



Review

Emerging contaminants—pesticides, PPCPs, microbial degradation products and natural substances as inhibitors of multixenobiotic defense in aquatic organisms

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Abstract

The environmental presence of chemosensitizers or inhibitors of the multixenobiotic resistance (MXR) defense system in aquatic organisms could cause increase in intracellular accumulation and toxic effects of other xenobiotics normally effluxed by MXR transport proteins (P-glycoprotein (P-gps), MRPs). MXR inhibition with concomitant detrimental effects has been shown in several studies with aquatic organisms exposed to both model MXR inhibitors and environmental pollutants. The presence of MXR inhibitors has been demonstrated in environmental samples from polluted locations at concentrations that could abolish P-gp transport activity. However, it is not clear whether the inhibition observed after exposure to environmental samples is a result of saturation of MXR transport proteins by numerous substrates present in polluted waters or results from the presence of powerful MXR inhibitors. And are potent environmental MXR inhibitors natural or man-made chemicals? As a consequence of these uncertainties, no official action has been taken to monitor and control the release and presence of MXR inhibitors into aquatic environments. In this paper we present our new results addressing these critical questions. Ecotoxicological significance of MXR inhibition was supported in *in vivo* studies that demonstrated an increase in the production of mutagenic metabolites by mussels and an increase in the number of sea urchin embryos with apoptotic cells after exposure to model MXR inhibitors. We also demonstrated that MXR inhibitors are present among both conventional and emerging man-made pollutants: some pesticides and synthetic musk fragrances show extremely high MXR inhibitory potential at environmentally relevant concentrations. In addition, we emphasized the biological transformation of crude oil hydrocarbons into MXR inhibitors by oil-degrading bacteria, and the risk potentially caused by powerful natural MXR inhibitors produced by invasive species.
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1. Introduction

Numerous studies performed during the last decade support the proposed role of the multixenobiotic

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resistance (MXR) [1] mechanism as a general, broadly distributed biological system in aquatic organisms used as a “first line of defense” [2] against endogenous and exogenous toxicants. MXR results from the rapid efflux of a wide variety of potentially toxic xenobiotics out of the cell by various transmembrane transport proteins. But a problem with this defense system is its remarkable sensitivity to environmental chemicals which can act as specific inhibitors. These chemicals have the potential to block this active efflux of xenobiotics, causing a significant increase in their intracellular accumulation. From an ecotoxicological viewpoint, the main consequence of inhibition is an increase in chemosensitivity of aquatic organisms toward the many xenobiotics typically present in aquatic environments. Based on these characteristics, these seemingly innocuous chemicals can be envisioned as a new class of environmentally hazardous chemicals which are termed MXR inhibitors or chemosensitizers [3,4].

Although MXR inhibitors are present in the environment at concentrations that could have ecotoxicological impact [3–7], no action has been taken in the regulatory arena to monitor and control their release and presence in aquatic environments. Two factors are responsible for this lack of recognition. The first is the still embryonic nature of work on MXR. Although several labs working in this area has provided much evidence about MXR in aquatic organisms [8] many crucial molecular biological and mechanistic features of MXR in aquatic life are still unknown. Another reason is the lack of *in situ* scientific evidence about the ecotoxicological relevance of MXR function and/or inhibition.

The second factor is that the presence of MXR inhibitors has been mainly demonstrated in environmental samples from polluted locations. It is thus not clear whether the observed inhibition is a consequence of competitive saturation of MXR transport proteins by the large number of chemicals present in polluted waters [9] or whether it results from the presence of only a few powerful MXR inhibitors. It is also unclear whether potent chemosensitizers are natural products or man-made substances still unrecognized for their unique property to inhibit this crucial cellular defense system. Moreover, are they conventional or are they rather so-called emerging contaminants [10]?

In this article we present our results addressing these issues. Special emphasis was given to the

demonstration of the ecotoxicological significance of MXR transporters as well as to identification of MXR inhibitors among anthropogenic contaminants. New research needed for scientifically reliable explanation of a general role of MXR and significance of chemosensitizers is proposed and methods and approaches for the determination of MXR inhibitory potential of chemicals and environmental samples are discussed.

2. MDR/MXR phenotype in aquatic organisms

The MXR phenotype in aquatic organisms is mediated by the transport activity of transmembrane proteins belonging to the ATP binding cassette (ABC) [11,12] superfamily, which are primarily involved in the active, ATP-dependent transport of biological molecules across plasma membranes. The best studied ABC protein is the P-glycoprotein (P-gp), a large protein found in the cell membrane and capable of transporting drugs and other xenobiotic compounds out of the cell conferring what is now termed the multidrug resistance (MDR) phenomenon [13,14]. Since the phenomenon of P-gp-mediated MDR is a major obstacle in cancer chemotherapy, it has been characterized in detail [15,16].

One of the most remarkable features of P-gp is its broad substrate recognition. The list of compounds transported by P-gp currently includes many structurally unrelated drugs and anticancer agents such as the vinca alkaloids and anthracyclines, drugs against human immunodeficiency virus (HIV), fluorophores [16] as well as typical environmental pollutants [3,8]. The basis for such broad substrate specificity has not yet been elucidated and the only common features of P-gp substrates appear to be moderate hydrophobicity, low molecular weight and the presence of a basic nitrogen atom in generally cationic or neutral molecules [15–17]. As a consequence, the number of potential P-gp substrates could be extended by several hundreds [18].

The second major ABC protein involved in MDR, described in 1992 by Cole et al. [19] is the multidrug resistance-associated protein (MRP) which has only 15% amino acid homology with P-gp. Nine MRP homologues termed MRP1-9 have since been identified [20]. As with all ABC transporters, the

actual mechanism of the MRP-mediated transport is not fully understood and its substrate specificity is similarly difficult to define. Apart from some overlapping in substrate specificity with P-gp, MRPs appear to be most effective in effluxing organic anions, glutathione, glucuronide and sulfate-conjugated compounds [16,21]. The best understood members of the MRP family are MRP1 and MRP2 and studies of their functional properties show that MRP1 transports mainly glutathione conjugates, whereas MRP2 is more specific for organic anions. Evidence from in vitro and in vivo investigations indicate that like the P-gp, MRP1, MRP2 and possibly other MRPs have a role in protecting tissues from toxin-induced damage, with active efflux through MRPs as an integral and important aspect of the cellular detoxification machinery preventing accumulation of putatively toxic conjugated and unconjugated compounds [16,22].

During the last decade it was realized – through identification of its elements by immunochemical and genetic approaches as well as direct assays of transport activity – that many aquatic organisms express one or more elements of the classical MDR phenotype. As a result, P-gp-related genes, proteins and/or the classical pattern of verapamil-sensitive transport activity have been found in almost 40 marine or freshwater species [8]. Importantly, these MDR-related elements have been detected in tissues most exposed to, or critical for, the detoxification or excretion of endo- or exogenous xenobiotics (gills, liver, hepatopancreas, kidney, intestine). Moreover, numerous laboratory or field studies have reported induction of P-gp transport activity and/or protein titer in a response to organic pollution [23,24].

