

Natural organic matter (NOM) has the potential to modify the multixenobiotic resistance (MXR) activity in freshwater amphipods *Eulimnogammarus cyaneus* and *E. verrucosus*

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Abstract

Based on the chemical features of natural organic matter (NOM) with its variety of functional groups, we hypothesized that NOM will modify the multixenobiotic-resistance (MXR) of an organism as xenobiotic chemicals do. The MXR system is a general first rather non-specific line of defense against environmental contaminants. The aim of this study was to compare the impacts on MXR activity in amphipod species (*Eulimnogammarus cyaneus* and *E. verrucosus*, from Lake Baikal) stressed by cadmium chloride or dissolved NOM for 24 h. NOM exposure concentrations were environmentally realistic. MXR activity was assessed based on rhodamine B efflux; its specificity was proven by a verapamil inhibition assay. It was shown that both NOM and CdCl₂ lead to substantial reduction of the rhodamine B efflux. This suggests that NOM may be regarded as a chemosensor which is able to reduce the efficiency of the MXR system. Possible mechanisms of direct NOM impact on MXR processes are discussed, such as peroxidation of the membranes (including P-glycoproteins) or internal blockage of the MXR pump by bioconcentrated NOM. In general, our results show that well-developed depuration pathways of freshwater organisms in contaminated environments may be impaired by strong chemical stressors and, more important, by natural biogeochemical matrices such as humic substances — humic substances are present in all freshwater systems.

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1. Introduction

Natural Organic Matter (NOM) has a wide distribution in aquatic environments. Humic substances (HS) are the major component of NOM representing more than 60–80% of the total dissolved organic carbon (Thurman, 1985; Steinberg and Münster, 1985). Recently, several reports on the function of NOM as environmental signals to freshwater organisms have been published (Steinberg et al., 2006). Münster (1985) was the first to postulate that NOM compounds may have the potential to

penetrate biomembranes. Later Petersen and Persson (1987) reported on such direct adverse effects of dissolved NOM on aquatic organisms and discussed the pH-dependent lipophilicity of at least one fraction of NOM as the potential mechanism. It was also shown that NOM could modulate the activity of biotransformation enzymes of organisms and also directly inhibits photosynthetic oxygen production of plants (Pflugmacher et al., 1999, 2001, 2006). Wiegand et al. (2004) found elevated levels of the chaperon HSP70 in fish after exposure to isolated HS; the impact of some NOM to several freshwater amphipods resulted in changes of peroxidase activity and HSP70 expression (Timofeyev et al., 2004). Steinberg et al. (2003) categorized the effects on aquatic plants and animals as (i) non-specific, such as expression of heat shock proteins (HSP) and

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modulation of biotransformation enzymes, or (ii) specific, such as inhibition of photosynthetic oxygen release in plants, or changes in the sex ratio of fishes and amphibians. Basic ecotoxicological requirements are fulfilled: several mechanisms apply to a variety of aquatic organisms, dose–response relationships and quantitative structure effect relationships may be established where applicable. It was concluded that HS are natural environmental chemicals that exert a chemical stress and, thus, are able to structure aquatic guilds by various modes of action (Steinberg et al., 2003). Furthermore, evidence is accumulating that HS even have the potential of hormone-like compounds (Meinelt et al., 2004; Steinberg et al., 2004; Lutz et al., 2005). Very recently, it was shown that both an NOM source and a synthetic humic-like substance act as an attractant for the nematode *Caenorhabditis elegans* and induce genes encoding for antioxidant and biotransformation enzymes (Menzel et al., 2005).

