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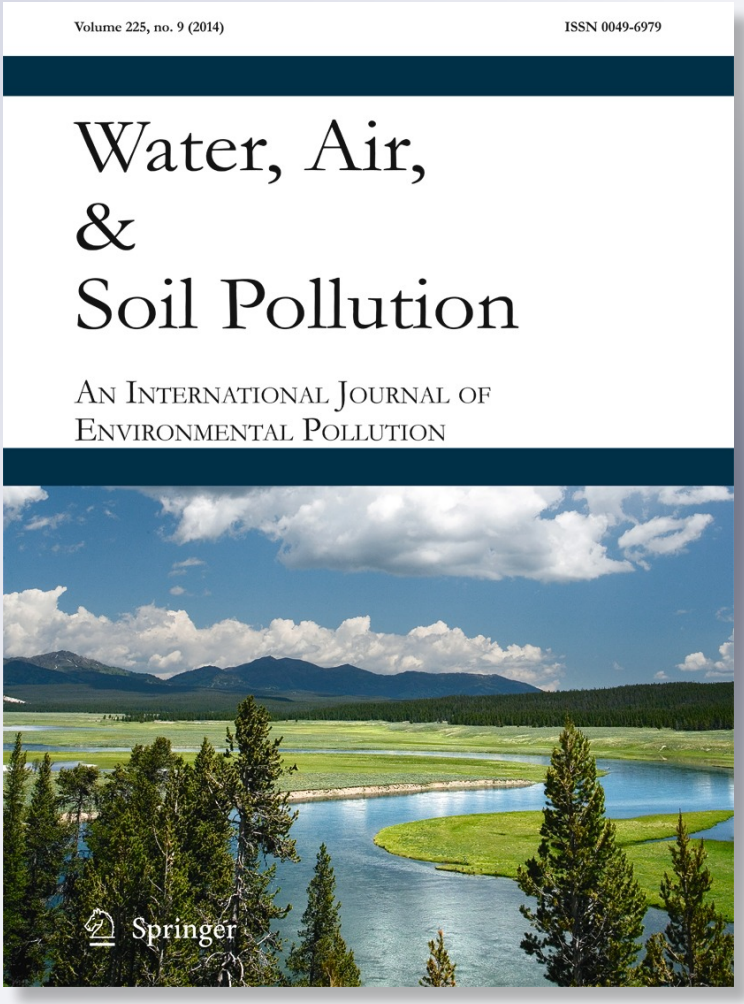
Water, Air, & Soil Pollution
An International Journal of Environmental Pollution

ISSN 0049-6979
Volume 225
Number 11

Water Air Soil Pollut (2014) 225:1-13
DOI 10.1007/s11270-014-2164-x



Springer

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The Multixenobiotic Resistance Mechanism in Species of Invertebrates Associated to an Urban Stream in the Patagonia Mountain

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Received: 28 May 2014 / Accepted: 16 September 2014

Abstract There are multiple stressors derived from urbanizations that result in frequent disturbances on streams and rivers reducing water quality and threatens aquatic biota. P-glycoprotein (P-gp)-mediated multixenobiotic resistance (MXR) is a defence mechanism analogous to multidrug resistance (MDR), which has been demonstrated in several aquatic organisms. This system protects cells against the entry and the accumulation of xenobiotics and has been proposed as a biomarker for pollution assessment. We conducted a study in a post-urban reach of Esquel stream (Chubut Province) downstream a wastewater treatment plant, in order to assess the presence and activity of MXR in five freshwater macroinvertebrate species (*Helobdella michaelseni*, *Helobdella simplex*, *Patagoniobdella variabilis*, *Hyalella curvispina* and *Chironomus riparius*). We measured the accumulation of the model P-gp substrate rhodamine B (RB) in organisms previously exposed to pollution. Our results described the activity of the MXR system in the three species of leeches suggesting their suitability as the in vivo biomonitoring. We also identified a dependence of the transporter activity with the development stage in *H. simplex*, highlighting the importance of using organisms of similar size classes since it may affect observed results. Finally, we concluded that benthic freshwater macroinvertebrates possess different species-specific levels of MXR activity possibly influencing their natural distribution as well as their survival.

Keywords MXR · P-glycoprotein · Macroinvertebrates · WWTP · Hirudinea · Hyalellidae · Chironomidae

1 Introduction

All over the world, there is a strong concern in urbanization phenomena because it results in frequent disturbances on streams and rivers reducing water quality and threatens aquatic biota. There are multiple stressors derived from urbanizations that produce pervasive effects on river health (Meyer et al. 2005; Collier and Clements 2011). The runoff from urbanised surfaces and the discharges of municipal wastewaters and industrial facilities frequently result in increased loading of nutrients, metals, pesticides, and other contaminants altering severely the biological communities inhabiting watercourses (Paul and Meyer 2001).

Pollution effect on aquatic ecosystems can be assessed on different levels of biological organization: molecular, cellular, individual, population, and community (Rosenberg and Resh 1993; Hickey 2000; Walters et al. 2009). Among communities macroinvertebrates are one of the most extensively used in biological assessment. As indicators of water quality and stream conditions have been employed worldwide in stream

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Published online: 02 October 2014

monitoring for governmental agencies (Barbour et al. 1999; European Union 2000). In fact, the level of tolerance of macroinvertebrate species to several kinds of contaminants is largely known, and scientists have recognised and classified several species as tolerant or intolerant to different substances. Tolerant organisms seem to display different adaptations that allow them to survive under harmful conditions (Mason 1991). Some of these strategies are related with induced variations in cellular or biochemical processes that protect organisms from environments containing multiple anthropogenic pollutants or natural product toxins (Kurelec 1992). Since many of these mechanisms involve the synthesis of proteins induced by xenobiotics, its measurement can serve as a biological marker that constitutes a valuable tool in ecological studies (Fent 2004; Hyne and Maher 2001; Livingstone 2001).

