



# First evidence of the presence of Multixenobiotic Resistance Mechanism activity in freshwater invasive species, signal crayfish *Pacifastacus leniusculus* (Dana, 1852)

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## Abbreviations:

MRP – multidrug resistance-associated protein  
MXR – multixenobiotic resistance  
P-gp – P-glycoprotein  
RB – Rhodamine B  
VER – Verapamil

**Key words:** heavy metals, multixenobiotic resistance mechanism, *Pacifastacus leniusculus*, signal crayfish

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## ABSTRACT

**Background and Purpose:** The signal crayfish *Pacifastacus leniusculus* (Dana, 1852) is one of the most successful invasive species of crayfish in European freshwaters, an extremely diverse though endangered group of ecosystems. The main goal of this study was to functionally characterize multixenobiotic resistance (MXR) mechanism defense activity in *P. leniusculus* tissues for the first time. MXR mechanism protects the cell from a wide variety of toxic compounds, and it is mediated by the transport activity of ATP-binding cassette (ABC) proteins.

**Materials and Methods:** MXR transporter activity dye assay was performed by using fluorescent model substrate rhodamine B (RB) in combination with inhibitors of MXR efflux pumps: MK571 and Verapamil, known to inhibit multidrug resistance-associated proteins (MRP) and P-glycoprotein (P-gp), respectively. In this assay, the increase in intracellular fluorescence of the substrate dye, indicates inhibition of MXR efflux protein pumps. The assay was performed in three different tissues (gills, hepatopancreas, tail muscle). Additionally, tissues were exposed to selected heavy metals – mercury (HgCl<sub>2</sub>) and zinc (ZnCl<sub>2</sub>), known to occur in open freshwaters as pollutants.

**Results:** Optimal time for RB accumulation in gills and hepatopancreas was determined to be 30 minutes. RB efflux in gills was inhibited by MK571 and in hepatopancreas by Verapamil, suggesting that multidrug resistance-associated proteins are dominant in gills of *P. leniusculus*, and P-glycoprotein in hepatopancreas. Finally, inhibitory effect of mercury (HgCl<sub>2</sub>; 10 and 20 μM) and zinc (ZnCl<sub>2</sub>; 5–20 μM) on multixenobiotic resistance mechanism activity in gills, and only mercury in hepatopancreas, was detected.

**Conclusions:** The results for the first time demonstrate the presence of multixenobiotic resistance mechanism efflux activity as an important tissue specific defense mechanism in *P. leniusculus* and provide the basis for future molecular and toxicological studies of this invasive and adaptable species.

## INTRODUCTION

Freshwaters are extremely diverse and vulnerable ecosystems that have been impacted globally by a suite of anthropogenic pressures including pollution (e.g. heavy metals), overexploitation, physical alternation

and damming and introduction of non-native species (1). Crayfish, one of the most successful aquatic invasive species, have adverse impacts on native crayfish populations and other biota (2, 3) as well as on the physical environment of streams and rivers (4). The success of invasive species is often attributed to specific life history and ecological traits, such as fast growth rate, high fecundity, early maturation, flexibility to water quality and habitat requirements, and tolerance to pollution (5–7).

In order to cope with contamination, aquatic organisms have to rely on the efficiency of cellular »chemical defense« mechanisms represented, among other proteins, by ATP-dependent efflux transporters (8). The key importance in this »first line of defense« has multixenobiotic resistance (MXR) mechanism mediated by the group of ATP-binding cassette (ABC) transporter proteins (9, 10). These membrane proteins use ATP to pump a wide variety of both endogenous and exogenous compounds out of cells against their concentration gradient, reducing their accumulation, and therefore potential toxic effects for the organism. Members involved in efflux of contaminants include the ABCC/multidrug resistance-associated proteins (MRPs) and ABCB/P-glycoprotein (P-gp) (9). Substrates of MRPs are mainly metabolites in the form of glutathione, glucuronic or sulfate conjugates, as well as exogenous compounds after metabolization. On the other hand, P-gp is primarily involved in the efflux of unmodified compounds, mainly xenobiotics (11, 12). Interestingly, both MRPs and P-gp are involved in protection against heavy metal toxicity in many aquatic organisms (13, 14). It is important to note that some environmental pollutants can inhibit ABC transporter proteins and therefore make the organism/cell more susceptible to a range of other xenobiotics (15, 16).