If HS are natural environmental chemicals and interact with the biotic defense systems (induction of stress proteins, biotransformation and antioxidant enzymes), we also should anticipate that they may interfere with the depuration (elimination) mechanisms. Many aquatic species are able to survive in environments which contain anthropogenic pollutants or natural toxins, and their resistance to pollution may be due, at least in part, to the function of their membrane glycoprotein extrusion pumps. The mechanism of this multixenobiotic resistance (MXR) (Kurelec and Pivcevic, 1989) is similar to the mechanism involved in multi-drug resistance (MDR) found in tumor cells (Juliano and Ling, 1976) and is associated with P-glycoproteins (P-gp). These proteins belong to a superfamily of membrane transporters termed ABC-proteins or traffic-ATPases (Ames et al., 1990; Higgins, 1992). P-gp acts as an energy-dependent pump to translocate a wide variety of structurally and functionally diverse compounds. P-gp-like proteins have been described as first line of defense against toxins (Epel, 1998) and detected in a variety of aquatic organisms including sponges, mussels, oysters, worms, and fishes (Waldmann et al., 1995; Smital and Kurelec, 1997; Minier et al., 1999; Kurelec and Pivcevic, 1991; Bard, 2000).

Taking into account the potential role of NOM as possible natural chemical with a variety of functional groups, we hypothesize that dissolved NOM may modify MXR activity as some xenobiotics would do. The aim of this study is to compare the modification of MXR activities in two freshwater amphipod species from the Siberian Lake Baikal, *Eulimnogammarus cyaneus* (Dyb) and *E. verrucosus* (Gerst.), stressed by presence of dissolved NOM components. CdCl₂ served as a representative of potentially toxic heavy metals and verapamil as a positive control.

2. Materials and methods

2.1. Animals

For this study, two freshwater amphipod species, *E. cyaneus* (Dyb) and *Eulimnogammarus verrucosus* (Gerst.) were used. These species are typical representatives of the fauna of the

upper littoral zone in Lake Baikal. The specimens were collected at the shoreline in the area of the settlement Bol'shie Koty (southern Baikal). Before the experiments, the animals were maintained (pre-acclimated) in aerated aquariums at 6–8 °C at least for two, some for three days. Commercial food was used (Tetra-Min, Tetra GmbH, Germany) *ad libitum*. The water was continuously aerated, and the oxygen content was between 8–9 mg L⁻¹. The size of the collected individuals was 22–25 mm (*E. verrucosus*) and 15–18 mm (*E. cyaneus*). Age and sex of amphipods were not determined (in order to minimize handling of the delicate organisms). However, we assumed that most of the amphipods were adults because their size was close to maximal body size known for this species 30 mm for *E. verrucosus* and 20 mm for *E. cyaneus* (Bazikalova, 1951). For experiments only well-active animals were used.

2.2. Isolation of natural organic matter (NOM)

NOM was isolated by reverse osmosis from Lake Schwarzer See (a brown-water lake, Brandenburg State, north-eastern Germany) according to Serkiz and Perdue (1990); as a modification, we applied the sodium cation exchanger. The NOM comprised 24% organic carbon; the chemical characteristics of Lake Schwarzer See water and its NOM isolate are given in Table 1. The DOC concentrations were determined by high-temperature combustion (Shimadzu TC 5000) after acidification with phosphoric acid to remove inorganic carbonates (DIN EN 1484). The DOC was fractionated by a liquid chromatography fingerprint-technique with simultaneous UV- and DOC-detection according to Sachse et al. (2001, 2005) and classified as follows: HS 87.5%; low-molecular-weight acids 9.2%; polysaccharides 2.6%; low-molecular-weight substances 0%; specific UV absorption of 5.1 L/(mg m). Approximately 90% of the DOC was comprised of HS. For the sake of comparison, we applied this somewhat exotic NOM, because there have been several ecophysiological and ecochemical studies with this NOM (review: Steinberg et al., 2006; gammarids: Timofeyev

