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Is the step-wise tiered approach for ERA of pharmaceuticals useful for the assessment of cancer therapeutic drugs present in marine environment?

G.V. Aguirre-Martínez [a,b,*], C. Okello [a,c], M.J. Salamanca [a], C. Garrido [b], T.A. Del Valls [a], M.L. Martín-Díaz [a,b]

[a] Department of Physical-Chemistry, Faculty of Marine and Environmental Sciences, University of Cadiz, Campus of International Excellence of the Sea (CEIMAR), Polígono Río San Pedro s/n, Puerto Real, 11510 Cádiz, Spain
[b] Andalusian Center of Marine Science and Technology Puerto Real Campus, Río San Pedro , Puerto Real, 11510 Cádiz, Spain
[c] Integrated Geoscience Research Group (IGRG), Interdepartmental Centre for Environmental Sciences Research (CIRSA), Ravenna Campus, University of Bologna, Via S. Alberto 163, 48100 Ravenna, Italy

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ABSTRACT

Methotrexate (MTX) and tamoxifen (TMX) cancer therapeutic drugs have been detected within the aquatic environment. Nevertheless, MTX and TMX research is essentially bio-medically orientated, with few studies addressing the question of its toxicity in fresh water organisms, and none to its' effect in the marine environment. To the authors' knowledge, Environmental Risk Assessments (ERA) for pharmaceuticals has mainly been designed for freshwater and terrestrial environments (European Medicines Agency-EMEA guideline, 2006). Therefore, the purpose of this research was (1) to assess effect of MTX and TMX in marine organism using the EMEA guideline, (2) to develop an ERA methodology for marine environment, and (3) to evaluate the suitability of including a biomarker approach in Phase III. To reach these aims, a risk assessment of MTX and TMX was performed following EMEA guideline, including a 2-tier approach during Phase III, applying lysosomal membrane stability (LMS) as a screening biomarker in tier-1 and a battery of biochemical biomarkers in tier-2. Results from Phase II indicated that MTX was not toxic for bacteria, microalgae and sea urchin at the concentrations tested, thus no further assessment was required, while TMX indicated a possible risk. Therefore, Phase III was performed for only TMX. *Ruditapes philippinarum* were exposed during 14 days to TMX (0.1, 1, 10, 50 $\mu g\ L^{-1}$). At the end of the experiment, clams exposed to environmental concentration indicated significant changes in LMS compared to the control ($p < 0.01$); thus a second tier was applied. A significant induction of biomarkers (activity of Ethoxyresorufin O-deethylase [EROD], glutathione S-transferase [GST], glutathione peroxidase [GPX], and lipid peroxidation [LPO] levels) was observed in digestive gland tissues of clams compared with control ($p < 0.01$). Finally, this study indicated that MTX was not toxic at an environmental concentration, whilst TMX was potentially toxic for marine biota. This study has shown the necessity to create specific guidelines in order to evaluate effects of pharmaceuticals in marine environment which includes sensitive endpoints. The inadequacy of current EMEA guideline to predict chemotherapy agents toxicity in Phase II was displayed whilst the usefulness of other tests were demonstrated. The 2-tier approach, applied in Phase III, appears to be suitable for an ERA of cancer therapeutic drugs in the marine environment.

1. Introduction

Several publications have indicated the presence of pharmaceuticals within the aquatic environment at the $ng\ L^{-1}$ to $\mu g\ L^{-1}$ range, due to either direct discharge or even post waste water treatment process (Andreozzi et al., 2002; Gros et al., 2007, 2009, 2010; Quinn et al., 2008a; Zuccato et al., 2004, 2005). In addition, it has been demonstrated that at these concentrations, some pharmaceuticals produce acute and chronic effects on aquatic organisms (Fent et al., 2006; Fent, 2008; Ferrari, 2003; Quinn et al., 2009; Hernando et al., 2006; Martín-Díaz el al., 2009; Aguirre-Martínez et al., 2013a, 2013b among others). Nevertheless, for most pharmaceuticals the effect which they have on aquatic biota

* Corresponding author at: Andalusian Center of Marine Science and Technology (CACYTMAR), Puerto Real Campus, Río San Pedro,Puerto Real, 11510 Cádiz, Spain.
E-mail address: gabriela.aguirre@uca.es (G.V. Aguirre-Martínez).

