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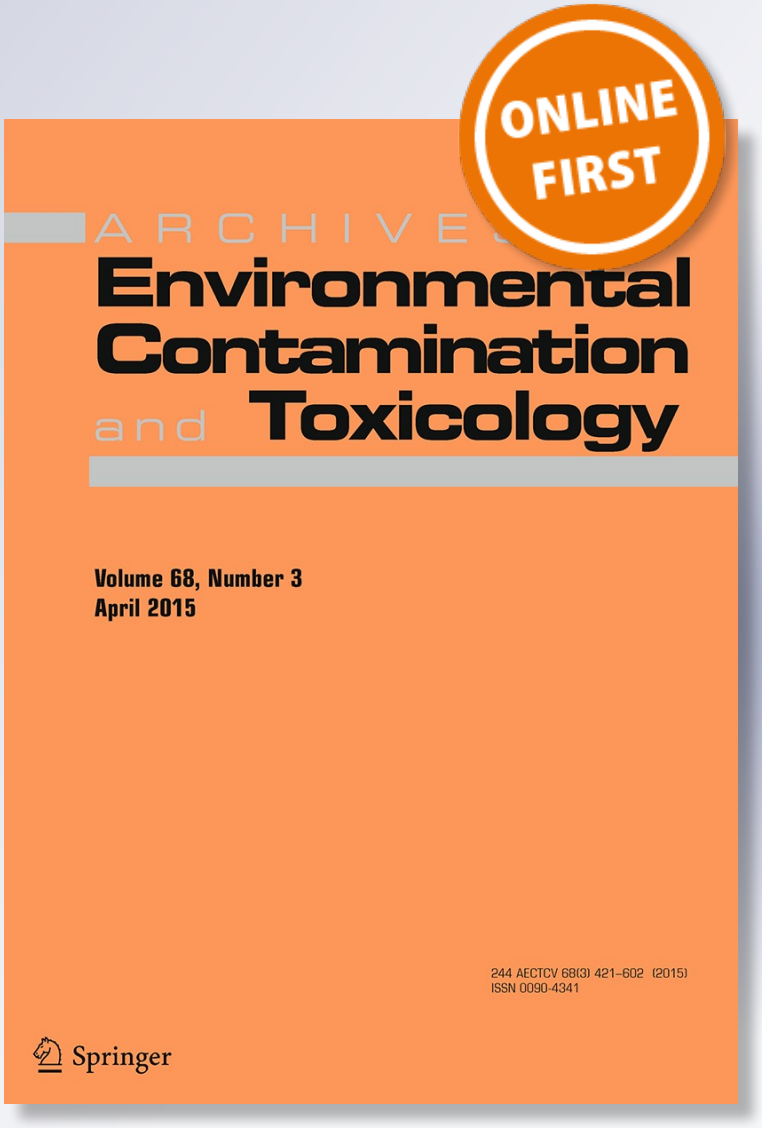

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Evaluation of Multixenobiotic Resistance in Dreissenid Mussels as a Screening Tool for Toxicity in Freshwater Sediments

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Abstract The multixenobiotic defense mechanism (MXR) in aquatic organisms was recognized as a first-line defense system, and its potential use as an early biomarker of exposure to environmental stress has raised attention in the last two decades. To evaluate the relevance of this biomarker in the freshwater mussel *Dreissena polymorpha*, we studied its responsiveness within laboratory exposures to contaminants sequestered in freshwater sediments affected by moderate anthropogenic impact. The effectiveness of this biomarker was assessed by comparing the MXR-transporter activities determined in bivalves first with toxicity scores recorded with the *D. rerio* embryo developmental assay. Both bioassays were applied in the sediment contact test format. As a second evaluation approach, MXR activities determined in exposed mussels were compared with sediment-contamination data integrated into toxic units on the basis of acute toxicity to *Daphnia magna*. In *D. polymorpha* subjected to acute exposure with moderately polluted sediments, we detected limited (22–33 %) but statistically significant induction of MXR activity. Mean MXR activities significantly correlated with TU values computed for test sediments. MXR activities in mussels showed strong positive correlation with the metal load of sediments and proved to be unrelated to the contamination with polycyclic aromatic compounds. MXR activity in laboratory-exposed mussels showed low variability within treatments and thus reliably reflected even low contaminant differences between the negative reference and moderately polluted harbor sediments. The strong correlation found in this study between the MXR-transporter activity in exposed mussels and environmentally realistic sediment contamination underscores the fairly good sensitivity of this biomarker in laboratory testing conditions to signal the bioavailability of sediment bound contaminants, and it may also anticipate even the incidence of toxicity to biota.

In assessing the quality status of aquatic habitats, it has long been shown that particular attention must be paid to the chemical and ecotoxicological characterization of sediments (Cleveland et al. 1997; Pederson et al. 1998; Ghirardini et al. 1999; Davoren et al. 2005) because the sediment compartment acts as a sink and source of internal contaminant load posing a permanent threat to resident biota. Contaminants accumulated in the sediment have the potential to remobilize, and thus become bioavailable, after chemical (e.g., pH, salinity fluctuations), physical (e.g., dredging), or biological (e.g., bioturbation) processes (Davoren et al. 2005). For the ecotoxicological evaluation of sediments, various acute and chronic bioassays have been developed, standardized, and successfully applied: Toxicity tests with bacteria and unicellular algae, crustacean bioassays, tests based on sediment- dwelling organisms, fish toxicity tests, etc. (Lappalainen et al. 1999; Algaltoxkit 1996; Nebeker et al. 1988; Phipps et al. 1993; Franco et al. 2006). Moreover, many alternative biotests making use of organisms belonging to various taxonomic groups and trophic levels proved to be useful tools in detecting toxicity risks posed by sediment-bound

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contaminants (Ingersoll et al. 1995, 1997; Chapman et al. 2002). In addition to biotests, molecular and cellular biomarker responses have been successfully applied to evaluate the relevance of risks posed by complex contaminant mixtures well before implications would evolve at population and community levels (Cajaraville et al. 2000; Behrens and Segner 2005).