Table 1
Chemical characteristics of Lake Schwarzer See water and the NOM isolate from September 09, 2003

| Lake Water, $\mu\text{mol L}^{-1}$ | NOM features, ‰ | | |
|------------------------------------|-----------------|-------------|------|
| Total phosphorus | 4.6 | Ash content | 76 |
| Total nitrogen | 140 | C | 204 |
| Sulfate | 250 | H | 25.2 |
| Chloride | 280 | N | 5.8 |
| Dissolved silica | 24.3 | S | 47.6 |
| Dissolved inorganic carbon | 790 | Fe | 0.44 |
| Dissolved organic carbon | 2,260 | Mn | 0.91 |
| Sodium | 200 | Zn | 0.05 |
| Potassium | 82 | Cu | 0.06 |
| Magnesium | 100 | Al | 0.12 |
| Calcium | 570 | Na | 157 |
| Aluminum | n.d. | K | 11.9 |
| | | Mg | 8.3 |
| | | Ca | 53.6 |

n.d.=not determined.

and Steinberg, 2006; Timofeyev et al., 2006a,b; macrophytes: Pflugmacher et al., 2006; stable organic radicals: Paul et al., 2006).

2.3. Animal exposure, sample preparation, and measurements

The MXR activity assessment is based on the uptake/efflux of rhodamine B (Smital and Kurelec, 1998), which was slightly modified in this study. The rhodamine B efflux was determined by its residual content in the tissues of the animals after specific periods of time. The comparison with the verapamil positive control allows the conclusion, whether or not the observed efflux modification in exposed animals is due to an impact on the MXR system. Verapamil specifically blocks the MXR pump (Smital and Kurelec, 1997; Eufemia and Epel, 1998); it was added at 50 $\mu\text{mol/L}$. In addition to the determination of residual rhodamine B concentrations, its release into the water from the animals was assessed by measuring the fluorescence of the released rhodamine B in the aqueous phase.

The animals were kept in the water with dissolved rhodamine B at a concentration of 1 mmol/L for 1 h. During this pre-exposure phase, rhodamine B was allowed to accumulate in the organisms. After pre-exposure, the animals were removed from the solution and rinsed in clean water at least three times. In every experiment five pre-exposed animals were deep frozen (test 0 h), the remaining animals were placed in clean water (control group) or exposed. The NOM effect upon the MXR system was assessed at the environmentally realistic NOM concentrations of 0.6 and 1.2 mmol/L. The NOM effect were compared with the subacute toxic stress by dissolved cadmium chloride (CdCl_2 ; 0.22 and 2.2 $\mu\text{mol/L}$); cadmium is often used for toxicity studies (Veldhuizen-Tsoerkan et al., 1991; Mesna et al., 2000; Mounaji et al., 2003). As in the NOM experiments, this exposure was performed for 24 h, too.

To determine the internal (residual) rhodamine B content, at least five animals for each test were removed from the experimental aquariums after 1, 3, 6, 12, and 24 h and deep frozen. Later, the fixed animals were simultaneously dried at 30 °C for 24 h and homogenized in re-distilled water. The homogenate was centrifuged at 1000 rpm for 3 min (Eppendorf Minispin). The rhodamine B content (expressed in $\mu\text{g/g}$ dry weight) was determined in the supernatant by fluorescence measurement using a 535 nm filter for excitation and a 590 nm filter for emission (Shimadzu RF-5000 spectrophotometer). In another series of experiments, the concentration of rhodamine B was measured in the ambient water itself in order to assess the efflux rates. The water samples were taken each hour, and rhodamine B fluorescence (expressed in nmol/L) was determined. Concentrations of rhodamine B were calculated using calibration curve.

At least 30 animals were used for each experiment. For each analysis, five individuals were taken. All experiments were replicated at least three times. All data were analyzed statistically using the Statistica 5.0 software (StatSoft). The analysis of variance (ANOVA) was used for comparison. In all figures, values are presented as means \pm standard deviation. Stars indicate significant differences from the controls at $p < 0.05$.

