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Inducibility of the P-glycoprotein transport activity in the marine mussel *Mytilus galloprovincialis* and the freshwater mussel *Dreissena polymorpha*

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Abstract

Previous investigations directed to the determination of the P-glycoprotein (Pgp) expression in aquatic organisms have indicated the possibility of the multixenobiotic resistance mechanism (MXR) induction as a response to organic pollution. However, in numerous cases no significant and/or no clear relationship between Pgp contents and pollution level was detected. Concerning these discrepancies the results of an extensive, 3-year study of the Pgp mediated MXR induction in the selected freshwater (*Dreissena polymorpha*) and marine (*Mytilus galloprovincialis*) bivalves are presented here. The main goals of the study were to ascertain the rate-dynamic, level, as well as the possible usability of MXR in environmental biomonitoring. Since the primary result of MXR induction should be the decreased intracellular accumulation of xenobiotics, the determination of MXR induction was performed using the measurement of Pgp transport activity. We measured the accumulation or the efflux rate of the model Pgp substrate rhodamine B (RB) in gills of the mussels previously exposed to pollution. The study was performed in several steps: from the exposure experiments in laboratory, using model inducers rhodamine 123 (R123) and water extract of Diesel-2 oil (D2), to the final in situ testing in real environmental conditions. Our results confirmed that Pgp activity is induced/induces according to the level of pollution, and that 4-days period was already long enough for the significant induction and deinduction of MXR activity. However, the inducibility of Pgp transport activity was significantly limited—the maximal level of induction obtained in this study resulted in 50–60% lower RB accumulation in the gills of induced specimens (laboratory or in situ exposed to pollution), when compared to control, non-induced animals. The obtained level of Pgp related MXR induction, resulting in halfway lesser accumulation of a model pollutant (RB), extrapolated to the similar scenario with toxic xenobiotics may have significant environmental relevance. However, our results also suggest that for the use of the MXR as a relevant biomarker the Pgp transport activity should be measured along with the determination of DNA, mRNA or/and protein expression. Based on the data from this study several factors that may have had critical influence on the effectiveness and the level of MXR induction are additionally discussed.

Keywords: Multixenobiotic resistance (MXR); P-glycoprotein (Pgp); Induction; Biomarker; *Dreissena polymorpha*; *Mytilus galloprovincialis*; RB accumulation

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1. Introduction

Aquatic organisms use various defense strategies to avoid potentially harmful effects of numerous xenobiotics present in their natural habitats. Since the water pollution is by definition caused by a complex mixture of structurally different chemicals, their existence indicates the presence of defense system(s) directed against many xenobiotics simultaneously. In agreement to this hypothesis the multixenobiotic resistance (MXR; Kurelec, 1992) mechanism in aquatic organisms represents a defense system directed against numerous endogenous and exogenous xenobiotics, preventing their intracellular accumulation and potentially toxic effect.

The molecular basis of MXR mechanism in aquatic organisms is similar to the well-known multidrug resistance (MDR) phenomenon involved in tumour cell lines resistant to chemotherapeutic drugs. MDR activity is mediated by the expression of a variety of transmembrane transport proteins. The most common among them is a 170 kDa transmembrane P-glycoprotein (P170 or Pgp) which uses energy (ATP) to pump xenobiotics out of cells (Gottesman and Pastan, 1993). The list of Pgp substrates is enormous and includes a wide variety of xenobiotics with different chemical structures and modes of action (Ambudkar et al., 1999).

Numerous lines of drug binding, immunological cross-reactivity, or functional-activity studies, along with toxicological evidence strongly indicate that (1) the Pgp is frequently present in aquatic organisms, and (2) the presence, as well as the differences in Pgp mediated MXR activity may critically influence the susceptibility of populations to xenobiotics. However, the main disadvantage of this mechanism is its fragility. Some chemical agents, recently named MXR inhibitors or chemosensitizers, may inhibit MXR activity. The consequences of such inhibition are enhanced intracellular accumulation and adverse effects of other xenobiotics present in aquatic environment (Epel, 1998; Smital and Kurelec, 1998; Bard, 2000).

Ecotoxicologically important characteristic of defense systems present in aquatic biota is the inducibility as a response to chemical stress. Basic questions regarding possible MXR induction are directed to the determination of the level, dynamic and the possible influence of environmental factors on these processes. Moreover, if the MXR activity is inducible, another interesting question is whether and how it could be used as a biomarker.

The results of some investigations clearly indicate the possibility of MXR induction as a response to organic pollution. In several studies significant correlation have been indicated between the amount of Pgp, measured immunologically using Western blotting technique, and organic pollution. Similar pattern of induction was found in gills of freshwater and marine snails or bivalves collected along the well-defined scale of pollution—the accumulation of model xenobiotics collected at polluted sites was significantly lower in comparison to specimens from less polluted locations (for review, see Minier et al., 1999).

However, in some investigations no significant and/or no clear relationship between Pgp contents and pollution level was detected. This was the case in oysters *Crassostrea virginica* collected from polluted and unpolluted sites in Charleston Harbor, SC, USA (Kepler and Ringwood, 2001), or in *Crassostrea gigas* collected along the French south-western coast (Minier et al., 1999). As the authors suggested, these results could indicate differences among species or interaction of other factors such as interindividual, interpopulational and interspecies differences in Pgp activity, seasonal variations in protein titer, a possible role of other transport proteins, or the effects of MXR inhibitors. Although these data support a general prediction concerning the possibility of MXR induction, significant differences obtained in these investigations make the drawing of ecotoxicologically relevant conclusions more difficult.

Considering described discrepancies, the primary aim of our investigation was to conduct an extensive study of Pgp mediated MXR induction in both freshwater and marine invertebrates, which would ascertain the rate-dynamic, as well as the possible usability of MXR induction in environmental biomonitoring.

Although it is possible to determine the presence and expression of Pgp at the level of mRNA, DNA, or at the protein level, we decided to express the Pgp activity by its functional, i.e. transport efficiency. Namely, if the basic MXR hypothesis is correct, the primary result and physiological benefit of MXR induction should be the decreased intracellular accumulation (i.e. increased elimination) of xenobiotics. The method chosen was the accumulation and the efflux

version of the method with rhodamine B (RB) as the model Pgp substrate. The usability of these techniques was recently demonstrated in several studies (Smital and Kurelec, 1997, 1998; Smital et al., 2000; Eufemia et al., 2002).

Two species of aquatic invertebrates were selected as test organisms in this study: the freshwater mussel *Dreissena polymorpha* and the marine mussel *Mytilus galloprovincialis*. Both selected species (or at least their subspecies) are widespread, easily available, and sedentary organisms. They are relatively resistant to organic pollution, suitable for laboratory maintenance, and have high filtration rates that favor the uptake and bioconcentration of xenobiotics. And the most important, due to their inherently high basal level of Pgp activity (Smital et al., 2000) these bivalves are very suitable for the determination of MXR induction using the described transport-activity measurement methods.

The study was performed in several successive steps—from exposure experiments in laboratory using model MXR inducers, to the final in situ testing in real environmental conditions. We also examined the contribution of some environmental factors that cause general seasonal variations and/or for which we assume that they could have had critical influence on the dynamic and the level of Pgp transport activity. Those were the degree of pollution, water temperature, hydrological dynamic (for freshwater ecosystems), general fitness of the exposed animals and especially the concentration and the type of MXR inhibitors.

The complete report on this extensive, 3-year study based on described principles is presented here. The conclusions of this investigation generally confirm earlier observations. Pgp transport activity is induced/induces in the presence of pollution, and the dynamic of induction/deinduction processes is comparable with the induction kinetic of other well-known defensive mechanisms. However, our results also indicate that this inducibility is significantly limited. As measured using the described transport-activity tests, the maximal level of induction resulted in 50–60% lower basal RB accumulation in the gills of induced specimens (laboratory or in situ exposed to pollution), when compared to control non-induced animals held in unpolluted water. The environmental factors that may have had the crucial influence on the described measurement of MXR induction are additionally discussed.

2. Materials and methods

2.1. Chemicals

The sources of chemicals used were as follows: verapamil (VER), ethylene glycol-bis(β-aminoethyl ether)*N,N,N′,N′*-tetraacetic acid (EGTA), ouabain, DL-dithiothreitol (DTT), β-[*N*-morpholino]propanesulfonic acid (MOPS), sodium azide, adenosine 5′-triphosphate (ATP), rhodamine 123 (R123), rhodamine B (RB), sodium dodecyl sulfate (SDS) and tris-(hydroxymethyl)-aminomethane (TRIS) were from Sigma, St. Louis, USA; Serdolit AD-7 0.3–1.0 mm (XAD-7 resin) was from Serva, Heidelberg, Germany; cyclosporin A (sandimmun optoral) (CA) was from Sandoz, Nürnberg, Germany; Diesel-2 oil (D2) was from INA, Zagreb, Croatia; ascorbic acid, dimethyl sulphoxide (DMSO), methanole, acetone, $MgCl_2$, $CaCl_2$, KCl, ammonium molybdate, antimony potassium tartarate and H_2SO_4 were from Kemika, Zagreb, Croatia.

2.2. Water sampling

The water samples for the in vivo determination of the concentration of MXR inhibitors as well as for the in vitro determination of the type of inhibitors were collected during the periods of in situ induction/deinduction experiments. The sampling periods that include autumn, spring and summer season were April, May, August, September and October 1998, 1999 and 2000; and April, May and August 1999, 2000 and 2001. The freshwater samples were collected from the following locations: the Sava River upstream (S-u; slightly polluted with household waste) and 8 km downstream (S-d) from the discharge of industrial sewage waters of the Zagreb city (1 000 000 population) (heavily polluted water), and the Lake Jarun (LJ; Zagreb, recreational area—unpolluted water) (Fig. 1A). Although the sampling periods encompassed different seasons, these samples were collected mostly during periods of the lowermost water level and the highest concentrations of pollutants, respectively.