Within biomarker-based assessment schemes, analysis of biomarker responses in molluscs have been extensively used to evaluate site impact in freshwater and marine ecosystems both by active monitoring with transplanted mussels (Quinn et al. 2004; Pampanin et al. 2005; Guerlet et al. 2007; Lekube et al. 2014) and in laboratory tests (Eertman et al. 1995; Binelli et al. 2010; Sundt et al. 2011). Typical ecotoxicological endpoints considered are usually cellular reactions (e.g, apoptosis), or—at a protein level—the modulation of biotransformation and antioxidative enzymes. Although very informative, accurate analysis of a high number of complex sets of cytochemical, biochemical, and physiological data might be problematic; therefore, multixenobiotic resistance (MXR) as a general marker of chemical stress was in some studies related to pollutant exposure (Kurelec 1992; Epel 1998; Bard 2000; Smital et al. 2003). The cellular MXR system represents a broad-scale defense mechanism protecting cells and organisms against both endogenous and environmental toxicants (Epel et al. 2008). MXR is mediated by membrane-transport proteins from the ABC (ATP binding cassette) protein family, which recognize a wide variety of potential xenobiotics as substrates, pumping them out of the cell in an energy-dependent, ATP-driven process (Navarro et al. 2012). Induction of the multixenobiotic defense mechanism (MXR) by chemical and physical stressors has previously been shown in bivalves as a general stress response (Eufemia and Epel 2000; Lüdeking and Köhler 2002, 2004).

The main goal of our study was to evaluate the potential of MXR-transporter activity in *Dreissena polymorpha* exposed to sediments to indicate the presence of bioavailable toxic contaminants sequestered in sediments in a dose-related way. More specifically, we examined whether MXR activity measured in mussels in our exposure conditions allow discriminating relatively small differences of contaminant burdens in sediments. In addition, we also checked the feasibility of using MXR activity in mussels exposed in laboratory conditions as predictors of the incidence and relevance of toxicity risks posed by sediment-bound chemicals. For this purpose, MXR activities measured in exposed mussels were correlated first with sediment toxic units as mixture toxicity estimates of sediment-bound contaminants. Sediment toxic units (TUs) were calculated based on the concentrations of pseudo total metals and polycyclic aromatic hydrocarbons (PAHs) measured in test sediments and then scaled by their 48-hour acute toxicity (LC_{50} values) toward *Daphnia magna* (Sprague 1970; Von der Ohe et al. 2009). As a second evaluation approach, the quality of linkages between MXR activities and toxicity data recorded for the sediment samples in the *Danio rerio* embryotoxicity assay was evaluated. The toxicity of sediments on *D. rerio* embryogenesis was investigated in sediment-contact test format.

For this purpose, we used "in situ-formulated" sediments moderately loaded with metals and PAHs collected from harbors and remote areas of Lake Balaton (the largest shallow lake in Central Europe). We considered these two contaminant classes because, in accordance with the results of previous studies, only metals and PAHs showed significant enrichment in harbor sediments (Kiss et al. 1997; Hlavay and Polyák 2002; Bodnár et al. 2005; Nguyen et al. 2005).

Materials and Methods

Sediment Characterization

Sediment samples were collected in four harbors (H1 through H4) of Lake Balaton (Hungary) in parallel with sediments from two unpolluted sites designated in the open area of the lake (0.5–1.0 km far from harbor entrances) (R1, R2). The BCR 701 freshwater sediment was used as positive reference in bioassays. At each sampling location, sediment samples were collected using a Plexiglass corer (three sediment cores/location; internal diameter = 10 cm; depth = 20 cm) within a circle with a diameter of approximately 30 m with a central point fixed by geographical coordinates. The samples were immediately placed in polyethylene bags and transported to laboratory in coolers. After homogenation of pooled sample replicates per location, sediments were air-dried for 10 days in a controlled and clean environment. Dried sediment samples were grinded to obtain fine particle-size fractions (<63 μm), and organic matter content (% OM) was estimated by measuring the loss of weight on ignition at 550 °C for 24 h.

The metals cadmium (Cd), chromium (Cr), copper (Cu), nickel (Ni), lead (Pb), and zinc (Zn) were extracted in concentrated HNO_3 overnight (Austen and Somerfield 1997). One gram of sediment sample (size fraction < 200 μm) was placed in 250-ml Pyrex digestion tubes, and extraction was run at room temperature for 16 h with 15 ml of 53 % HNO_3. Then the suspension was filtered through an ashless Whatman 41 filter, diluted to 50 ml with doubly distilled water, and stored in polyethylene bottles at 4 °C for analysis. The detection was performed by inductively coupled plasma-optical emission spectromtery (ICP-OES; Spectro Flame

Modula E, Spectro GmbH, Germany). The analytical quality was checked by analysing blanks, duplicates, and the certified reference material BCR 701 freshwater sediment (Community Bureau of Reference). PAHs were analyzed according to the procedure applied by Kiss et al. (1997) and Bodnár et al. (2005). Separation and quantification of 15 PAHs (naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benz[*a*]anthracene, chrysene, benzo[*b*]fluoranthene, benzo[*k*]fluoranthene, benzo[*a*]pyrene, dibenz[*a,h*]anthracene, benzo[*g,h,i*]perylene, and indenopyrene) were performed by high-performance liquid chromatography (HPLC) with fluorescence detection. For the analysis of PAHs, HPLC-grade acetonitrile (Romil) and water (Milli-Q water purification system; Waters, Milford, Massechusetts, USA) were used. The eluent was composed as follows: linear gradient from 40 (v/v) % acetonitrile in water to 100 % acetonitrile in 20 min and then 100 % acetonitrile for 15 min with a flow rate of 1 ml/min. Separation was performed on a LiChroCART C18 PAH column (l = 250 mm, ID = 4 mm, d_p = 5 µm). Standards as PAH mixture of 610-M (Merck) were used. Blanks were performed without PAHs, and concentrations were found to be lower than the limit of detection (LoD). Recovery of spiked PAHs in acetone ranged from 58 % (fluorene) to 77 % (phenanthrene), and the results of samples were adjusted accordingly (Bodnár et al. 2005).