The multixenobiotic resistance (MXR) mechanism found in aquatic organisms represents a defence system that is similar to the phenomenon termed multidrug resistance (MDR) first characterised in mammalian tumour cells resistant to chemotherapeutic drugs (Kurelec 1992; Bard 2000). The pharmacological basis for this resistance appears to be associated with the overexpression of P-glycoprotein (P-gp). This protein belongs to the ATP-binding cassette (ABC) transporter proteins and is an ATP-dependent pump that transports a wide variety of structurally unrelated compounds out of cells, leading to a reduction in intracellular drug accumulation and hence impairing its efficacy (Borges-Walmsley et al. 2003; Loo and Clarke 2005).

Induction of P-gp expression after field (i.e. polluted with industrial and communal waste) and laboratory xenobiotic exposures have been demonstrated in many aquatic organisms. In view of the fact that the activity of this defence system appears to be proportional to the level of pollution, MXR was proposed by several authors as a biomarker of exposure (Kurelec et al. 1996; Minier and Moore 1996; Epel 1998; Eufemia and Epel 1998; Smital and Kurelec 1998; Smital et al. 2003; Amé et al. 2009). Since the first observation of the environmental relevance of these transporters by Kurelec and Pivcevic (1989), the MXR transporter was mainly described in mussels, sponges, oysters and fishes (Kurelec 1992; Kurelec et al. 1996; Smital et al. 2000; Bard 2000). However, studies on freshwater macroinvertebrates (excluding molluscs) are scarce. In this sense, the expression of P-gp was demonstrated in a Mesostominae flatworm (De Jong et al. 2008) and also in *Chironomus* gr. *riparius* larvae providing evidence of their localisation in the anal papillae organ and its potential use a biomarker (Podsiadlowski et al. 1998; Moreau et al. 2008). Although MXR emerges as a general biological defence mechanism for the protection of organisms against toxicants, this system may not be a sensitive indicator of contaminant exposure in certain species (Damaré et al. 2009; Hamdoun et al. 2002). For this reason, it becomes important to characterise the MXR system in the species of interest, especially those widespread distributed.

There is an increasing concern in fluvial ecosystems in Patagonia because they are being endangered by human actions including those derived from a rapid urban expansion (Miserendino and Brand 2009). The Esquel stream (Northwest Patagonia) drains into an urbanised catchment (Esquel City), and studies have confirmed the poor ecological status of some stream reaches (Miserendino et al. 2008, 2011). Recently, the first evidence of the P-gp-like transport system in a freshwater snail (*Physa acuta*) from Esquel stream was presented, suggesting its role as a defence mechanism (Assef et al. 2014). In the present study, we employed a combination of functional assays that measure bioaccumulation of rhodamine B (RB) and molecular approaches to examine the MXR system in the most common and abundant macroinvertebrate species inhabiting a post-urban reach affected by discharges from a local wastewater treatment plant. The knowledge of the expression and functioning of this mechanism on the selected species will be useful as a biomarker environmental tool, particularly to those studies dealing with the assessment of anthropogenic effects on aquatic environments associated to treatment urban wastewater plants.

2 Materials and Methods

2.1 Study Area and Environmental Characterisation

The stream selected for the study flows through the Esquel City (32,016 inhabitants), in Chubut Province, Argentina. Esquel stream in the Futaleufú-Yelcho basin is third order above the town but fourth order below it as the Valle Chico stream (third order) enters it within Esquel. The selected "urban" site (42° 58′ 32″ S, 71° 23′ 47″ W) is located 5 km downstream the wastewater treatment plant (WWTP) and have been visited in several occasions as part of different scientific projects

(Miserendino and Pizzolón 2000; García Sotillo 2011). Previous works in the area have documented the environmental characteristics and analysed the macroinvertebrates community including tolerant and intolerant species from the urbanised section of Esquel stream (Pizzolon and Miserendino 2001; Miserendino et al. 2008).

The WWTP (phytoremediation by modules of *Phragmites communis*) at Esquel was built in 2001, but facilities were successively expanded to accomplish with the volume of effluents received by the domestic net. In the last few years, Esquel has experienced a strong growth in population (1992, 17,000 to 2010, 32016) and as observed by the physicochemical conditions as well as indicators based on macroinvertebrate communities, the ability of the plant to cope with the volume of waste produced appears to be inadequate (Miserendino et al. 2008).

The study was conducted from March 20 to May 8, 2013. Substratum composition was estimated visually and recorded as percentages of boulders, cobbles, gravel, pebbles, and sand. Current speed was measured in mid-channel on three occasions by timing a float (average of three trials) as it moved over a distance of 10 m (Gordon et al. 1994). Average depth was estimated from five measurements along one transect across the channel with a calibrated stick. Wet and dry widths of the channel were also determined. Discharge was obtained by combining depth, wet width, and current velocity as in Gordon et al. (1994). At each site, air and water temperature were measured with a mercury thermometer.

On each sampling occasion, specific conductance ($\mu S_{20} cm^{-1}$), pH, turbidity (NTU), and dissolved oxygen (mg $O_2 l^{-1}$) were obtained with a multiparameter probe (Hach SensION 156). Same instrument was employed for measurements of these variables of medium (dechlorinated water) used in laboratory. At two occasions, water samples (2 l) were collected below the water surface and kept at 4 °C for nutrient analyses. At the laboratory, total nitrogen (TN), nitrate plus nitrite nitrogen (NO_3-NO_2), ammonia (NH_4), total phosphorus (TP), soluble reactive phosphate (SRP) (±0.1 μg l^{-1}), and total suspended solids (TSS) (±0.1 mg l^{-1}) were analysed following APHA (1994).

2.2 Animal Sampling

Selected species for experiments were all those species of macroinvertebrates showing high densities in the period in which the study was conducted (March–May 2013) and which are also present throughout the year (Miserendino et al. 2008; Miserendino and Gullo 2014).

Specimens of macroinvertebrates—the hirudineans *Helobdella michaelseni*, *Helobdella simplex* (Glossiphoniidae), *Patagoniobdella variabilis* (Semiscolescidae), the crustacean *Hyalella curvispina* (Amphipoda, Hyalellidae) and the insect *Chironomus* gr. *riparius* (Diptera, Chironomidae)—were collected in the urban section of Esquel stream. Organisms living attached on rocks (*H. michaelseni*, *H. simplex* and *P. variabilis*) were sampled manually from 0.2- to 0.6-m depth. To obtain an appropriate number of specimens of *Hyalella curvispina* and *C. riparius*, a Surber sampler (0.09 m^2; 250-μm pore size) was employed at different habitats: runs, riffles, pools, and macrophytes. All organisms were carried alive to the laboratory in the water of origin. Macroinvertebrate species were identified using regional available keys (Fernández and Domínguez 2001) or sent to specialists.