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is largely unknown.

During 2006, the European Medicines Agency (EMEA) released a guideline describing how to evaluate the potential risks of pharmaceuticals products entering the environment. However, it is only focused on the environmental risks associated with the use of pharmaceuticals and not from storage, disposal, synthesis or the manufacture of these substances. The guidelines describes a step-wise tiered procedure for Environmental Risk Assessment (ERA) of pharmaceuticals with two phases. Briefly, the Phase I is a pre-screening assessment, which aims at a first estimation of exposure with an action limit of 0.01 $\mu g\ L^{-1}$. This guideline indicates that if the predicted environmental concentration (PEC) of a pharmaceutical of surface water is below this limit, it is assumed that the compound is unlikely to represent a risk for the environment. However, in some cases, the action limit may not be applicable, for example regarding endocrine disrupting compounds. If the PEC is equal to, or above 0.01 $\mu g\ L^{-1}$ then a Phase II environmental fate and effect analysis should be performed. Phase II is further divided into Tier A which gives a rapid prediction of environmental risk based on screening data. If the risk is identified at this level, then a Tier B should be performed; this tier requires extended ecotoxicity data to reduce uncertainty, this is the ultimate step in risk assessment of the EMEA guideline (Grung et al., 2008; Kampa et al., 2010).

Nevertheless, this guideline specifies that only newly authorized pharmaceuticals require an environmental assessment, and to this respect, there is little knowledge concerning the environmental risk for most chemotherapeutic agents released to the market before 2006 (Besse et al., 2012; Johnson et al., 2008). This is the case of two frequently used cancer therapeutic drugs methotrexate (MTX) tamoxifen (TMX). Methotrexate (4-amino-10-methyl-folic acid) is a commonly used anti-metabolite (folic acid antagonist) in cancer treatment and is also applied as an anti-rheumatic drug. It is not normally sold in pharmacies; but its use in medicine is widespread. This substance interacts with cell proliferation, blocking the folate dehydroreductase enzyme disrupting the synthesis of nucleic acid, which is responsible for the purine and pyrimidine synthesis (Trigg and Flanigan-Minnick, 2011). It is eliminated virtually unchanged by the kidneys (Fent et al., 2006). MTX has been found in effluents from hospital and waste water treatment plants at a concentration from 0.0021 to 0.25 $\mu g\ L^{-1}$ (Table 1). Tamoxifen is an anti-estrogen, a non-steroidal triphenylethylene derivative, which is widely and successfully used in the chemotherapy and chemoprevention of primary and recurrent breast cancer (Bergh, 2003; Custodio et al., 1993; Jordan et al., 1977; Nayfield et al., 1991; Osborne, 1998; Powles et al., 1994). More recently, this drug has been used as a prophylactic agent in women who are considered to be at a high risk of developing the disease (DellaGreca et al., 2007; Fisher et al., 2005). Like many other pharmaceuticals, it can enter the aquatic environment through municipal sewage effluents and cause adverse effects (Ashton et al., 2004; Hilton and Thomas, 2003; Mater et al., 2014; Sun et al., 2007). This is of importance since TMX has been proposed for use as a growth-promoting agent in aquaculture (Park et al., 2003) and in this context would pose an additional risk to aquatic organisms (Sun et al., 2007, 2009; Mater et al., 2014). TMX has been included on the prioritization list of bioaccumulable potential in the human body and probably in aquatic organisms (Jean et al., 2012). Assessment for this drug has been suggested by the Oslo and Paris Commission (OSPAR, 2003), Environment Agency from the U.K. (Hilton et al., 2003), and Environment Canada (2014). Moreover, as potential endocrine disruptor in European water sources, the Institute of Environment and Health (U.K.) have suggested an ERA for this drug (IEH, 2012). TMX has been found in aquatic environment at concentrations ranging from 0.004 to 0.21 $\mu g\ L^{-1}$, and in effluents from waste water treatment plants and hospital at 0.0002 and 0.037 $\mu g\ L^{-1}$ (Table 1). In addition, MTX and TMX are both included in the list of drugs that should be handled as hazardous (NIOSH, 2012, 2014).