Bioassays

Exposure of D. polymorpha to sediments

Specimens of *D. polymorpha*, 16 ± 1-mm shell length, were collected in September 2013 from reed stems in a highly protected remote area of Lake Balaton (Hungary) from 0.5 to 1 m depth. Mussels were transferred to the laboratory in lake water tanks and maintained in glass aquaria (approximately 1000 individuals/aquarium) with a capacity of 120 L in a flow of well-aerated unfiltered water (T = 18 ± 2 °C) directly taken from the Lake [flow rate approximately700 mL/min, *i.e.*, 1 L/(mussel day)]. Mussels were given no food during the adaptation (5 days before experiments) or the experimental periods.

Toxicity tests were performed in 2-L glass aquaria. A layer of 200 g of sediment (2-cm deep) was placed in each vessel before 1.5 L of fresh lake water was added to assure a water column depth of 15 cm above the sediment–water interface. Sediments were allowed to settle for approximately 1 h before 10 healthy *D. polymorpha* were added to each test vessel. Mussels were exposed to the sediments for 72 h at 18.0 ± 0.5 °C with a natural photoperiod. The exposure duration in definite experiments was selected based on 7-day pre-exposure trials to the same sediment samples, in which MXR induction reached its peak level after 3e days. All vessels were aerated by way of glass Pasteur pipettes at a level where the sediment surface was not disturbed ensuring that oxygen was never limited. Experiments were performed in duplicate in three independent experiments, resulting in six pooled tissue replicates per treatment. Negative procedural control was represented by exposure to lake water only.

Basic physicochemical parameters of the overlying water were recorded at the start and immediately before termination of the test. Salinity and pH were measured using a WTW 350i multiparameter instrument, and ammonia, nitrite and nitrate were measured using water-quality test kits (Aquarium Pharmaceuticals, UK Ltd.). During exposure the quality of the overlying water remained basically unchanged (data not shown).

MXR-Transporter Activity Assessment

Induction of MXR-transporter activities in exposed mussels were monitored by the rhodamine-B accumulation assay according to Smital et al. (2003) with modifications. The assay is based on the property of certain fluorescent dyes (rhodamine B in our case) to act as substrates of ABC-efflux transporters. The dye is kept out of cells if efflux transporters are active and increasingly accumulate inside the cells if transporter activities are disrupted by transporter-inhibiting chemicals. As model disruptor of ABC-like efflux activities in mussels, we used the model inhibitor verapamil. Mussels exposed to sediment samples were subjected to a depuration phase of 3 h and then were loaded with rhodamine for 1 h in 5 µM of rhodamine B solution (50 ml/single mussel). The loading was followed by three washing steps with deionized water and sampling of the whole soft tissue of five mussels in duplicate per exposure. Tissue samples were weighed, homogenized, and centrifuged for 10 min at 10000 g (Biofuge; Heraeus Instruments, Germany). The fluorescence of the supernatants was measured at λ_{ex} = 535 nm, λ_{em} = 590 nm with a Shimadzu F-4500 spectrophotometer. Data are expressed in fluorescence units (f.u.) of RB accumulated/g of whole soft-tissue homogenate.

Sediment Contact Test With *D. rerio*

The zebrafish (*D. rerio*) facility established at the Hungarian Academy of Sciences, Centre for Ecological Research provided the embryos used in this study per the method prescribed by the Organisation for Economic Co-operation and Development (OECD) 236 (2013) guideline–fish embryo acute toxicity test.

The sediment-contact test with *D. rerio* embryos was performed according to Tuikka et al. (2011) with modifications. Three grams of air-dried sediments (grain size <300 μm) were mixed with 10 ml of artificial water (ISO 1996) and transferred into six-well polypropylene plates. The sediment was allowed to settle for 24 h and then 10 eggs/well were placed on the surface of the sediment and incubated for 96 h at 26.5 °C with a 12:12-h light-to-dark cycle. Water-quality parameters, e.g., dissolved oxygen, ammonia, pH, in the overlying water were measured at the start and end of exposure to ensure that the potential toxic effects were caused by particle-bound contaminants. At the end of exposure these parameters varied within the following ranges: oxygen saturation 68–73 %; NH_4^+ and NO_2^- < 0.001 mg L^{-1}; pH 7.8–8.3; and hardness 170–235 mg L^{-1}. Each treatment was assessed in duplicate within three independent experiments. Scoring of effects after 96 h of exposure was performed based on the cumulative mortality and hatching success. Mortality criteria after 96 h of exposure were cumulated coagulation of embryos, lack of somite formation, nondetachment of the tail, and lack of heartbeat (OECD 236 [2013]).