The multixenobiotic defence system was then assessed using the bioaccumulation method (Sect. 2.4). In order to evaluate if different activity of the MXR system occurred between juvenile and adult instars in species of *H. michaelseni*, *H. simplex* and *P. variabilis*, two sets of organisms were analysed. Organisms were separated by measuring specimens (alive) as follows: *H. michaelseni* juveniles (wide 1.17±0.28 mm, large 0.84±0.12 cm) and adults (wide 2.25±0.46 mm, large 1.38±0.27 cm); *H. simplex* juveniles (wide 2.81±0.57 mm, large 0.89±0.21 cm) and adults (wide 3.91±0.41 mm, large 1.14±0.22 cm); and *P. variabilis* juveniles (wide 2.59±0.58 mm, large 1.36±0.20 cm) and adults (wide 4.00±0.55 mm, large 1.95±0.25 cm).

2.3 Measurement of MXR Activity

The principle of this assay is the in vivo measurement of the accumulation level of P-gp substrate, the fluorescent dye RB, in aquatic organisms after the exposure to the dye without (control) and with addition of model MXR inhibitor such as verapamil. This assay was performed with minor modifications to previously published assays to obtain the clearest results; this included the determination of optimal incubation times, as well as the working dilutions of drugs (Smital and Kurelec 1997; Kurelec et al. 2000; Assef et al. 2014). Animals were placed into a light-protected Petri dish (10–20 specimens/glass, depending on the specie) in 50 ml of

dechlorinated tap water, supplemented with 5 μM RB (Sigma, St. Louis, MO, USA) in absence or presence of 30 μM verapamil (Sigma) and incubated at room temperature (18–22 °C) for 4 h. Incubation of specimens in a medium containing RB enabled the probe to cross the cellular membranes by passive diffusion and to accumulate within the organism. To discard a transport system saturation that could mask the MXR activity, the specimens were incubated in the medium with lower concentrations of RB, ranging from 0.1 to 5 μM (Toomey and Epel 1993; Galgani et al. 1996).

After the exposure period, specimens were washed three times in 100 ml of dechlorinated tap water using a tea strainer. The entire body from each specimen was weighed, transferred to a flat-bottomed tube containing 0.5 ml of distilled water, and homogenised for 15 s (Pro200 Homogenizer, Pro Scientific Inc., USA). Homogenates were centrifuged at 3,000×*g* for 5 min, and the supernatants were carefully transferred to clean tubes. The fluorescence of accumulated dyes in the entire organism was measured immediately using a fluorometer (QuantiFluor-TM, Promega, USA). The fluorometer was calibrated with known solutions of RB at the beginning of each working day; thus, the fluorescence data was provided in concentration units. Data were expressed in picomoles of accumulated RB per gram of organism (pmol g^{-1}).

The primary criterion for the quantification of the level of MXR activity was the ratio (R) previously described by Smital et al. (2000). The R-value was calculated by dividing the amount of RB accumulated into de total body of the organisms without the addition of verapamil (control), with the amount of dyes accumulated in the presence of inhibitor. Thus, the theoretically maximal R-value is 1 while the minimal *R* tends to be 0 (that means theoretically maximum of MXR activity).

The assay was carried out within 24 h of collection, but not before the 2 h needed for the release of environmental pollutants (MXR substrates) previously bound on active sites of P-gp (Kurelec et al. 2000). During the whole procedure, all materials were light protected to avoid the possible loss of the RB fluorescence intensity caused by direct exposure to light.

2.4 Laboratory Depuration

A depuration of experimental organisms in clean water is often performed to assess the background or baseline level of MXR transporters (Smital and Kurelec 1997; Smital et al. 2000; Pain and Parant 2007). After capture, a group of 10–20 specimens was isolated and kept in dechlorinated clean water at 8–12 °C for 10 days to determine the basal level of MXR activity as described above. The medium was changed every 2 days. Features of water employed in maintenance of organisms were the following: conductivity 213±2 (μS cm^{-1}), salinity 0.1‰, dissolved oxygen 7.9±1.0 (mg l^{-1}) and pH 7.3± 0.7.

Animals were given no food during the laboratory depuration period. At the time of the experiment, animals were still in good condition assuring that the MXR level measured was not an artefact due to decreased body fitness.

2.5 Western Blot Analysis

Specimens from the selected macroinvertebrate species were gently isolated, washed two times in dechlorinated clean water, transferred to flat-bottom tubes in 0.5 ml of lysis buffer (250 mM NaCl, 50 mM HEPES, 1 mM EDTA, 1 % NP-40, 1 mM PMSF, 1 mM DTT and protease inhibitor cocktail; Sigma), and homogenised for 15 s on ice (Pro200 Homogenizer, Pro Scientific Inc., USA) to prepare total protein extracts. Homogenates were then centrifuged at 6,000×*g* for 10 min at 4 °C, and the supernatants were carefully transferred to clean tubes. Total protein in each sample was quantified according to the Bradford method and stored at −20 °C until use. Protein extract from *Physa acuta* snails was run on all gels as a positive control. The presence of P-gp in *P. acuta* was previously studied in relation to the multidrug-resistant K562 human leukemic cells overexpressing P-gp, and is also the only aquatic organisms tested so far in Patagonia for the expression of this protein (Assef et al. 2014). All these homogenates were prepared with pooled samples (total protein extracts from at least five specimens).