Recent studies demonstrate MRP-related genes or proteins in fish species such as Atlantic killifish (*Fundulus heteroclitus*), dogfish shark (*Squalus acanthias*) and red mullet (*Mullus barbatus*) [25–27]. Using a standard calcein-AM transport assay [52] we also found that the resistance of sea urchin embryos is likely to be mediated by an MRP-like transporter. Calcein-AM enters unfertilized eggs indicating no MRP activity and within 25 min of fertilization the calcein accumulation stops. However, calcein accumulation can then be restored by treating the embryos with the MRP-specific inhibitor MK571 (Fig. 1). These results suggest that MRP transporters appear in sea urchin embryos after fertilization and may have a role in embryo protection.

These observations suggest that the various MDR efflux transporters (P-gps, MRPs, and possibly others) simultaneously contribute to resistance towards many xenobiotics. It is based on this evidence that the MDR-like phenotype in aquatic organisms is referred to as MXR. Thus, MXR may be related to the fact that many aquatic species survive and reproduce successfully in polluted environments, often with significantly lower tissue levels of contaminants than the surrounding environment.

3. MDR/MXR inhibition

The MXR defense is compromised by its sensitivity to inhibition by its substrates. Although broad substrate specificity appears to be advantageous in dealing with a wide range of potentially toxic chemicals, this property contains the danger of saturation of

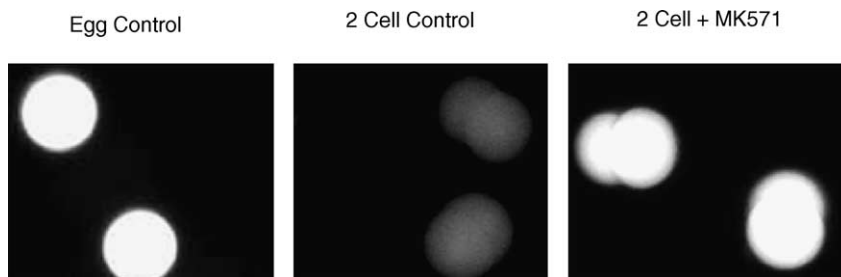


Fig. 1. A standard calcein-AM (Ca-AM) transport assay demonstrates that multidrug transport is initiated at fertilization in the sea urchin. Non-fluorescent extracellular Ca-AM (250 nM) is an MRP substrate that accumulates and is converted to its fluorescent form (calcein) by intracellular esterases in eggs. In two cell embryos, the same concentration of Ca-AM is excluded from cells, but that exclusion is reversed by addition of the MRP-specific inhibitor MK571 (10 μ M).

the transporter when too many substrates are present which is then evidenced as an inhibition of activity. Thus, using in vitro and in vivo screening, the presence of substances which inhibit P-gp transport has been found in environmental samples such as marine and freshwater concentrates; sediment, soil, and industrial or household waste extracts; hospital waste; Diesel-2 oil and algal extracts [3,7,28–30].

The MDR/MXR inhibitors can be divided into two main classes. The first are competitive inhibitors which act as substrates with high affinity for P-gp binding sites, preventing binding and active transport of other substrates/xenobiotics. A typical example is verapamil. The second class are non-competitive inhibitors which can act in various ways, for example by blocking the P-gp ATPase activity that is necessary for ATP-mediated transport of xenobiotics across the plasma membrane. A typical example is cyclosporine A [31,32]. Based on these features verapamil and cyclosporin A are frequently used as positive controls in P-gp transport activity experiments.

4. Ecotoxicological significance of MXR inhibition

Regardless of the type of MXR inhibitors, the primary consequence of their presence in the aquatic environment is an increase in intracellular accumulation of other xenobiotics (of both endogenous and exogenous origin). The addition of even one non-toxic compound, either an extremely good substrate for P-gp or an effective non-competitive inhibitor of Pgp-ATPase, to an already polluted ecosystem could cause an increase in toxic effects in the exposed species. The critical point is that such toxic effects would be unexpected or unexplainable because the levels of known toxic substances were constantly below the established toxic thresholds.

Several studies with aquatic organisms clearly demonstrate that this might be the case: both model MDR inhibitors and environmental pollutants can cause a significant increase in the accumulation of P-gp substrates. In vivo experiments with clams, mussels, sponge cubes, marine worms, sea urchins, etc., demonstrated that exposure of aquatic organisms to P-gp inhibitors such as verapamil, cyclosporin A or staurosporine, or the model pollutant Diesel-2

oil, leads to multifold increases in accumulation of model substrates such as vincristine, rhodamine 123, rhodamine 6G, rhodamine B or calcein-AM. Even exposure to polluted water or extracts from polluted waters was able to significantly elevate the accumulation of model substrates [3,8], confirming the presence of MXR inhibitors in the environment.

Evidence showing laboratory or in situ enhancement of MXR inhibition with concomitant detrimental effects has been provided in several studies. For example, staurosporine (0.5 μM) inhibited MXR in a fresh water clam *Corbicula fluminea* and switched the no observed effect concentrations (NOEC) of acetylaminofluorene (AAF, 0.01 μM), as measured by alkaline filter elution detection of single strand breaks (SSB) in DNA, to the observed effect concentrations (OEC) equivalent to that caused by an order of magnitude higher (0.1 μM) concentration [33]. Clams freshly collected from the Rhine River, in contrast to control clams (held in fresh aquarium water for 6 weeks), did not induce SSB when exposed to OEC of AAF (0.01 μM), probably due to a lower accumulation of AAF. In another study, exposure of sponge tissue (*Geodia cydonium*) to seawater spiked with AAF for 2 h induced more AAF–DNA adducts in the presence of 5 μM verapamil than in sponge exposed to AAF without verapamil [34].