Sediment TUs

Contaminant concentrations exceeding the LoD sediment samples were converted into summed toxic units (TU_{sum}) based on laboratory-derived acute toxicity data (LC_{50}) for *D. magna* (Sprague 1970; Von der Ohe and Liess 2004). The concentrations of pseudo total metals and the relatively mobile fractions of PAHs were considered because these two prevailing contaminant classes proved in previous studies to be of relevance for Lake Balaton (Kiss et al. 1997; Hlavay and Polyák 2002; Bodnár et al. 2005; Nguyen et al. 2005). According to Von der Ohe and Lies (2004), TU estimates were computed separately for the two classes of contaminants. Because LC_{50} values for *D. magna* were obtained in aqueous exposures, contaminant concentrations determined in the sediments were converted to "porewater" concentrations as described in detail by Höss et al. (2011). In brief, based on the equilibrium-partitioning concept, porewater concentrations of contaminants measured in sediments were calculated according to formula:

$$C_{PW} = C_S \times K_P$$

where C_{PW} is the porewater concentration of the contaminant, C_S the contaminant concentration determined in the sediment, and K_P is the partitioning coefficient.

As for nonionic organic chemicals, organic matter is assumed to be the major binding phase in sediments (Di Toro et al. 1991) K_P is calculated by the following equation:

$$K_P = f_{OC} \times K_{OC}$$

Here, f_{OC} is the (w/w) fraction of total organic carbon measured in the sediment sample, and K_{OC} is the octanol–carbon coefficient.

For metals, in the absence of established mechanistic approach of calculation, we followed the general practice and used K_P values experimentally as determined by Van Der Kooij et al. (1991).

The summed TUs per contaminant class (metals, PAHs), as well as total TU estimates, were calculated according to formula:

$$TU_{sum} = log \sum_{i=1}^{n} \frac{C_i}{LC_{50i}}$$

,where i is the compound, C_i is the measured concentration of compound i in the sediment, LC_{50i} is the respective acute lethal concentration in the standard 48-hour toxicity test for *D. magna*, and n is the number of compounds considered. In addition, maximal TUs were also determined to evaluate the expected effect of the most potent toxicant present in the sediment samples.

Statistics

The significance of differences in the MXR-transporter activity in bivalves subjected to various treatments was evaluated by applying one-way analysis of variance followed by Tukey's post hoc test with Bonferroni correction. For the *D. rerio* assay, Mann–Whitney test was used to evaluate the significance of differences between the effects exerted by polluted, reference, and control sediments. Associations between bioassay endpoints and TU estimates (TU_{sum} *D. magna*) were tested for significance using Pearson's product moment correlation analysis. In statistical evaluations, significance was accepted when $p < 0.05$. Statistics and graphical plotting were performed using the OriginPro software package (9.0, OriginLab Corporation, USA).

Results

Physicochemical Characteristics and Toxicity Estimates of Sediment Samples

Environmental sediment samples used in this investigation did not significantly differ in terms of grain-size composition and organic matter content (Table 1). Moreover, they were found to have a relatively low level of anthropogenic contamination because according to the consensus-based sediment-quality criteria of McDonald et al. (2000), none of the investigated contaminants exceeded the threshold

Table 1 Sediment characteristics for the different sediment samples

Properties	R1	R2	H1	H2	H3	H4	BCR		
% <63 µm	15	29	31	34	30	16	n.a.		
TOC (%)	5	5	10	5	10	5	10		
PAHs (µg kg^{-1})								LOQ	TEC
Naphthalene	4.46	1.20	3.38	0.65	5.33	1.14	2.58	1.252	
Acenaphthene	1.71	1.59	1.52	1.26	1.91	1.28	9.86	2.500	
Fluorene	1.75	0.47	3.80	1.92	2.98	1.28	1.30	0.100	
Phenanthrene	6.05	4.26	40.05	12.06	25.67	12.32	19.67	0.401	
Anthracene	0.54	0.28	2.74	1.66	2.27	1.83	2.23	0.050	
Fluoranthene	9.37	4.58	37.78	20.52	23.02	23.94	37.23	0.497	
Pyrene	6.80	4.51	40.63	25.41	35.79	25.34	38.18	0.996	
Benz[*a*]anthracene	1.94	0.49	9.79	10.40	8.80	8.01	16.28	0.124	
Chrysene	3.66	1.03	11.87	10.81	11.28	8.92	18.25	0.050	
Benzo[*b*]fluoranthene	9.20	n.d.	30.61	13.76	25.17	13.34	44.96	0.050	
Benzo[*k*]fluoranthene	2.69	0.93	10.66	7.47	10.02	6.56	15.35	0.020	
Benzo[*a*]pyrene	4.65	1.24	16.08	12.63	16.58	12.23	25.22	0.050	
Dibenz[*a*, *h*]anthracene	0.58	0.39	2.27	1.26	2.03	1.12	3.42	0.200	
Benzo[*g,h,i*]perylene	5.67	1.78	12.39	13.93	21.00	10.88	32.03	0.199	
Indenopyrene	5.18	1.30	17.34	11.81	17.57	10.67	30.49	0.125	
∑PAH (µg kg^{-1})	64.24	24.06	240.90	145.55	209.41	138.86	297.06		1600
Elements (mg kg^{-1})								BCR-certified values	TEC
Pb	7.00	6.75	21.63	24.89	20.68	15.95	130.20	138.48	36
Cd	0.08	0.08	0.31	0.21	0.31	0.16	13.02	11.38	0.99
Cr	3.25	2.50	5.00	4.75	7.00	5.25	168.00	190.96	43
Cu	6.00	6.25	20.25	18.50	17.50	10.25	277.80	228.5	32
Ni	7.50	6.25	5.49	3.11	6.34	6.68	52.02	57.3	23
Zn	3.00	3.00	20.62	61.25	27.87	11.07	389.40	364.70	121
∑Me	26.83	24.83	73.31	112.71	79.71	49.36	946.44	991.32	
TUs									
$LogTU_{sum}$ *D. magna*$_{metal}$	−1.905	−1.917	−1.426	−1.419	−1.445	−1.652	−0.270		
$LogTU_{sum}$ *D. magna*$_{PAH}$	−3.883	−4.038	−3.366	−3.582	−3.557	−3.574	−3.669		
$LogTU_{max}$ *D. magna*$_{metal}$	−2.108	−2.090	−1.580	−1.619	−1.643	−1.875	−0.442		
$LogTU_{max}$ *D. magna*	Cu	Cu	Cu	Cu	Cu	Cu	Cu		
$LogTU_{Total}$ *D. magna*	−1.901	−1.914	−1.421	−1.416	−1.442	−1.647	−0.442		