For Western blot analyses, samples were dissolved in loading buffer (50 mM Tris, pH 6.8, 2 % (*w*/*v*) sodium dodecyl sulfate (SDS), 10 % (*v*/*v*) glycerol, 100 mM DTT and 0.2 mg ml^{-1} bromophenol blue). The heating step was omitted to minimise membrane protein aggregation. Then, 50 μg of total protein was separated by 8 % SDS polyacrylamide gels and electrotransferred onto nitrocellulose membrane (Hybond ECL, Amersham, GE Healthcare, Buckinghamshire, UK). Five microlitres of molecular weight marker

(Promega) was also migrated. The membrane was then blocked with 10 % dry milk in Tris–saline buffer containing 0.05 % Tween 20 (T-TBS) and then incubated with P-gp specific C219 monoclonal antibody (Calbiochem, USA) diluted 1:1,000 for 3 h at room temperature. We determined optimal working dilutions and found that the chosen incubation conditions gave the clearest results. The membranes were washed five times with T-TBS and then incubated with horse anti-mouse immunoglobulin G (IgG) antibody conjugated to horseradish peroxidase (Vector Laboratories, Burlingame, CA, USA) diluted 1:5,000 for 1 h at room temperature. After washing five times with TBS, positive reactivity was detected using the ECL Western Blotting Analysis System (Amersham, GE Healthcare, Buckinghamshire, UK). The chemiluminescence reaction was visualised on AGFA Medical X-Ray films (Agfa-Gevaert S.A., Argentina).

2.6 Statistical Analysis

The resulting data were analysed separately using a variety of statistical tools according to the type of experiment. All data are given as mean±standard deviation (SD). Mean values were calculated from the results obtained for at least five groups of 10–20 specimens, and *n* indicates the number of groups used in each experiment. A paired Student's *t* test was used for comparison between two groups. One-way analysis of variance (ANOVA) was performed to determine if significant differences between the developmental stages for each specie existed and also to analyse collection/depuration data. Differences were considered significant when $P<0.05$ (Sokal and Rohlf 1995).

3 Results

3.1 Environmental Features of the Esquel Stream

Main characteristics of the sampled reach during the study are presented in Table 1. Hydraulic variables as current velocity, depth and discharge indicated that the sampling was coincident with the low water period. Pollution effects were evidenced in a strong deficit of dissolved oxygen and high values of main nutrients, particularly in extremely elevated levels of ammonia.

Table 1 Environmental features of the study site a post-urban reach from Esquel stream (Patagonia, Argentina) during the study period ((a) March 20 to (b) May 21, 2013)

Physical variables		
Elevation (m a.s.l.)	491	
Stream order	5	
Dry width (m)	10.5	
Wet width (m)	8.40	
Depth (cm)	16.08	
Current velocity (m s^{-1})	0.55	
Water temperature (°C)	14.4 (11.9–16.3)	
Discharge (m^3 s^{-1})	0.74	
Chemical variables		
pH	7.2 (6.8–7.7)	
Dissolved oxygen (mg l^{-1})	6.29 (3.80–8.13)	
Oxygen percentage	65.3 (42.2–85.0)	
Conductivity (μS cm^{-1})	333.2 (272.9–370.9)	
Total dissolved solids	203.9 (178.7–219.9)	
Alkalinity (mEq l^{-1})	4.36	
	a	b
Nitrate plus nitrite-nitrogen (μg l^{-1})	40.9	151.5
Ammonia (μg l^{-1})	10,628.9	6,188.1
Total nitrogen (μg l^{-1})	11,018.2	7,948.1
Soluble reactive phosphate (μg l^{-1})	1,413.1	852.5
Total phosphorus (μg l^{-1})	1,230.0	711.0

Variables with more than one record in the period are consigned as mean value and range in parenthesis ($n=4$). For nutrient values, first column (a) and second column (b)

3.2 Identification of MXR Activity in Different Macroinvertebrate Species

The activity of the MXR system was tested in the leeches *H. michaelseni* and *H. simplex* by way of the bioaccumulation of RB method. We incubated the specimens in the medium with 5 μM of RB in the absence or presence of the MXR inhibitor verapamil. Figure 1 shows that the MXR transporter was effective at exporting the fluorescent probe in the two species using specimens of all developmental stages. Accumulations of RB in control condition were 353.0±29.1 and 388.1±36.3 pmol RB g^{-1} for *H. michaelseni* and *H. simplex*, respectively. In the presence of verapamil (30 μM), the accumulation of RB increased by 1.9- and 1.7-fold in comparison with the corresponding control group of animals for *H. michaelseni* and *H. simplex*, respectively ($P<0.05$). The ratio R (control/verapamil), calculated from the RB accumulation data, constitutes a primary

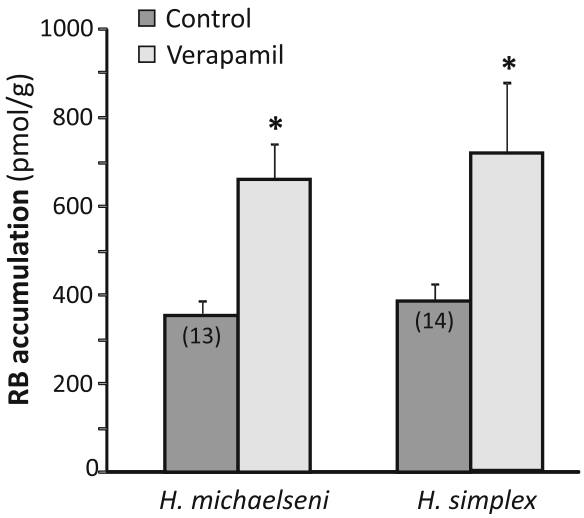

Fig. 1 Activity of the MXR system in *Helobdella michaelseni* and *Helobdella simplex* tested within 24 h of collection. The accumulation of RB has been assessed in control leeches and after inhibition with 30 μM verapamil. Data are expressed in picomoles of accumulated RB per gram of entire leech body weight. *Asterisk* indicates a significant difference ($P<0.05$) compared to the corresponding control. The number of experiments is consigned *inside the bars*

criterion for the quantification of the level of MXR activity. The R-values for Figs. 1, 2 and 3 are presented in Table 2.

We evaluated possible differences in the transporter activity profiles in relation to the instars of the organisms. Figure 2 shows a significant increase in the RB accumulation in the presence of verapamil for *H. michaelseni*, regardless of their developmental stage. However, a different pattern of MXR activity was observed in *H. simplex* where the increment in the accumulated RB after the addition of verapamil was only observed in the adult specimens.