Despite detected concentrations in the environment, most MTX and TMX research is essentially bio-medically orientated with few papers addressing the question of toxicity in aquatic organisms (Besse et al., 2012; Mater et al., 2014; Orias and Perrodin, 2013; Sun et al., 2007, 2009). Knowing that these pharmaceuticals are widely used, have been found in the environment, and are of special interest, there is a need to analyze the type of effect that they might produce in aquatic biota, taking into account their distinctive mode of action. Besse et al. (2012) suggest that these drugs should be screened and assessed for environmental risk according to the EMEA guideline released in 2006, since there is a lack of information of their ecotoxicity and more specific knowledge is required regarding the marine environment.

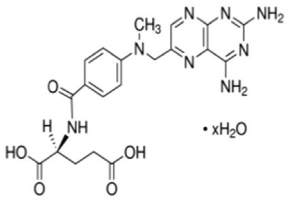
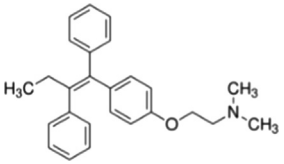
As previously mentioned, ERA for human pharmaceuticals should be performed according to EMEA Guideline (2006) proposed for freshwater and terrestrial environments (McVey, 2012). Nevertheless, authors believe that research should be focused on developing a risk assessment methodology in which marine environment components are included. In contrast to other pollutants, pharmaceuticals are specifically designed to have pharmacological and physiological effects on their target (i.e. humans or animals under veterinary treatment) species. However, their effects on non-target (environmentally exposed) species are difficult to predict and may often be detrimental (Hampel et al., 2014). The aims of this study were the following: (1) to assess the effects of MTX and TMX in marine organisms using the EMEA guideline, (2) to develop an ERA methodology in which marine organisms are included, (3) to evaluate the suitability of including a biomarker approach for the last phase (Phase III).

2. Materials and methods

2.1. Environmental Risk Assessment (ERA)

The ERA of the EMEA guideline use a step-wise structure (Fig. 1), including a **Phase I.** This first step is the estimation of the exposure by calculation of a predicted environmental concentration (PEC). Nevertheless, in the present study, the measured environmental concentration (MEC) was applied, which was obtained from reported data of MTX and TMX found in municipal effluents, sewage treatment plants, surface water, etc. described in Table 1. The use of MEC allows establishing more realistic ERA than PEC (Blasco and DelValls, 2008). If MEC exceeds the action limit, then further testing is required. Leading onto **Phase II,** this second step corresponds to an initial prediction of the risk applying a set of acute toxicity tests towards three species from different phyla: bioluminescence on *Aliivibrio fischeri* (Proteobacteria), growth inhibition on microalgae *Isochrysis galbana* (Haptophyta), and on sea urchin *Paracentrotus lividus* (Echinodermata) during their early life stage. In this step, the predicted no effect concentration (PNEC) is extrapolated by dividing the EC_{50} by an assessment factor of 1000. If the ratio MEC/PNEC is < 1, then no further assessment is necessary. If MEC/PNEC is > 1, an ecological hazard may occur, and so further assessment should be performed (Quinn et al., 2008a). **Phase III** includes long term exposure; in this step sensitive endpoints are included in order to evaluate the chronic effects of drugs. A 2-tier approach is applied following the methodology proposed by Viarengo et al. (2007), which was then applied to marine crabs by Aguirre-Martínez et al. (2013a, 2013b). In **Tier 1,** a sensitive, low-cost biomarker is used as "early warning" to indicate the level of stress of the organisms exposed to the contaminant. A lysosomal membrane stability test (LMS) is

Table 1
Characteristics of chemotherapeutic agents selected for this study and measured environmental concentrations (MEC) in municipal effluent (ME), sewage treatment plant effluent (STP), waste water treatment plant effluent (WWTP).

