

Sea Urchin Embryotoxicity Test for Environmental Contaminants—Potential Role of the MRP Proteins

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Abstract The main goals of this study were (1) to standardize a simple and reliable embryotoxicity test for environmental contaminants and (2) to evaluate the presence and possible protective role of the multidrug resistance-associated (MRP) protein-mediated multi-xenobiotic defense in two Mediterranean sea urchin species, *Paracentrotus lividus* and *Arbacia lixula*. Toxic end-point used was the success of the first cell division in sea urchin embryos. Embryotoxicities of three environmentally relevant contaminants: mercuric chloride (HgCl_2 , 0.05–6 μM), trybutiltin (TBT, 2.5–500 nM), and oxybenzone (OXI, 0.1–100 μM); as well

as seawater samples collected from the polluted and unpolluted locations, were determined and compared. *A. lixula* embryos were more sensitive to all three toxic compounds, and both *P. lividus* and *A. lixula* embryos were highly sensitive to TBT at nanomolar concentrations (EC_{50} 49 \pm 5 and 36.8 \pm 3 nM, respectively). Inhibition of MRP protein by specific inhibitor MK571 caused significant increase in embryotoxic potency of HgCl_2 (EC_{50} 0.697 \pm 0.03 and 0.245 \pm 0.04 μM , respectively), TBT (EC_{50} 24 \pm 3 and 7.4 \pm 1 nM, respectively) and polluted seawater sample, but not of OXI or unpolluted, natural seawater. Therefore, our results demonstrated for the first time the protective relevance of MRP proteins in early development of these two Mediterranean sea urchin species. Finally, the embryotoxicity protocol described in this study represents a simple and rapid bioassay for determination of environmentally relevant seawater contamination.

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1 Introduction

In contaminated environments, aquatic organisms use multixenobiotic resistance (MXR) mechanism as an important cellular defense against toxic compounds (Kurelec 1992; Epel 1998). Mediated through transport activity of the ATP-binding cassette (ABC)

proteins, it reduces intracellular accumulation and potentially toxic effects of a wide variety of environmental compounds (Smital et al. 2004; Epel et al. 2008). In synergy with phase I and II enzymes, the ABC transporters represent an integral part of cellular detoxification machinery, extending the range of natural product toxins or anthropogenic pollutants that can be processed (Kobayashi and Okamura 2002; Leslie et al. 2005; Hamdoun and Epel 2007; Epel et al. 2008).

The function of ABC transporters has been most studied in mammalian eukaryotic cancer cells. Often described as key mediators of the multidrug resistance (MDR) phenotype, they enable resistance of cancer cells to various anticancer drugs. The best known MDR efflux transporters are the P-glycoprotein (MDR1/ABCB1/P-gp), multidrug resistance-associated proteins 1–3 (MRP1–3/ABCC1–3) and the breast cancer resistance protein (BCRP/ABCG2; Gottesman 2002; Leslie et al. 2005). For long it has been considered that the primary MXR transporter in aquatic organisms and their embryos is the P-gp. However, MRP proteins have also been recently recognized as major components of MXR defense in many aquatic organisms, both freshwater and marine, including mussels, sea urchins, and fish (Hamdoun et al. 2004; Sauerborn et al. 2004; Kingtong et al. 2007; Luckenbach and Epel 2008; Bošnjak et al. 2009). The MRP proteins transport organic and inorganic anions, which are direct products of phase I and II metabolism present in the form of glutathione, glucuronic, or sulfate water soluble conjugates (Ballatori et al. 2005; Cole and Deeley 2006). Studies have shown that MRP proteins are also capable to transport heavy metals including inorganic mercury (Aleo et al. 2005; Kingtong et al. 2007; Bošnjak et al. 2009).

Because of their rapid early development that can be easily monitored, sea urchin embryos and larvae are a valuable model frequently used for determination of toxic potential of contaminants present in marine environments (Marin et al. 2000; Kobayashi and Okamura 2002; Bellas et al. 2005; Salamanca et al. 2009). In addition, several recent studies had offered functional and molecular evidence for acquisition of MDR/MXR transporter-mediated efflux activity as a consequence of fertilization in sea urchin embryos. Using specific inhibitors of efflux transporters, Hamdoun et al. (2004) showed that the major efflux activity is mediated by one or more MRP-like transporters. Moreover, recent analysis of the sea urchin *Strongylocentrotus purpuratus* genome

revealed the presence of more than 400 genes and/or homologs of gene families thought to protect against chemical stressors (Goldstone et al. 2006). ATP-dependent efflux transporters are shown to be important part of these “chemical defense genes”, along with cytochromes P450 and other oxidases, various conjugating enzymes, oxidative detoxification proteins, and transcription factors that regulate these genes. More than half of the defense genes are expressed during embryonic or larval life stages, indicating their importance during development.