The presence of an active MXR transport system was also evaluated in *P. variabilis* (Fig. 3). The MXR activity assay indicated that the level of accumulated dye was increased 1.50±0.19-fold after verapamil exposition, relative to the value obtained on control conditions ($P<0.05$). There was no dependence of the transporter activity with the developmental stage in this specie, which is clearly indicated by the R-values (Table 2).

The activity of MXR transport system was not detected in some species of macroinvertebrates inhabiting this urban stream such as *C.* gr. *riparius* and *Hyalella curvispina*. Figure 4a shows similar values of accumulated RB in the absence (control) or presence of verapamil ($P>0.05$). The calculated R-values were close to one in both cases (1.09±0.08 and 1.07±0.08 for *C.* gr. *riparius* and *Hyalella curvispina*, respectively). The accumulation of RB within the organisms increased with higher concentrations of the dye in the loading medium, showing not saturation of the transport system up to 5 μM of RB (Fig. 4b). However, these values were

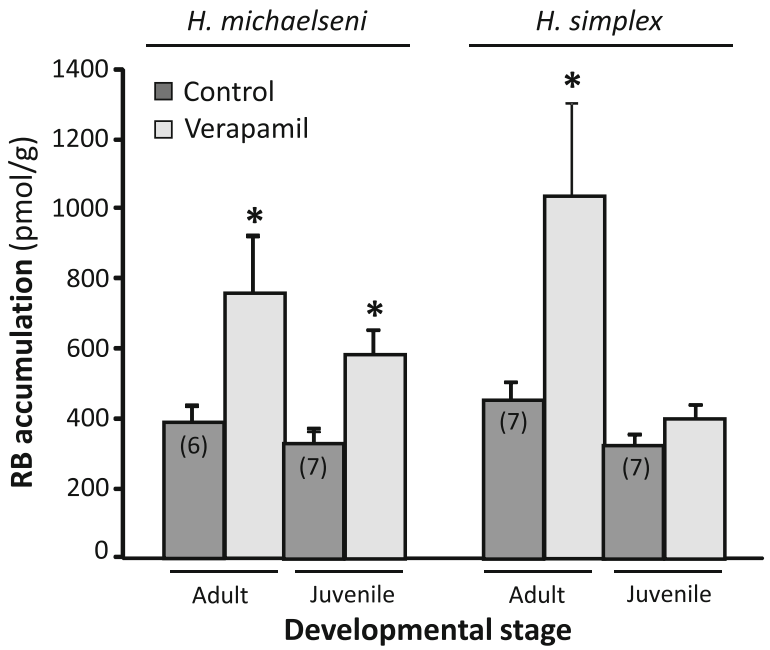

Fig. 2 Measurement of MXR activity from leeches (*Helobdella michaelseni* and *Helobdella simplex*) in different developmental stages tested within 24 h of collection. The accumulation of RB has been assessed in the presence and absence of 30 μM verapamil in juvenile and adult leeches. *Bars* represent standard deviation of the mean. Data are expressed in picomoles of accumulated RB per gram of entire leech body weight. *Asterisk* indicates a significant difference with respect to the other values for the same species ($P<0.05$). The number of experiments is consigned *inside the bars*

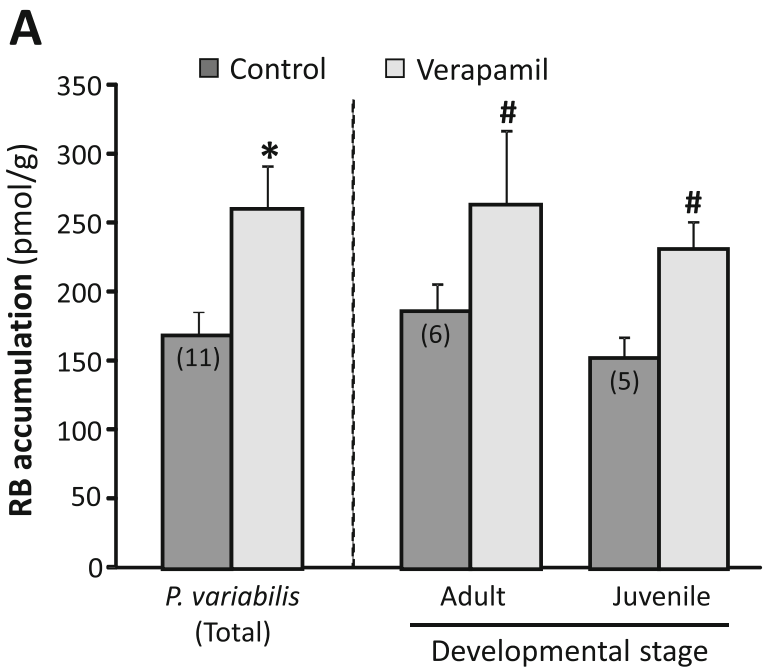

Fig. 3 Activity of the MXR system in *P. variabilis* tested within 24 h of collection. The accumulation of RB has been assessed in the presence and absence of 30 μM verapamil. The *right section of the panel* shows the results for leeches separated according to their developmental stage. Data are expressed in picomoles of accumulated RB per gram of the entire leech body weight. *Asterisk* indicates a significant difference ($P<0.05$) compared to the control and the *number sign* denotes significant difference between these bars and the other two. The number of experiments is consigned *inside the bars*

not modified in the presence of MXR inhibitor (30 μM verapamil) for none of the tested concentrations and for none of the two studied species ($P>0.05$).

3.3 Immunochemical Detection of P-gp

To assess the expression of P-gp in the selected species, Western blot analysis was conducted in total tissue homogenates using the monoclonal antibody (MAb) C219. Figure 5a shows the Western blot analysis for the species with demonstrated activity of the MXR system by the bioaccumulation assay. The presence of an immunoreactive band at ~160 kDa was observed in *P. variabilis* homogenates, at slightly lower molecular weight than the band obtained in *P. acuta* total extracts used as a positive control. Proteins isolated from *H. simplex* and *H. michaelseni* cross-reacted only at a detection-limit level, showing a very weak band that appears between 150 and 170 kDa. The MAb C219 antibody failed to show the expression of P-gp in samples from *Hyalella curvispina* and *C. riparius*, which are species with no apparent activity of MXR (Fig. 5b).