The marine water samples were collected at two sites along a well-defined scale of pollution in the aquatorium of Rovinj, Northern Adriatic, Croatia (Fig. 1B). These were the Molo site (M; a “mixing zone” of a waste from a local cannery—polluted

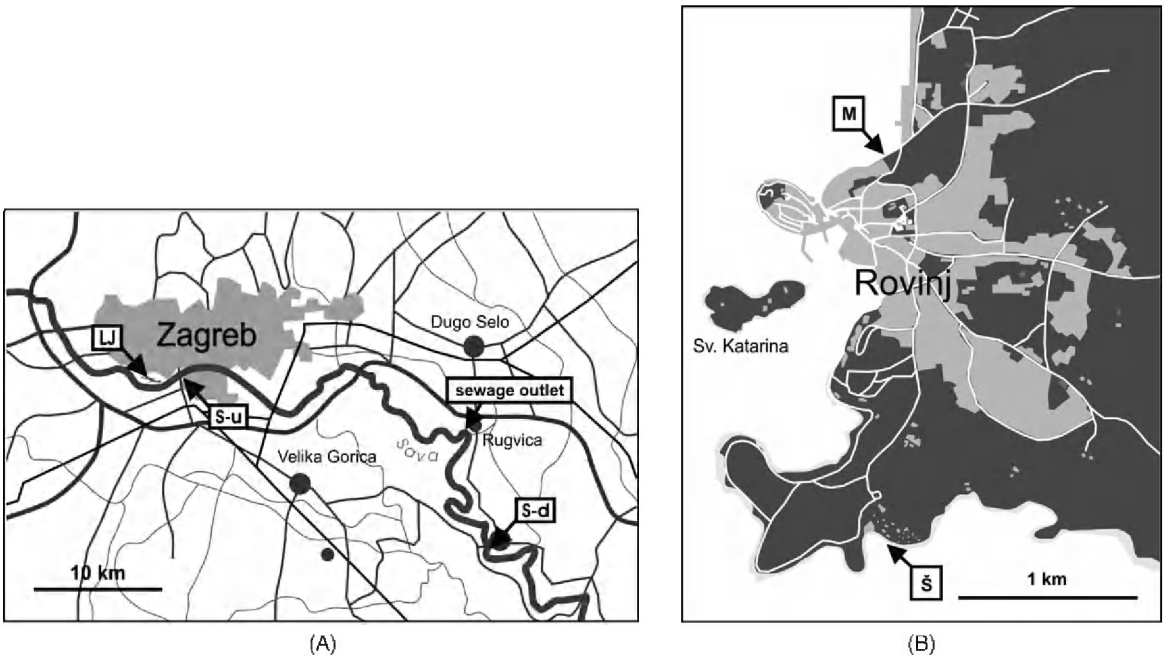

Fig. 1. Maps of research areas with locations used in this study. (A) Freshwater sites in the area of the Zagreb city: LJ, Lake Jarun; S-u, the Sava River upstream from the main sewage outlet; S-d, the Sava River downstream from the outlet. (B) Marine sites in the area of the town of Rovinj: M, the Molo site next to the local cannery; S, the Skarabe site.

water) and the Skarabe site (S; a site 3 km upstream from the town of Rovinj—unpolluted water).

All samples were collected in glass 25 l bottles from 0.5 m depth. The bottles were held at 4–10 °C in handy coolers until returning to the laboratory. Then the water samples were filtrated through nylon nets (pore size 500 μm) in order to remove detritus and large particles. After this step the water samples were immediately (within 3 h from the time of collection) in vivo tested for the presence (concentration) of MXR inhibitors, and the XAD-7 extraction procedure was performed simultaneously.

2.3. Definition of pollution degree

The degree of pollution in investigated waters was characterized by several parameters: (1) dissolved organic carbon values were 0.8 for the LJ site, 2.4 for the S-u and 76.4 mg/l for the S-d site (Kurelec et al., 1998); (2) the activity of benzo(*a*)pyrene monooxygenase (BaPMO) in the livers of nose carp, *Chondrostoma nasus*, caught at the S-d site was 69.7 ± 9.8 pmol of BaPOH/mg protein/min, i.e. two to seven times higher than in nose carp from the S-u site (Britvić et al., 1993); (3) high-resolution capillary GC coupled with MS analysis identified >200 specific organic compounds in Sava River waters, including several polycyclic aromatic hydrocarbons at concentrations of 10–100 ng/l (Ahel and Giger, 1985); (4) a 10-year follow-up study of mutagenic potential in the waters of Sava River never detected mutagens or promutagens in the Ames test in aliquots of 51 of water (either as hexane extracts, or as XAD-2 or Blue Cotton-concentrates) per plate (Kurelec, 1986); (5) ^{32}P postlabeling analysis of DNA adducts in fish from Sava River failed to show any pollution-related DNA adducts (Kurelec et al., 1989); (6) the activities of BaPMO in the livers of small-territory fish *Blennius pavo* were 31.2 ± 6.4 for fish caught at the M site and 6.1 ± 1.3 pmol of BaPOH/mg protein/min for fish caught at the S site (Kurelec et al., 1977); (7) the level of immunochemical expression of P-glycoprotein (the intensity of Western blot Pgp bands) was significantly higher in membrane vesicles isolated from

pooled samples of gills from mussels living at the M site when compared with mussels living at the S site; Kurelec et al., 1996a); (8) the frequency of chromosomal aberrations (FCA) in mussels native to these sites (the S site 2.9% and the M site 9.6%; Al-Sabti and Kurelec, 1985); and by (9) the MFO-induct test, i.e. the level of BaPMO induction in the livers of carp after i.p. application of hexane extracts of 1 l of seawater (S site 2.1; M site 46.1 pmol BaPOH/mg protein/min; Kurelec et al., 1979). And finally, the levels of BaPMO and/or EROD activity in the livers of fish from investigated waters, monitored on a seasonal basis, have not been significantly changed during the last 15 years (unpublished results).

2.4. Preparation of XAD-7 water concentrates

The extraction of water samples using XAD-7 resin was performed according to the procedure described by Yamasaki and Ames (1977). Briefly, water (25 l) was passed (flow rate 80 ml/min) through a column of XAD-7 resin (particle size: 0.3–1.0 mm, 10 g), the resin was eluted with 30 ml acetone, the acetone was evaporated on a rotary evaporator, and the dry residue dissolved in DMSO (25 l/500 μl).

2.5. Experimental organisms

Specimens of *D. polymorpha*, 20–30 mm long, were collected from April 1998 to August 2001 from the accumulation of the hydroelectric power plant Varaždin at the Drava River, Croatia, from 0.5 to 2 m depth. Depending on the season at the time of collection the water temperature was 14–24 °C. Specimens were taken to the laboratory and maintained at 21 °C in glass aquaria (~300 individuals per aquarium) with a capacity of 36 l (20 cm high × 60 cm long × 30 cm wide) in a flow of dechlorinated, well-aerated water (flow rate ~200 ml/min, i.e. 1 l per mussel per day). Mussels were given no food during the adaptation (at least 3 days before experiments) or the experimental periods.

For the determination of MXR inhibitors and laboratory induction experiments, specimens of juvenile *M. galloprovincialis*, 35–50 mm long, were collected at aforementioned periods in a highly protected area of Lim Channel (Limski Kanal), on the west coast of Istra Peninsula, Northern Adriatic. For in situ MXR induction/deinduction experiments specimens of the same age and size were collected at the M and S site. The water temperature was 12–26 °C. After collection the animals were maintained in aquaria with flowing filtered seawater until they were used for laboratory experiments.

2.6. The measurement of the level of Pgp transport activity

The in vivo measurement of the level of Pgp activity, for the purpose of both determination of induction/deinduction of Pgp activity and for determination of the concentrations of MXR inhibitors in exposure waters, was performed using the accumulation and the efflux version of the method with rhodamine B as the model Pgp substrate. Both methods were recently developed in our laboratory and their usability and significance demonstrated and described in details in several papers (Smital and Kurelec, 1997; Kurelec et al., 2000; Smital et al., 2000). Briefly:

2.6.1. Accumulation version of the method

The principle of the bioaccumulation assay is the measurement of the level of accumulation of the model fluorescent P-glycoprotein substrate in the gills of aquatic organisms after the exposure with or without model MXR inhibitor. For the purposes of this study we used following procedure: mussel specimens were placed into 1000–2000 ml glass containers (10 specimens per glass) containing 250–500 ml (depending of the size of specimens) of either analyzed water (test sample) supplemented with 2.5 μM RB, or dechlorinated tap water, or filtered seawater supplemented with 2.5 μM RB without (control) or with addition of cyclosporin A (10 μM, positive control). A Petri dish with a stirring rod was stuck at the centre of each glass. Specimens were arranged around the Petri dish. Containers were placed on a multiple magnetic stirrer. Animals were exposed in corresponding medium for 2 h (stirring speed 350 rpm). Under such conditions, 5–10 min after initiation of exposure, all animals must be opened, indicating active filtering. If it were not the case, the experimental conditions would not be optimal. In that case the oxygen content (aeration/steering speed) and water temperature were adjusted. If the described conditions were not met, the particular experiment would be repeated with new animals.

After the exposure period each specimen was washed in 100–200 ml of either dechlorinated tap water or filtered seawater using a tea-net. Gills from individual specimens were gently isolated, weighed, transferred to flat-bottom tubes in 0.5 ml of distilled water, and homogenized for 15 s. Homogenates were centrifuged at 3000 × *g* for 7 min, the supernatants carefully transferred to a 96-well flat-bottom dark microplate (100 μl per well, each supernatant in duplicate), and the fluorescence of accumulated dyes measured immediately.

2.6.2. Efflux version of the method

The principle of the efflux assay is to measure the rate of efflux of the previously accumulated fluorescent model Pgp substrate from the gills of aquatic organisms during the exposure in the medium with or without addition of model MXR inhibitor. All specimens needed for the experiment planned (10 specimens per sample) were placed into 1500–2000 ml glass containers in either dechlorinated tap water or filtered seawater (30–50 ml water per specimen), supplemented with 5 μM RB. Animals were exposed to the corresponding medium for 1 h, under well-aerated conditions provided with an aeration-stone placed at the bottom. Under such conditions, 5–10 min after initiation of exposure all animals must be opened, indicating active filtering.

