compounds relevant for the *in vivo* situation and may therefore be adequate for an ecotoxicological assessment of a chemosensitizing compound when aiming to assess concentrations occurring in the field. Although we identified candidate proteins likely to mediate ABCB1- and ABCC- type efflux activities in the gill tissue, it is of interest whether other Abcb and Abcc paralogs also contribute to the overall efflux activities of either type. Results on chemical-efflux transporter interference appear to be species-specific. Thus it may be important to perform case-specific

experiments with potential target species. The experiments performed here, in addition to published data, indicate that environmentally relevant chemosensitizing compounds mainly affect ABCB1-type efflux activity; however, considering the comparatively small number of compounds so far tested this needs to be further explored.

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Table 1. Parameters determined for concentration-effect relationships according to the HILL model (eq. 1) for the six studied compounds applied singly in Ca-AM uptake assays. Regression parameters included maximum calcein fluorescence increases depicted as fold changes -1 (Max), estimated median concentration effects (IC50), HILL coefficient (p), regression coefficient of determination (r^2). Except for H-HEX, regression coefficients for the tested compounds were significantly different from 0 (Student's t test, $P<0.05$) and $P<0.001$ for r^2 of all regressions. SE and N are standard error and sample size, respectively. Lowest effect concentration values (LOEC) and observed maximal fold increase of calcein fluorescence (fold changes-1) (MaxOb) are also depicted.

	Max	SE	IC50	SE	p	SE	r^2	LOEC	MaxOb	SE	N
REV	3.20	0.2	4.40 µM	0.51	1.67	0.35	0.88	1 µM	2.94	0.07	30
MK	13.08	2.2	24.86 µM	8.54	1.24	0.34	0.81	5 µM	10.61	0.46	38
VER	2.96	0.53	8.73 µM	2.35	1.71	0.54	0.81	1 µM	2.56	0.17	38
KET	1.39	0.23	6.21 µM	1.26	2.05	0.58	0.75	3 µM	1.21	0.05	38
XYL	0.48	0.03	3.99 µM	0.57	2.23	0.62	0.74	2 µM	0.47	0.05	30
H-HEX	0.83	0.11	9.84 µM	3.58	1.12	0.41	0.65	5 µM	0.80	0.05	33
PCP	0.84	0.18	8.85 µM	2.06	2.66	1.11	0.76	8 µM	0.78	0.06	38

Table 2. IC50 values for the binary combinations of test compounds according to the model of eq. 1 based on experimental data (IC50 exp., including 95% CI) and according to predictions based on CA and IA concepts (eqs. 2, 3). Effect values were converted to fold inhibition changes -1, thus control values (corresponding to min in eqs. 1, 2) are set to 0. For each binary mixture relative proportions p_i (%) = c_i/c_{mix}, of each mixture constituent (c_i) to the total concentration of the mixture (c_{mix}) is also provided. In the column headed with "pi (%)" the first value refers to the mixture constituents MK571 or REV205 and the second value refers to each of the second mixture constituents (REV205, KET and PCP for the mixtures with MK571; and VER, KET and PCP for the mixtures with REV205). For further details on the regression parameters Max, r^2 and N refer to the supplementary Table S7.

		pi (%)	**IC50 (exp.)**	**95% CI**	**IC50 (pred.)**	
					IA	**CA**
MK571/	REV205	35/65	13.3 µM	9.1-17.5	12.8 µM	4.9 µM
	KET	22/78	14.8 µM	12.6-17.1	12.5 µM	5.0 µM
	PCP	9/91	17.4 µM	15.9-18.9	14.8 µM	6.2 µM
REV205/	VER	34/66	4.7 µM	3.9 - 5.5	8.2 µM	6.4 µM
	KET	20/80	4.1 µM	3.2 - 4.9	9.5 µM	4.7 µM
	PCP	14/76	5.0 µM	4.0 - 5.9	12.7 µM	5.4 µM

Table 3. Overview on occurrence of chemosensitizing effects by compounds tested in this and in other studies. The table shows whether the experiments performed in the different studies indicated efflux transporter inhibition (i.e. chemosensitization) by the listed compounds. Effects of compounds on efflux transporters from different species were examined. + : chemosensitizing effect by test compound; n.e.: no effect. Light grey: detected effects correspond to findings in this study; dark grey: findings do not correspond to this study.