3.4 MXR Laboratory Depuration

Following a depuration period of 10 days, the accumulation of RB experiment was assessed in the species that previously showed MXR transport activity

Table 2 MXR activity of Hirudinea species expressed as R-values for total and separated organisms according to their developmental stage

	Total	Adult	Juvenile
Glossiphoniidae			
Helobdella michaelseni	0.57±0.04	0.55±0.05	0.59±0.06
Helobdella simplex	0.66±0.06	0.51±0.07	0.81±0.04[a]
Semiscolecidae			
Patagoniobdella variabilis	0.79±0.11	0.86±0.18	0.70±0.10

These values were calculated from data of RB accumulation assays shown in Figs. 1, 2 and 3. Data are consigned as mean value±standard deviation

[a] Denotes significant difference with respect to other values obtained from *Helobdella* species

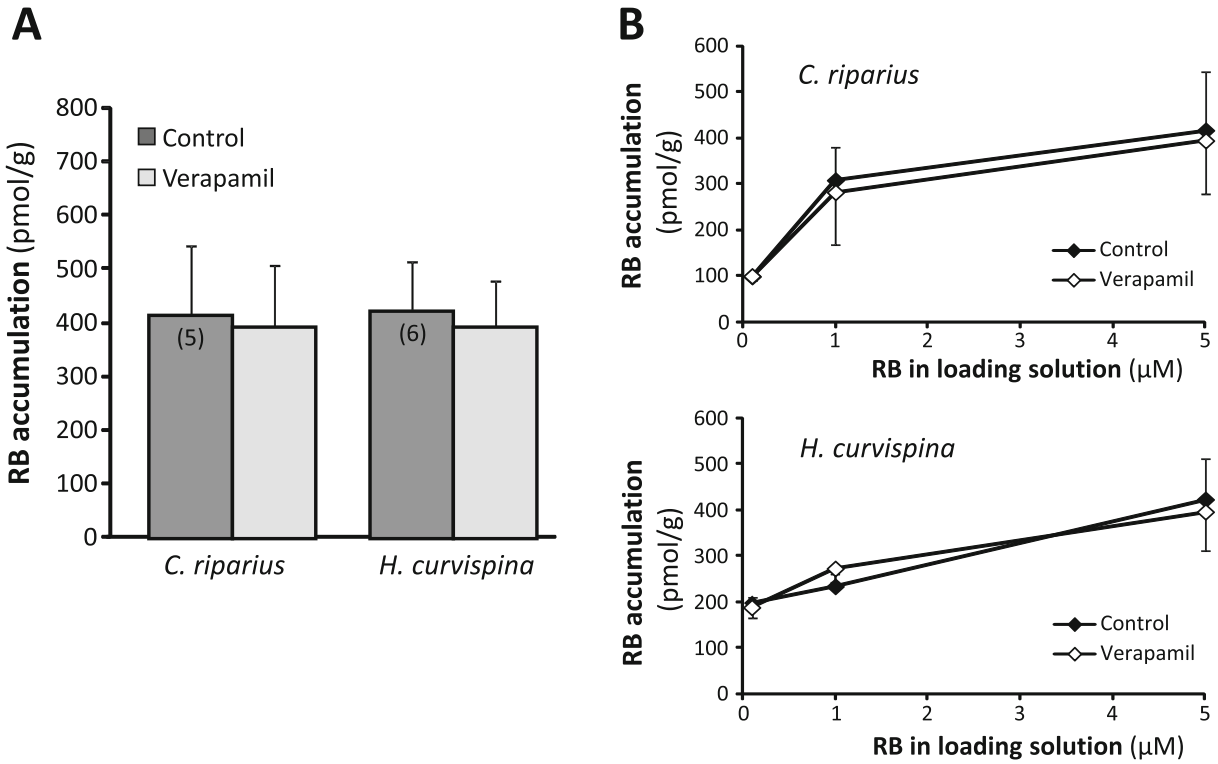

Fig. 4 Determination of MXR activity in *Chironomus riparius* and *Hyalella curvispina* tested within 24 h of collection. **a** The accumulation of RB has been assessed in control macroinvertebrates and after inhibition with 30 μM verapamil. The number of experiments is consigned *inside the bars*. **b** The RB accumulation of has also been measured at different concentrations of RB in the loading solution with or without 30 μM verapamil (n=5). In all cases, data are expressed in picomoles of accumulated RB per gram of the entire body weight (mean±SD)

(*H. michaelseni*, *H. simplex* and *P. variabilis*). The level of MXR activity deinduction in leeches exposed to unpolluted water was expressed as a percentage of the increase in RB accumulation in comparison to the

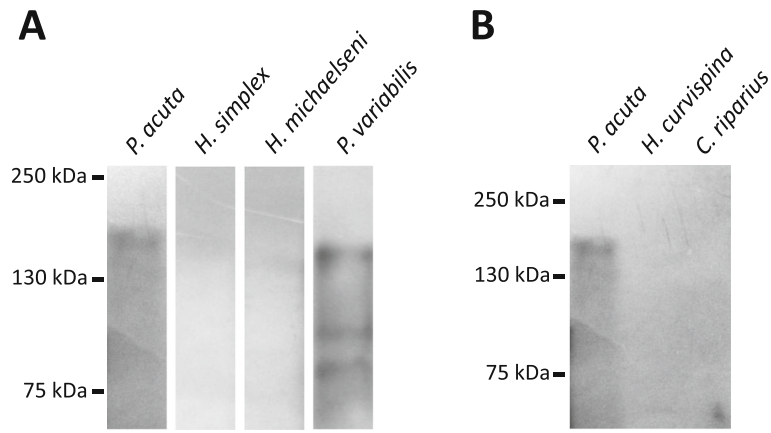

Fig. 5 Detection of P-gp in five species of macroinvertebrates. **a** Western blot was probed with anti-P-gp C219 antibody which reacted with a band at ~160 kDa in *Patagoniobdella variabilis* samples. Proteins isolated from *Helobdella simplex* and *Helobdella michaelseni* cross-reacted only at a detection limit level. Total protein extracts from *Physa acuta* snails were used as a positive control. Positions of molecular weight markers in kilodaltons (kDa) are indicated on the *left*. **b** Immunoblot analysis of 50 μg of the whole protein extracts obtained from *Hyalella curvispina* and *Chironomus riparius* specimens with the C219 antibody. This figure is representative of at least four independent trials