When this "loading" period is done, the RB-medium was decanted and the mussels rapidly washed three times in the same volume of water. Specimens were then again transferred to 1500–2000 ml glass containers in either dechlorinated tap water or filtered seawater (25–50 ml water per specimen) for a 5 min "pre-efflux" period. This "pre-efflux" period is necessary to allow the animals to release the captured volume of loading medium (high concentration of RB), since it causes a high "background noise" at later fluorescence measurements of effluxed dyes. After the "pre-efflux" period specimens were transferred (10 specimens per sample) to 1000 ml glass containers containing 250–500 ml (depending of the size of specimens) of either analysed water (test sample), or dechlorinated tap water or filtered seawater with or without addition of CA (5 μM). A Petri dish with a stirring rod was stuck at the centre of each glass. Specimens were arranged around Petri dish. Containers were placed on a multiple magnetic stirrer. Animals were exposed in corresponding medium for 1 h (stirring speed 350 rpm). The media from each glass were transferred (200 μl per well) to a 96-well dark microplate every 10 min during a 60 min exposure period, avoiding touching or disturbing the mussels. After the last collection the fluorescence of effluxed dyes were immediately measured.

During the procedure (of both the accumulation and the efflux version of the method) glasses, gills, vials/homogenates/supernatants and microplates were light protected (with aluminium foil), because of the possible loss of the RB-fluorescence intensity caused by the direct exposure to light.

2.7. Determination of the MXR inhibitors in corresponding fresh and marine waters

2.7.1. Concentration of MXR inhibitors

The concentration of MXR inhibitors in investigated waters was also determined using accumulation and the efflux version of the method with RB. The amount of accumulated or effluxed RB was proportional to the concentration of inhibitors in the analyzed water sample. The determined MXR inhibitory potential of investigated waters was compared with the inhibitory effect of growing concentrations of model MXR inhibitor CA (standard curve). The chemosensitizing effects of investigated waters were read from the standard curve for CA and the concentrations of MXR inhibitors were expressed in μM of CA-equivalents.

2.7.2. Type of MXR inhibitors

For the determination of the dominant type of MXR inhibitors present in the water samples we used the method based on the measurement of the P-glycoprotein specific ATPase activity in membrane vesicles rich in Pgp. Verapamil-type of inhibitors (competitive or direct type) stimulate so-called vanadate-sensitive Pgp-ATPase activity, contrary to cyclosporin A-type (non-competitive or non-direct type) of inhibitors that inhibit corresponding activity. The vanadate-sensitive Pgp-ATPase activity was estimated by measuring inorganic phosphate (Pi) liberation using the method described by Sarkadi et al. (1992).

The membrane vesicles rich in Pgp, isolated from Sf9 (*Spodoptera fugiperda*) insect cells infected with recombinant baculovirus carrying the human *MDR*1

gene, were obtained (4.3 mg/ml of reaction medium) from Dr. Balasz Sarkadi from the National Institute of Hematology, Blood Transfusion and Immunology, Budapest, Hungary. Twenty micrograms of membrane vesicles (10 μl/0.15 ml of reaction medium) were incubated for 20 min at 37 °C in 0.15 ml of reaction medium containing 40 mM Tris–MOPS, pH 7.0; 0.5 mM EGTA–Tris, pH 7.0; 50 mM KCl; 5 mM sodium azide; 2 mM DTT, and 1 mM ouabain. This media provide optimal reaction conditions and almost completely inhibit all other present ATPases except vanadate-sensitive Pgp-ATPase. The ATPase reaction was started by the addition of MgATP, final concentration of 5 mM (10 μl of 50 mM MgATP, pH 7.0/0.15 ml of reaction medium). The indicated drugs (VER and CA, as the positive control for the stimulation and the inhibition of Pgp-ATPase activity, respectively) or XAD-7 water extracts (1 μl of extract = equivalent of 200 ml of water) were added in DMSO (final concentration of DMSO in assay medium was less then 1%). Preliminary experiments showed that this level of DMSO does not have any significant effect on the ATPase activity. The reaction was stopped by addition of 0.1 ml of 5% SDS solution and the amount of inorganic phosphate in the reaction mixture was determined immediately. Inorganic phosphate was measured by the simple colorimetric method from Murphy and Riley (1962). ATPase activity was calculated by the difference in Pi levels obtained before (reaction stopped at zero-time with SDS) and after 20-min incubation period, and expressed in nmol/Pi/mg of protein/min. All samples were tested in triplicate.

2.8. Pgp activity induction/deinduction experiments in laboratory conditions

Groups of mussels (n = 130–150) (*D. polymorpha* or *M. galloprovincialis*) were exposed in glass aquaria in 5 l of well aerated either dechlorinated tap water or filtered seawater, with (control) or without the addition of model inducers. As model Pgp inducers either a well-known model Pgp substrate rhodamine 123 (0.1, 0.5, 1 or 3 μM) or Diesel-2 oil, a typical environmental pollutant (at the concentration of hydrocarbons equivalent to 15, 50, 105 or 350 ppb of Kuwait oil) were used. The saturation of water with D2 was achieved by passing the inflow tap water through a vertical glass tube (R = 4 cm) mounted in the middle of the aquaria and filled with a layer of D2 (2–50 ml). The layer of D2 was renewed daily in order to maintain the same D2 concentration as in the beginning of the exposure. A water fraction was collected and the concentration of D2 hydrocarbons in the experimental aquaria was determined after purification and separation of water samples through an acid alumina column according to the standard Intergovernmental Oceanographic Commission method (IOC, 1982) modified by Picer (1985). The exposure period was 6 days, the water temperature was about 21 °C, and the water in all aquaria was changed every other day. The concentrations of R123 and D2 were fluorometricaly controlled on daily basis.

During the exposure period the rate and the level of Pgp activity were measured using previously described accumulation and efflux version of the method with RB.

2.8.1. Accumulation method

In order to measure the induced level of Pgp activity during the period of exposure to model inducers, on days 2, 4 and 6 of the exposure period 10 mussels from each group (control, R123, D2) were pretransferred into 60 l basin with a rapid flow of well-aerated dechlorinated tap water or filtered seawater. The mussels were held in corresponding water for a period of 2 h in order to enable the detachment of substrates from the active sites of Pgp. After this step, the specimens were transferred into glass aquaria in 3 l of well aerated either dechlorinated tap water or filtered seawater with the addition of 2.5 μM RB, for a period of 2 h. Following this accumulation period, the mussels were further treated according to the described procedure for the accumulation version of the method with RB.

For the measurement of the rate and the level of deinduction of Pgp activity after the entire exposure period (6 days), 10 mussels from each group were transferred into 60 l basin with a rapid flow of dechlorinated tap water or filtered seawater. Then, on days 8, 10 and 12 (counting from the start of experiment) the level of Pgp activity was determined using the same RB accumulation procedure.

2.8.2. Efflux method

On days 2, 4 and 6 of the exposure period 10 mussels from each group (control, R123, D2) were

pretransferred into 60 l basin with a rapid flow of dechlorinated tap water or filtered seawater. Then, after detachment of substrates from the Pgp active sites the mussels were transferred into glass aquaria in 3 l of well aerated either dechlorinated tap water or filtered seawater with the addition of 5 μM RB, for a period of 1 h. Following this loading period the mussels were further treated according to the described procedure for the efflux version of the method with RB.

For the measuring of the dynamic of Pgp activity deinduction process on days 8, 10 and 12, the level of MXR activity was determined using the same procedure for the RB efflux method.

2.9. Pgp activity induction/deinduction experiments in situ

2.9.1. Freshwater (D. polymorpha)

Investigations of the rate and dynamic of the induction of Pgp activity in the freshwater mussel *D. polymorpha* were performed in April, May, August, September and October 1998, 1999 and 2000; and in April, May and August 2000 and 2001. Before the start of the in situ experiments mussels were held in laboratory in 60 l basin with a rapid flow of well-aerated dechlorinated tap water for a period of 10 days. Following this step, the mussels were transferred to the described freshwater locations (Fig. 1A). The exposure experiments were performed mostly during periods of lowermost water level and the highest concentrations of pollutants, respectively. Specimens ($n = 50$–250) were exposed in steel cages (50 cm × 30 cm × 20 cm; hole diameter 1 cm) at 0.5–0.8 m depth. Depending on the season and the location water temperature was 6–21 °C. According to the type of experiment the exposure periods lasted 2–14 days. The level of the induction of Pgp activity was determined using the following procedure.

After 2–14 days of the exposure 15 specimens from each location (5 for the accumulation and 10 for the efflux version of the method) were transferred to the laboratory in glass aquaria with a constant flow of well-aerated dechlorinated tap water. The mussels were held in aquaria for a period of 12 h, in order to enable the releasing of substrates (i.e. environmental pollutants) from the Pgp active sites and the accommodation to laboratory conditions. Note that this period is short enough to avoid any significant deinduction of the Pgp activity. After the end of this period the level of induction of Pgp activity was determined using both the RB accumulation and the efflux method.

The level of Pgp related MXR induction in mussels exposed to differently polluted waters was expressed as a percentage of the decrease in RB accumulation in comparison to the control group of mussels (non-induced, unexposed mussels held in the laboratory = 100% basal accumulation of RB).

The rate and dynamic of the deinduction of Pgp activity in *D. polymorpha* after the induction, by the exposure to the environmental pollution, was investigated in two ways:

(1) In laboratory conditions—after the end of exposure period(s) (7–10 days) mussels were transferred from the corresponding locations to the laboratory. Following the accustomed period needed for the accommodation to the laboratory conditions (12 h) and for the releasing of substrates from Pgp active sites the level of Pgp activity was measured as described above.
(2) In the real environmental conditions—i.e. transferring the mussels from the polluted to unpolluted location. For this purpose caged specimens of *D. polymorpha* were exposed (7–10 days) at the most polluted location (the S-d site). After significant induction of Pgp activity (tested using RB accumulation method) the mussels were transferred to the unpolluted location (the LJ site). Then, during the exposure of these "induced" mussels the deinduction of Pgp activity was monitored every other day (days 2, 4 and 6) using RB accumulation method.

The level of Pgp activity deinduction in mussels exposed to unpolluted water was expressed as a percentage of the increase in RB accumulation in comparison to the control group of mussels.

All induction/deinduction experiments that were performed in situ were repeated several times (mostly 3–4 times) depending on weather conditions and water level at the time of exposure.