3. Results and discussion

3.1. Rhodamine B assay

The results of *E. cyaneus* are presented in Fig. 1 and of *E. verrucosus* in Fig. 2. The upper diagrams (Figs. 1A, 2A) show the decreasing residual contents of rhodamine B with time. The animals were allowed to accumulate rhodamine B during pre-exposure, and subsequently released this dye after they were transferred into clean water. In the control groups of both species, the rhodamine B levels decreased already within the first 6 h. After 24 h, the residual content of rhodamine B has decreased to 44% (*E. cyaneus*) and 40% (*E. verrucosus*) of the initial level. Simultaneously, the dissolved rhodamine B content in the water increased steadily (Figs. 1B, 2B). There were no distinct differences between the two amphipod species studied. To demonstrate that the observed rhodamine B efflux was mediated specifically by the MXR activity and not simply by diffusion, specimens were exposed to verapamil as a positive control of MXR inhibition. Indeed, verapamil inhibited the rhodamine B efflux almost completely (Figs. 1A, 2A) and reduced the detectable rhodamine B concentration in the ambient water to minimal levels (Figs. 1B, 2B). Thus, we may conclude that the mechanism of MXR is present in *E. cyaneus* and *E. verrucosus*, and rhodamine B is gradually and actively released from intact animals.

In the NOM-exposed animals, the intensity of the rhodamine B excretion is obviously impaired (Figs. 1A, 2A). A significant efflux by the animals was not detected before 6–12 h. Moreover, an NOM-concentration dependent diminished release of rhodamine B to the surrounding water (Figs. 1B, 2B) reflects this finding distinctly: the 1.2 mmol/L DOC exposure reduced the efflux more than the 0.6 mmol/L DOC exposure. Hence, rhodamine B release of *E. cyaneus* and *E. verrucosus* is obviously inhibited by Lake Schwarzer See NOM, but not completely blocked.

3.2. Efflux inhibition with CdCl_2

In a second experimental approach, we compared the NOM impact on rhodamine B efflux with the impact of cadmium chloride; the obtained results are presented in Fig. 3. The development of the controls of both species (Fig. 3A and B) is almost identical with the results of the first experiment, showing a gradual decrease of residual rhodamine B content in the animals. Neither CdCl_2 exposure was lethal within the exposure period; also, we did not observe a reduced activity of the animals. In those animals exposed at 0.22 $\mu\text{mol/L}$ CdCl_2 , the reduction of the rhodamine B efflux occurred more slowly and less strong than in the 2.2 $\mu\text{mol/L}$ exposure. A significant decrease was registered after 6 h. In the 2.2 $\mu\text{mol/L}$ CdCl_2 exposure, the residual rhodamine B level did not change significantly from initial levels over the whole experiment in both species, resembling the verapamil experiment. Hence, measurements of rhodamine B concentration in the surrounding water of cadmium exposed animals show only after 12 and 24 h marginal increases, whereas in the control animals the rhodamine B