Taking into account the described state of knowledge, the main goals of this study were to standardize a simple, rapid, and reliable embryotoxicity test for environmental contaminants, and to evaluate the presence and possible protective role of MRP proteins in sea urchin embryos. Toxic end-point used in our assay was the success of the first cell division in sea urchin embryos upon addition of model contaminants and MK571, a specific inhibitor of MRP-mediated transport activity, respectively. Two urchin species—*Paracentrotus lividus* and *Arbacia lixula*—were used as model organisms. Both species inhabit benthic coastal regions (0–50 m) of the Adriatic Sea, and are widely spread across Mediterranean and Eastern Atlantic region. To establish the assay we have exposed the embryos to three environmentally relevant contaminants often found in marine environments: heavy metal mercuric chloride (HgCl_2); antifouling agent trybutiltin (TBT); and finally oxybenzone (OXI), one of the main substances of sun block creams, recently shown to have estrogenic activity in fish (Kingtong et al. 2007). Further, in order to validate the assay as potentially relevant tool for toxicity screening of environmental samples, we have tested its reliability by exposing the embryos to the seawater samples collected either from the Vranjic location—a highly polluted part of the Kaštela Bay next to the city of Split, Croatia; or to the water collected at the Hvar Bay—an unpolluted, natural seawater location close to the Hvar Island, Adriatic Sea, Croatia.

2 Materials and Methods

2.1 Reagents

In this study following reagents have been used: MK571 was obtained from Cayman Electron Micros-

copy Sciences (Ann Arbor, MI, USA). Oxybenzone (2-ethylhexyl 4-(dimethyl-amino)benzoate) (OXI), tributyltin chloride (TBT), 96% mercuric chloride (HgCl_2), potassium chloride (KCl), paraformaldehyde, and dimethyl sulphoxide (DMSO) were purchased from Sigma-Aldrich (St. Louis, MO, USA). DMSO was used as a solvent for all stock solutions except inorganic mercury stock, which was prepared in 1- μm filtered seawater (FSW).

2.2 Assessment of Animals and Seawater Samples

Adult sea urchins *Paracentrotus lividus* and *Arbacia lixula* were collected in June and August 2008 in coastal region (1 to 5 m depth) of Marjan Peninsula (city of Split, Croatia; Fig. 1) and placed in flow-through seawater tanks at the Institute of Oceanography and Fisheries (Split, Croatia). Gametes were collected by intracoelomic injection with 0.5 M KCl. Eggs were collected by separately placing each spawning female in 250-mL beakers with 1 μm FSW. Egg maturity was checked under the microscope and three to five female specimens were selected for each experiment. Selected eggs were filtered through nylon mesh (120 μm) and diluted in order to obtain the final concentration of 1,000 eggs/100 mL (stock solution). “Dry” sperm from each male was collected with an automatic pipette and stored at 4°C in a sterile tube. Sperm mobility and amount was checked under the microscope prior to each experiment, as well as the success of fertilization. Only batches of embryos with >90% successful fertilization were used. In order to simulate real environmental

conditions during the time of sampling and prevent the fertilization failure, all experiments were conducted at 24°C.