Pharmaceutical	Molecular weight	Structure	CAS No.	Product No.	Purity	Color and form	MEC μg L^{-1}	Place of study	Reference
Methotrexathe(MTX)	454.44		133073-73-1	M9929	Meets United States Pharmacopeia (USP) testing specifications	Powder	0.0021	WWTP	Negreira et al. (2013)
							0.0200	WWTP	Negreira et al. (2013)
							0.0500	STP	Stackelberg et al. (2004)
							< 0.0	STP	Zuccato et al. (2005)
							0.1200	Hospital	Catastini et al. (2009)
							0.245	Hospital	Yin et al. (2010)
Tamoxifen (TMX)	371.5		10540-29-1	T5648	> 99%	A white to white with a yellow cast powder	0.0002	Hospital	Liu et al. (2010)
							0.0010	WWTP	Verlicci et al. (2012)
							0.0020	WWTP	Lara-Martín et al. (2014)
							0.0020	Hospital	Verlicci et al. (2012)
							0.0035	WWTP	Negreira et al. (2013)
							0.0040	Estuary	Thomas and Hilton (20040
							0.0058	STP	Coetsier et al. (2009)
							< 0.0058	River	Coetsier et al. (2009)
							0.0082	Hospital	Liu et al. (2010)
							0.0100	STP	Ashton et al. (2004)
							0.0170	WWTP	Negreira et al. (2013)
							0.0250	River	Coetsier et al. (2009)
							0.0270	River	Roberts and Thomas (2006)
							0.0420	STP	Ashton et al. (2004)
							~0.0650	Hospital	Langford and Thomas (2009)
							0.0740	Estuary	Thomas and Hilton (2004)
							0.1000	STP	Coetsier et al. (2009)
							0.2100	River	Roberts and Thomas (2006)
							0.3700	WWTP	Roberts and Thomas (2006)