	this study*	Cornwall et al., 1995#	Galgani et al., 1996 ‡	Smital et al., 2004¥	Bain and Le Blanc, 1996α	Stevenson et al., 2006#	Luckenbach and Epel, 2005#
aroclor 1254	n.e.	n.e.	+				
DDT	n.e.	n.e.	+		n.e.		
Endosulfan	n.e.			+	+		
Ivermectin	n.e.				+		
pentachlorophenol (PCP)	+	+	+		n.e.		
Galaxolide	n.e.						+
Tonalide	n.e.						+
musk ketone (KET)	+						+
musk xylene (XYL)	+						+
alpha-Hexylcinnamaldehyde (H-HEX)	+						
perfluorooctanoic acid (PFOA)	n.e.					+	
perfluorooctanesulfonate (PFOS)	n.e.					(+)	

Experimental species in respective studies:
**Dreissena polymorpha*
 # Mytilus californianus
‡ Mytilus galloprovincialies
¥ Aquatic invertebrates – Review
α *Homo sapiens*

Figure captions

Fig. 1: Phylogenetic analysis of *Dreissena polymorpha* Abcc1 and Abcb1 and related Abcc and Abcb transporter proteins from a range of invertebrate and vertebrate species. The protein names in the phylogenetic tree correspond to those in the data bank records (refer to Table S2 for names and respective NCBI accession numbers). "MRP" (Multidrug Resistance associated Protein) is used for Abcc transporters associated with the cellular multidrug resistance phenotype; MRP1 ≙ ABCC1, MRP2 ≙ ABCC2 etc.. "P-gp", "permeability glycoprotein", "Mdr" and "MDR" are names also used for Abcb1 homologs. Amino acid sequences were aligned using the program TCoffee (Notredame *et al.*, 2000) and a phylogenetic tree constructed using the neighbor-joining method as implemented in the software MEGA6 (Tamura *et al.*, 2013). The percentage concordance based on 1,000 bootstrap iterations is shown at the nodes. Trees obtained with the alternative maximum likelihood and minimum evolution methods had very similar topologies (data not shown), indicating that the results are robust.

Fig. 2. Concentration-dependent effects of standard transporter inhibitors REV205, VER and MK571 and of environmentally relevant compounds KET, XYL, H-HEX and PCP on efflux of Ca-AM in *D. polymorpha* gill tissue. Values are fold changes of calcein fluorescence in treatments vs. the respective controls minus one. Each symbol represents a single data point. Regression curves were fitted to values depicted as filled triangles with the HILL model (eq. 1). For regression parameters refer to Table 1. The open triangles represent measured values at higher concentrations that were descending from the maximum and were not included in the concentration-response regression analysis.

Fig. 3. Concentration dependent effects of binary mixtures of test compounds on efflux of Ca-AM in *D. polymorpha* gill tissue. Pairings were MK571 with REV205 (A), KET (B) and PCP (C), respectively, and REV205 with VER (D), KET (E) and PCP (F), respectively.

Concentrations of single constituents in the mixtures were according to the fixed ratio design, i.e., we kept the ratio of the mixture components constant and varied the concentration of the mixture as we do in individual compound-response studies as shown in Table 2 (for further explanations please refer to Altenburger et al. 2003). The depicted values are fold changes of calcein fluorescence in treatments vs. the respective controls minus one. Black triangles are single observations for mixture treatments and dashed black lines represent the according regression curves (based on eq 1). Straight grey and black lines mark joint effects predicted by IA and CA models, respectively (eq 2 and 3). Dashed grey lines 1, 2, 3, 4, 5 are for MK571, REV205, KET, PCP and VER, respectively, and represent regressions for effects of the respective single mixture constituents (see also Figure 2). For regression parameters refer to Table 2.