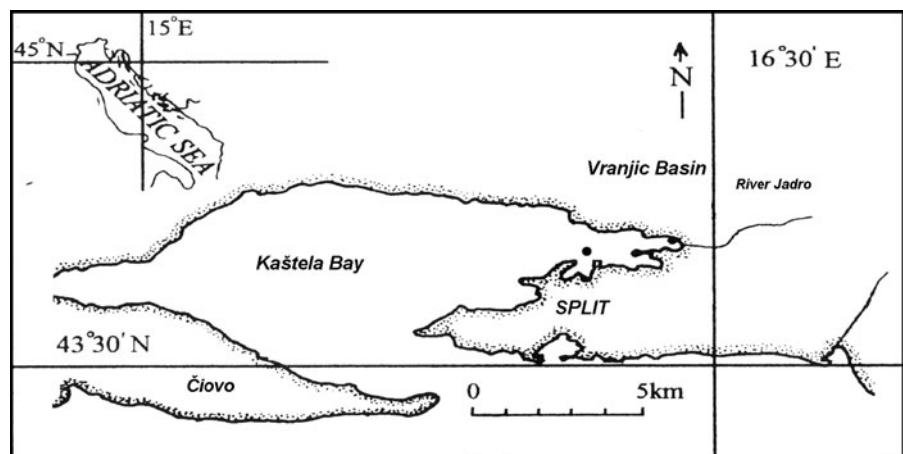
Seawater samples were collected in August 2008 from two locations. Vranjic location (43°31'52.84" N; 16°27'56.58" E, 1 m deep) is a heavily polluted site in the Kaštela Bay, next to the city of Split (Fig. 1). This location was characterized by significant load from different industrial and domestic sources: ironworks, petrochemical industry, cement and asbestos production, industrial harbor activities, repair-shipyard facilities, along with household waste from local urban areas (Marasović et al. 2005; Lovrenčić et al. 2005). A water sample collected at the pristine, open sea location close to the Hvar Bay, (43°13'1" N; 16°49'32" E; 77 m deep) was used as a reference unpolluted sample. Seawater samples were kept at 4°C and related exposure experiments were performed within 2 days from sample collection.

2.3 Embryotoxicity Protocols

In embryotoxicity protocols, 1 mL of sperm (diluted 1:3,000 in FSW) was added to 100 mL of egg stock solution (1,000 eggs/100 mL FSW). Five minutes after the sperm addition, fertilization success was verified by the presence of the fertilization membrane in a random sample of 100 embryos. The excess of sperm was removed by washing the embryos with FSW.

For the exposure to toxic compounds, 20 min after fertilization aliquots of 3 mL of fertilized embryo culture were transferred into wells of polystyrene 12-

Fig. 1 Map of the Kaštela Bay in the city of Split area, Croatia, SE Europe



well plates. Since the previous study has shown that multidrug efflux activity is up-regulated 25 min after fertilization in *S. purpuratus* embryos (Hamdoun et al. 2004), the start of exposure of embryos to HgCl_2 , TBT, or OXI \pm inhibitor (MK571) was set to 30 min post-fertilization. The final concentrations of HgCl_2 were 0.05, 0.5, 0.75, 1, 1.5, 2, 3, and 6 μM ; for OXI 0.1, 0.5, 1, 5, 10, 25, 50, and 100 μM ; and for TBT 2.5, 5, 10, 25, 50, 100, 250, and 500 nM. The corresponding wide range of concentrations for all three toxic compounds was chosen in order to facilitate proper fitting of the dose–response curves and enable reliable determination of EC_{50} concentrations (the concentration causing 50% of embryos not completing the first cell division). The concentration of MK571 used in all experiments was 5 μM ; representing concentration sufficient to completely inhibit the MRP-mediated transport activity in *S. purpuratus* embryos (Hamdoun et al. 2004).

For exposure to the seawater samples, dilution series of seawater samples were prepared by mixing the original sample with FSW, reaching the final concentration/portion of the original sample in range of 0–100%, as indicated in related figures. Two 12-well plates were prepared for each sample and for each experiment, without and with model inhibitor MK571 at final concentration of 5 μM . Ten minutes after fertilization, 3 mL of embryo culture was transferred per well, and embryos were allowed to settle down. Thirty minutes post-fertilization the embryos were transferred to 12-well plates with corresponding seawater samples dilution series \pm MK571. Embryos were then incubated at 24°C with gentle shaking on the shaker to provide even mixing in wells. When >90% of control embryos reached the two-cell stage, approximately 55 min for *P. lividus* embryos, and approximately 60-min post-fertilization for *A. lixula* embryos, cell division was stopped by addition of one drop of 3.2% paraformaldehyde/well. The success of development to the two-cell stage was then determined from 100 embryos analyzed per each well on a dissection microscope.

Each exposure experiment was performed at least three times.