The standard functional assay for determination of MXR activity in tissues is based on the measurement of accumulated level of specific fluorescent dye (e.g. rhodamine B) in the presence or absence of specific MXR transporter inhibitors (e.g. Verapamil – specific P-gp inhibitor or MK571 – specific MRP inhibitor) (13, 15–17). Fluorescent dye substrate is actively effluxed from the cells by MXR transporters and addition of inhibitors causes its higher accumulation in tissues. Therefore, dye efflux assay indicates the presence and activity of MXR efflux pumps in the tissue. Such assays have been reported for several species of crustaceans: common lobster *Homarus gammarus*, common shrimp *Crangon crangon*, isopod *Idotea emarginata* and flea cladoceran *Daphnia magna* (17, 18), but the MXR efflux activity has not yet been investigated in a decapod invasive species.

In this study, we report the first evidence of defense activity of MXR mechanism in tissues of the signal crayfish *Pacifastacus leniusculus* (Dana, 1852), a freshwater benthic macroinvertebrate and one of the most successful invasive species of aquatic ecosystems in the Europe (2).

Interestingly, invasive success of this species was suggested to be correlated with its tolerance to pollution (7). To determine whether MXR transporters are active in *P. leniusculus* we performed dye efflux assay in three different tissues (gills, hepatopancreas, tail muscle), using rhodamine B and compounds known to inhibit MXR transporters in other organisms (MK571 and Verapamil). Further, we showed that specific heavy metals could inhibit ABC efflux transporters, leading to increased sensitivity toward other pollutants.

## MATERIALS AND METHODS

### Reagents

MK-571 sodium salt hydrate (MK571), Verapamil hydrochloride (VER), rhodamine B (RB), Coomassie Brilliant Blue G-250 (CBB), bovine serum albumine (BSA) and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA), and mercuric chloride (HgCl<sub>2</sub>) and zinc chloride (ZnCl<sub>2</sub>) were from Kemika (Zagreb, Croatia). DMSO and 96% ethanol were used as solvents for MK571 and VER stock solutions, respectively. RB and heavy metal stock solutions were prepared in distilled water. Phosphate buffered saline (PBS: NaCl 0.138 M; KCl 0.0027 M; pH 7.4) was prepared with chemicals obtained from Sigma (St. Louis, USA).

### Animal and tissue collection

Adult female signal crayfish (10–15 cm total length – from the tip of the rostrum to the end of the telson) were caught using baited crayfish traps from the Korana river near Karlovac, Croatia (45°23'29.84" N, 15°35'41.28" E), and were immediately transported to aerated freshwater tanks (tank size: 80 × 100 × 50 cm, water temperature: 20±1 °C). During the captivity, animals were kept in separate boxes and were given no food during the adaptation (at least 3 days before the experiments) or the experimental periods. Animals were humanely euthanized (19): in order to lower their activity they were first cooled for 5–10 min at –20 °C and then immediately (within 10 seconds) the chains of nerve centers on the central ventral length of their body were cut with dissection knife.

Three different tissues were collected during dissection: gills, hepatopancreas and tail muscle (Figure 1). Each tissue was kept separately in cold PBS (4 °C) and sliced by dissection knife into cubes (tail muscle) or by dermatology biopsy punches (Acuderm, Fort Lauderdale, FL) into disks (gills and hepatopancreas) of app. equal size (2 × 2 mm; 40 mg in average). Cubes/disks were rinsed thoroughly in ice cold PBS.

For each experiment, we pooled tissue cubes/disks from 6 different animals. This was done separately for each of the three examined tissues. For each experimental

treatment, we took 4 samples from the pool of cubes/disks, each sample consisting of three individual cubes/disks randomly selected from the pool. Samples were incubated in 1 ml of PBS with addition of different MXR substrates, inhibitors or heavy metals. All incubations were performed with gentle shaking in the dark for 30 min at  $20 \pm 1$  °C. Each experiment was done in triplicate.