NA not applicable, *ND* not detected, *PHEN* phenanthrene

effect concentration (TEC) below which no biological effect can be expected. However, distinctly greater contaminant loads were characteristic for harbor sediments as shown by the 1.1- to 2.6-fold increase for summed metal concentrations and the 3.2- to 5.5-fold increase for summed PAH concentrations compared with the samples collected in open areas. The greatest contaminant loads for both the metals and PAHs were recorded in the BCR 701 reference sediment used as positive control in exposures.

Based on the TU approach for both contaminant classes, the harbor sediments were estimated to be more toxic than the sediment samples from open areas (Table 1). Of greater relevance, the metal load of the sediments was apparent as proven by the log TU_{sum} *D. magna*$_{metal}$ values exceeding the −2 threshold (this corresponds to 1/100 of the acute LC_{50} value). Thus, for the sediments used in our investigation, the metals seem to be the most important pollutants that might cause sublethal toxicity for invertebrate communities (Tuikka et al. 2011). The greatest toxicity potential was apparent for the BCR 701 reference sediment, for which the log TU_{sum} *D. magna*$_{metal}$ was −0.270. For each sediment sample tested, Cu proved to be the most critical pollutant of concern as shown by the maximal TU estimates ($logTU_{max}$ *D. magna*) (Table 1). Overall, the

total TU estimates (TU_{Total} *D. magna*) outlined the relatively low differences in pollution of test sediments sampled from Lake Balaton and the distinctly more polluted BCR 701 sediment used as positive reference.

MXR-Transporter Activity in Mussels Exposed to Sediment Samples

In the whole soft-tissue homogenates of mussels exposed for 3 days to sediments from remote areas (R1, R2), the level of rhodamine dye accumulated did not significantly differ from that measured in mussels exposed to unpolluted lake water only (Fig. 1). In mussels exposed to harbor sediments (H1 through H4), significantly ($p < 0.001$) lower accumulation of rhodamine dye (by 23–33 %) than in the tissues of mussels exposed to sediments used as controls (R1, R2) (Fig. 1) was recorded. The lowest rhodamine accumulation (by 57 % compared with control sediments) was detected for mussels exposed to the BCR 701 sediment used as positive control. The difference between rhodamine accumulation in mussels exposed to BCR 701 sediment proved to be statistically significant for the H1, H2, and H3 harbor sediments but at the $p < 0.05$ level only. The decreased dye uptake in mussels exposed to harbor sediments and the BCR 701 reference versus exposure to sediments from remote areas suggests induction of MXR-transporter activity because its activation results in decreased substrate accumulation. In mussels exposed for 3 days to verapamil, a 2.6-fold increase of rhodamine concentration compared with unexposed mussels was recorded.

The Toxic Effects of Sediments on D. rerio Embryogenesis

In the *D. rerio* embryo toxicity assay, scoring of effects was performed based on the cumulative mortality and hatching success after 96 h of exposure. In addition, the rate of developmental alterations as sublethal end point was also evaluated. For control exposures, the mean cumulative mortality (six replicates within three independent exposures) was <10 %, whereas the hatching success was approximately 90 % (Fig. 2). Thus the main requirements of the OECD 236 (2013) protocol for a valid test were always met, and the effects recorded in the exposures to test sediments could be attributed to their contaminant loads.

Distinct alterations in embryogenesis as function of sediment quality were recorded in the sediment-contact tests. Even exposure to unpolluted sediments (R_{1-2}) caused some insignificant increase in mortality, and certain negative effects on embryogenesis were observed as the number