2.4 Data Analysis and Presentation

Measure of HgCl_2 , TBT, or OXI toxicity was determined by the success of the first cell division

expressed as the percentage of two-cell embryos. Data from each set of individual experiments were first analyzed with the Kolmogorov–Smirnov test for normality and then with Bartlett test for homogeneity of variances; all data presented were normally distributed and had homogeneous variance. The data represent three to five replicates (i.e., three to five individual batches of embryos). Mean, standard deviation, EC_{50} values, and the trendlines of mean values of these replicates experiments were calculated and plotted using the Hill equation (nonlinear regression) and the Sigma Plot software (Version 8.0, Systat Software Inc., Richmond, CA, USA). All data are represented as mean \pm standard deviation.

3 Results

3.1 Toxic Compounds Effect the First Cell Division

Our primary goal was to determine the toxicity of three prototypic, environmentally relevant contaminants— HgCl_2 , TBT, and OXI—toward the early development of sea urchin embryos. In order to rapidly assess the sensitization of sea urchin embryos to these compounds we measured the success of the first cell division. Embryos were exposed to the wide concentration range of HgCl_2 , TBT, or OXI, and the percentage of embryos successfully finishing the first cell division was monitored. As seen in Fig. 1a–f, *A. lixula* embryos were more sensitive to all three toxic compounds in comparison to *P. lividus* embryos. Nevertheless, both *P. lividus* and *A. lixula* embryos were highly sensitive to TBT, as even nanomolar concentrations have blocked the first division of embryos (Table 1; Fig. 2c and d).

In comparison to TBT, *P. lividus* and *A. lixula* embryos were less sensitive to HgCl_2 , although HgCl_2 still inhibited cell divisions in micromolar range. Calculated EC_{50} concentrations causing 50% of embryos not completing the first cell division are given in Table 1. *A. lixula* embryos were in general three times more sensitive to HgCl_2 than *P. lividus* embryos (Fig. 1a and b).

Contrary to the toxic effect of HgCl_2 and TBT, the exposure of *P. lividus* embryos to OXI did not result in blockage of the first cell division at concentrations up to 100 μM (Fig. 2f). *A. lixula* embryos showed

Table 1 Embryotoxicity of HgCl₂ and TBT as determined with and without addition of the MRP inhibitor (5 μM MK571) in *P. lividus* and *A. lixula* embryos, respectively

Toxic compound	MK571	<i>A. lixula</i> (EC ₅₀)	<i>P. lividus</i> (EC ₅₀)
HgCl ₂	Absent	0.53±0.08 <i>n</i> =5	1.53±0.1 <i>n</i> =5
	Present	0.245±0.04 <i>n</i> =5	0.697±0.03 <i>n</i> =5
TBT	Absent	36.8±3 <i>n</i> =5	49±5 <i>n</i> =3
	Present	7.4±1 <i>n</i> =3	24±3 <i>n</i> =5

Data are presented as mean EC₅₀±SDs (μM—HgCl₂; nM—TBT) from three to five independent experiments (*n* denotes the number of experiments)

certain sensibility to OXI, although even at 100 μM concentration more than 60% of embryos have reached the two-cell stage (Fig. 2f).

3.2 Inhibition of MRP Increases Potency of TBT and HgCl₂

The next step was to initially test the presence and activity of MRP-mediated MXR defense in early sea urchin embryos against all three toxic compounds, HgCl₂, TBT, and OXI. In order to do that the embryos of both species were exposed to tested contaminants in the presence or absence of MK571, specific inhibitor of MRP-like transport (Gekeler et al. 1995). As previous, the success of the first cell division toward the sensitization of sea urchin embryos was measured.

The addition of 5 μM MK571 caused significant increase in toxic potency of both HgCl₂ and TBT. Over fivefold and twofold increase in TBT toxicity for *A. lixula* and *P. lividus* embryos, respectively, was observed (Fig. 2a and b; Table 1). In case of HgCl₂, the inhibition of MRP efflux activity caused more than twofold increase in toxicity for both *P. lividus* and *A. lixula* embryos (Fig. 2c and d; Table 1). In both experiments the addition of 5 μM MK571 alone had no effect on the first cell division (Fig. 2). Addition of MK571 did not have any influence on the toxic potency of OXI toward *P. lividus* embryos (Fig. 2c), but potentiated toxicity of OXI in *A. lixula* embryos (Fig. 2f).