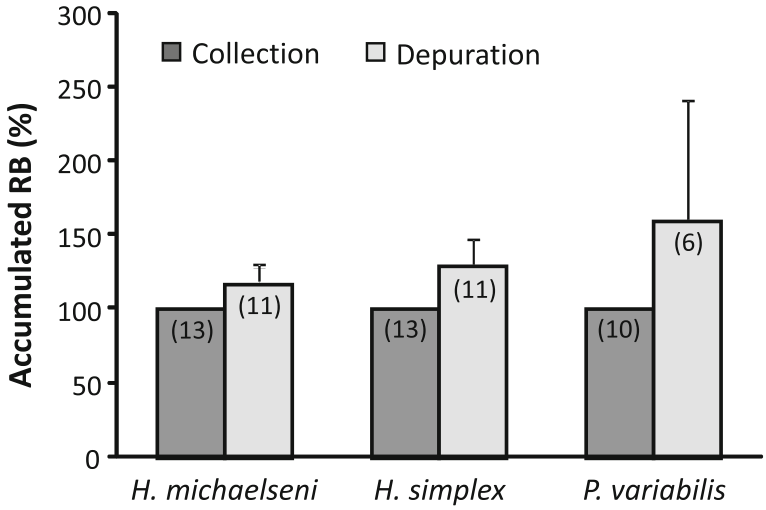

Fig. 6 Activity of the MXR system in leeches after a 10-day period of laboratory depuration. Accumulation of RB assay was performed in macroinvertebrate species that previously showed MXR transport activity on collection day (*Helobdella michaelseni*, *Helobdella simplex* and *Patagoniobdella variabilis*). Data are expressed as percentage of control values on collection day (mean±SD). The number of experiments is consigned *inside the bars*

control group (=100 % basal accumulation of RB). Although we observed a weak increase in the RB accumulation in control conditions relative to the values obtained on the collection day, these differences were not statistically significant ($P>0.05$) (Fig. 6). This assay was performed in the absence or presence of verapamil to calculate the R-values after a laboratory depuration period. Similarly to results obtained with the accumulated RB, the R-values show a weak but non-significant increase relative to those obtained on the collection day. For depurated organisms, R-values were 0.64±0.06, 0.69±0.04 and 0.68±0.19 for *H. michaelseni*, *H. simplex* and *P. variabilis*, respectively ($P>0.05$).

4 Discussion

The present study analysed the expression and activity of the MXR transport system in benthic macroinvertebrates inhabiting an urban stream of Patagonian Andes and for the first time, is demonstrated in Hirudinea species. The extremely high level of nutrients especially of ammonia values found in the site during this study were 60 to 100 times higher than previously reported by Miserendino et al. (2008) for the same place and season, indicating a strong deterioration of the system. The marked increase in organic enrichment and in nutrient level of this part of the system has been noticed recently by García Sotillo (2011). Comparing to previous work carried out in the area, macroinvertebrate community resulted impoverished in terms of species richness and composition since community was highly simplified and dominated by tolerant taxa from moderate to heavy organic pollution such as *Helobdella* sp., *Hyalella* sp. and *Chironomus* sp.

Among the studied organisms, *H. michaelseni*, *H. simplex* and *P. variabilis* were the species that displayed activity of the MXR transport mechanism. In all cases, a low accumulation of RB dye was observed immediately after collection which resulted significantly increased after the addition of a transport inhibitor, verapamil. Our results suggest that there was no dependence of the transporter activity with the developmental stage in *H. michaelseni* and *P. variabilis*. MXR transporters display a similar pattern of expression in embryos of the marine worm *Urechis caupo*, suggesting that juvenile and adult worms may also need this mechanism to develop normally in that apparently noxious environment (Toomey and Epel 1993). However, we found a differential expression in *H. simplex* where the MXR activity was only detected in adult specimens. Since all organisms were collected at the same impaired site, this result can be explained based on physiological differences related to the size of these animals. This observation is in line with the results of Zilberberg and co-workers (2011) who detected a variation in the expression of a stress-related protein used as a biomarker in mussels of different size classes. The authors speculate that physiological differences related to the size of these animals could explain their results including dissimilar metabolic

rates, stress responses due to age and the level of exposition to natural and/or anthropogenic impacts.

The activity of the MXR system observed in the three species of leeches by the functional assay seems not to be reflected by the direct immunochemical measurement of P-glycoprotein levels using Western blotting technique. For example, the expression of P-gp in *H. michaelseni* and *H. simplex* were unexpectedly low compared to the results obtained in *P. variabilis*, since the three species showed similar activities of MXR by the RB accumulation assay. Although a low expression of the protein could be sufficient to produce an appropriated activity, the measurement of P-gp level in these species appears a less reliable biomarker. Our results confirmed the observations and problems emerged from some previous studies showing that this approach was neither a reliable biomarker of exposure of mussel to xenobiotics nor an indicator of the actual state of pollution in a particular environment (Kurelec et al. 1996; Epel 1998; Smital et al. 2000). Another plausible explanation of our results might be the existence of other members of the ABC transporter family such as multidrug resistance-related proteins (MRPs) that also provide aquatic organisms with resistance to chemicals in a polluted environment. It has been demonstrated that rectal gland of the dogfish shark is capable of active and specific excretion of xenobiotics and that such transport is mediated by an analogue of MRP2, but not by P-gp (Miller et al. 1998). Also, the identification of two MRP isoforms in the blue mussel *Mytilus edulis* confirms the expression of these transporters in aquatic organisms (Luedeking et al. 2005).