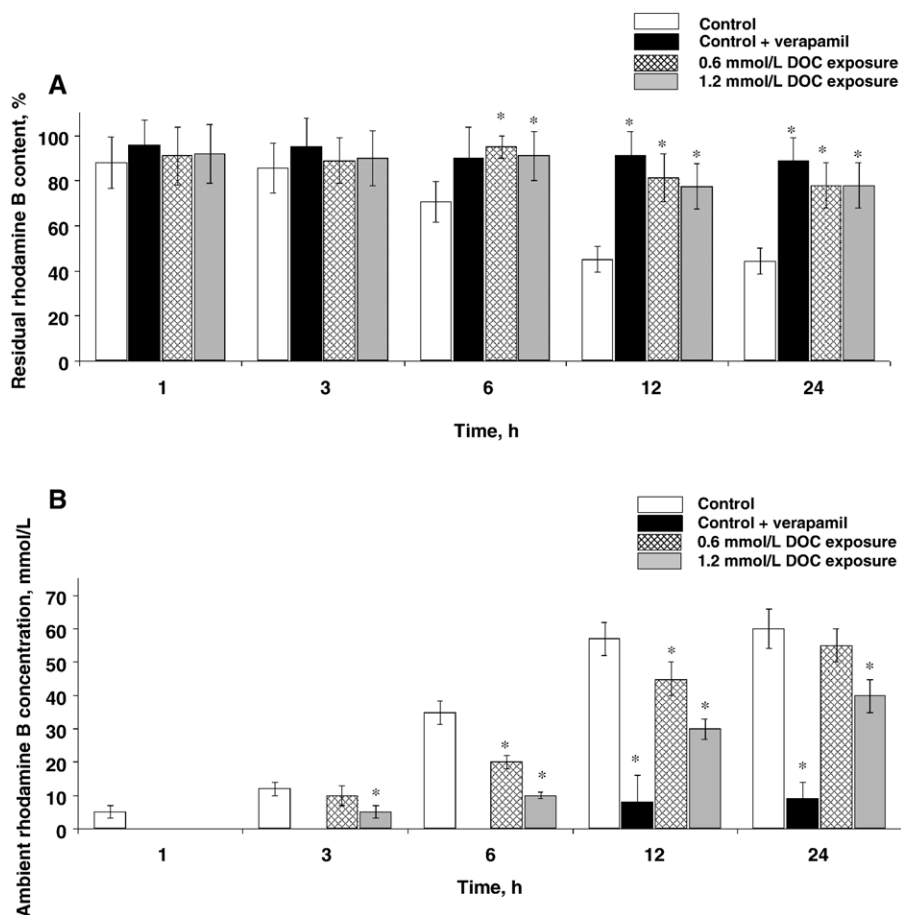


Fig. 1. Rhodamine B efflux in the amphipod *Eulimnogammarus cyaneus* exposed at two environmentally realistic NOM concentrations. A. Residual rhodamine B inside the amphipods. B. Rhodamine B concentration in the ambient water. * $p < 0.05$; $n = 5$.

release was much higher with a gradual increase over the time (Fig. 3C).

There are several mechanisms, how organisms may cope with sublethal environmental pollution. One of these mechanisms is the multixenobiotic resistance (MXR), which is similar to the mechanism involved in multi-drug resistance in tumor cells (Juliano and Ling, 1976). The first evidence of a MXR mechanism was derived from marine invertebrates (Eufemia and Epel, 1998). In the meantime, evidence is accumulating that this mechanism is also present in freshwater animals: zebra mussel *Dreissena polymorpha* (Smital and Kurelec, 1998; Britvic and Kurelec, 1999; Smital et al., 2003), Asiatic clam *Corbicula fluminea* (Waldmann et al., 1995; Legeay et al., 2005; Tran et al., 2005), and – without any environmental realism – isolated trout hepatocytes (Sturm et al., 2001). In the present study, we show that this mechanism also applies to freshwater amphipods. To our knowledge, this is the first proof of presence of the MXR system in freshwater amphipods. Furthermore, based on the reports mentioned above and on our study we may hypothesize that also in freshwater animals, the MXR system is a general first line of defense against any kind of environmental contaminants (xenobiotic chemicals, toxins, and allelochemicals).

With the simple rhodamine B technique (Smital and Kurelec, 1998), we were able to show that the exposed gammarids

(*E. cyaneus*, *E. verrucosa*) were able to eliminate this dye by more than 50% within 24 h. The blocking of this elimination mechanism by the specific P-glycoprotein inhibitor verapamil supports previous findings (mentioned above) that this mechanism is due to the activity of a P-glycoprotein with broad substrate specificity (Eufemia and Epel, 1998).

Although exposures to soft chemical stresses, as with NOM, may be more common in the environment than strong chemical ones, yet, chemical hot spots do still occur. But, how does the MXR mechanism respond to the exposure to strong chemicals, such as heavy metals? Will it eliminate the metal burden or will it be blocked itself, due to intoxication? This problem has been approached by exposing the gammarids to CdCl_2 . Actually, this exposure caused a strong inhibition of the rhodamine B excretion (Fig. 3); with both animal species, we observed a concentration-dependent inhibition, with the 2.2 $\mu\text{mol/L}$ exposure being even more pronounced after 6 h than the corresponding results of the verapamil assay.