In freshwater mussel *Dreissena polymorpha* exposed to water spiked with 2-aminofluorene (AF) in the presence of 10 μM cyclosporin A, a non-competitive, P-gp-ATPase inhibitor, production of mutagens in exposure medium was enhanced 460% compared to mussels exposed to AF without cyclosporin A [35]. In a recent study, we observed almost the same pattern with the marine mussel *Mytilus galloprovincialis*. The exposure of *M. galloprovincialis* to AF resulted in a time- and concentration-dependent excretion of mutagenic metabolites into the surrounding water (as assessed in hexane concentrates of the water). However, when mussels were exposed for 1–3 h to water spiked with the same AF concentration (50 μM) in the presence of the MXR inhibitors verapamil and cyclosporin A, the amount of mutagens excreted was increased by 3- to 5.5-fold in a time-dependent manner (Fig. 2A). And this increase in production and excretion of mutagens was clearly dependent on the concentration of MXR inhibitors (Fig. 2B). Presumably, the inhibition of P-gp

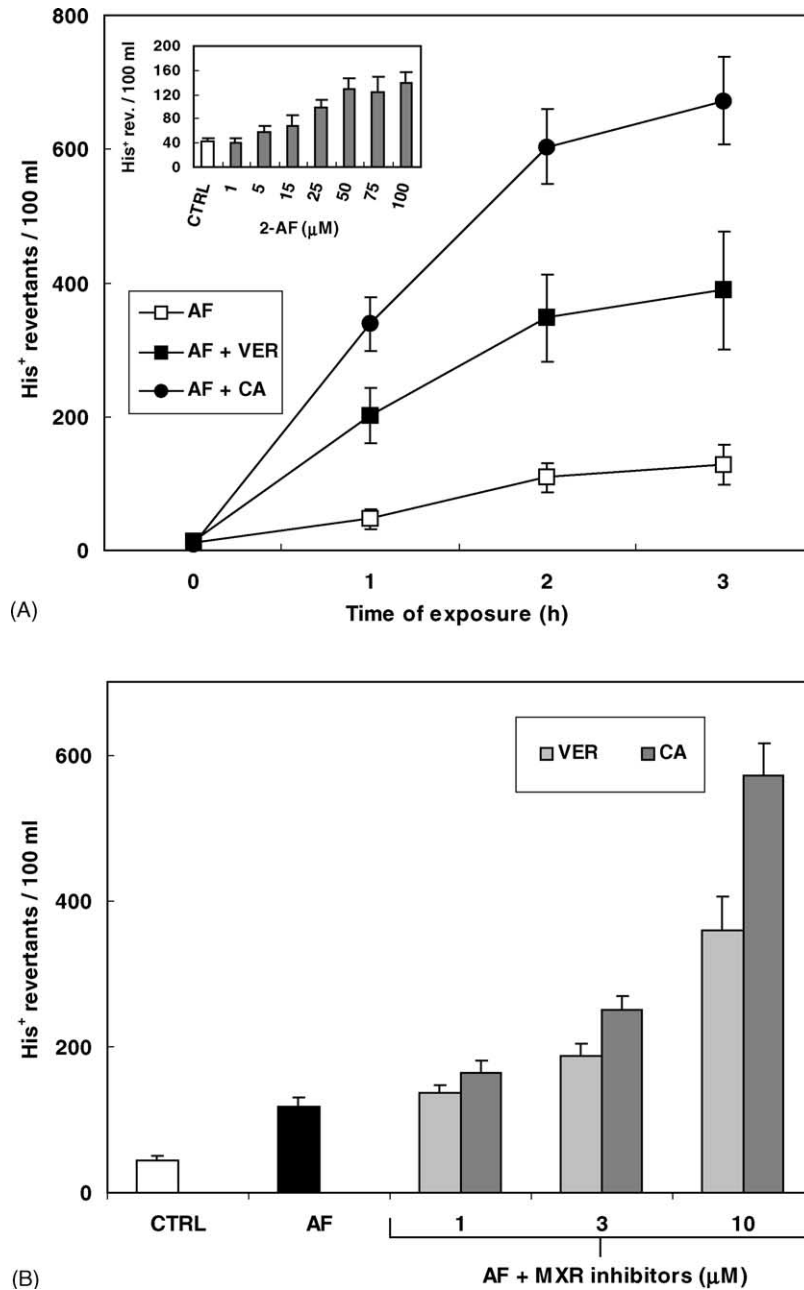


Fig. 2. The modulation of the genotoxic effect of AF in the presence of model MXR inhibitors. (A) The effects of verapamil (VER, 10 µM) or cyclosporin A (CA, 5 µM) on the production/excretion of mutagens into exposure-water was determined after exposure (1–3 h) of 10 specimens of the marine mussel *M. galloprovincialis* in seawater (1 L) spiked with AF (50 µM); Dependence of the excretion of *M. galloprovincialis* mutagens (2 h exposure) on the concentration of AF (inner figure). (B) Dependence of the excretion of mutagens into the exposure water spiked without (CTRL) or with AF (50 µM) on the concentration of VER or CA. In all cases mutagenicity of hexane extracts of exposure-water was determined by the Ames-mutation test. Data are expressed in the number of *S. typhimurium* TA98 His⁺ revertants per 100 ml of exposure water with determinations done in triplicate. Heights of bars (mean ± S.D.) represent the average results from two independent experiments.

transport activity resulted in increased intracellular concentration of AF, which in turn allowed more AF metabolic conversion into a more hydrophobic mutagenic metabolite(s) [36] that was readily excreted to the surrounding water. These results are in accordance with previous studies that demonstrated a high FAD-containing monooxygenase activity in the post-mitochondrial fractions of the marine mussel *Mytilus edulis* or *M. galloprovincialis* digestive gland. These fractions expressed the potential to bioactivate aromatic amines (like AF) to bacterial mutagens, and this potential was inhibited by methimazole, a selective FAD-dependent monooxygenase inhibitor [37,38]. Therefore, the presence of MXR inhibitors in waters already polluted with pre-mutagenic xenobiotics might ultimately enhance not only the genotoxic risk in the exposed mussels but also the mutagenic risk in the environment in which the exposed organisms live.

A second line of evidence comes from investigations that analyzed the consequences of MXR inhibition during embryonic development of the marine worm *Urechis caupo*. In these studies, exposure of embryos to emetine or vinblastine concentrations that normally do not affect cell division resulted in significantly lower cell division rates in the presence of verapamil. Embryos exposed to 0.2 μM emetine in the presence of verapamil showed a 2- to 3-fold decrease in cell division, and 4 μM emetine depressed cell division by 4-fold. The effect of vinblastine in the presence of verapamil was even stronger: co-incubation of embryos with verapamil and 0.5 μM vinblastine caused a 350-fold decrease in the cell division rate [28]. Similar deleterious effects were observed in *M. edulis* embryos exposed to vinblastine, mitomycin-C, cytochalasin D, chloroquine, or colchicines: exposures in the presence of verapamil increased the frequency and severity of embryonic deformities [39].

Other studies have used apoptosis as a critical effect endpoint. One study by Schröder et al. [40] showed the induction of apoptosis in the marine sponge *Geodia cydonium* by exposure to normally non-toxic doses of the common water pollutant tributyltin (1 μM) in the presence of non-toxic doses of an extract of the marine algae *Caulerpa taxifolia*. These extracts were previously found to inhibit the P-gp-mediated efflux of rhodamine B from a MDR cell line [38] and presumably the enhancement of apoptosis ensues from this P-gp inhibition.