### Basal fluorescent dye accumulation

Prior to MXR transporter activity assay we determined the optimal time of exposure of crayfish tissue samples to fluorescent dye RB in order to achieve stable accumulation of the dye in the tissue. RB enters the cells passively (by diffusion). Fresh solution of 1  $\mu$ M RB in PBS was prepared and tissue pieces were incubated for 5, 10, 20, 30, 45, 60 and 120 min.

In the same manner we have tested R123, another substrate of MXR efflux pumps, but could not observe any increase in hepatopancreas intracellular fluorescence, even after we increased R123 concentration from 1 to 10  $\mu$ M and prolonged incubation to 150 min. Moreover, accumulation of R123 was more than two times lower than RB accumulation in all three tissues (data not shown). Therefore, R123 was not used in further experiments.

### MXR transporter activity assay

In MXR transporter activity assay RB, a model MXR substrate and an indicator of MXR efflux transporter activity (20), was used in combination with one of the two MXR inhibitors – VER and MK571 (1, 5, 10, 20 and 50  $\mu$ M), or heavy metals – Hg and Zn (1, 5, 10 and 20  $\mu$ M). The dye is effluxed out of cells if MXR transporters are active. Contrary, the inhibition of MXR transporter activity is indicated by increased accumulation of RB in the signal crayfish tissue samples (i.e. increased fluorescence when compared to controls, exposed only to 1  $\mu$ M RB and DMSO in the MK571 inhibitor setup or RB and ethanol in Verapamil setup). Therefore, lower fluorescence measured in cells/tissues indicated higher efflux transporter activity and vice versa.

Tissue samples were placed in small glass beakers with fresh PBS and inhibitors or heavy metals. Reaction was started with the addition of RB to a final concentration of 1  $\mu$ M, followed by 30 min incubation. In pre-exposure experiments the samples were first exposed to MXR inhibitor or heavy metal for 30 minutes after which RB was added and followed by additional 30 minutes of exposure.

### Measurement of fluorescence

Incubation was stopped by transferring tissue cubes/disks in fresh PBS to remove external RB (washing). Then, samples were transferred into 500  $\mu$ l of cold PBS and sonicated for 30 sec by Ultra-Turrax T25 homogenizer (IKA, Germany). Sonicates were centrifuged at 13,000

x g for 5 min at 4 °C and supernatant containing RB was transferred to black 96-well microplates (Nunc, Thermo Fisher Scientific, Denmark). Fluorescence was measured in triplicates using FLUOstar OPTIMA platerreader (BMG Labtech GmbH, Offenburg, Germany): excitation at 544 nm, emission at 590 nm. Rhodamine extracts were kept in the dark and on ice during the experiment.

### Protein concentration measurement

Protein concentration in the samples was determined using previously described method (21) with some modifications. BSA was used as a standard (linear range from 0.025 to 0.25 mg ml<sup>-1</sup>). Reaction mixtures containing 2  $\mu$ l of sample, 13  $\mu$ l of PBS and 10  $\mu$ l of protein reagent [0.05% (w/v) CBB, 4.7% (w/v) ethanol, 8.5% (w/v) phosphoric acid] were prepared in duplicates and incubated at room temperature for 10 minutes. Absorbance at 595 nm was measured using NanoDrop 2000c (Thermo Fisher Scientific, Waltham, MA, USA).

### Data analysis and presentation

Prior to statistical analysis of accumulated fluorescence in treated tissues each sample was normalized relative to non-treated controls (i.e. background fluorescence value was subtracted from each reading). Background fluorescence values of tissues with no RB were the same as those of the PBS extraction medium ( $p < 0.05$ ). All experiments were performed at least three times and mean values and SDs were determined. Data from each set of individual experiments were first analyzed with the Shapiro-Wilk test for normality and then with Levene's test for homogeneity of variances; all data presented were normally distributed and had homogeneous variance. Values were expressed as FU-mg-1 of protein (Figure 2), or as fold increase in comparison to control values (tissue incubated only with RB in the presence of solvents; Figures 3–5). Significant differences were determined using unpaired Student's *t*-test ( $p < 0.05$ ). Plots were made using Sigma Plot software (Version 8.0, Systat Software Inc., Richmond, CA, USA).