2.9.2. Marine (M. galloprovincialis)

Investigations of the rate and dynamic of the induction of Pgp activity in marine mussel *M. galloprovincialis* were performed in April, August, September

and October 1998, 1999 and 2000. Specimens (n = 50–250) were exposed to water at the described locations (the M and the S site) in nylon nets (20 l volume; hole diameter 1 cm) at 1.0–1.5 m depth. Depending on the season water temperature was 15–26 °C. The duration of exposure periods was 2–5 days. The level of the induction of Pgp activity during the exposure to environmental pollution was determined after accommodation to laboratory conditions in a flow of filtered seawater, using RB accumulation and efflux method.

The rate-dynamic of the deinduction of Pgp activity in *M. galloprovincialis* were examined only in situ. For this purpose, mussels that normally live on the M site (polluted location) were transferred to the S site (unpolluted location) and inversely. Before the transfer (day 0), the basal level of Pgp activity in mussels from each location was tested using RB accumulation and efflux method. The mussels were exposed on the described locations for a period of 5 days. After the end of exposure period (day 5), the level of the deinduction of Pgp activity in mussels transferred to the unpolluted site as well as the level of the induction in mussels transferred to the polluted site was measured using both methods.

2.10. Fluorescence measurement

Fluorescence of accumulated or effluxed RB was measured on a Fluorometer 1000 fluorometer (Dynatch, Chantilly, VA, USA) using 535 nm (excitation) and 590 nm (emission) filters. The same filters were used for the daily testing of D2 concentration in experimental basins. The daily testings of R123 concentration in experimental aquaria were performed using 485 nm (excitation) and 530 nm (emission) filters.

2.11. Statistical analysis

The resulting data were grouped according to the type of experiment and analyzed separately using a variety of statistical tools. Normality was tested using Smirnov–Kolmogorov non-parametric test for each of the data groups. For data comparison between tests and control groups the two-independent sample t-test with assumed equal variance of samples, further confirmed with a Mann–Whitney–Wilcoxon non-parametric test for equal medians was used. One-way analysis of variance (ANOVA) was performed to determine if significant differences between the tested groups existed, followed by one-way analysis of means (ANOM) in order to indicate exactly which groups were different. For statistically significant difference the rejection/acceptance of null hypothesis was taken into account at a risk of less than a 5% (*), 1% (**) and 0.1% (***). Minimal significant detectable difference between estimated (x) and true (μ) mean of population, as well as between two estimated sample means was calculated according to two iterative procedures, whose code was self written in Microsoft Excel Visual Basic (Formula 1 and 2), as follows:

$$n \geq \frac{\mathrm{var}^2 \times t_{a,df}}{d^2} \quad (1)$$

$$n \geq \frac{2 \times \mathrm{var}_p \times (t_{\alpha,df} + t_{\beta,df})^2}{d_{\bar{x}_1 - \bar{x}_2}} \quad (2)$$

where n is minimal sample size, var the variance, t the t-coefficient, α the confidence interval, df degree of freedom, $d_{\bar{x}_1 - \bar{x}_2}$ the distance, i.e. difference of samples averages, d the acceptable error, β the confidence interval for β-type error and var_p is the pooled variance.

Data storing, data processing, as well as the calculation of critical tests values were performed by Microsoft Excel 2002 spreadsheet software.

2.12. Presentation of data

The data shown in the figures were based on at least three independent experiments per year. The results shown are those of one representative experiment and are presented as mean values, with S.D. range indicated in figure legends.

3. Results

3.1. Concentrations of MXR inhibitors in the exposure waters

MXR inhibitory potential of the corresponding marine and freshwater was determined using the accumulation and the efflux version of the method with RB. Although within this study the water samples were collected and tested several times during different years and seasons, statistically significant seasonal difference in concentration of MXR inhibitors were

not observed. Therefore, the results shown in the figures represent average values obtained in course of the multiple testing.

3.1.1. Freshwater

Among the freshwater samples tested using the RB accumulation method, water samples collected on the S-d site showed the highest MXR inhibitory potential. RB accumulation in gills of *D. polymorpha* specimens exposed to this water was on the average 233% higher in comparison to the control group of mussels exposed to dechlorinated tap water (Fig. 2A). Water samples collected from the location S-u (upstream from the discharge of sewage waters) showed lesser, but significant inhibitory effect (80% increase in RB accumulation), whereas water sample from the LJ site contained no significant concentrations of MXR inhibitors (Fig. 2A).

The concentrations of MXR inhibitors in corresponding samples were then expressed in μM of the model inhibitor cyclosporin A-equivalents, i.e. relative to the inhibitory effect of 10 μM CA (positive control) tested in each experiment, as well as based on the CA-standard curve for the RB accumulation method in *D. polymorpha*, respectively (Kurelec et al., 2000). According to the described calculation the average concentrations of MXR inhibitors were: in water samples from the S-d site 7.35 μM of CA-equivalents, from the S-u site 2.14, and from the LJ site 0.84 μM of CA-equivalents (Table 1).

Table 1
The concentration and the type of MXR inhibitors determined in marine and freshwater samples collected from locations used in this study (the S-d, S-u, LJ, M and S site)

Location	MXR-inhibitory potential (μM of CA-eq.)		Type of MXR-inhibitors
	Accumulation method	Efflux method	
S-d	7.35 ± 1.40	6.28 ± 1.19	VER
S-u	2.14 ± 0.36	1.09 ± 0.23	VER
LJ	0.84 ± 0.14	0.83 ± 0.15	–
M	5.79 ± 1.11	6.77 ± 1.49	CA
S	0.62 ± 0.14	1.03 ± 0.26	–

Concentrations of MXR inhibitors were determined using RB accumulation and efflux method and expressed as MXR inhibitory potential, in μM of cyclosporin A (CA) equivalents, based on the CA standard curve for both methods. Type of MXR inhibitors in the used locations was determined using the measurement of the P-glycoprotein specific ATPase activity in Pgp rich membrane vesicles and expressed either as competitive, verapamil-like (VER) or non-competitive, cyclosporine A-like type of MXR inhibitors predominantly present in the analyzed water samples.

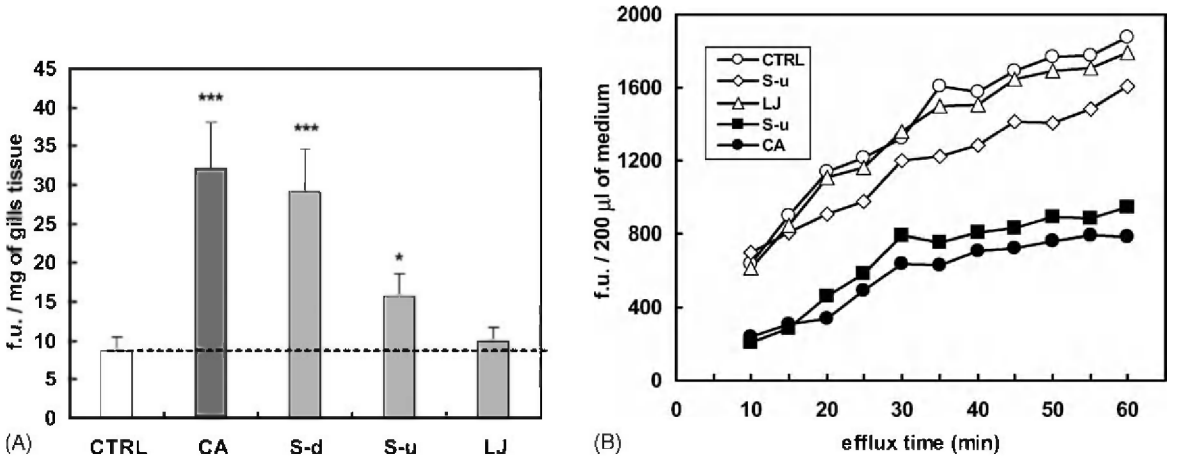

Fig. 2. MXR inhibitory potential of water samples collected from the freshwater locations. The concentration of MXR inhibitors was determined using the accumulation (A) and the efflux (B) version of the method. In the presence of RB, specimens of *D. polymorpha* were exposed to dechlorinated tap water with (CA) or without (CTRL) the addition of model MXR inhibitor CA, or to investigated water samples collected from the following locations: the Sava River upstream (S-u), downstream (S-d) from the discharge of industrial sewage waters of the Zagreb city, and the Lake Jarun (LJ). The accumulation version data are expressed in fluorescence units (f.u.) of RB accumulated/mg of gill tissue (mean ± S.D. from decaplicate determination), and the efflux version data are expressed in f.u. of RB effluxed per 200 μl of exposure medium (means from duplicates obtained from replica wells: S.D. < 5%). $^{*}P < 0.05$, $^{***}P < 0.001$.

Testing of the freshwater samples using the efflux version of the method revealed a significant correlation with the results of RB accumulation method. The highest inhibitory potential was observed in the S-d water samples (50% decrease of the RB efflux rate from gills of *D. polymorpha* specimens exposed to this water when compared to the control group of mussels exposed to dechlorinated tap water; Fig. 2B). Water samples collected from the S-u showed significantly lesser inhibitory effect (15% decrease in RB efflux rate), and water samples from the LJ site showed no significant MXR inhibitory potential (Fig. 2B).

The concentrations of MXR inhibitors expressed in μM of CA-equivalents, based on the CA-standard curve for the RB efflux method in *D. polymorpha*, were: 6.28 μM (S-d site), 1.09 μM (S-u site) and 0.83 μM (LJ site) of CA-equivalents (Table 1).

3.1.2. Marine water

Results of the testing of selected marine water locations using RB accumulation method with marine mussel *M. galloprovincialis* also revealed the significant correlation between the level of pollution and concentration of MXR inhibitors. The exposure of mussels to water from the M site (the referent polluted site) resulted in the average 232% increase in RB accumulation in gills of *M. galloprovincialis* when compared to the control group of mussels exposed to filtered seawater, whereas the mussels exposed to water from the S site (the referent unpolluted site) showed only minor (10%) increase in RB accumulation (Fig. 3A). Following the described methodology for the testing of freshwater samples, calculated concentrations of MXR inhibitors were: 5.79 μM (M-site) and 0.62 μM (S-site) of CA-equivalents (Table 1).