We recently found that sea urchin embryos demonstrate a higher incidence of cell death when inducers of apoptosis are combined with model MXR inhibitors, such as verapamil and reversin 205. Etoposide, a topoisomerase II inhibitor and classical apoptosis inducer, shows almost no effect on embryo DNA quality at 5 μM concentrations, but when added in combination with low concentrations of verapamil (0.1–1 μM) or reversin 205 (0.1–2 μM), there are dramatic alterations of blastomere DNA, including fragmentation and chromosome bridging. Additionally, co-exposure experiments show greater than a 10-fold increase in the number of embryos with apoptotic cells (Fig. 3A and B). These findings indicate that a functional apoptotic system is present in embryos and that the apparent resistance to cell death from nominal apoptosis-inducing agents might result from a vigorous efflux drug transporter system present in these embryos. MXR transporters may therefore be one mechanism responsible for apoptosis-resistance during early development of sea urchins and indeed may be a general mechanism in embryos to avoid genotoxic damage during development.

5. MXR inhibitors among conventional and emerging contaminants

All these examples support the general hypothesis that MXR inhibitors are a possible threat to the stability of aquatic ecosystems and this is further supported by studies demonstrating high concentrations of MXR inhibitors in environmental samples. Expressed in μM equivalents of model MXR inhibitors (verapamil or cyclosporin A) these concentrations are comparable to levels used in laboratory experiments that resulted in almost complete inhibition of P-gp transport activity and large increases in intracellular accumulation and effects from toxic xenobiotics [3].

These studies still do not tell us anything about the chemical identity of MXR inhibitors in environmental mixtures. Is measured inhibitory potential the additive effect of many P-gp substrates present in a given sample, resulting in a saturation of efflux transporters and subsequent increase in accumulation of model substrates? Or does inhibition result from the presence of one or a few high affinity competitive or efficient non-competitive MXR inhibitors?

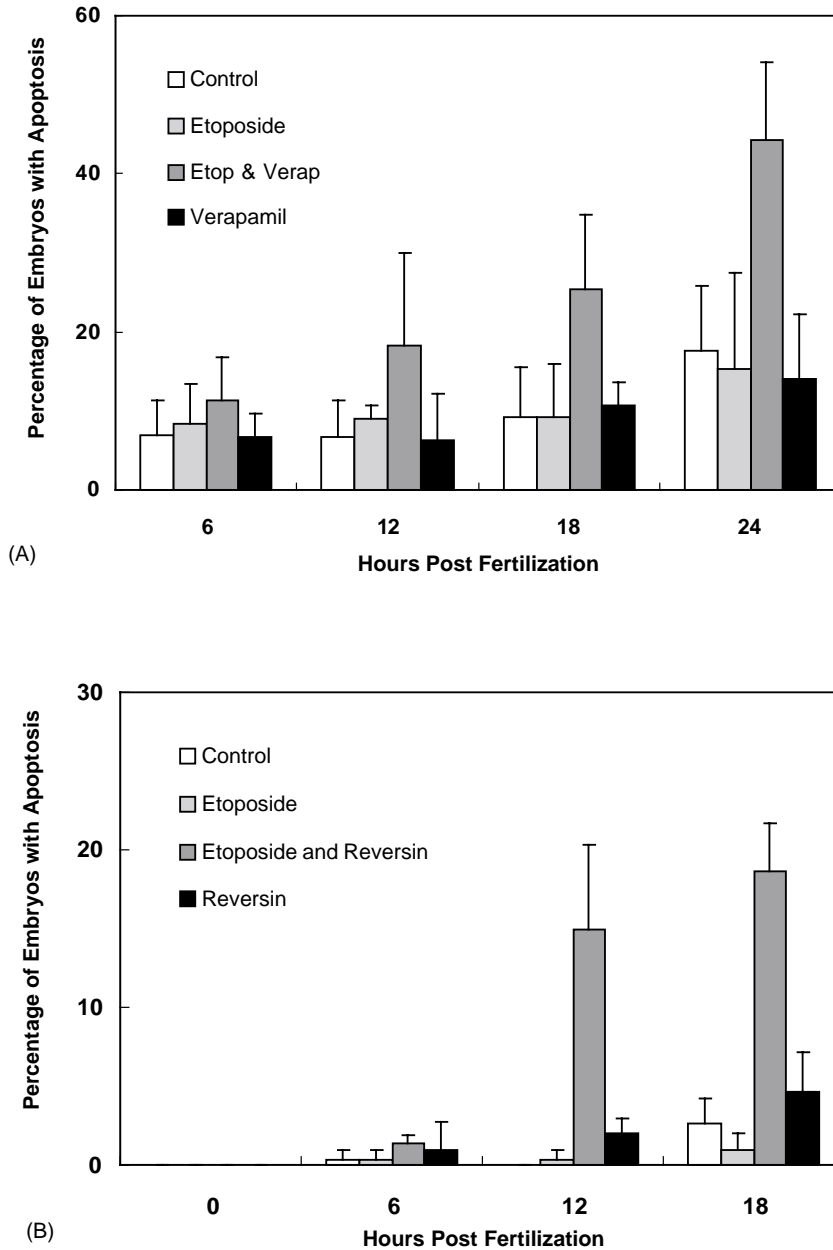


Fig. 3. The effect of model MXR inhibitors on apoptosis in sea urchin embryos. Four-cell embryos were exposed to etoposide (5 μM), a topoisomerase II inhibitor and well known apoptosis inducer alone and in combination with (A) verapamil (0.75 μM) or (B) reversine 205 (1 μM). Embryos were collected and fixed in 4% formaldehyde, 80% sea water, and 100 mM HEPES every 6 h after fertilization until the hatching stage. Two hundred fixed larvae were assayed at each time point for both DNA fragmentation (TUNEL) and caspase activation. Additionally, embryos were scored for DNA alterations such as chromosome bridges and mitotic catastrophe.

Both possibilities are equally important from an ecological viewpoint. However, looking from the regulatory and legislative perspective it is far more important to find out whether or not powerful MXR inhibitors (1) exist in aquatic environments and (2) if they are of anthropogenic origin. And finally, if the answers are positive, are they present in conventional priority pollutants (agrochemicals and various persistent industrial intermediates), and/or in pharmaceuticals and personal care products (PPCPs) as so-called emerging contaminants as well? In the next section, we present results that address these issues and show that potent MXR inhibitors are indeed present in both categories.