3.3 Toxicity of Seawater Samples

In order to validate potential environmental relevance of the assay as a tool for rapid screening of complex environmental samples, *P. lividus* and *A. lixula* embryos were exposed to the seawater samples collected from the well-characterized polluted (Vranjic Bay, Fig. 1) and unpolluted location (Hvar Bay), respectively. Embryos were exposed to a range of seawater samples diluted with laboratory prepared FSW. Our results showed that, as the percent of the seawater from heavily polluted Vranjic location increased, the first cell division of both *P. lividus* and *A. lixula* embryos was affected, with a dramatic effect when the exposure medium contained more than 30% of the original seawater (Fig. 2b). *P. lividus* embryos were more resistant and only when 90% of original seawater was added, the embryos did not divide (Fig. 3a). Furthermore, the addition of MK571 highly increased sensitivity of both *P. lividus* and *A. lixula* embryos toward the seawater from polluted Vranjic location (Fig. 3a and b), indicating the presence of toxic substrates normally effluxed by the MRP protein(s).

The same experimental setup was applied on the reference unpolluted seawater sample collected from the open sea location at the Hvar Bay. The first cell division in both *P. lividus* and *A. lixula* embryos was not affected upon this exposure, and over 90% of embryos reached the two-cell stage regardless of the dilution range or the presence of MK571 in the exposure medium (Fig. 3c and d).

4 Summary and Discussion

Sea urchin embryos have rapid development that can be easily monitored under the microscope. Due to this feature, over the last few decades they were frequently used as a relatively simple and tractable research model system for toxicity experiments directed to evaluation of biological effects of contaminants in marine environments. One of the earliest reported bioassays was the sea urchin sperm bioassay originally developed by Dinnel and Stober (1987). The majority of toxicity experiments, however, utilize sea urchin larval growth and morphology as the toxic endpoint(s) (Marin et al. 2000; Kobayashi and Okamura 2002; Bellas et al. 2005; Salamanca et al. 2009),