As in the case of freshwater samples, the results obtained using RB efflux method were in concordance with the results obtained with the accumulation version. Water samples from the M site caused 46% decrease of the RB efflux rate from gills of *M. galloprovincialis*, whereas the exposure to water samples collected from the S site caused weak inhibitory effect (5% decrease in RB efflux rate) (Fig. 3B). Calculated concentrations of MXR inhibitors for the efflux version of the method were 6.77 μM (M site) and 1.03 μM (S site) of CA-equivalents (Table 1).

3.2. Type of MXR inhibitors in the exposure waters

The type of MXR potential of the investigated marine and freshwaters was determined using in vitro method originally designed by Sarkadi et al. (1992).

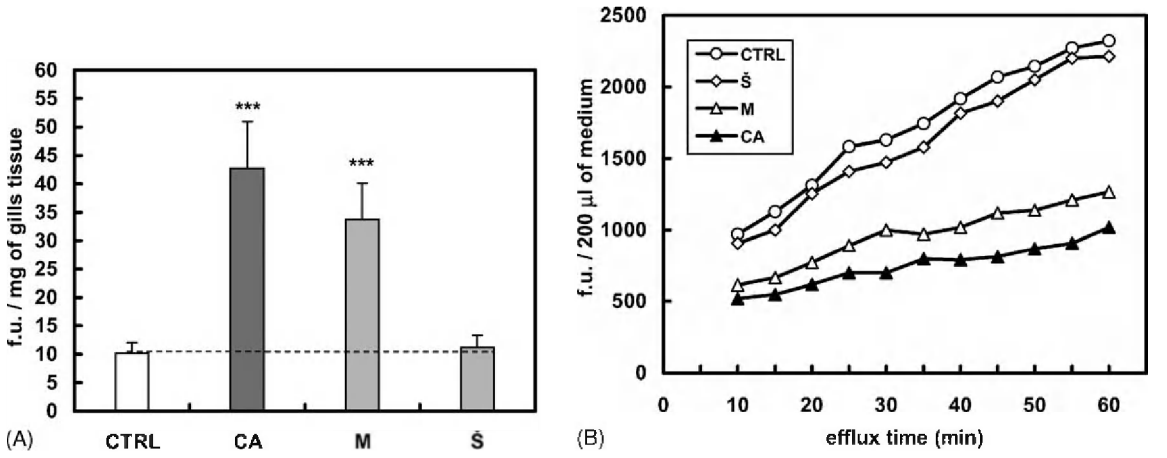

Fig. 3. MXR inhibitory potential of water samples collected from the seawater locations. The concentration of MXR inhibitors was determined using the accumulation (A) and the efflux (B) version of the method. In the presence of RB, specimens of *M. galloprovincialis* were exposed to filtered seawater with (CA) or without (CTRL) the addition of model MXR inhibitor CA, or to investigated seawater samples collected from the Molo (M) and the Skarabe site (S). The accumulation version data are expressed in fluorescence units (f.u.) of RB accumulated/mg of gill tissue (mean ± S.D. from decaplicate ($n = 10$) determination), and the efflux version data are expressed in f.u. of RB effluxed per 200 μl of exposure medium (means from duplicates obtained from replica wells: S.D. < 5%). *** $P < 0.001$.

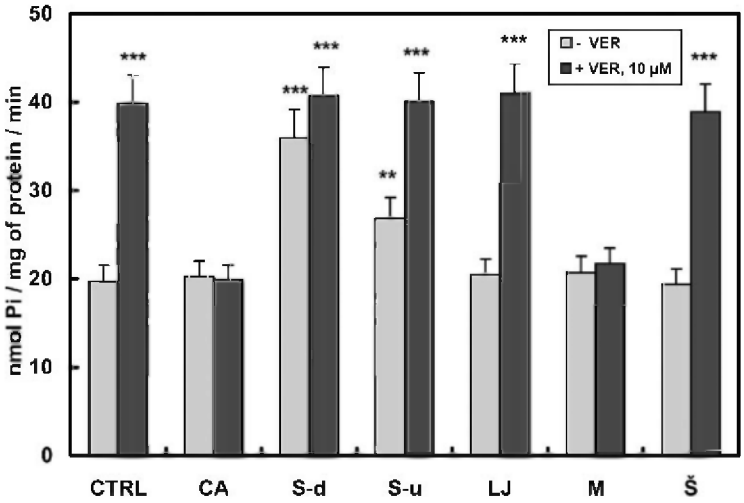

Fig. 4. The determination of the type of MXR inhibitors present in investigated marine and freshwaters. P-glycoprotein rich membrane vesicles were incubated with either XAD-7 extracts of water samples collected from investigated locations (the S-u, S-d, LJ, M and S sites) or cyclosporin A (CA, 5 μM), without (−VER) or in the presence of verapamil (10 μM, +VER) for 20 min at 37 °C, as described in Section 2. Vanadate-sensitive ATPase activity of P-glycoprotein was expressed in nmol/Pi/mg of protein/min (mean ± S.D. from triplicate obtained from replica wells). $^{**}P < 0.01$, $^{***}P < 0.001$.

Among the freshwater samples, the addition in the incubation medium of XAD-7 extract of water samples collected from the location S-d resulted with 82% stimulation of Pgp-ATPase activity, and the extract from the S-u site caused 36% stimulation of ATPase activity. In both cases, the addition of VER (10 μM) into the medium (in the presence of extract) resulted with the maximal stimulation of Pgp-ATPase activity. These data clearly indicate that MXR inhibitors presented in the Sava River, at least predominantly, belonged to the VER, competitive type of MXR inhibitors (Fig. 4; Table 1).

However, the addition of XAD-7 marine water extract from the M site (referent polluted location) caused the inhibition of VER-stimulated Pgp-ATPase activity comparable to the effect of CA. Even the addition of VER (in the presence of extract) to the incubation medium did not result in a significant increase of ATPase activity. Consequently, we concluded that the MXR inhibitory potential of marine water samples collected from the M site could be qualified as CA or non-competitive type of inhibition (Fig. 4; Table 1).

XAD-7 extracts from the LJ site and from the marine S site (referent unpolluted locations) did not show any statistically significant effect on the Pgp-ATPase activity. These results are in concordance with the data obtained using RB accumulation and efflux method that detected only low concentrations of MXR inhibitors in these waters (Figs. 2A,B and 3A,B; Table 1).

3.3. Induction/deinduction of Pgp activity by the laboratory exposure to R123 or D2

The primary goal of these laboratory experiments was to determine the rate-dynamic of MXR induction/deinduction in controllable conditions of laboratory exposure to well-defined model inducers.

Model inducers selected for the laboratory exposure were rhodamine 123 and Diesel-2 oil. R123 is a well-known model fluorescent Pgp substrate so we presumed that in our test-system, it would effectively induce the Pgp activity in *D. polymorpha*. R123 fluoresces at different wave lengths then RB, which allowed us the determination of Pgp activity during induction/deinduction periods using the described accumulation and efflux versions of the method with RB as the model Pgp substrate. The second model inducer used was the water extract of Diesel-2 oil as a typical environmental pollutant.

Using RB accumulation version of the method the statistically significant induction of Pgp activity was

determined after 2 days of exposure in mussels exposed to 0.5, 1 and 3 μM R123 (11–22% decrease in basal RB accumulation; Fig. 5A). The induction was continuing till the 6th day when the highest level of induction was determined in mussels exposed to 3 μM R123 (45% decrease in basal RB accumulation; Fig. 5A). Clear dose-response relationship was also obtained in mussels exposed to D2 and the highest level of induction was determined in mussels exposed to D2 at concentration equivalent to 350 ppb of Kuwait oil (49% decrease in basal RB accumulation; Fig. 5B).

Transfer of these, "induced mussels", in aquaria with a constant flow of the clean, well-aerated dechlorinated water resulted in the deinduction of Pgp mediated MXR activity approximately with the same dynamic as in the case of induction process. After 12 days of the experiment, i.e. after 6 days of deinduction, the level of MXR activity returned to the level measured in control group of mussels (Fig. 5A and B).

This induction/deinduction processes in all groups had also been monitored using the RB efflux method. Induction of Pgp activity after 6 days of exposure caused significant decrease in RB accumulation, which was observed by the trend of RB efflux, as well as by the increased amount of RB effluxed in the media, i.e. retained in the gills of exposed mussels. After 6 days of exposure level of RB retention measured after 60 min efflux period was 41% lower in mussels exposed to 3 μM R123 (Fig. 6A) and 43% lower in group exposed to D2 (350 ppb eq. of Kuwait oil), respectively (Fig. 6B). As in the case of RB accumulation measurement, the transfer of the induced mussels to clean water lead to the successive deinduction and after 6 days the basal activity was the same as in control group of *D. polymorpha* specimens (Fig. 6A and B).

The obtained dose-response relationships revealed that almost maximal level of Pgp transport activity was induced by exposure to either 1 μM R123 or D2 at concentration equivalent to 105 ppb of Kuwait oil. The exposure to higher concentrations of model inducers did not result in significantly higher level of induction. Regarding this fact in further experiments, primarily directed to the investigations of rate-dynamic of Pgp mediated MXR induction, we decided to use only one concentration of model inducers: 1 μM R123 as the concentration high enough for the effective induction, but at the same time not too high concentration that could result in a long lasting competitive inhibition of RB binding to the Pgp active sites, and D2 concentration equivalent to 105 ppb of Kuwait oil, which represents the environmentally relevant concentration (Kurelec et al., 1996b).

The rate-dynamic and level of Pgp activity induction in laboratory conditions were also analyzed in marine mussel *M. galloprovincialis*. The results of these experiments clearly showed nearly the same pattern of the induction as it was the case with *D. polymorpha*. As it was obtained using both versions of RB method, the level of Pgp activity, induced either by R123 (1 μM) or D2 (105 ppb), reached its maximum after 5 days of exposure (Fig. 7A and B). Though, the dynamic of induction was faster and the level slightly higher than in the case of *D. polymorpha*. The laboratory experiments of the deinduction of Pgp activity were not performed within the "marine part" of this study. Nevertheless, the similarity between the "freshwater" and "marine" results implicated that the period needed for the effective deinduction of MXR activity might be the same, or at least very close to the period needed for the maximal induction, i.e. 5–7 days.