5.1. Pesticides

Although pesticides are among the most important environmental pollutants, produced and released into the environment in huge quantities, interactions between P-glycoprotein and pesticides have only recently been reported. In vivo studies by Schinkel et al. [42] showed that knockout mice lacking the *mdr1a* gene were sensitized to the neurotoxic pesticide ivermectin. The increased toxicity resulted in higher pesticide accumulation in both the brain and the liver and a decreased rate of elimination compared to control mice with normal *mdr1a* function. In another study, treatment of the tobacco budworm *Heliothis virescens*, which is resistant to most pesticides, with the P-gp inhibitor quinidine in the presence of the pesticide thiodicarb strongly decreased the LD₅₀, suggesting the involvement of P-gp in pesticide resistance [43,44]. Using a fluorescent dye assay, Galgani et al. [45] showed that four moderately hydrophobic pesticides (dachtal, chlorbeside, sulfallate and pentachlorophenol) inhibited the efflux of dye from the gills of the marine mussel *M. galloprovincialis*, indicating their interaction with MXR transport system in this organism. In 1996 Bain and LeBlanc [46] conducted an extensive in vitro study using murine melanoma cells transfected with the human *MDR1* gene. Thirty-eight pesticides, representing several classes of compounds, were evaluated for their potential to bind to P-gp. Although their results demonstrate that binding to P-gp does not necessarily imply transport by P-gp, they clearly showed that some pesticides have high MXR inhibitory potential.

Based on those data we recently chose 15 commonly used pesticides from several chemical classes and with different mode of action (Table 1), to assess their ecotoxicological relevance as MDR inhibitors. However, our approach was in several important ways different from that used by Bain and LeBlanc [46]. First, we used an ecotoxicologically more reliable in vivo method for the determination of P-gp transport efficiency. Second, the concentrations of pesticides used in our study were environmentally more realistic and never exceeded declared LD₅₀ values (Table 1). And third, we compared effects of the chemically pure substances with the commercially available pesticides that contain the active compound in combination with the carrier, prepared according to manufacturer's protocols. Our results revealed high MXR inhibitory potential of several pesticides. As can be seen in Fig. 4, exposure of *D. polymorpha* specimens to pirimicarb, endosulfan, chlorpyrifos-methyl, malathion or dichlorvos resulted in a rapid increase in rhodamine B accumulation in gills, in some cases even at concentrations far below established LD₅₀ values. Other pesticides (diazinon, phosalone, dimethoate, glyphosate, methomil, propiconazole, 2,4-D, malic hidrazid, fenoxycarb, furathiocarb) showed moderate or weak MXR inhibitory potential. Finally, some pesticides expressed higher MXR inhibitory effect as commercial mixtures than when tested in pure chemical form (Table 1). This observation raises the disturbing question regarding unknown synergistic interactions between "carrier" substances and active chemicals and this possibility should be the subject of additional research.

None of the above studies have yet addressed the role of MRP-type xenobiotic transporters for pesticide resistance in aquatic organisms. GSH conjugates of many agrochemicals could be substrates or inhibitors for MRP transporters. Supporting this idea, Leslie et al. [22] demonstrated that metolachlor-SG, a herbicide used in large quantities in the US, is a competitive inhibitor of leukotriene C₄ which is a high affinity MRP1 and MRP2 substrate.

5.2. Fragrances

Synthetic musk fragrances, widely used as inexpensive fragrances in personal care products (cleaning agents, air fresheners and other hygiene and house-

Table 1
Commercially used pesticides tested for MXR inhibitory potential

| Name | Target (mode of action) | Reported LD ₅₀ for aquatic organisms | Maximum experimental concentration (μM) | Relative MXR inhibitory potential commercial/pure |
|---------------------|-------------------------|---|---|---|
| Diazinon | i | ^a 10.5–77 μM ^b 8.5–10.5 μM ^c 8.2–23.5 μM | 7 | ++/∅ |
| Diclorvos | i | ^a 1.03 μM ^b 0.9 μM ^c 0.045–0.32 μM | 1 | ++/++ |
| Chlorpyrifos-methyl | i | ^b 8.6–31.4 μM | 20 | ++/++ |
| Phosalone | i | ^b 0.82–1.71 μM | 1 | ++/+ |
| Malathion | i | ^a 7.57 μM ^b 0.52 μM ^c 0.1–9.1 nM | 3 | ++/++ |
| Dimethoate | i | ^a 20.28 μM ^b 37.1 μM ^c 27.9 μM | 15 | +/∅ |
| Glifosphate | h | ^b 0.31–1.3 mM ^c 4.61 mM | 100 | ++/ND |
| Methomil | i | ^b 20.96 μM ^c 0.28 μM | 3 | +/∅ |
| Pirimicarb | i | ^b 121.8 μM | 100 | ++/++ |
| Endosulfan | i | ^a 3.68 nM ^b 73.7 nM ^c 0.15–1.82 μM | 0.1 | ++/++ |
| Propiconazole | f | ^a 292.2 μM ^b 58.44 μM | 50 | +/∅ |
| 2,4-D | h | ^a 23.1–434.3 μM ^b 1.2 mM ^c 0.16–1.76 mM | 100 | ∅/∅ |
| Malic hidrazid | r | ^b 8.92 mM ^c 8.92 mM | 100 | +/+ |
| Fenoxycarb | i | ^a 34.2 μM ^b 5.3 μM ^c 1.33 μM | 10 | +/+ |
| Furathiocarb | i | ^a 0.31 μM ^b 0.08 μM ^c 4.7 nM | 0.1 | ∅/∅ |

i, Insecticide; h, herbicide; f, fungicide; r, growth regulator. Relative MXR inhibitory potential was expressed as the increase (% above control) in rhodamine B accumulation. In all experiments model MXR inhibitor cyclosporin A was added as a positive control. All pesticides are tested both as commercial mixtures prepared according to manufacturers protocols and as pure chemicals, respectively. Symbols (++) denote ≥ 70%; symbols (+) denote 30–69%, and symbol (∅) denote 0–29% increase in rhodamine B accumulation; ND, no data.

^a Common carp (*Cyprinus carpio*), 96 h acute toxicity test.

^b Rainbow trout (*Oncorhynchus mykiss*), 96 h.

^c *Daphnia magna*, 48 h.

hold products), are ubiquitous pollutants in aquatic environments and have received increasing attention in recent years [47,48]. Although of low toxicity, their persistence and ability to bioaccumulate have raised concerns about them as environmental pollutants and also for human health. Their main entrance path into the environment is through sewage treatment plants.

Since synthetic musks are small, moderately hydrophobic compounds, they are potential candidates as substrates or inhibitors of MXR transporters [10,49].

To test this hypothesis, gill tissue of the California mussel (*Mytilus californianus*), which contains a high titer of the MXR transporter P-glycoprotein, was incubated with musks and rhodamine B.