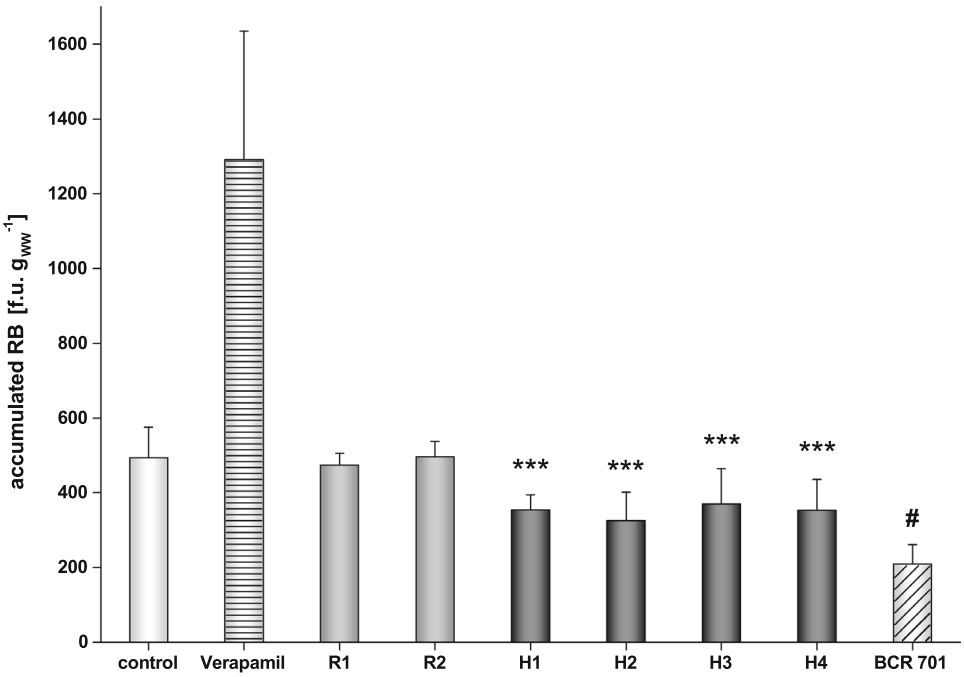

Fig. 1 MXR-transporter activity in mussels exposed for 72 h to selected sediment samples and the model inhibitor verapamil (10 μM). MXR activity was determined by the accumulation version of the model P-gp substrate rhodamine B expressed as fluorescence units of RB per g wet weight mussel soft tissue (f.u. mean ± SD, $n = 6$–10). Control = mussels exposed to clean unfiltered lake water; R_{1-2} = exposure to unpolluted sediments; H_{1-4} = exposure to harbor sediments; BCR 701 = exposure to positive reference lake sediment. The significance of differences between RB accumulation in mussels exposed to reference and harbor sediments (***$p < 0.0001$); significant difference in RB accumulation in mussels exposed to harbor sediments and BCR 701 reference (#$p < 0.05$)

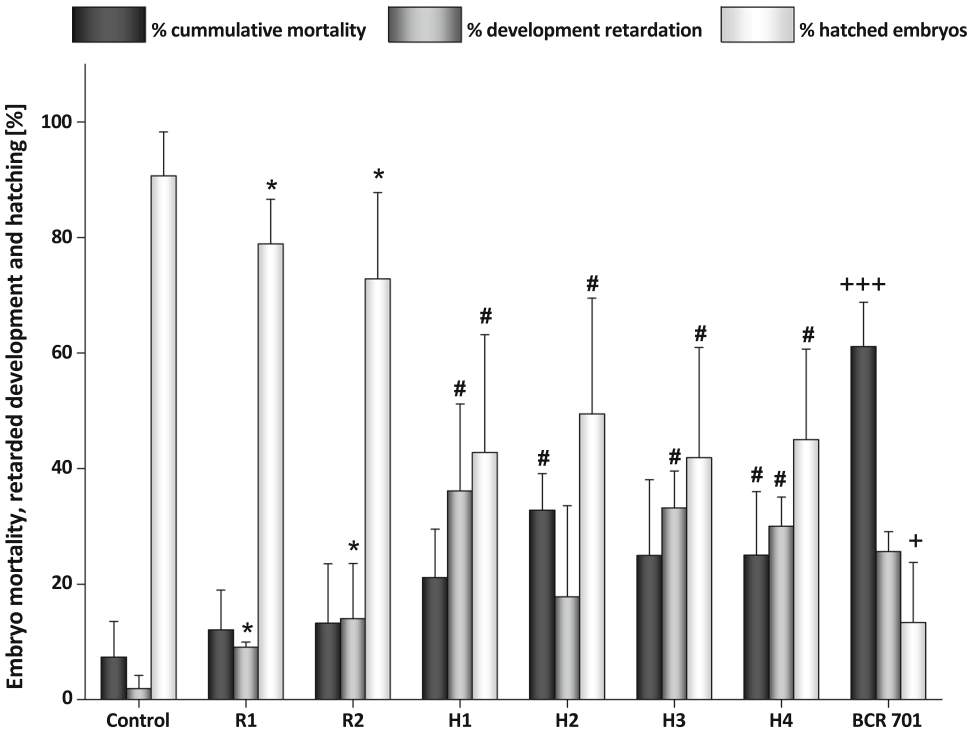

Fig. 2 Cumulated mortalities, developmental retardation, and hatching success of *D. rerio* embryos exposed for 96 h to selected sediments. Data correspond to mean percentages of six replicates (run in three independent experiments) ± SD. *Significant differences between R_{1-2} treatments and control ($p < 0.05$); #significant differences between H_{1-4} versus R_{1-2} treatments ($p < 0.05$); +significant differences between H_{1-4} treatments and BCR 701 positive reference ($^{++}p < 0.001$; $^{+}p < 0.05$)

of embryos with delayed development significantly increased (by 5 %) and, accordingly, the hatching success of embryos significantly decreased (approximately 11 %; $p < 0.5$) (Fig. 2). Exposure to harbor sediments (H_{1-4}) implied further increase in mortality (significant *vs.* control but insignificant compared with exposures to reference sediments), whereas the incidence of delayed embryonic development and decrease of hatching success was more accentuated (18 and 32 % respectively) compared with the effects of unpolluted sediments ($p < 0.5$). The greatest toxicity was observed for the BCR 701 sediment causing a mean mortality rate of 61 ± 7.7 %, a development-retardation rate of 26 ± 3.5 %, and a hatching rate of only 13 ± 10.4 %.

Correlations Between Bioassay Endpoints and TU Unit Estimates

The feasibility of using MXR activity as an indicator of sediment contamination and moreover, as predictor of toxicity risks, was evaluated by performing Pearson's product moment correlation analysis between the mean bioassay end point values: MXR activity in exposed mussels, *D. rerio* embryo mortality, development retardation and hatching, and the estimated toxicity of test sediments to *D. magna* ($\log TU_{sum}$ *D. magna*) (Table 2).

This statistical evaluation showed a fairly good negative correlation ($r = -0.920$, $p < 0.01$) between RB accumulation in mussels, thus the induction of MXR activity, and the metal load of test sediments (Fig. 3).

The strength of this association was comparable with that recorded for the mortality and hatching success of *D. rerio* embryos, where mortality positively correlated with the summed TUs calculated for metals ($r = 0.973$, $p < 0.001$), and the hatching rate significantly decreased ($r = -0.912$, $p < 0.01$). Regarding summed TUs computed for PAH compounds, only the rate of development retardation of fish embryos showed a significant positive association ($r = 0.841$, $p < 0.05$).