3.4. Pgp activity induction/deinduction experiments in situ

3.4.1. Freshwater (D. polymorpha)

Based on the results of laboratory experiments with *D. polymorpha* we concluded that an optimal period needed for the maximal induction is about 6–7 days. However, it was necessary to test these observations in more realistic environmental conditions. Therefore, we selected three locations with different but well-defined levels of pollution in the area within and around the Zagreb city. The mussels were cage-exposed on these locations and the basal level of Pgp activity was measured using only the accumulation version of the method, primarily because of its better accuracy in comparison to the efflux version. Results of these experiments are presented in Fig. 8A and B. The rate-dynamic of induction of the Pgp activity in real environmental conditions was clearly visible—after 5 days of exposure the level of Pgp activity was almost maximal. On the 7th day, the activity reached the maximum and further exposure had no additional effect. The highest induction was determined in the mussels exposed at the S-d site. This

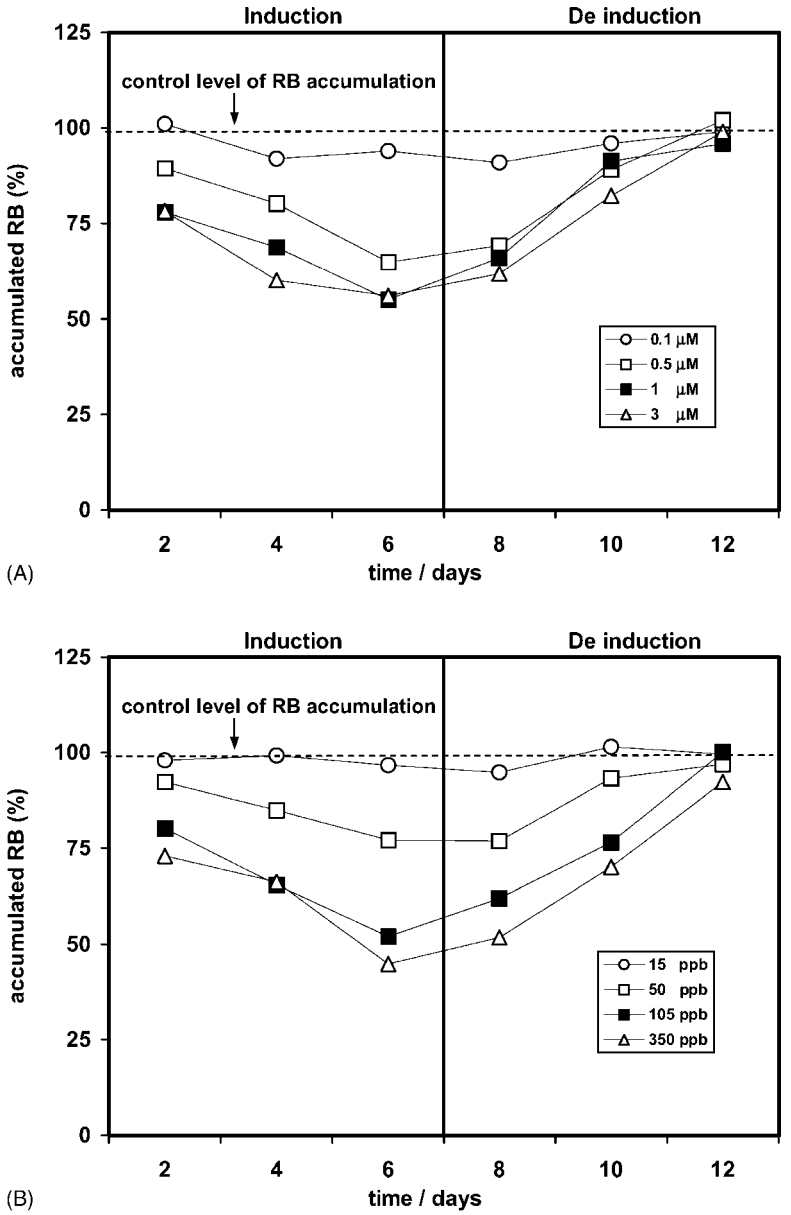

Fig. 5. Determination of the induction and deinduction of Pgp transport activity in *D. polymorpha* using the RB accumulation method after laboratory exposure to model Pgp inducers. The mussels were exposed in aquaria in well-aerated water spiked with either rhodamine 123 (A) or the water extract of Diesel-2 oil (B) (induction), followed by the exposure to dechlorinated tap water only (deinduction). The level of Pgp activity was determined every other day during both exposure periods and expressed as mean percentage of basal RB accumulation determined in the control group (non-exposed mussels). The control level of RB accumulation was considered to be 100%. S.D.s determined from decaplicate ($n = 10$) determinations ranged 16–22%.

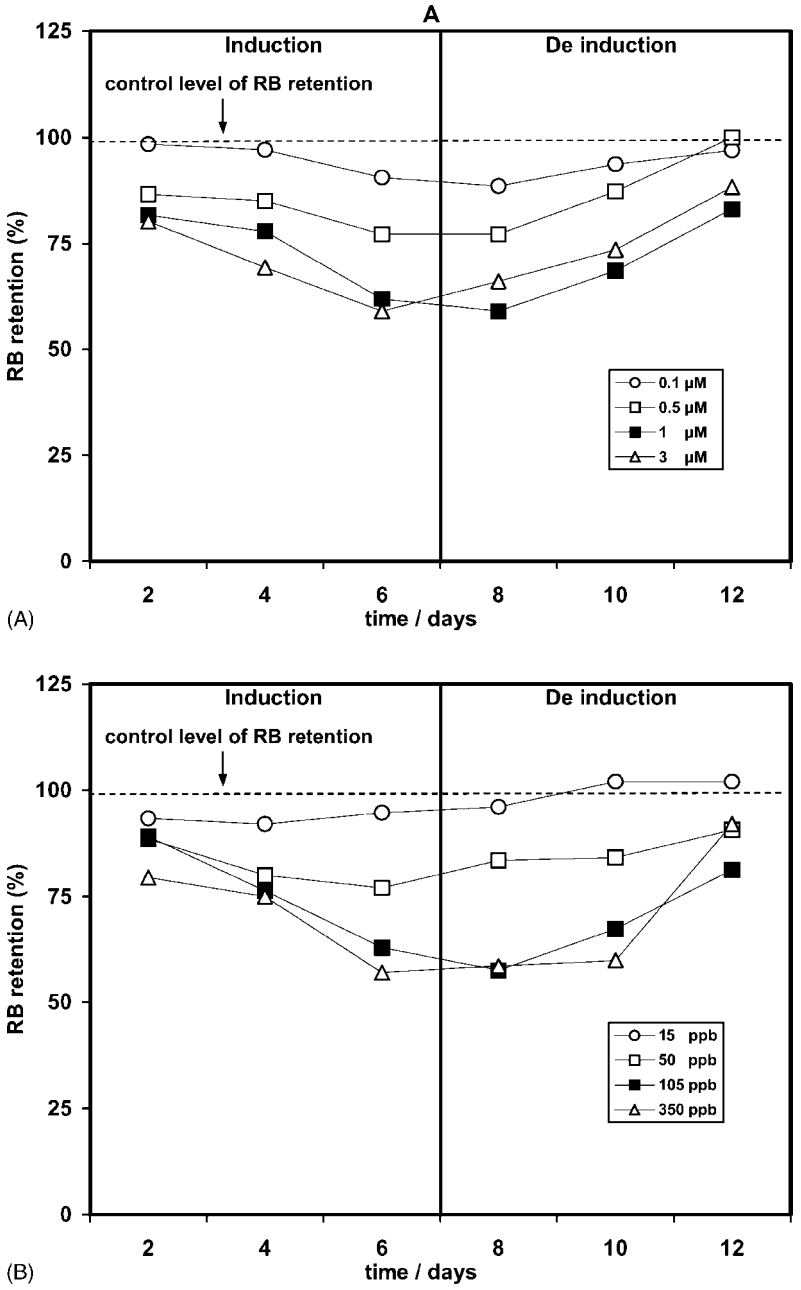

Fig. 6. Determination of the induction and deinduction of Pgp transport activity in *D. polymorpha* using the RB efflux method after laboratory exposure to model Pgp inducers. The mussels were exposed in aquaria in well-aerated water spiked with either rhodamine 123 (A) or the water extract of Diesel-2 oil (B) (induction), followed by the exposure to dechlorinated tap water only (deinduction). The level of Pgp activity was determined every other day during both exposure periods and expressed as mean percentage of basal RB retention determined in the control group (non-exposed mussels). The control level of RB retention was considered to be 100%. S.D.s determined from duplicates obtained from replica wells were less than 5%.

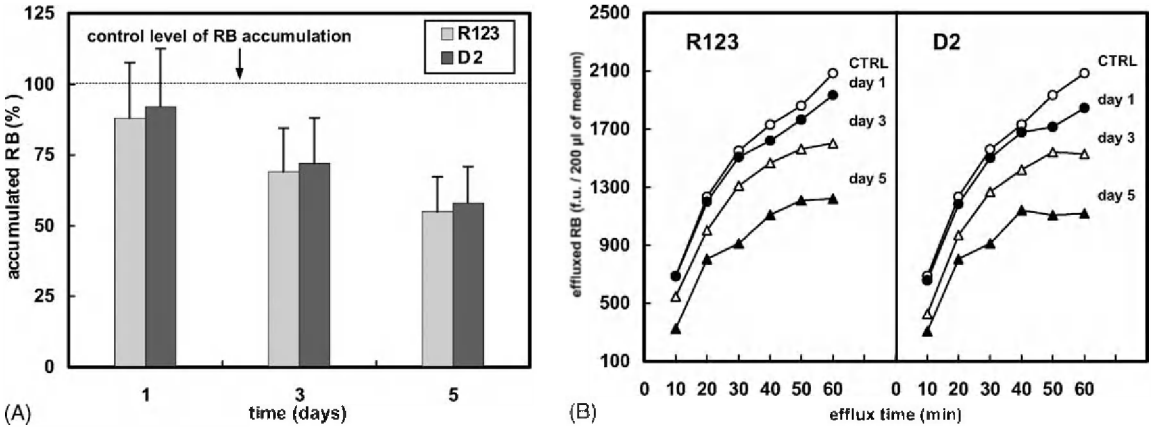

Fig. 7. Determination of the induction of Pgp transport activity in *M. galloprovincialis* using the RB accumulation (A) and efflux (B) method after laboratory exposure to model Pgp inducers. Mussels were exposed in aquaria in well-aerated water spiked with either rhodamine 123 (R123, 1 μM) or the water extract of Diesel-2 oil (D2, 105 ppb eq.). The level of Pgp activity was determined every other day during the 5-day exposure period and expressed in: (A) percentage of basal RB accumulation (mean ± S.D. from decaplicate ($n = 10$) determination), and (B) in fluorescence units (f.u.) of RB effluxed per 200 μl of exposure medium (means from duplicates obtained from replica wells; S.D. < 5%) regarding the control level of RB efflux determined in the control group (non-exposed mussels; CTRL).

induction resulted in 57% decrease in RB accumulation in the gills of the exposed *D. polymorpha* specimens in comparison to the control (unexposed) group (Fig. 8A). Significant induction was also determined in specimens exposed at the S-u site (38% decrease in RB accumulation), whereas the water of the Lake Jarun (LJ) had almost no detectable induction effect.