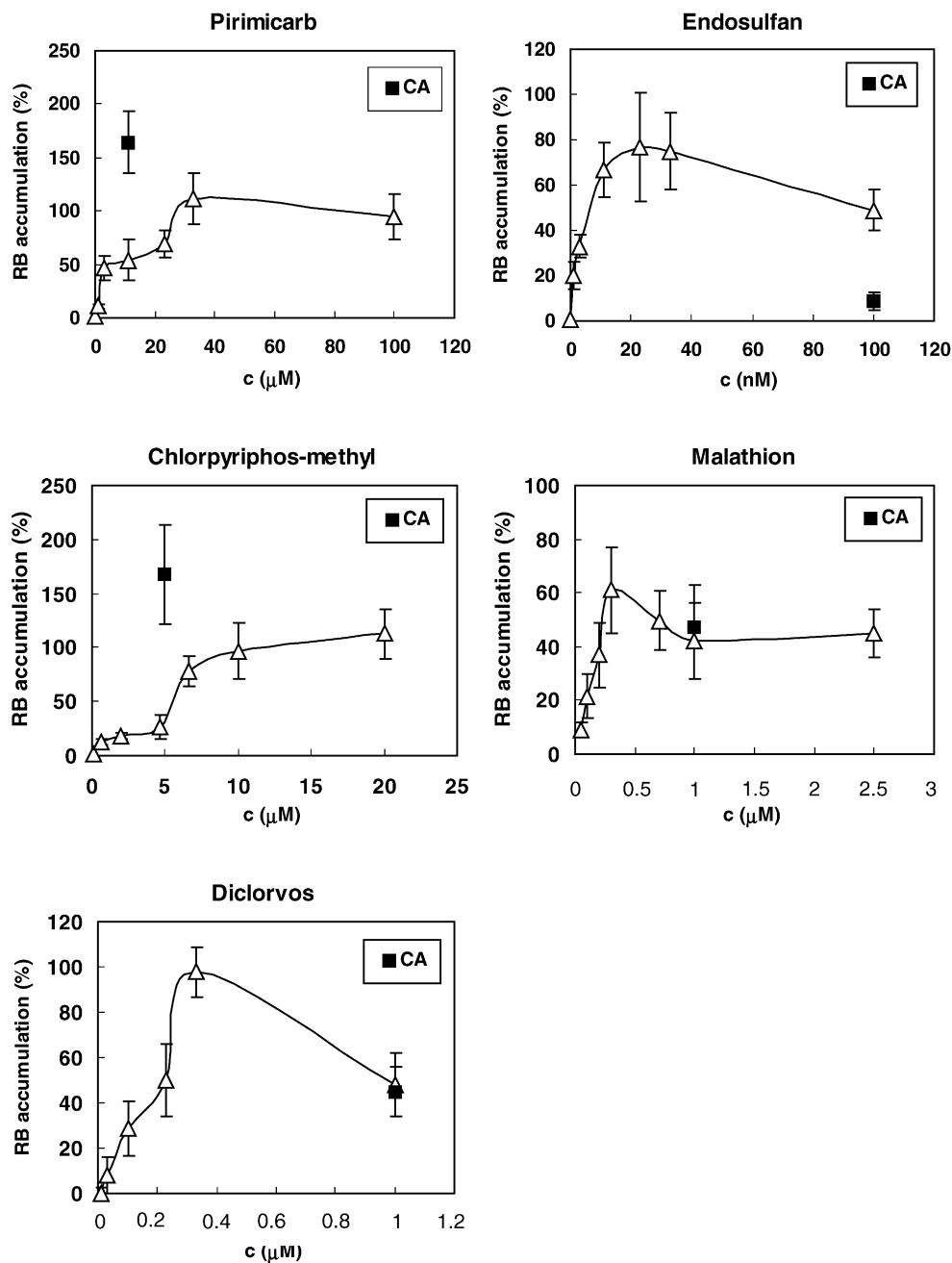


Fig. 4. The determination of the MXR inhibitory potential of pesticides. The MXR inhibitory effect was determined by the exposure (2 h) of the freshwater mussel *Dreissena polymorpha* ($n = 7$) in dechlorinated tap water (150 mL) spiked with fluorescent MXR substrate rhodamine B (RB, 2.5 μM), without (control) or with the addition of indicated concentrations of pesticides or model MXR inhibitor cyclosporin A (CA). The amount of RB accumulated in gills tissue was fluorescently determined at the end of the exposure period. MXR inhibitory effect of pesticides or CA resulted in increase in RB accumulation above control level (considered as 0%). Each pesticide was tested in 3–4 independent experiments. Symbols (mean \pm S.D.) represent results of a typical experiment.

In the presence of all musks tested (musk ketone, musk xylene, Galaxolide™ (1,3,4,6,7,8-hexahydro-4,6,6,7,8,8-hexamethylcyclopenta(g)-2-benzopyrane; HHCB), Celestolide™ (4-acetyl-1,1-dimethyl-6-*tert*-butylindan; ADBI), Tetralide™ (7-acetyl-1,1,3,4,4,6-hexamethyl-1,2,3,4-tetrahydronaphthalene; AHTN), Traseolide™ (5-acetyl-1,1,2,6-tetramethyl-3-isopropylindane; ATII), accumulation of rhodamine B in the gill tissue significantly increased, indicating inhibition of MXR transporter activity by the musks. Effective concentrations were at the 10^{-6} M range which is similar to quinidine, a known MXR inhibitor [50]. Thus, inhibition of MXR transporter activity by synthetic musks may enhance the toxic potential of other MXR substrates by enabling them to accumulate in the cell.

Furthermore, we found that inhibitory effects in gill tissue persisted 1–2 days after exposure to synthetic musks (Fig. 5). Long term inhibition of MXR transporter activity by environmental pollutants has not

been shown before and is an unanticipated and important aspect when evaluating environmental pollutants such as MXR specific chemosensitizers. These data additionally support the need to test “non-toxic” chemicals for indirect actions, e.g. as chemosensitizers, to fully understand their potential environmental impact.

5.3. Microbial degradation products as MXR inhibitors

Most compounds are relatively unstable in the environment and undergo physical and biochemical transformations that dramatically alter their bioavailability and toxicity [51]. In addition to relatively predictable transformations that occur as a consequence of exposing chemicals to air, heat, light and water, natural compounds and xenobiotics in the aquatic environment also undergo transformations as a consequence of their metabolism by bacteria. One