Discussion

Alterations of biochemical markers in molluscs have been extensively used to evaluate the pollution status of aquatic ecosystems. Within biochemical endpoints, MXR as a general marker of chemical stress proved to be a powerful tool in toxicity risk-evaluation studies (Contardo-Jara and Wiegand 2008; Luckenbach and Epel 2008; Faria et al. 2011).

Modulation of the MXR defense system in bivalves, specifically induction, was related to exposure with diesel-2 oil (Smital and Kurelec 1998), to organophosphorus and organochlorine pesticides, as well as to PCBs and PAHs (Bard et al. 2002), whereas for a range of chemosensitizers (as reviewed by Bard 2000), the inhibition of this defense mechanism was reported. More recently, P-glycoprotein-mediated MXR induction in mussels was reported for the cyanobacterial toxin microcystin-LR by Contardo-Jara and Wiegand (2008), whereas downregulation of P-gp was

Table 2 Pearson's product moment correlation coefficients (r) between bioassay endpoints (MXR activity in mussels and *D. rerio* bioassay endpoints (mortality, development retardation, hatching) and TU estimates ($logTU_{sum}$ *D. magna*$_{metal}$, $logTU_{sum}$ *D.magna*$_{PAH}$)

	MXR activity (RB accumulation)	*D. rerio* bioassay endpoints (96-h exposure)		
		Mortality (%)	Developmental retardation (%)	Hatching (%)
$LogTU_{sum}$ *D. magna*$_{metal}$	−0.920**	0.973***	0.359	−0.912**
$LogTU_{sum}$ *D.magna*$_{PAH}$	−0.612	0.294	0.841*	−0.621

Asterisks indicate the significance level of associations between variables: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$

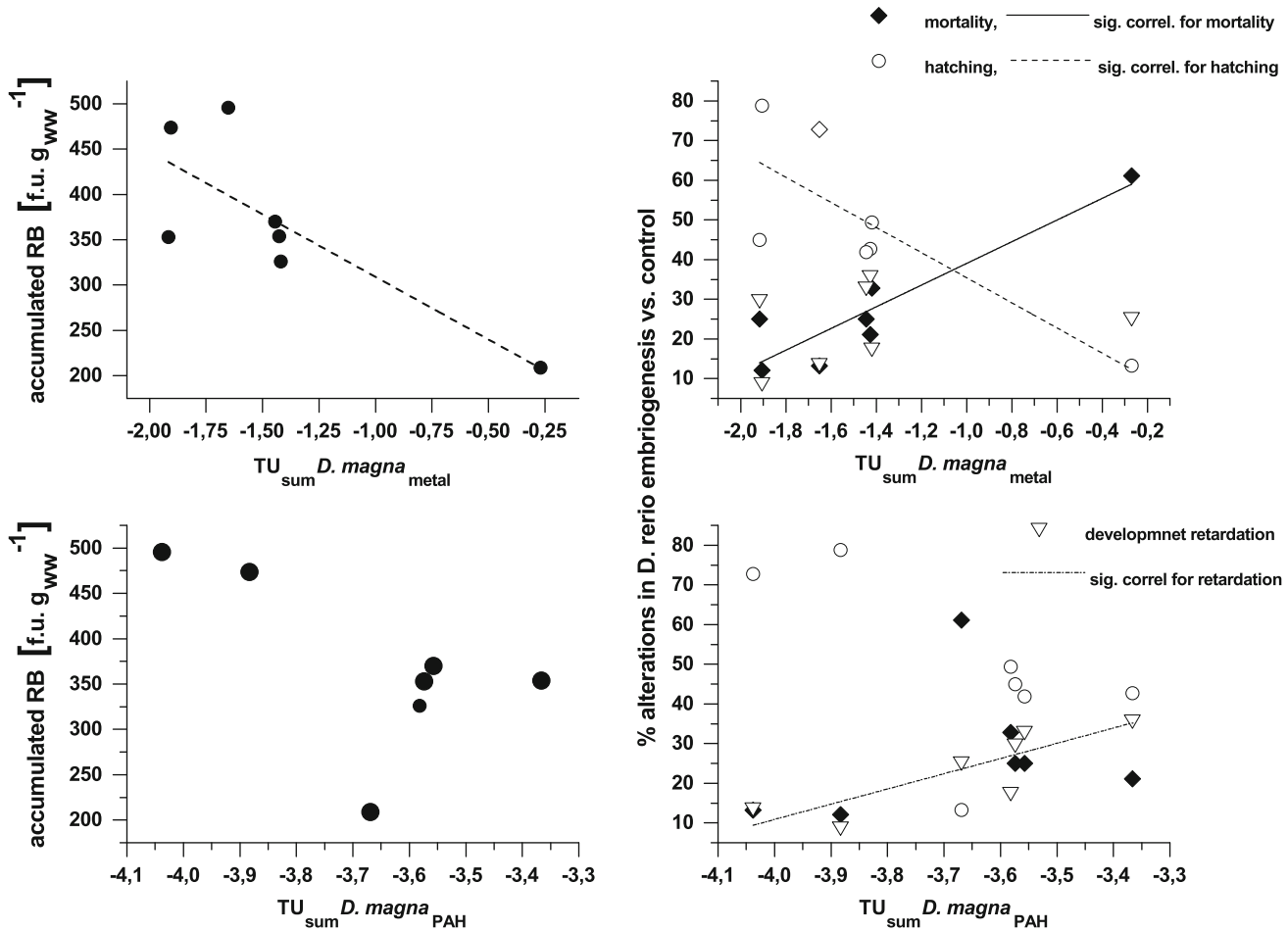

Fig. 3 Pearson's product moment correlation between MXR activities in mussels expressed as fluorescence units of RB per g wet weight mussel soft tissue (f.u. mean ± SD) (**a** and **b**) and *D. rerio* bioassay endpoints: mortality, developmenal retardation, and hatching (% alterations *vs.* controls) (**c** and **d**) and TU estimates ($logTU_{sum}$ *D. magna*$_{metal}$, $logTU_{sum}$ *D.magna*$_{PAH}$). Regression slopes were plotted only for significant relationships ($p < 0.05$)

observed in the digestive glands of mussels exposed to certain pharmaceuticals (Contardo-Jara et al. 2011). Clear relationships between MXR induction and contamination by Cd, mercury, Zn, Cu have been also reported (Minier et al. 2000; Eufemia and Epel 2000; Achard et al. 2004; Franco et al. 2006).