In order to monitor the deinduction of Pgp activity, following the 10-day induction period each group of mussels (from all locations) was transferred into laboratory basins. During the entire 10-day deinduction period the mussels pre-exposed to the unpolluted water from the LJ site retained the Pgp activity on the same previously detected non-induced level. On the contrary, mussels pre-exposed to the polluted water at the S-d or S-u site needed 5–7 days (depending on the level of induction) for the complete restoration of Pgp activity to the control level (Fig. 8B).

The described results of the in situ induction/deinduction investigations were well correlated with laboratory experiments. The maximal level of induction of Pgp activity observed in environmentally exposed mussels (exposed at the S-d location) was to some extent higher than in the case of the laboratory exposure to model inducers (57% versus 49%).

In the described experiments, the rate-dynamic and the level of Pgp mediated MXR deinduction were always determined after the return of environmentally induced specimens to the laboratory basins. In the third step of this study, we intended to monitor the induction and deinduction processes entirely in the real environmental conditions, without any laboratory exposure steps. Additionally, based on the previously obtained data we tried to rationalize the experimental conditions, to shorten the exposure period and to minimize the disturbance of mussels, i.e. to avoid the manipulation with cages every other day in order to determine the rate of induction/deinduction.

For this purpose, two groups of *D. polymorpha* specimens were cage-exposed for 7–10 days at the most polluted location (S-d) and at the referent-unpolluted location (the LJ site), respectively. After this period (day 0) the level of Pgp activity in specimens from both groups was tested using the accumulation and the efflux method. Following this step the mussels from the corresponding polluted location were transferred to the unpolluted location and inversely. After 5 days of exposure the level of MXR activity was again measured in both groups. As measured using both methods, the mussels that significantly induced their Pgp activity during the previous exposure to polluted water, 5 days after the transfer to the unpolluted location deinduced the Pgp activity to the level that was compatible to the activity of

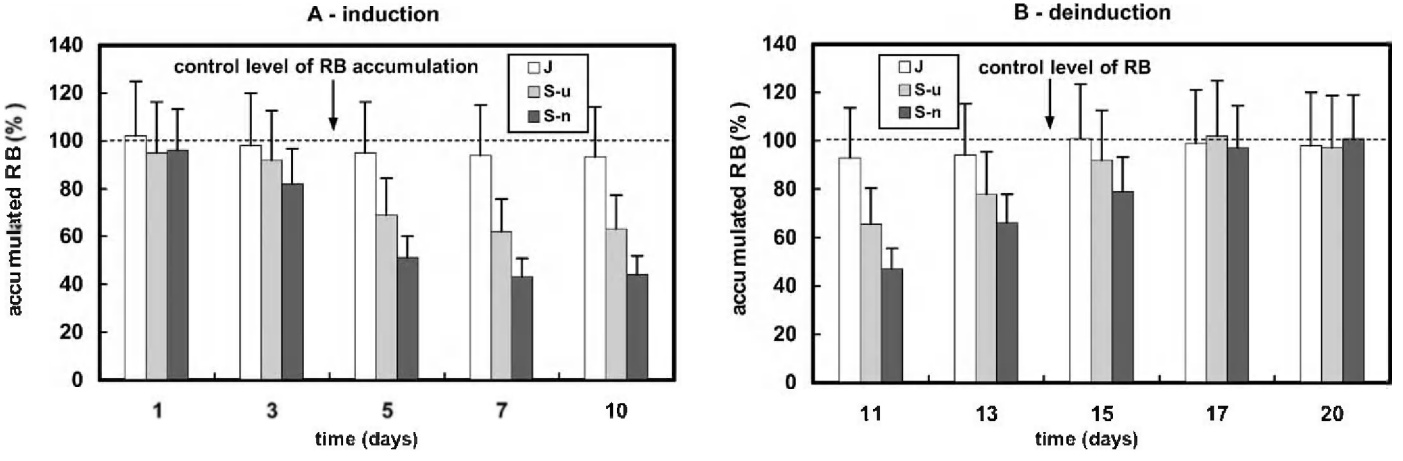

Fig. 8. Determination of the induction and deinduction of MXR activity in *D. polymorpha* using the RB accumulation method after in situ exposure on the investigated freshwater locations. Mussels were cage-exposed exposed on the chosen locations (the S-u, S-d and LJ site) (A: induction), followed by the laboratory exposure to dechlorinated tap water (B: deinduction). The level of Pgp activity was determined every other day during both exposure periods and expressed as described in Fig. 5.

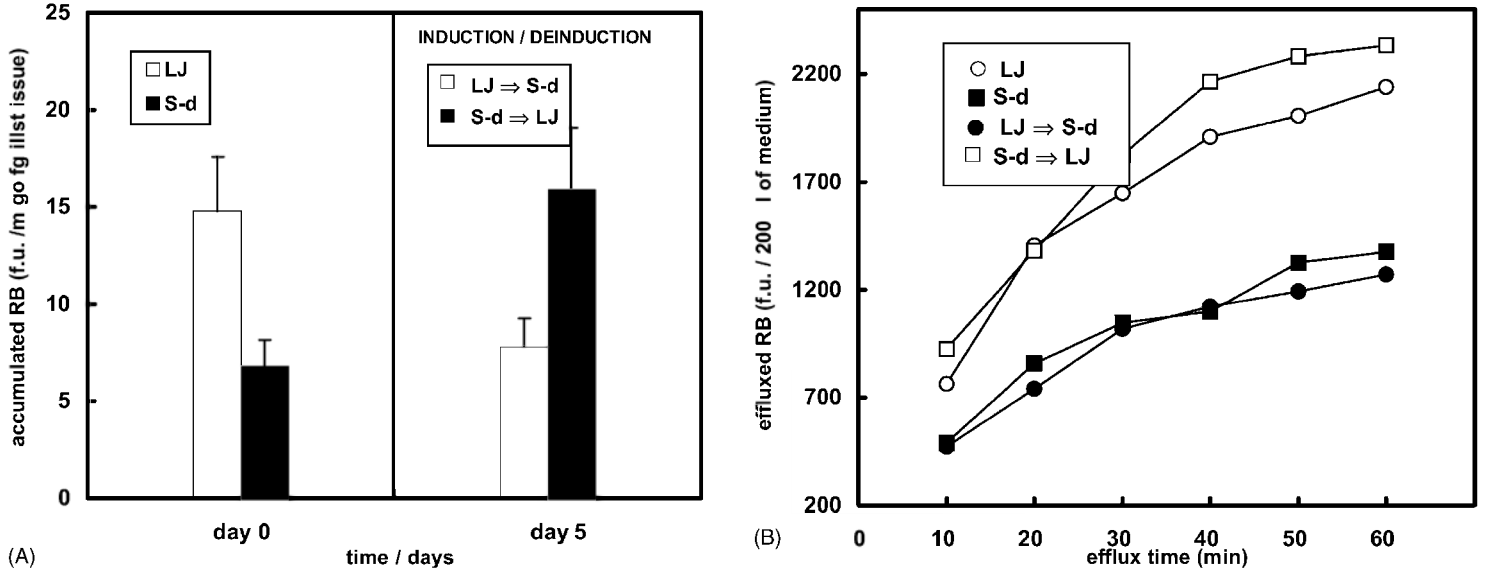

Fig. 9. Determination of the induction and deinduction of Pgp transport activity in *D. polymorpha* exposed in real environmental conditions. The level of Pgp activity was measured in mussels cage-exposed on the most polluted (the S-d site) and unpolluted (the LJ site) freshwater locations, respectively, and in specimens transferred from the S-d site to the LJ-site and inversely, as described in Section 2. Pgp activity was determined using accumulation (A) and efflux (B) version of the method and the results are expressed in fluorescence units (f.u., mean ± S.D., $n = 10$) of RB accumulated/mg of gills tissue (A) or (B) in f.u. of RB effluxed per 200 μl of exposure medium (means from duplicates obtained from replica wells; S.D. < 5%).