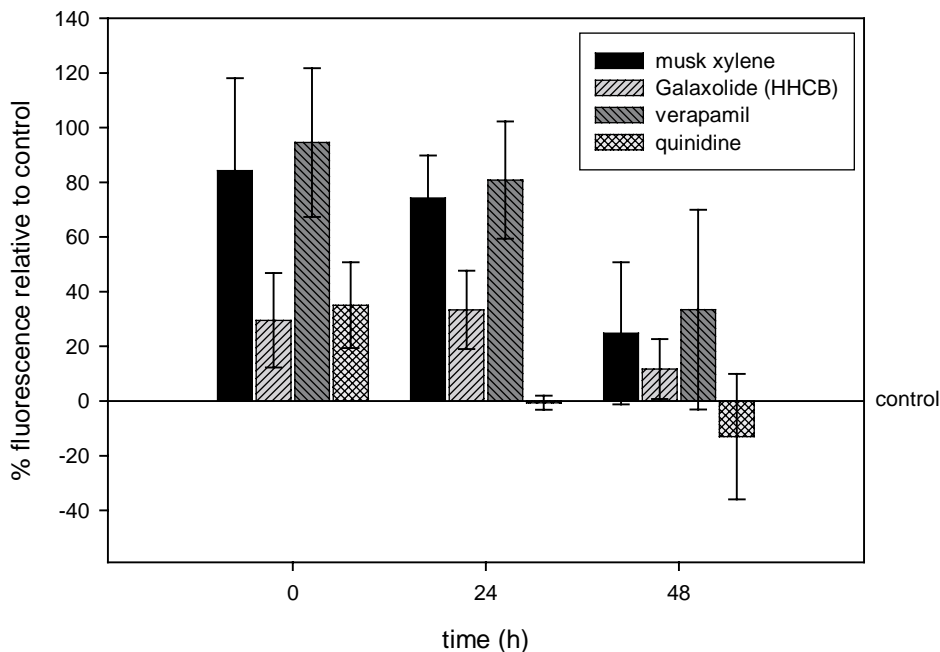


Fig. 5. Longterm inhibition of MXR transporter activity in gill tissue of the California mussel (*Mytilus californianus*) by synthetic musks and the known MXR inhibitors verapamil and quinidine. The values are relative to the control (set at 0). Values > 0 indicate increased fluorescence due to higher accumulation of rhodamine B in the tissue and therefore lower transporter activity. Gill tissue discs were incubated in filtered seawater (FSW) with or without test compounds at $1 \mu\text{M}$ for 2 h at 15°C . MXR transporter activity was measured in treated and untreated tissue discs immediately after the exposure (0h) and after 24 h and 48 h of washing in FSW. To measure MXR transporter activity, gill tissue discs were exposed to $1 \mu\text{M}$ rhodamine B for 90 min. The amount of rhodamine accumulated in the tissue was determined by measuring the fluorescence of a butanol extract of the tissue discs at 545 nm (excitation) and 575 nm (emission).

example is the biological transformation of crude oil hydrocarbons into MXR-reversing compounds by oil-degrading bacteria [52]. These hydrocarbons enter the marine environment as a result of natural seepage and as a consequence of human activities. Naturally occurring bacteria rapidly convert these large, water-insoluble hydrocarbons into smaller, more water-soluble byproducts that are potent cytotoxins [52,53]. Paradoxically, many organisms such as those living around crude oil seeps tolerate these compounds [54] and MXR-like mechanisms might be part of this protection. We found that microbially degraded oil hydrocarbons are high affinity P-gp substrates in *U. caupo* larvae (Fig. 6) and are tolerated by these embryos and larvae, but not by sea urchin embryos with low levels of P-gp activity [52]. This finding illustrates the importance of the efflux transporters and also is consistent with the life history of *Urechis* which involves close association with marine sediments rich in microbes. Consistent with the hypothesis that substrates might be produced by bacteria, extracts of gut-associated bacteria in adult *U. caupo* also produce potent P-gp inhibitors [29]. High levels of P-gp are found in the gut of *Urechis* and it has been postulated that these might be there to protect the organism from toxicants in their food. This work suggests that these transporters might protect the host organism from bacterial products produced by their symbiotic gut bacteria.

5.4. Natural MXR inhibitors as emerging contaminants—unexpected consequences of biological invasions

Detoxification mechanisms most likely evolved as adaptations to export natural endo- and exogenous xenobiotics out of the cell. Apart from endogenous metabolites, most of these compounds are products of the chemical warfare and competition between and among animals and plants. This evolutionary driving force presumably stimulated development of MXR defense and the observed protection against man-made chemicals is simply an additional feature and the result of a broad substrate specificity of transport proteins included in this phenomenon. Therefore, it is reasonable to expect the existence of potent natural MXR inhibitors in the aquatic environment. Regardless of their animal or plant origin, if they are present in the ecosystem within which they originated and are constantly co-evolving with their opposite defense mechanisms, these substances cannot be considered as “contaminants” that pose a threat for the ecosystem. However, if they are transferred from/to another ecosystem, as happens in species invasions mediated by anthropogenic vectors, they might be considered a potential risk.

An example of the transfer of substances to a new environment comes from studies on the invasive tropical green alga *C. taxifolia*. Accidentally introduced

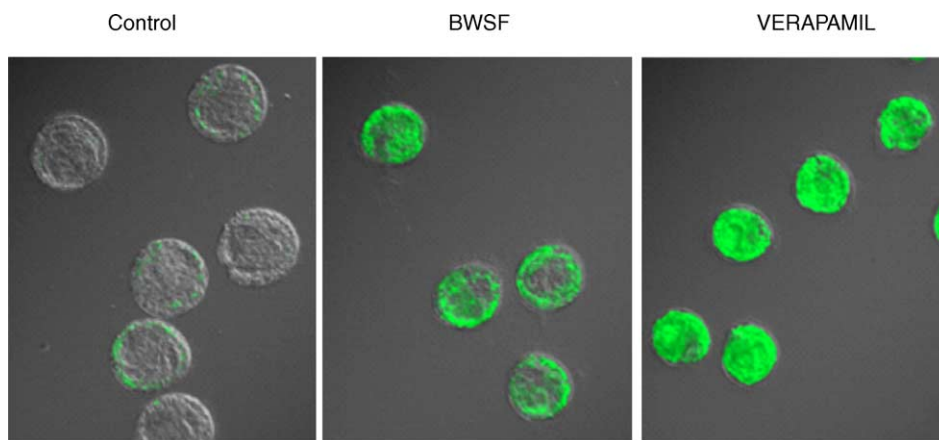


Fig. 6. Figure shows fluorescence micrograph overlays of trocophore *Urechis caupo* embryos treated with 250 nM calcein-AM (Ca-AM) alone or Ca-AM with 2.2 ppm microbially biodegraded water soluble fractions (BWSF) of crude oil or 2.2 ppm verapamil. The increase in fluorescence in drug treated embryos indicates competitive inhibition of their multidrug pumps.

into the Mediterranean in the mid-1980s, it has dramatically changed the biodiversity of littoral algal communities, since it can reproduce and spread much more efficiently than native plant species. Apart from the fact that there are no animal species that feed on *Caulerpa* in the Mediterranean, the reason(s) for this remarkable success is still not fully explained. We hypothesize – based on the results described below – that at least part of its success might come from production and excretion of powerful MXR inhibitors. One indication of this is that MDR-overexpressing cells in culture show reversal of MDR in the presence of *C. taxifolia* extracts [41]. These results were later confirmed in in vivo experiments with *D. polymorpha* by Schröder et al. [40].