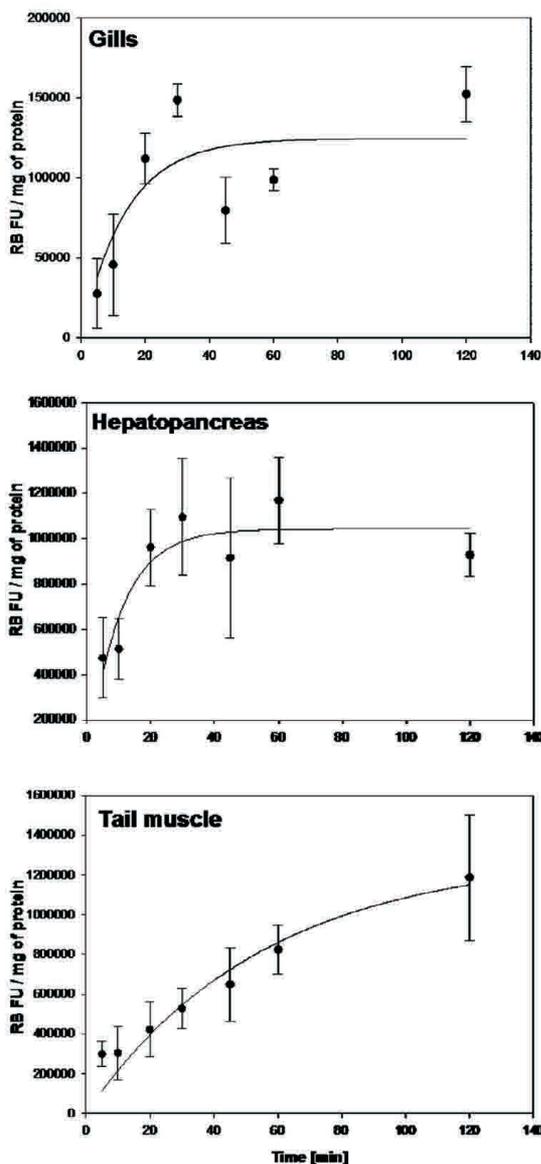
## RESULTS

### Basal fluorescent dye accumulation

To establish optimal conditions for dye efflux assay in crayfish we measured accumulation of model MXR substrate RB in different tissues (Figure 1). The accumulation of RB in gills and hepatopancreas was linear until 20 min and reached the plateau level (i.e. saturation of MXR efflux activity) at 30 min (Figure 2). In comparison, accumulation of RB in tail muscle was much slower and did not reach the plateau even after prolonged incubation (120 min). Based on this, in further experiments we did not work with tail muscle tissue but only with gills and hepatopancreas. The incubation time of gills and hepatopancreas



**Figure 1.** Dissected signal crayfish *P. leniusculus* showing target tissues: (a) gills, (b) hepatopancreas, and (c) tail muscle.



**Figure 2.** Time dependent RB accumulation in *P. leniusculus* tissues. Tissues were incubated in  $1 \mu\text{M}$  RB. Mean values of fluorescence  $\pm$ SD are shown;  $n=6$ .

pancreas with RB in the following experiments was 30 min, i.e. at the maximum basal level of the MXR efflux activity.

### Inhibition of MXR transporter activity

We detected dose responded tissue-specific inhibition of MXR efflux activity using both MK571 and VER (Figure 3). After 30 min of incubation MK571 showed statistically significant inhibition only in gills (1.3 to 1.4-fold increase in comparison to control, when using  $5\text{--}50 \mu\text{M}$  MK571), while VER showed more pronounced effect in hepatopancreas (1.7 and 1.3-fold increase when using 20 and  $50 \mu\text{M}$  VER, respectively).