The next step to more environmental realism takes into account that single-compound exposure is rare in the environment; multiple exposure is the more common situation, an aquatic organism has to face. What happens with the MXR mechanism, if several compounds from various chemical classes and from different origins (natural and man-made chemicals) act in concert? This problem has been simulated by exposing the

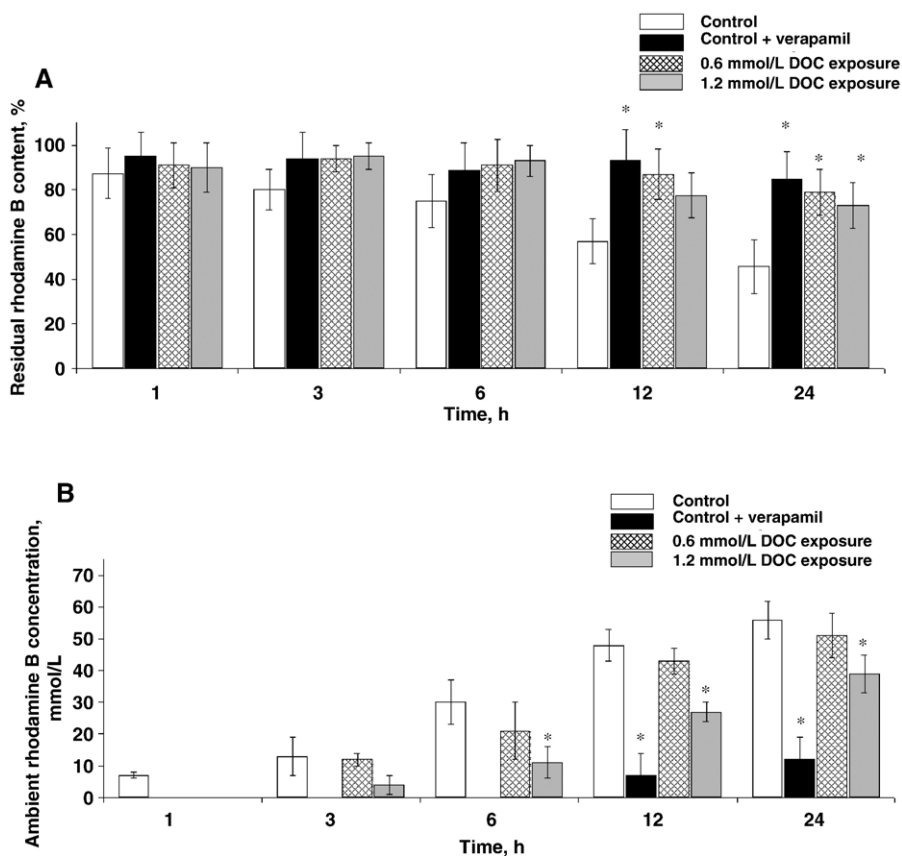


Fig. 2. Rhodamine B efflux in the amphipod *Eulimnogammarus verrucosus* exposed at two environmentally realistic NOM concentrations. A. Residual rhodamine B inside the amphipods. B. Rhodamine B concentration in the ambient water. * $p < 0.05$; $n = 5$.

gammarids to NOM, which we consider natural environmental chemicals. In fact, also the exposure of the animals in the NOM solution substantially inhibits the rhodamine B efflux (Figs. 1, 2). Phenomenologically, the impact of the 1.2 mmol/L NOM exposure was comparable to that one of 0.22 $\mu\text{mol/L}$ CdCl₂.

The MXR inhibition by NOM appears to be the first consolidated finding on the inhibitory impact of NOM or dissolved HS on the MXR-based efflux mechanism of bioaccumulated (xenobiotic) chemicals. Because NOM is present in all freshwater systems, this finding means that this biogeochemical matrix may have the potential to inhibit one major depuration mechanism of xenobiotic chemicals. Future studies have to assess, whether or not the inhibitory effect caused by various dissolved HS can be statistically related to specific (effective) structures of the HS, or if the MXR system is that non-specific that it responds to any xenobiotic compound, be it of industrial or natural origin. Furthermore, additional studies have to include, whether the observed effects upon NOM exposure are controlled transcriptionally.