In this study, we assessed the incidence of MXR-transporter activity induction in laboratory-exposed mussels to contaminated sediments by the accumulation version of the fluorescent P-gp substrate rhodamine B in whole tissue homogenates. The basal level of MXR activity in the soft tissues of control *D. polymorpha* (494 ± 82 f.u. g_{ww}^{-1}) was comparable with that recorded by Contardo-Jara and Wiegand (2008) in the gills of this species (approximately 900 f.u. g_{ww}^{-1}). The choice of measuring MXR-transporter activity in whole tissue homogenates was selected to significantly decrease the tissue preparative work; thus, the processing of much greater volume of samples is feasible.

In *D. polymorpha* subjected to acute exposure with moderately polluted sediments, we detected limited (22–33 %) but statistically significant induction of MXR activity, and somewhat greater expression (57 %) was observed for the BCR 701 reference sediment. Concomitantly, MXR activity levels in mussels showed a strong correlation with the metal load of sediments and proved to be unrelated to the level of contamination with PAHs. This particular association with these two chemical classes most presumably relates to the fact that contamination with metals of the test sediments was of greater environmental relevance than that with PAHs. The significance of metal load in the test sediments was supported also by the TU estimates calculated for *D. magna* ($\log TU_{sum}$ *D. magna*$_{metal} > -2$ threshold), which highlighted the probability of significant metal-toxicity risks for this bioindicator organism. The strong correlation between MXR transporter activity in exposed mussels and environmentally realistic sediment contamination underlines the fairly good sensitivity of this biomarker in laboratory testing conditions to signal the bioavailability of sediment-bound contaminants and may also even anticipate the incidence of toxicity to biota.

Moderate expression of P-gp activity, thus moderate induction of MXR transporter activity in bivalves to environmental contaminants, has already been reported both for laboratory tests and in situ surveys (Eufemia and Epel 2000; Smital et al. 2003). Epel et al. (2008), in their review article, commented that this phenomenon is an indication of several alternatives: (1) increased P-gp (MXR) levels are not an important protective response; (2) the level of transporter activity is already set to the expected historical load of xenobiotics; and (3) a small increase in transporter activity is adequate to protect the organism. Our results seem to support the latter hypothesis, but to prove this in additional studies with more complex polluted sediments must be performed.

MXR activity levels measured in mussels for each exposure condition showed quite low variability within replicates (as indicated by the relatively small SD values) and thus reliably reflected even low contaminant differences between the sediments from remote areas and moderately polluted harbor sediments. The fairly high precision in the measurement of MXR activities per treatment, as indicated in previous studies (Bodin et al. 2004; Pain and Parant 2007), most presumably relates first to the fact that the test organisms belonged to a genetically uniform population and were collected from an anthropogenically undisturbed habitat. Second, in the previous exposure, the mussels were acclimatized in unpolluted and unfiltered lake water (with food-deprivation) for 5 days. This period proved to be sufficient for the MXR system to reach the baseline levels in zebra mussels (Smital et al. 2000; Pain and Parant 2007); thus, a kind of synchronization of the test organisms was achieved.

The strong correlation recorded between the MXR-transporter activities in exposed mussels and contaminant loads detected in sediments, as well as with the relative toxicities detected in the *D. rerio* bioassay, and, moreover, the low variability of this biomarker within treatments, supports the feasibility of its application in the quality assessment of sediments.

Exposure of *D. rerio* embryos showed a certain inhibitory effect even for the sediments from remote areas as evidenced by the statistically significant delay in development and consecutive decrease in the hatching rate of embryos (by 5 and 11 %, respectively). Such slight inhibitory effect of unpolluted sediments related to certain natural characteristics (grain size, aromaticity) in different sediment-contact test assays have been previously reported (Tuikka et al. 2011; Wolfram et al. 2012). However, the strong positive correlation for mortality rate ($p < 0.001$) and negative correlation for hatching rate of embryos ($p < 0.01$) with sediment TU estimates ($\log TU_{sum}$ *D. magna*$_{metal}$) was evidenced. It is worth mentioning the significant positive correlation between PAH contamination of sediments and the increase in incidences of developmental delay of fish embryos. This could be related to the fact that PAHs have been proven to alter heart morphology, impair heart looping, and cause atrioventricular conduction block in fish well before morphological abnormalities become apparent (Luckenbach et al. 2001; Incardona et al. 2004; Garner and Di Giulio 2012).

Conclusion

Screening MXR-transporter activity in *D. polymorpha* for assessing the anthropogenic pollution load of freshwater sediments in laboratory conditions reliably indicated environmentally realistic toxicity risks, and could discriminate even relatively low differences of contamination. Observations on ABC transporter interactions obtained on environmental samples suggest that efflux transporters significantly contribute to cellular defense against toxic metals in bivalves. In terms of cost- and time-effectiveness, screening P-gp expression in mussels appears to be a powerful tool for the preliminary toxicity testing of aquatic sediments due lower vulnerability of test organisms to experimental conditions.

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