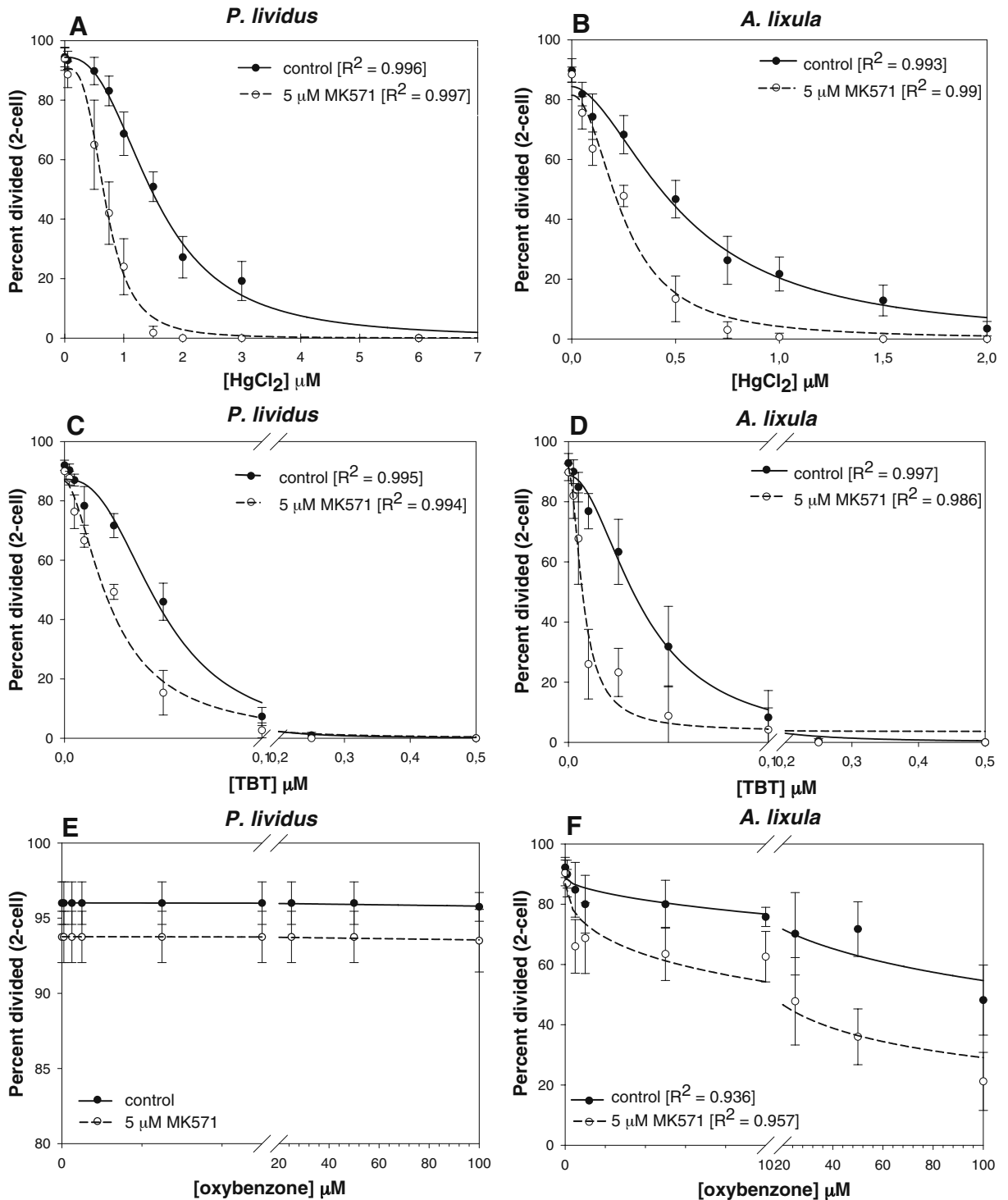


Fig. 2 Potency of the tested contaminants to inhibit the first cell division in *P. lividus* (a, c, e) and *A. lixula* (b, d, f) sea urchin embryos in the absence (solid line) or presence (dotted line) of the MRP inhibitor MK571. Data are expressed as percentage of embryos reaching two-cell stage, 55 (*P. lividus*)

or 60 min (*A. lixula*) post fertilization, and represent means±SDs of five (HgCl₂ and OXI—*A. lixula* and *P. lividus*; TBT—*A. lixula*), four (OXI—*P. lividus*), and three (TBT—*P. lividus*) independent experiments, respectively