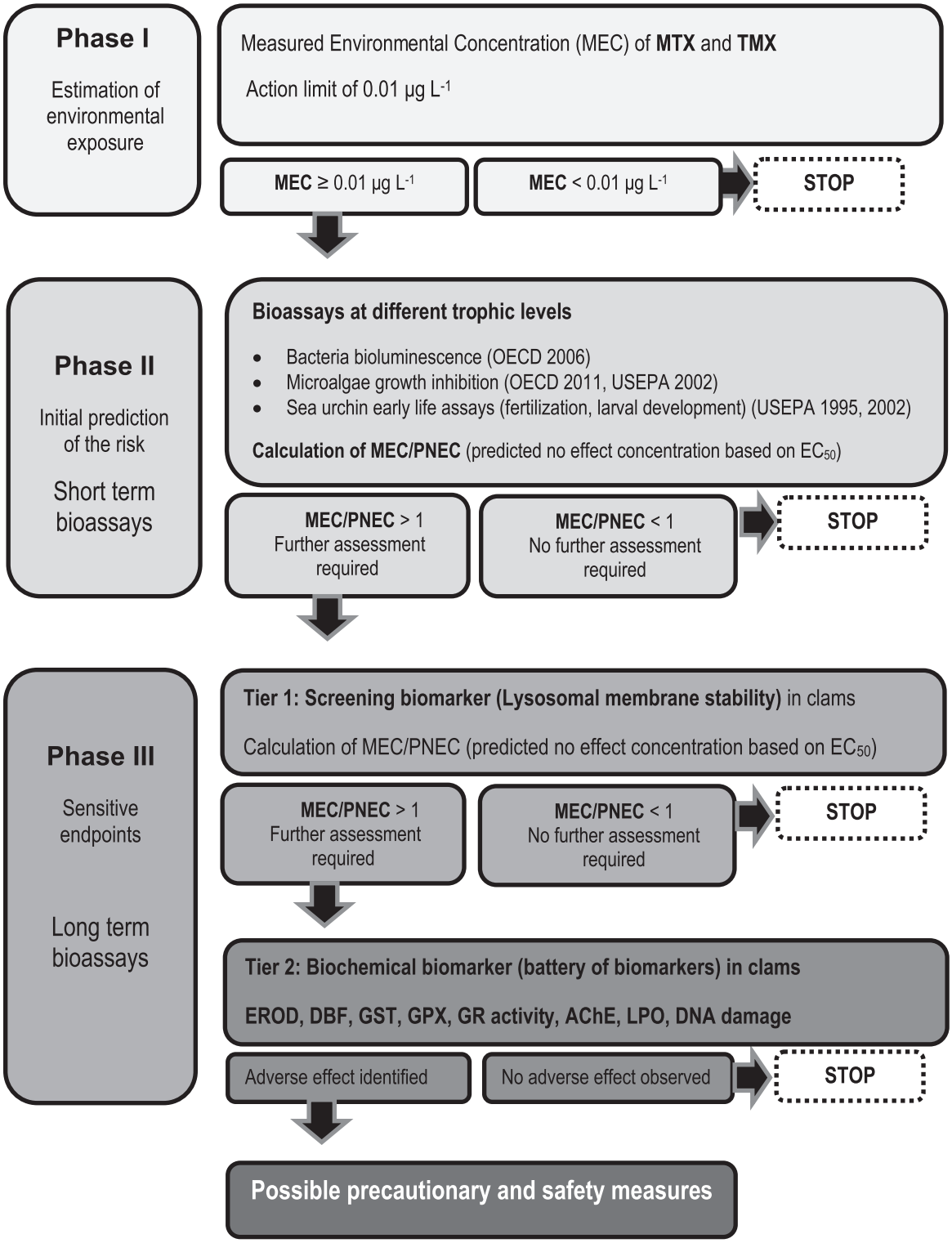


Fig. 1. Workflow for the ERA (Environmental Risk Assessment) of MTX (methotrexate) and TMX (tamoxifen) based on the EMEA Guideline (2006) and the 2-tier approach proposed by Viarengo et al. (2007). EC_{50} (Effect concentration for 50% of the test population), EROD (Ethoxyresorufin O-deethylase), DBF (Dibenzylfluorescein dealkylase), GST (Glutathione S-transferase), GR (Glutathione reductase), AChE (Acetylcholinesterase), LPO (Lipid peroxidation).

evaluated in *Ruditapes philippinarum* (clam) following the methodology applied by Aguirre-Martínez et al. (2013c). If organisms indicate stress (based on evident changes in the LMS) and no mortalities are observed, then the second tier should be applied. **Tier 2** includes a complete battery of biochemical biomarkers evaluated in these species, to assess the levels of pollutant-induced stress syndrome.

MTX and TMX were purchased from Sigma-Aldrich, Spain characteristics of these compounds are indicated in Table 1, Spain. Storage and temperature conditions were followed according to recommendations from Sigma-Aldrich. MTX was stored at −20 °C and TMX at 4 °C under dark conditions. Stock solutions of both drugs were freshly prepared in dimethyl sulfoxide (1 mg mL^{-1} DMSO) in glass vials. All bioassays performed in this study

included a control consisting of seawater without any toxicant, and a solvent control (DMSO) tested at 0.001% v/v to ensure there were no solvent effects (Eades and Waring, 2010; Quinn et al., 2008a, 2008b).

2.2. Phase II

2.2.1. Bacteria bioluminescence inhibition

This is a microbial bioassay proposed by OECD (2006) based on the natural luminescence of the marine bacteria *A. fischeri.* This test was performed using standard methods according to the manual (Azur Environmental, 1998) and follows the basic test procedure (liquid phase) for Microtox®. The materials for analysis (test reagent, diluents, osmotic adjusting solution and reconstituting solution) were supplied by Azur Environmental (Carlsbad, CA. USA). The bacteria was exposed to a control sample and nine serial dilutions of the sample solution of MTX (starting concentration 2.5×10^6 μg L^{-1}) and TMX (starting concentration 8.33×10^5 μg L^{-1}). Sample dilutions were incubated with luminescent bacteria for 5, 15 and 30 min intervals at 15 °C. The reduction in intensity of light emitted from the bacteria was measured along with standard solutions and control samples. The change in light output, and concentration of the toxicant produced a concentration/response relationship. The results were normalized and the EC_{50} (effect concentration of a test chemical which causes a 50% inhibition of bacteria luminescence) was calculated at 5, 15 and 50 min. The test was performed using a Microtox® Model M500 analyzer Azur Environmental (Carlsbad, CA).