The excretion of highly efficient MXR inhibitors into the surrounding seawater by *C. taxifolia* was demonstrated in our recent studies using the rhodamine 123 and calcein-AM accumulation assay with the marine mussel *M. galloprovincialis*. The exposure of mussels in seawater spiked with the *C. taxifolia* extract resulted in a significant increase in rhodamine B and calcein-AM accumulation in the gills of exposed

animals, similar to the effect of model inhibitors, verapamil and cyclosporin A (Fig. 7). Caulerpenine (the main component of *C. taxifolia* extract), and its synthetic congener 10,11-epoxycaulerpenyne, did not influence the rate of accumulation indicating that these were not the active substances. However, the exposure of mussels to “*C. taxifolia* seawater”, prepared by incubating 2 kg of fresh algae for 18 h in 12 L of well aerated seawater at 18 °C, resulted in strong increase in dye(s) accumulation, pointing to the presence of yet unidentified MXR inhibitors excreted from the algae into surrounding water (Fig. 7).

Therefore, this study with *C. taxifolia* incident showed that invasive species can affect biodiversity through excretion of MXR substrates which inhibit the inherent MXR defense of native species. In this case, natural MXR inhibitors might even be considered as emerging contaminants. Unfortunately, intensive tanker traffic across world seas increases the possibility of new invasions through their ballast waters often “collected” from the other side of the globe.

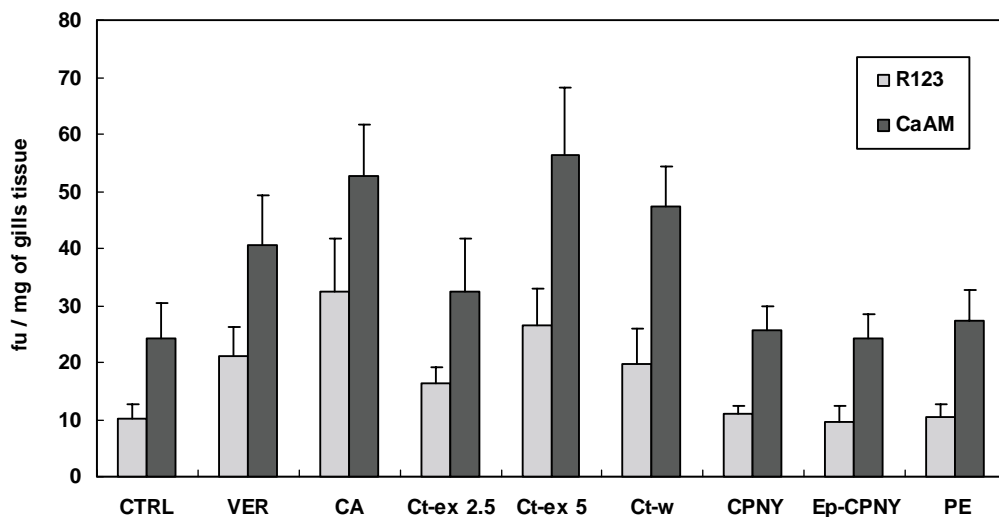


Fig. 7. The determination of the MXR inhibitory potential of a *Caulerpa taxifolia* extract. The effects of verapamil (VER, 10 μ M), cyclosporin A (CA, 5 μ M), *C. taxifolia* extract (Ct-ex, 2.5 and 5 μ g), caulerpenyne (CPNY, 5 μ g), epoxycaulerpenyne (Ep-CPNY, 5 μ g), or phytoplankton extract (PE, 5 μ g) were measured after exposure of *M. galloprovincialis* specimens ($n = 5$) for 2 h in 0.2 L of either filtered seawater alone (CTRL) or spiked with rhodamine 123 (R123, 3 μ M) or calcein-AM (Ca-AM, 0.25 μ M), or *C. taxifolia* seawater (Ct-w) spiked with R123 or CaAM. At the end of exposure period the fluorescence of accumulated R123 or CaAM in gills was measured. Data are expressed in fluorescence units (fu) of accumulated dyes per mg of gills tissue. Heights of bars (mean \pm S.D.) represent the average results from two independent experiments.

6. Overview and future directions

The results of the studies described in this article support the hypothesis that the MXR defense system plays a protective role in aquatic organisms. These studies also provide evidence on the ecotoxicological importance of MXR inhibition by natural or man-made substances. Finally, these studies show that highly efficient MXR inhibitors are likely to be present among both conventional and emerging contaminants. However, there are many fundamental issues that require explanation before the successful implementation of MXR as a tool in biomonitoring of the quality of aquatic environments and for use of this activity in regulatory decisions.

First, the discovery of new MDR transport proteins adds new questions to an already complex research area. New efflux transporters, like MRPs and other recently described proteins [16] could be present in aquatic taxa as well, which suggests that the MXR phenomenon is most likely mediated by several types of transport proteins. Obviously, research on the identification of these different types of transport proteins, along with their isoforms, is urgently needed and the relative contribution of each to transmembrane transport of xenobiotics has to be established. Consequently, considering the probable multifactorial nature of MXR in aquatic organisms, the possibility that these proteins may at the same time overlap and differ in terms of their physiological role, substrate specificities and sensitivity to inhibitors, makes the design of ecotoxicologically reliable high throughput screening (HTS) methods for the determinations of MXR inhibitors a difficult task. The use of radioactive compounds is generally not feasible in environmental monitoring so this approach cannot be used. Nevertheless, HTS might be feasible with the use of appropriate combinations of fluorescent model substrates and specific inhibitors [26,55,56] along with the determination of MXR inhibitory potential via specific (P-gp or MRP) ATPase activity [16,57,58].

Much more attention should also be directed to PPCPs as emerging contaminants, some of which are produced in quantities close to those of many agrochemicals. Some PPCPs, like fragrances, are persistent chemicals, and many pharmaceuticals resist intensive microbial biodegradation [10]. In the case

of PPCPs the persistence is not critical because the source is constant, resulting in chronic aquatic exposure. Therefore, PPCPs as well as untreated and treated sewage samples should be screened for MXR activity. However, the necessary prerequisite for an effective identification of chemicals expressing high MXR inhibitory potential within complex environmental samples is the combination of HTS and a toxicity identification and evaluation (TIE) procedure. To achieve this goal, an effective collaboration between environmental chemists and ecotoxicologists is urgently needed.

And the last, most critical issue is that we do not know the consequences of MXR inhibition at the population and ecosystem level. This is despite results from laboratory experiments that demonstrate significant increases in toxic effects by exposure to high concentrations of MXR inhibitors. Does the exposure to MXR inhibitors in real environmental situations correlate with the incidence of toxic effects? Or do we have “a cause without a disease”? Part of the problem is that it is difficult to address this question using established screening strategies. Toxic effects could be so subtle that they would escape any effort to detect them, making our current regulations largely useless [59]. As Kurelec has formalized in the so-called genotoxic disease syndrome (GDS) concept [60], it is time to make a shift from the present regulatory dogma of toxicity-, neoplasia-, or LD₅₀-directed endpoints to the measurement of a variety of endpoints that represents irreversible toxic events. To do so we strongly need more biomonitoring studies with multiple biomarker endpoints. Determination of environmental concentrations of MXR inhibitors should be among them.

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