Figure 1

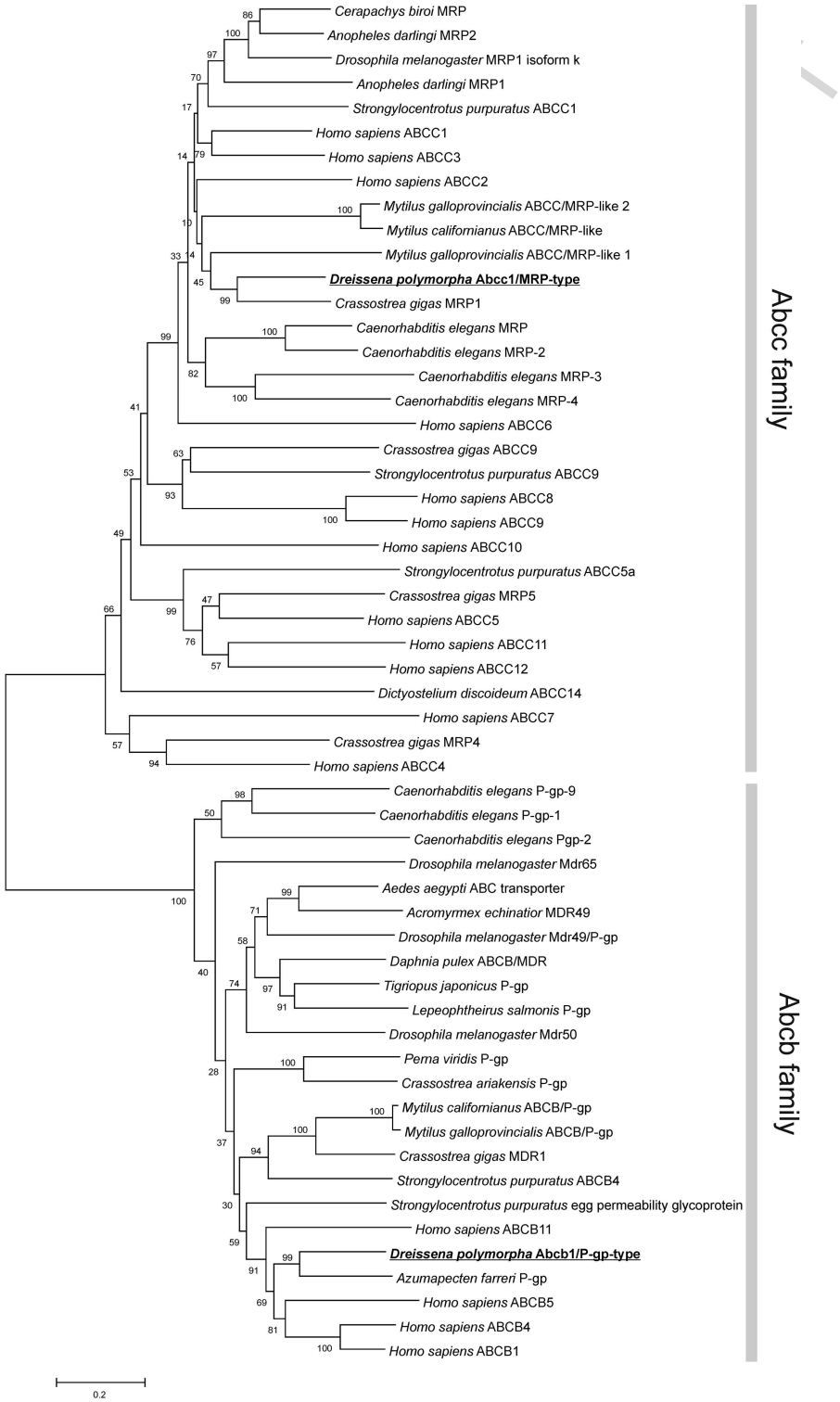


Figure 2

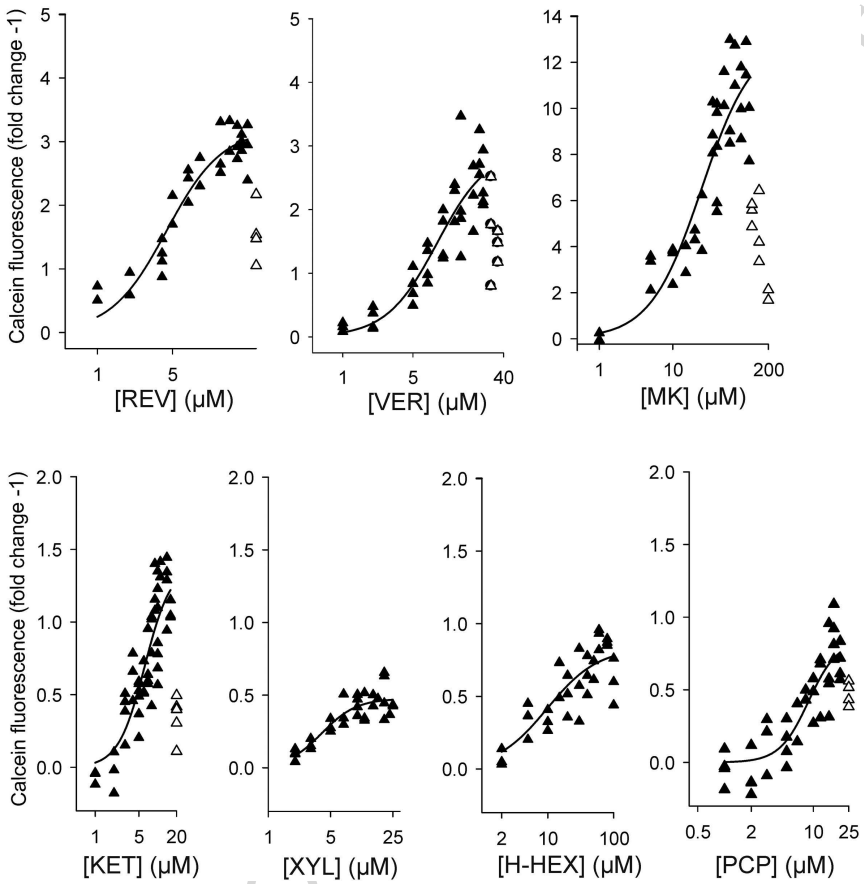
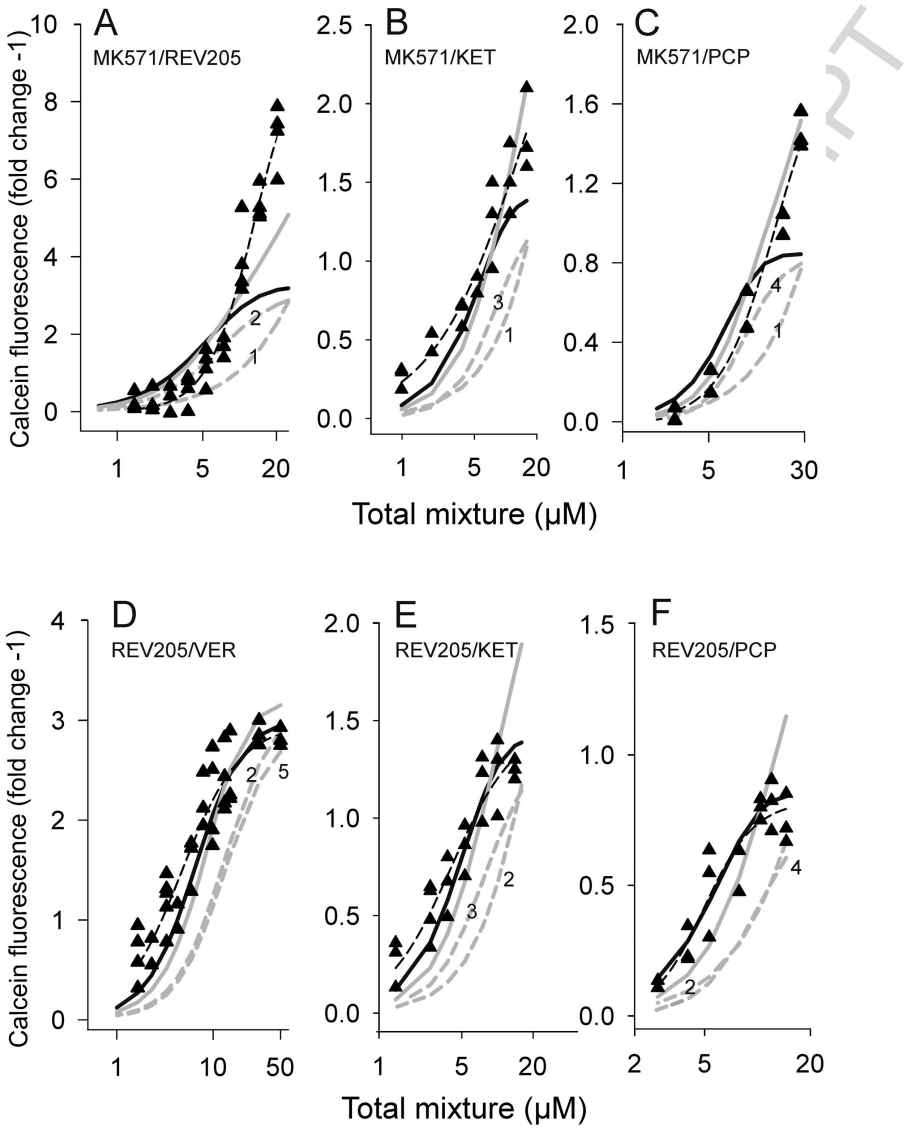


Figure 3



Highlights

- Sequences and function of ABC efflux transporters in bivalve gills were explored
- Full length *Dreissena polymorpha abcb1* and *abcc1* cDNA sequences were identified
- A mixture effect design with inhibitors was applied in transporter activity assays
- ABCB1- and ABCC-type efflux activities were distinguished in native gill tissue
- Inhibitory action of environmental chemicals targeted ABCB1-type efflux activity