### Effect of mercury and zinc on MXR transporter activity

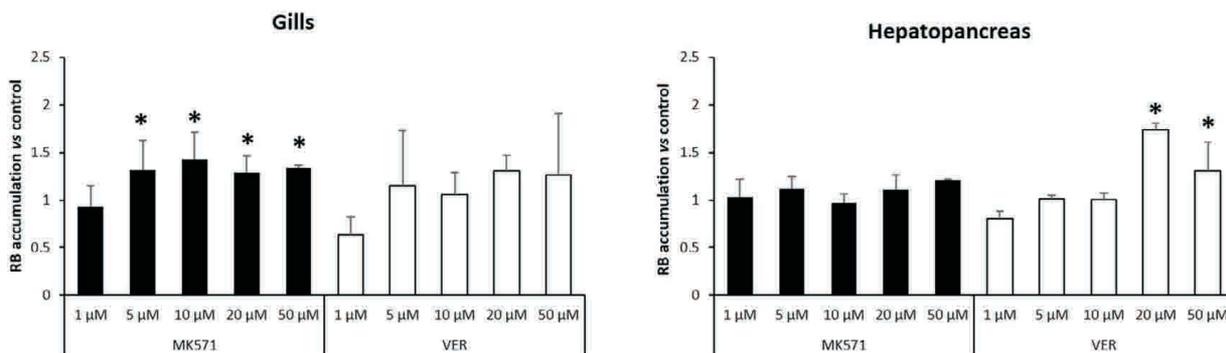
Both  $\text{HgCl}_2$  and  $\text{ZnCl}_2$  caused MXR inhibition in tested crayfish tissues (Figure 4) and the effect was more pronounced in gills where statistically significant increase in RB fluorescence ( $\sim 1.5$ -fold) was detected with 10 and  $20 \mu\text{M}$   $\text{HgCl}_2$  and with 5, 10 and  $20 \mu\text{M}$   $\text{ZnCl}_2$ . In hepatopancreas, significant increase in RB fluorescence ( $\sim 1.6$ -fold) was measured only after 30 min incubation with  $20 \mu\text{M}$   $\text{ZnCl}_2$ .

### Effect of pre-exposure on MXR efflux activity

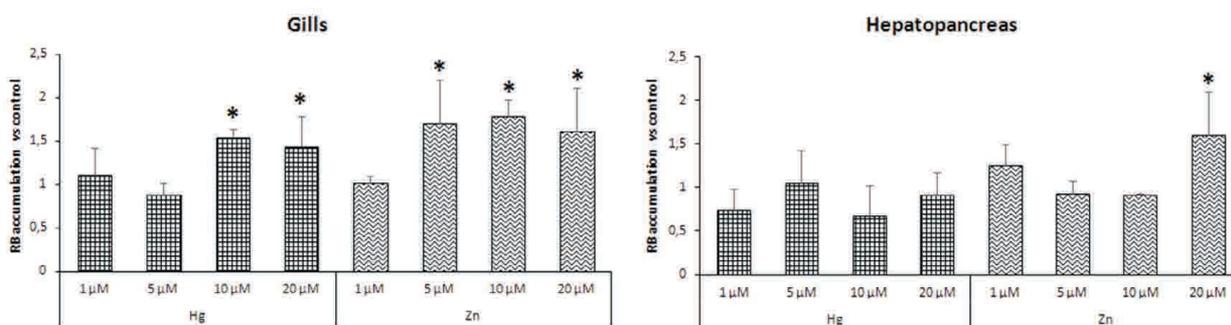
Finally, we tested the effect of pre-exposure on MXR transporter activity, i.e. MXR inhibitors or heavy metals were added 30 min before addition of RB to allow better penetration in crayfish tissues (Figure 5). To evaluate the effect of pre-exposure we compared results presented in Figures 3 and 4 (without pretreatment, i.e. co-exposure) with the results presented in Figure 5 (with pretreatment, i.e. pre-exposure). Inhibitory effect of  $10 \mu\text{M}$  MK571 on both tissues was more pronounced when pretreatment was used (1.4 vs. 2.1-fold increase in gills, and no increase vs. 2.0-fold increase in hepatopancreas, respectively). With  $10 \mu\text{M}$  VER pretreatment was effective in hepatopancreas (from no increase to 1.7-fold increase), while in gills VER did not cause increase in fluorescence with or without pretreatment. Inhibitory effect of heavy metals on gills was approximately the same with or without pretreatment. In contrast,  $\text{HgCl}_2$  inhibited efflux of RB from hepatopancreas when pretreatment was used (no increase in fluorescence without pretreatment and 1.6-fold increase after pretreatment).