However, taking into account existing evidence on direct and indirect effects of HS on the activation of biotransformation processes, activation of glutathione *S*-transferase, induction of HSPs synthesis, and the modulation of antioxidative enzymes in different amphipod species (Timofeyev et al., 2004, 2006a,b) and the nematode *C. elegans* (Menzel et al., 2005), the results of the present study are not rather surprising: HS interact within or

induce almost all pathways of biotransformation (Phase I and II enzymes, and now: including the MXR depuration pathway), oxidative stress defense (antioxidant enzymes), or general stress defense (induction of stress proteins).

Based on the presented material, we can conclude that NOM has the potential to modulate MXR activity in the studied amphipod species. The mechanism of this modulation, however, may not be fully understood from the obtained data yet. Based on previously published materials on direct NOM impact on freshwater animals we may hypothesize potential mechanisms of NOM action. The exposure of aquatic organisms to HS results in oxidative stress and induces the formation of reactive oxygen species (ROS) (Steinberg et al., 2003; Timofeyev et al., 2004, 2006a,b). Moreover, biomembranes exposed to HS are not only physically irritated, but also chemically (Steinberg et al., 2006). ROS species attack cell membranes and induce lipid peroxidation (Timofeyev et al., 2006a,b; Blokhina et al., 2003). It can be assumed that P-glycoprotein as a membrane protein will also be subjected to ROS. This impact can be associated with the direct destruction of the protein as well as with peroxidation and transformation of the surrounding cell membrane. Hence, the ROS and cell membrane/protein interaction should adversely affect the efficiency of P-glycoprotein-mediated excretion.

Another explanation of the efflux inhibition is the possible penetration of NOM particles into the cells. Based on several