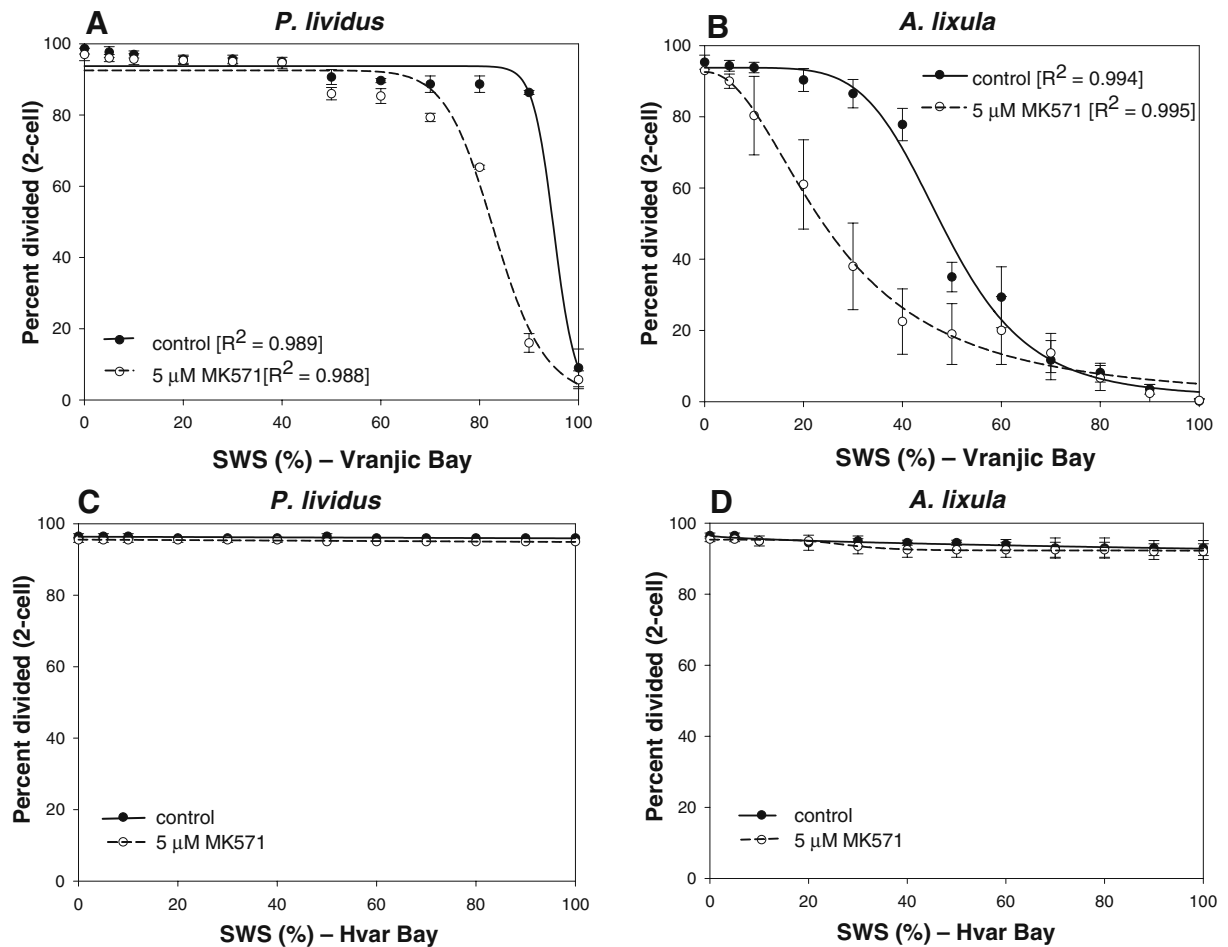


Fig. 3 Potency of the seawater samples (SWS) from the polluted Vranjic location (**a**, **b**) and referent unpolluted Hvar Bay location (**c**, **d**), respectively, to inhibit the first cell division in *P. lividus* or *A. lixula* embryos in the absence (solid line) or presence (dotted line) of the MRP inhibitor MK571. Percen-

tages of the original seawater sample in the exposure medium are indicated on x axis. Data are expressed as percentage of embryos reaching two-cell stage, 55 (*P. lividus*) or 60 min (*A. lixula*) post-fertilization, and represent means±SDs of three independent experiments

where typical laboratory time needed to perform such embryotoxicity tests last up to 48 h (four-arm pluteus larvae).

In this study, we evaluated a short-term version of the sea urchin embryotoxicity test, using the rate of the first cell division as the main toxic end-point that can be scored as early as 50–60 min post-fertilization. The environmental relevance and applicability of the short-term embryotoxicity test is justified on (1) the reported presence and functional activity of defense mechanisms in sea urchin embryos and (2) the synchronous culturing embryos conditions. Sea urchin eggs contain high 5 mM concentrations of glutathione and ovoidiol—the antioxidants that provide an efficient defense mechanism for embryo immediately after

the fertilization (Turner et al. 1987; Shapiro 1991). Furthermore, it is established that Pacific sea urchin *S. purpuratus* embryos, the best studied urchin species with a well-characterized genome (Sea Urchin Genome Sequencing Consortium 2006), have a large number of ATP-binding cassette or multidrug efflux transporter genes and a high level of the corresponding transport activity up-regulated 25 min after fertilization (Hamdoun et al. 2004; Goldstone et al. 2006; Sea Urchin Genome Sequencing Consortium 2006). The most abundant and active ABC transporters present in *S. purpuratus* embryos are proteins belonging to the MRP (ABCC) family. These proteins provide an important defense role against inorganic mercury (HgCl₂) but at the same time are

inefficient against organic mercury (CH_3HgCl) during the first cell division of *S. purpuratus* embryos (Bošnjak et al. 2009). Based on this knowledge, the protective role of MRP embryo transport system was evaluated in our embryotoxicity test, which is not possible in the additional rapid sea urchin sperm bioassay (Dinnel and Stober 1987) due to the lack of efflux transport system in the sea urchin gametes (Hamdoun et al. 2004).

The role of synchronous culturing embryos conditions is an important factor of our embryotoxicity test as it is based on the comparison of the success of the first cell division of the control group with the treated groups of embryos. The time limit set for the first cell division (approximately 55 min for *P. lividus* embryos, and approximately 60 min post-fertilization for *A. lixula* embryos) was long enough for >90% success of first cell division for control groups. The lack, or decrease, of success of the first cell division in the treated groups in comparison to the control group of embryos represents possible delay of the cell division due to the influence of the toxic compound or polluted seawater sample.