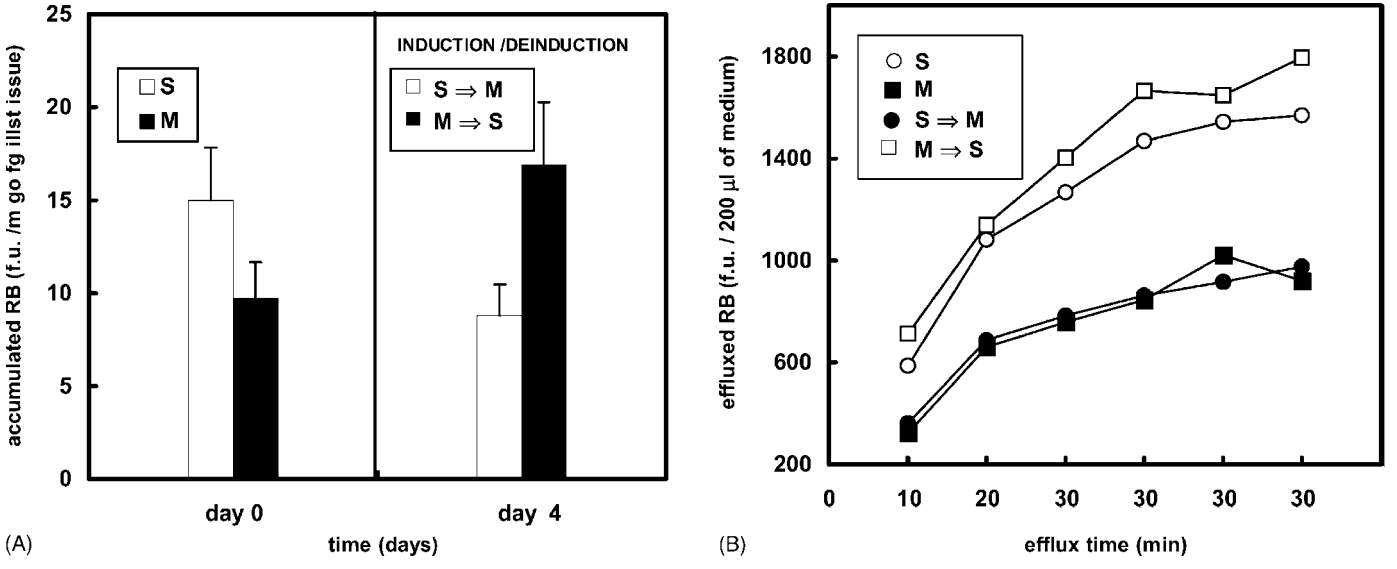

Fig. 10. Determination of the induction and deinduction of Pgp transport activity in *M. galloprovincialis* exposed in real environmental conditions. The level of Pgp activity was measured in mussels exposed on the polluted (the M site) and unpolluted (the S site) marine locations, respectively, and in specimens transferred from the M site to the S site and inversely, as described in Section 2. MXR activity was determined using accumulation (A) and efflux (B) version of the method and the results are expressed as described in Fig. 9.

mussels pre-exposed to the unpolluted water of the LJ site. And inversely, after the transfer to the polluted site mussels pre-exposed at the LJ site significantly induced the Pgp activity up to the level characteristic for the mussels pre-exposed to the polluted water at the S-d location (Fig. 9A and B).

3.4.2. Marine (*M. galloprovincialis*)

The in situ experiments of the induction and deinduction processes in environmental conditions with *M. galloprovincialis* were only performed using the last version of our experiments, i.e. the transfer of mussels from the polluted to the referent unpolluted location, and inversely. However, in comparison to the "freshwater" part of the study, the marine experiments had an important advantage. Namely, at our freshwater locations at the Sava River and the Lake Jarun, there is only a small number of *D. polymorpha* specimens available. Therefore, in order to perform our experiments we had to transfer mussels from our laboratory. But these animals were previously collected from their native habitat at the Drava River (north-western part of Croatia). Unlike that, in the area of the town of Rovinj native populations of *M. galloprovincialis* are abundant at our selected referent locations (the M and S sites). This fact allowed us to perform the induction/deinduction experiments in environmentally more reliable conditions in which our experimental organisms actually live.

The results of these experiments closely correlated with the data from the same type of freshwater experiments. Again, as measured using both methods, the mussels from the polluted M site, which naturally express highly induced Pgp activity, after 4 days of exposure to the unpolluted water at the S site deinduced their Pgp activity almost completely to the level measured at the day 0 in mussels that naturally lived at the S site. On the contrary, during the corresponding exposure period the mussels from the S site induced the Pgp activity up to the level inherent for mussels that naturally lived at the polluted M site (Fig. 10A and B).

4. Discussion

Summarizing all presented results with respect to the goals stated in the Section 1, we have to point out several conclusions that rose from this study. First, it is clear that the functional in vivo detection of Pgp transport activity generated results that almost without exception confirmed the correlation between the level of pollution and the basal level of Pgp activity. Both R123 and D2 were efficient as inducers of Pgp activity in our experimental organisms. As measured using the accumulation and the efflux version of method with RB as the fluorescent model Pgp substrate, the Pgp transport activity became significantly higher on the second day of the exposure and reached its maximum approximately on the days 6–7. The obtained Pgp related MXR induction resulted in significant decrease in the basal RB accumulation in gills of exposed specimens in comparison to the control group, and this process had clear influence on the trend of RB efflux from the gills (Figs. 5–7). These observations were further confirmed using different types of field experiments. The exposure of mussels to the waters at the referent unpolluted and polluted either freshwater (*D. polymorpha*) or marine (*M. galloprovincialis*) locations resulted in data that were well correlated with the results of the laboratory exposure experiments (Figs. 8–10).

Furthermore, the MXR mechanism in aquatic invertebrates was inducible and deinducible just as it is expected from the effective and adaptable defense mechanism. Only 4-day period was long enough for the significant induction and deinduction of Pgp activity, suggesting that a cell/organism will not keep the high level of Pgp activity if it is not necessary. Concerning some recent models that predict a ratio of approximately 1 ATP molecule hydrolyzed per substrate molecule transported (Shapiro and Ling, 1998; Wang et al., 2000), it is certainly a very appropriate strategy for a significant saving of metabolic energy needed for this, basically active, transport of potentially harmful xenobiotics against concentration gradient.

Another important task in this study was the analysis of the possible influence of MXR inhibitors on the induction process. The presence of MXR inhibitors may have a significant influence on the MXR induction and the determination of the dominant type of MXR inhibitors present in investigated waters may point to the source, amount and type of substrates/inhibitors, as well as to the expected induction intensity as well (Gant et al., 1995; Jette et al., 1996; Rekha and Sladek, 1997). However, we did not observe any significant differences in the rate of induction of Pgp activity concerning similar concentration, but different type of

MXR inhibitory potential determined in water samples from our referent polluted locations (Sava river versus Rovinj-Molo, i.e. VER versus CA type of inhibition, Table 1).

MXR phenotype can also be induced as a response to natural factors that are not necessarily related to pollution, like for example by seasonal temperature changes (Chin et al., 1990; Minier et al., 2000; Kepler and Ringwood, 2001), certain hormones (Quian and Beck, 1990), or endogenous substrates and metabolites (Toomey et al., 1996; Smital et al., 1996). In this investigation, which was performed during the 3 years in all seasons except in winter periods, significant differences in Pgp transport activity that could be clearly related to a particular seasonally variable environmental factor were not observed. Some of our data partially indicate that changes in water level in the Sava river and the Lake Jarun, as well as the water temperature shift generally affect the Pgp activity. However, statistically significant, year-by-year correlation between these variations was not observed (data not shown).

The most interesting result of this study is the fact that the relatively high concentrations of inducers used during the laboratory exposure caused no more than 45% (R123) or 49% (D2) decrease in the basal RB accumulation (Figs. 5–7). Comparing these data with in situ experiments with *D. polymorpha* specimens exposed at the referent polluted and unpolluted freshwater sites (Figs. 8 and 9), or with ecotoxicologically more relevant experiments with *M. galloprovincialis* specimens transferred from the unpolluted to the polluted location and inversely (Fig. 10), the level of MXR induction expressed as decrease in basal RB accumulation again was not higher than 50–60%.

This, in general halfway lesser accumulation of a model pollutant (RB) extrapolated to the similar scenario with toxic xenobiotics may have significant environmental relevance. Thou, when compared to well-established biomarkers of exposure, for example the measurement of CYP1A1 induction in fish that results in multiple increases in the enzyme activity, potential for routine use of the determination of Pgp transport activity as a biomarker of exposure seemed doubtful. Namely, considering the obtained "induction range" (0–60%) and the resolution of the method, the statistically correct and sensitive determination of exposure using the functional measurement of Pgp related MXR induction is possible only at locations with markedly different levels of pollution. For example, according to our preliminary calculations for *D. polymorpha* RB accumulation method, which are mainly based on the data obtained during this study (Table 2), using what we considered an acceptable sample size (up to 30 specimens) the minimal detectable difference for one mean is 1.12 fluorescence units (f.u.) and 0.58 f.u. for two means, respectively. It means 16.2 and 19.4% from the expected values by non-induced specimens, and 6.1 and 7.2% for induced specimens, respectively. With these values of minimal significant detectable differences, it is very difficult to make precise decision about possible environmental concentration of MXR inducers without better knowledge of the kinetic of MXR induction. Theoretically, a more sensitive determination would also be possible if we could greatly increase the level of sensitivity and reproducibility of transport measurements. Although it is reasonable to anticipate some methodological improvements, it is hard to expect that it is possible to match so strict goals. Primarily because of numerous reasons that account for the determined variability: among them there are not only technical errors but also the expected interindividual differences in Pgp activity, possible presence of other similar, non-Pgp transport proteins, and the influence of environmental factors as well. Additional possibility that might significantly improve the determination of Pgp mediated MXR induction would be the discovery or development of a better fluorescent model Pgp substrate that would rapidly accumulate in Pgp non-induced cells, to a high basal level so

Table 2
Estimated parameters for the induced and non-induced mussels according to results (expressed and calculated in fluorescence units of RB accumulated/mg of gill tissue) obtained in RB accumulation experiments with *D. polymorpha*

Parameter	Symbol	Induced mussels	Non-induced mussels
Average	$\bar{x}$	6.91	18.50
Variance	var	5.29	4.99
Standard deviation	σ	2.30 (33.3)	2.23 (12.1)
Standard error	σ_M	0.22	0.15
Min. detectable difference (one mean)	d	1.12 (6.1)	1.12 (16.2)
Min. detectable difference (two means)	$d_{\bar{x}_1-\bar{x}_2}$	0.58 (7.2)	0.58 (19.2)

Values in parenthesis are in percentage.

that decrease in its accumulation as a consequence of MXR induction could be measured within significantly greater induction range. Another option is the measurement of MXR induction on the level of basal Pgp specific ATPase activity, combined with the catalytic histochemistry methods for the determination of functionally active proteins (Bleeker et al., 2000).

As a general conclusion we can claim that although this investigation generally confirmed the potential of the described functional measurement of Pgp transport activity, these results also suggest that for the use of the MXR as a relevant biomarker the induction of this defense system has to be measured on various levels—combining the measurement of DNA, mRNA and/or protein expression with the determination of functional Pgp ATPase and/or transport activity.

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