2.2.2. Microalgae growth inhibition

Growth inhibition tests using microalgae have been recommended by USEPA (2002), OECD (2011), ECHA (2008) and TSCA (2003). Concentrated cultures of *I. galbana* were provided by the Laboratory of Marine Culture at the Marine and Environmental Sciences Faculty of the University of Cadiz. The cultures were maintained in the laboratory under aseptic conditions in a nutritive medium composed of synthetic sea water (USEPA, 2002) and supplied with nutrients and vitamins according to the f/2 medium (Guillard and Ryther, 1962). Microalgae toxicity tests were performed in transparent and sterilized vials of 15 mL borosilicate glass and sealed with aluminum capsules. The vials contained 2 mL of an inoculum of microalgae with optimal cellular density and 2 mL of a concentration of MTX (0.05, 0.5, 1, 5, 15, 50, 500, 5000, 50,000, 100,000, 500,000 μg L^{-1}) and TMX (0.001, 0.005, 0.01, 0.05, 0.5, 1, 5, 15, 50, 500, 5000, 50,000, 100,000, 500,000 μg L^{-1}). A more detailed description of the protocol can be found in Garrido-Perez et al. (2008). Tests were carried out under controlled conditions of continuous illumination (cold white light of 11.000 lux) and temperature (20 ± 1 °C) in a climatic test chamber. Biomass concentration was measured at 0, 24, 48, 72 and 96 h period in terms of optical density (USEPA, 2002) at a wavelength of 690 nm using a colorimeter Nannocolor PT-3 MACHEREY-NAGEL.

2.2.3. Sea urchin toxicity test

Tests on the early life stages of sea urchin have been recommended by USEPA (1995, 2002) and Environment Canada (2011). Sea urchins *P. lividus* during adult stage were collected from a clean site of the Atlantic Coast of Cádiz located between playa Getares (Algeciras) and Punta Carnero (Carnero's cape) 36° 5′ 8 59″ N and 5° 26′3 98″ from a rocky sub tidal environment where sea urchins are found at 2 m depth. Organisms were stored in a cooler box ready for transportation to the University of Cadiz (UCA) laboratory for the bioassays. Both sea urchin toxicity bioassays were carried out following the Fernandez and Beiras (2001) and Volpi-Ghirardini and Arizzi-Novelli (2001) protocol adapted from the national environmental agencies Environment Canada (2011) and USEPA (1995, 2002). Sea urchin bioassays were performed under controlled laboratory conditions using natural and filtered sea water; water quality parameters including temperature (17 ± 1 °C), salinity (33.8 ± 0.3‰), pH (7.7 ± 0.2) and dissolved oxygen (> 5 mg L^{-1}, 60% sat) were measured at the beginning and at the end of the bioassay to ensure acceptability of the tests (Salamanca et al., 2009).

2.2.3.1. Fertilization. Gametes were obtained by dissecting mature individuals, and then collected using the direct pipette extraction method. The extracted gametes were checked under the microscope for optimal conditions to ensure that the ovules were spherical and sperms motile. Ovules were then transferred into a sterile measuring cylinder containing naturally filtered seawater to recover them, whilst sperms were kept in dry and cold conditions. 25 μL of dry sperms were introduced to 10 mL vials containing concentrations of MTX and TMX (0.01, 0.005, 0.01, 0.05, 0.5, 1, 5, 15, 50, 500, 5000, 50,000, 100,000, 500,000, 1,000,000 μg L^{-1}). All samples were set in quadruplicates. After 60 min of exposure, a standardized ovule suspension (2000 eggs mL^{-1}) was introduced to each vial containing the sperms and treatment. Fertilization occurred after 15 min of ovule addition. The test ended with a drop of formalin (40%). Fertilization success was indicated by the presence of a fertilization membrane. The Percentage fertilization of each treatment was determined by counting 200 eggs.