Environmentally abundant contaminants, HgCl_2 , TBT, and OXI, used in this research were selected to evaluate the sensitivity of embryotoxicity test done on embryos of both Mediterranean urchin species—*A. lixula* and *P. lividus*. Inorganic mercury, HgCl_2 and TBT have already shown to be toxic for sea urchin embryos (Marin et al. 2000; Marc et al. 2002; Bošnjak et al. 2009) and so far there are no studies showing the toxicity of OXI on sea urchin embryo development.

EC_{50} concentrations for each of the three toxicants were determined (Table 1, Fig. 2). The same bioassay has already showed to be reliable for *S. purpuratus* embryos which were exposed to two different forms of mercury compounds (Bošnjak et al. 2009). As expected, both *P. lividus* and *A. lixula* embryos were the most sensitive to TBT and these results were in accordance to the study published by Marin and colleagues (2000). In comparison to TBT, urchin embryos were less sensitive to HgCl_2 although the obtained data for HgCl_2 were similar to the toxicity of HgCl_2 observed in *S. purpuratus* embryos (Bošnjak et al. 2009). The interesting fact was that *A. lixula* embryos showed less tolerance toward both TBT and HgCl_2 . Furthermore, this difference in sensitivity was highest upon exposure to OXI, towards which both *P.*

lividus and *A. lixula* embryos were the most resistant (Fig. 2e and f).

Putative protective role of MRP proteins during the first cell division of embryos was the next hypothesis addressed in this study. To achieve that, the embryos were simultaneously exposed to HgCl_2 , TBT, or OXI in the presence or absence of 5 μM MK571, a specific inhibitor of MRP transport system. The 5 μM MK571 concentration has been shown to be low enough not to cause any toxic effect on embryo development and at the same time high enough to efficiently inhibit the MRP embryo transport system (Bošnjak et al. 2009). Although previous studies have shown that MRP proteins can protect against heavy metals by expulsion of metal complexes, including HgCl_2 and tributyltin (Aleo et al. 2005; Kingtong et al. 2007; Bošnjak et al. 2009), until now there were no studies testing if MRP proteins are capable of effluxing OXI. As expected, the inhibition of MRP transport has significantly increased the potency of both TBT and HgCl_2 (Table 1, Fig. 2) and *A. lixula* embryos have again been more sensitive in comparison to *P. lividus* embryos. The toxic potency of OXI has just slightly been increased in the presence of 5 μM MK571, but not before the OXI concentrations were above 10 μM (Fig. 2f). This result indicates that the MRP efflux system probably has no role in protection against OXI, and that the potential toxic effect of OXI could be expected later in the development of sea urchin embryos, e.g., during the larval growth and differentiation.

In the final step of our work the reliability and potential environmental relevance of the assay was validated by exposure of *P. lividus* and *A. lixula* embryos to seawater samples collected at well-characterized polluted (Vranjic Bay, Fig. 1) and unpolluted location (Hvar Bay), respectively. Undiluted seawater sample from polluted site caused a complete stop of the first cell division in both *P. lividus* and *A. lixula* embryos. Again, *A. lixula* embryos were more sensitive, as the toxic effect was clearly seen when polluted water was diluted with more than 50% filtered seawater (Fig. 3b). The inhibition of MRP transporters by 5 μM MK571 caused a significant increase in potency of polluted seawater sample for both *P. lividus* and *A. lixula* embryos (Fig. 3c and d). These results indicate that MRP proteins are actively involved in protection of sea urchin embryos immediately after the fertilization.

In conclusion, our results demonstrated for the first time the presence and protective relevance of MRP proteins in early development of these two Mediterranean sea urchin species. A thorough molecular biological characterization and analysis of substrate specificities of MRP proteins included in the MXR mechanism in these species should be the next research goal needed for a reliable characterization of this protective system. In addition, this assay may be a useful tool for rapid, first check determination of the presence of inhibitors of MRP efflux transporters in marine water samples. Finally, the embryotoxicity determination protocol described in this study represents a simple, rapid, and high-throughput bioassay for determination of environmentally relevant seawater contamination.

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