## DISCUSSION

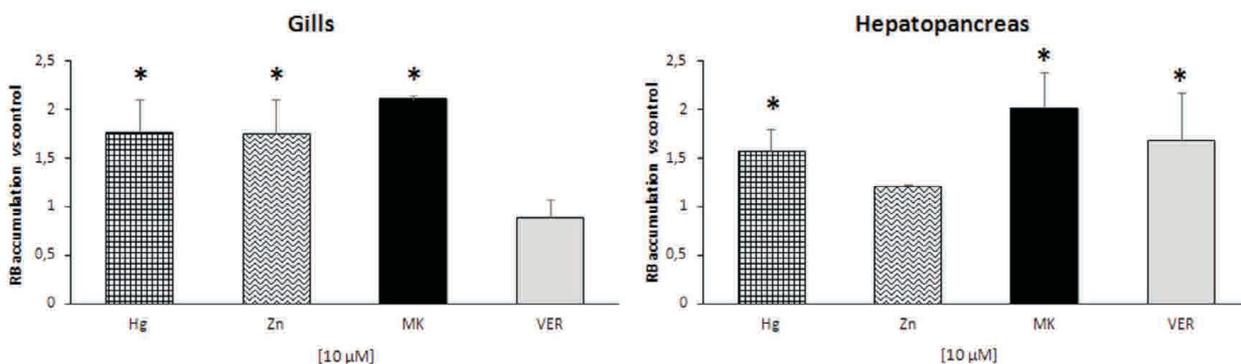
The aim of this study was to demonstrate the MXR efflux activity in three different tissues (gills, hepatopancreas and tail muscle) of signal crayfish, *P. leniusculus*. Namely, we evaluated ABC transport proteins activity by functional assay via the level of intracellular accumulation



**Figure 3.** RB accumulation in gills and hepatopancreas upon co-exposure with P-gp and MRP inhibitors (MK571 and VER). Bars represent substrate fluorescence levels in comparison to control (mean ± SD; n=6). Asterisk indicates significantly different accumulation in respect to control (p < 0.05).



**Figure 4.** Effect of heavy metals (mercury and zinc) on MXR transporter activity. Bars represent substrate fluorescence levels in comparison to control (mean ± SD; n=6). Asterisk indicates significantly different accumulation in respect to control (p < 0.05).



**Figure 5.** Effect of pre-exposure on MXR transporter activity. Bars represent substrate fluorescence levels in comparison to three different control groups: RB for Zn and Hg exposure; RB + DMSO for MK 571 exposure; and RB + ethanol for VER exposure (mean ± SD; n = 6). Asterisk indicates significantly different accumulation in respect to control (p < 0.05).

of MXR model substrate RB in the presence of known inhibitors (MK571 and VER), as well as in the presence of two heavy metals (HgCl<sub>2</sub> and ZnCl<sub>2</sub>). Three different tissues were analyzed since it is known that ABC transport proteins have different tissue distribution according to their protective role and involvement in adsorption, distribution, metabolism and elimination (12). Gill tissue

is frequently used in MXR efflux studies of aquatic organisms as it acts as the first barrier against entrance of compounds and is therefore greatly exposed to environmental pollutants (22, 23). Hepatopancreas, as a major digestion gland, is a metabolically active organ involved in detoxification (18, 24). Finally, the tail muscle represents metabolically more inert tissue. Accordingly, we have showed

that accumulation of RB in tail muscle is very slow (Figure 2) and therefore we excluded this tissue from further MXR transporter activity experiments.