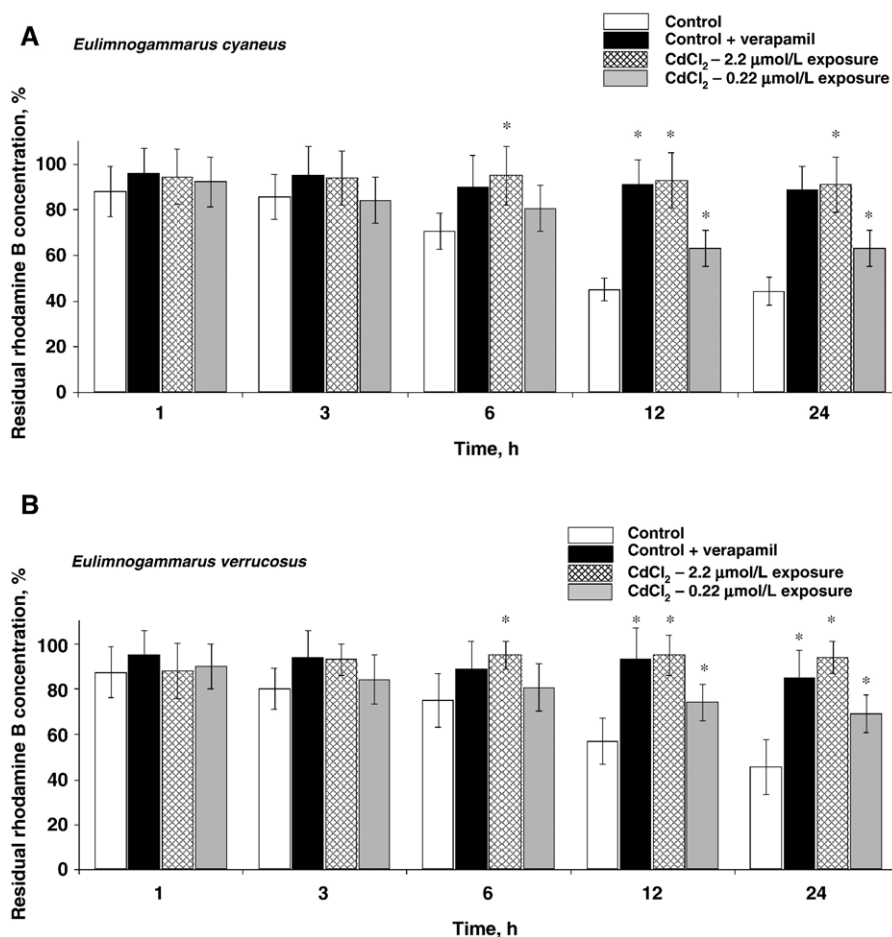


Fig. 3. Rhodamine B efflux activity in to amphipod species exposed at two CdCl₂ concentrations. A. Rhodamine B concentration inside *Eulimnogammarus cyaneus*. B. Rhodamine B concentration inside *Eulimnogammarus verrucosus*. C. Rhodamine B concentration in the surrounding water of amphipod species exposed to 5 μ/L CdCl₂. * $p < 0.05$; $n = 5$.

published results, this possibility appears feasible. In a cell culture study, Wang et al. (1999) showed that NOM or at least parts there of were found inside the cells and even in the nucleus. More recently, Nardi et al. (2002) showed that the physiological effects of HS on terrestrial plants depend on the source, concentration and molecular mass of the HS. The authors presented evidence that HS <3.5 kDa easily pass the plasmalemma of higher plant cells and, in part, were taken up. On the basis of recent geochemical papers (Hoque et al., 2003; Reemtsma and These, 2005), we can deduce that, due to the apparently low-molecular mass of the water-soluble and ionizable HS fraction (~0.5 kDa), this fraction of HS, as one major fraction of NOM, can easily be taken up by freshwater organisms (Steinberg et al., 2003). Hence, a penetration of at least an NOM fraction into the cells and their direct or indirect interaction in the efflux process are feasible, resulting in an inhibition of the MXR system in the exposed amphipods.

One significant outcome of this study is that the differences between the two *Eulimnogammarus* species studied are very small. This applies to the basic rhodamine efflux as well as to both exposure scenarios. We may propose that the MXR mechanism is not very specific and, more important, does not significantly vary over different species, especially in close

related species. If this assumption holds true even on the broader basis of species variety, shall be tested in futures studies.

3.3. Environmental significance

Freshwater organisms have to cope with a variety of chemical stressors, such as contamination. As a general first line of defense against any kind of environmental contaminants (metals, xenobiotic chemicals, toxins, and allelochemicals), organisms of various phylogenetic positions have developed the MXR system. However, this depuration system is clearly overtaxed by strong chemical stressors, such as toxic heavy metals. Furthermore, it is hampered, but not completely blocked, by environmental matrices, such as dissolved HS, which are present in all freshwater systems (Jones, 2005). Particularly, relatively low DOC concentrations appear to be active in this respect: In their review, Haitzer et al. (1998) found that 7 out of 27 studies reported an increase in bioconcentration of xenobiotics, when the animals were co-exposed to HS at concentrations below 1 mmol/L DOC. Although several hypotheses have been put forward and discussed (Haitzer et al., 1998, 2001), none appears to be convincing and a subsequent experimental verification failed (Steinberg, 2003). The

chemosensitization of the MXR pump by dissolved HS has not been discussed, however, and seems to be a plausible mechanism. The same mechanism may also apply to metal bioconcentration. For instance, John et al. (1987) showed that the cadmium uptake by Atlantic salmon (*Salmo salar*) is strongly dependent on the concentration of HS with a pronounced maximum at low (0.23 mmol/L) DOC concentrations. Elevated HS concentrations reduce the bioconcentration of cadmium, probably due to the formation of complexes or aggregates with decreased potentials to penetrate biomembranes. This is the well known picture; but environmentalist should pay more attention to the concerted action of metals and xenobiotic chemicals on the one hand and humic substances at environmental realistic concentrations on the other hand.

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