To our knowledge, this is a novel study that describes this mechanism of detoxification in Hirudinea species (leeches), in particular Glossiphoniidae and Semiscolecidae. Leeches have some advantages to be used as biomarker: worldwide distributed, sessile organisms easily available in freshwater habitat, suitable for laboratory maintenance and for the determination of MXR activity using the RB accumulation assay and have relatively small size for easy handling and lower cost of experiment. In particular, those from the genus *Helobdella* are widely distributed in South America being them available in a great number of environments and conditions (Christoffersen 2009). Interestingly, it has been observed a seasonal variation in the inducibility of MXR proteins in marine and freshwater mussels, being probably the most studied aquatic organisms in relation to the phenotype of MXR (Minier et al. 2000; Pain et al. 2007). As a way to circumvent this problem, it has been proposed to screen other organisms to find ones that are not so subject to seasonal variation. In this regard, benthic animals might be less susceptible to seasonal stresses than mussels that are typically harvested from littoral areas (Minier et al. 1999).

Measurements on organisms in the field indicate some correlation between the amount of MXR protein and organic pollution (Minier et al. 1999). Esquel stream is a well-characterised system that was subject of several studies tending to assess the urban impacts on water quality and benthic communities. The post-urban section, mostly located downstream the WWTP, displays high levels of nutrients including phosphorus and nitrogen compounds and high values of organic matter (Pizzolon and Miserendino 2001; Miserendino et al. 2008). Although municipal wastewaters seem to show a very complex composition containing a large number of regulated and non-regulated contaminants (Petrovic et al. 2008; Smital et al. 2011), the particular contaminants that have demonstrated the ability to induce the MXR system in studied organisms (Assef et al. 2014) have not yet been identified at Esquel stream.

It was observed a good correlation between the inherent level of the basal MXR activity in the aquatic organisms with the pollution degree present in their natural habitats, being this basal activity, at least partly, responsible for the resistance or for the sensitivity to organic pollution (Smital et al. 2000). The depuration period reported in the literature is highly variable, from several days to several months depending on the species studied (Smital and Kurelec 1997). However, 5–8 days has often been used to achieve the baseline level of an MXR system in several marine and freshwater Molluscs including *Mytilus galloprovincialis*, *Monodonta turbinata* and *Dreissena polymorpha*, among others (Smital et al. 2000; Pain and Parant 2007). Following the depuration period of 10 days, we have not found a significant reduction in MXR activity compared to data obtained immediately after collection. These results allow us to speculate that there might be further declines in MXR activity after this time, since we expected a decrease in MXR activity after the maintenance in free-xenobiotic water. However, this observation is consistent with the concept of that MXR represents the first line of protection in the defence system of aquatic organisms, prepared to act against incoming toxicants, and it may increase in response to higher concentrations of xenobiotics (Epel 1998).

It was hypothesised that MXR could occur in every living organism as a constitutive defence system (Kurelec 1992). However, no differences in the activity or expression of P-gp after verapamil exposure were measured in the freshwater species western mosquito fish and bluegill sunfish (Damaré et al. 2009). Another study shows that larvae of sea urchin (*Lytechinus anamesus*) seem not to express MXR efflux protein (Hamdoun et al. 2002). In our study, this detoxification mechanism does not appear as a sensitive indicator of exposure of contaminants in *C.* gr. *riparius* and *Hyalella curvispina*, at least with the techniques used in the present study. Podsiadlowski et al. (1998) confirmed the expression of P-gp only in the epithelia of the anal papillae of *C.* gr. *riparius* larvae. Since this organ is a small part of the whole organism, it is likely not to observe the activity of MXR transporters when performing functional assays with complete specimens just as we did. The larvae of *C.* gr. *riparius* are able to survive in rather polluted waters (Postma et al. 1996). Despite the MXR transporter expression is delimited to a single tissue, the required tolerance to xenobiotic agents may be mediated, at least in part by this detoxification system. Something similar may be occurring with *Hyalella curvispina*, although to our knowledge, there is no prior evidence of this mechanism in *Hyalella* species. Further studies using fluorescence microscopy will be need to confirm this hypothesis.

Therefore, the results presented demonstrate the expression and activity of the MXR mechanism in *H. michaelseni*, *H.simplex* and *P. variabilis*, suggesting their suitability as the in vivo biomonitoring. It will be important to include these selected species in future studies examining their MXR responses to other types of impairment detected in the aquatic systems (Miserendino et al. 2011) or on the contrary assessing this mechanism at less polluted sites.

In the last years, selected species expressing the MXR system are being used for the quantification of MXR inhibitors present in the environment, defined as "chemosensors." Many xenobiotics and also some natural products have the ability to modulate the MXR system, and it could potentially alter toxicity in aquatic organisms by decreasing their natural resistance (dos Santos and Martinez 2014; Epel et al. 2008; Kurelec et al. 2000; Smital et al. 2011). In this regard, it has been described that NOM has the potential to modify the MXR activity in freshwater amphipods *Eulimnogammarus cyaneus* and *Eulimnogammarus verrucosus* being dissolved humic substances a potential responsible for this inhibition (Steinberg et al. 2006; Timofeyev et al. 2007). Interestingly, specific inhibition of MRP protein caused a significant increase in toxic potency of polluted seawater samples in embryos of sea urchin (Bošnjak et al. 2011).

Our study demonstrated that benthic freshwater macroinvertebrates possess different species-specific levels of MXR activity which may have consequences in their natural distribution, as previously described for other aquatic organisms (Smital et al. 2000). This work underlined the importance of a better knowledge of the population of model organisms selected for experiments using MXR transport protein as biomarkers. In this context, our results also highlight the importance of using organisms of similar size classes since in certain species, it may affect the design and sampling strategies of future studies.

Acknowledgments We would like to thank Dr. Bettina S. Gullo from the Facultad de Ciencias Naturales y Museo, Universidad Nacional de La Plata, Argentina, for the taxonomic determination of Hirudinea species. We are grateful to the reviewer Dr. Marta M. Souza for her valuable comments that greatly improve the manuscript. This work has been supported in part by the National Research Council of Argentina (CONICET; Project Res No. 1735/11 and the PIP 11220080101907). This is Scientific Contribution No. 104 from LIESA.

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