Only two functional assays of MXR proteins were optimized previously for crustaceans. Lüder and co-authors used an alternative substrate Fura 2AM for hepatopancreas and brain tissues of *C. crangon*, *H. gammarus* and *I. emarginata* (18). Nevertheless, preferred incubation time was the same as determined here (30 min). The second study was not conducted on isolated tissues but used whole immobilized *D. magna* juveniles and RB, R123 and calcein-AM were used as MXR substrates (17).

Out of two candidate substrates, RB and R123, both commonly used in functional assays of MXR proteins in aquatic organisms (17, 25, 26), we have chosen RB, since with R123 we obtained lower tissue accumulation, also noticed previously (27). Further, no uptake of R123 could be measured in hepatopancreas, presumably because of pronounced endogenous emission of this tissue at 544 nm that coincides with emission wavelength of R123.

We indirectly demonstrated the presence of MXR transporters in crayfish gills and hepatopancreas by two known MXR inhibitors that caused accumulation of RB in living cells: MK571 is an inhibitor of MRP-like activity (28) and VER inhibits P-gp efflux pumps (29). Tissue-specific differences in transporter expression and/or specificity were detected, with MRP-like inhibition activity in both tested tissues of *P. leniusculus* and more pronounced P-gp-like inhibition activity in hepatopancreas. Therefore, our results confirm the presence of both type of MXR transporters in hepatopancreas that could be beneficial since it is the major energy reserve storage in crustaceans (24) and is highly sensitive to physiological and environmental changes (30). Inhibition of MRP-like activity by MK571 was also demonstrated in hepatopancreas of related decapod species *H. gammarus*, but not in decapod *C. crangon* and isopod *I. emarginata* (18). No comparison of our results with the results of Campos and co-workers on *D. magna* is feasible because they exposed the whole body of 4-day old juvenile daphnids, while we used isolated *P. leniusculus* tissues (17). In any case, we could not detect P-gp like activity in gills even when tissue was pre-exposed to VER. Additional gene expression and immunohistochemical studies should further confirm this result.

As stated earlier, MXR mechanism acts as one of the primary cell detoxification machineries. Many diverse environmental pollutants can be exported via ABC transporters, including heavy metals like mercury and zinc (31) that are often present in the freshwater body (32). These contaminants in water pose a serious risk to aquatic organisms as they are frequently incorporated into trophic chains, but have no biological role and are detrimental to cellular processes. Apart for being substrates for ABC efflux pumps, it is also known that mercury and zinc exposure can induce MXR protein expression in aquatic or-

ganisms (33) or that they can act as chemosensitizers, i.e. inhibit the ABC efflux pumps and therefore increase toxicity of other pollutants present in the environment (34). Here, we show inhibitory effect of mercury and zinc on MXR in gills and hepatopancreas of *P. leniusculus*. Inhibition with both metals was more pronounced in gills, possibly because of slower uptake of mercury and zinc into hepatopancreas. Further, it would be interesting to use longer exposure to lower concentrations of heavy metals that are more environmentally relevant (17, 35, 36). Using this approach it would be possible to see if mercury and zinc could also cause increase in ABC transporter gene expression, as it was seen previously (17, 33, 37). As an example, it was recently shown that 24 h exposure of *D. magna* juveniles to 1 or 10 nM mercury caused induction of ABC transporter activity (17). This effect was measurable when R123 and calcein-AM substrates were used, but not when RB was used.

In conclusion, we have optimized an experimental *in vitro* procedure for functional analysis of MXR mechanism activity in the invasive crayfish *P. leniusculus*. We recommend using both gill and hepatopancreas tissues, since differences in MXR efflux activity were detected. Also, pre-exposure is beneficial as it amplified tissue response for several tested compounds. A thorough molecular biological characterization and analysis of substrate specificities of these proteins is needed for a more detailed characterization of this protective system in the future. Finally, our results for the first time demonstrate the presence of MXR efflux protein activity in gills and hepatopancreas of freshwater signal crayfish.

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