2.2.3.2. Larval development. For this test, dry sperms (taken from the gonads) were introduced to the ovules in a 100 mL measuring cylinder, containing seawater which was then gently stirred to allow fertilization. Then, 25 μL of fertilized eggs were introduced to 20 mL vials containing concentrations of MTX and TMX (0.01, 0.005, 0.01, 0.05, 0.5, 1, 5, 15, 50, 500, 5000, 50,000, 100,000, 500,000 and 1,000,000 μg L^{-1}). All samples were set in quadruplicates. The vials containing the fertilized eggs were incubated at 18 °C for 48 h in darkness; since these conditions allowed complete development of embryo into pluteus larvae (Fernandez, 1999). After the incubation period, the test was completed by the addition of a drop of formalin (40%). The measured endpoint was the embryogenesis success measured in 100 pluteus larvae ($n = 100$) per replicate. The results were expressed as percentage of normal pluteus larvae (defined as those with four well developed arms) normalized to the corresponding control.

2.3. Phase III

2.3.1. Experimental design

Manila clam *R. philippinarum* (size and length 42 ± 0.9 mm) were purchased from an aquaculture farm located at a clean site on the Atlantic Coast of Southern Spain (Cadiz). Organisms were acclimatized for a week in the laboratory in tanks of 300 L capacity with filtered seawater, supplied with constant aeration. Conventional parameters including pH (7.8–8.2), T° (19 ± 1 °C), salinity (33.8 ± 0.3) and dissolved oxygen (> 5 mg L^{-1}, 60% sat) were strictly controlled and maintained under a 12 h light: 12 h dark regime. After the acclimation period, clams were then divided into 25 per aquarium (20 L glass aquaria) and exposed during 14 days to nominal concentrations of TMX (0.1, 1, 10 and 50 μg L^{-1}). Stock solution of TMX was freshly prepared in dimethyl sulfoxide (1 mg mL^{-1} DMSO) in glass vials every two days. A solvent control (DMSO) was tested at 0.001% v/v to ensure there was no solvent effects (Eades and Waring, 2010; Quinn et al., 2008a, 2008b). Each treatment of TMX was performed in duplicate, including sea water control, solvent control (DMSO) in a semi static renovation system. During the experiment, clams were fed every 48 h with phytoplankton (*Tetraselmis sp.* T-150 and *Chaetoceros sp.*). After the

feeding process, the water was siphoned out. Waste food, faeces and other debris were removed and water was completely renewed. Then a volume of the fresh stock solution of TMX was added to each aquarium in order to expose organisms to the nominal concentration required. Physical–chemical parameters during the experiment were similar to those applied in acclimation period.

2.3.2. Tier 1: screening biomarker

Lysosomal membrane stability (LMS) was evaluated at the end of the experiment (day 14) using the neutral red retention assay (NRRA) ($n=10$ clams per treatment), following the methodology described in detail by Aguirre-Martínez (2013c) including the LMS criteria applied for these species. The threshold values applied were as follows: clams were considered to be healthy if NRRT was ≥ 80 min; they were considered stressed but compensated if NRRT was <80 but ≥ 45 min; and to present diminished health status if NRRT was <45 min.

2.3.3. Tier 2: biochemical biomarkers

Clams were collected after 14 days of exposure to TMX spiked water. From each aquarium, digestive glands tissues from 10 clams were dissected and combined into 4 pools and stored at -80 °C prior to homogenization. Samples were homogenized following the procedure described by Lafontaine et al. (2000) and centrifuged to obtain supernatant fractions S_{15} (15.000*g* for 20 min at 2 °C) and S_3 (3.000*g* for 20 min at 2 °C). The total protein concentration (TP) (expressed as mg mL^{-1}) was determined following an adaptation of Bradfords' (1976) methodology. All biochemical biomarkers were measured using a kinetic microplate reader (Infinite® M200).

Ethoxyresorufin O-deethylase (EROD) activity (expressed as pmol/min/mg TP) was measured following Martín-Díaz et al. (2007). 50 μL of the S_{15} was added to 160 μl 7-ethoxyresorufin, and 10 μl reduced NADPH, in 100 mM KH_2 PO_4 buffer (pH 7.4). The reaction was initiated by the addition of NADPH, and was allowed to proceed for 60 min (10 min intervals) at 30 °C. 7-hydroxyresorufin was determined fluorometrically using 516 nm (excitation) and 600 nm (emission) filters. Calibration was then achieved through a standard calibration curve developed with concentrations of resorufin.

Dibenzylfluorescein dealkylase (*DBF*) *activity* (expressed as pmol/min/mg TP) was evaluated following the methodology described by Quinn et al. (2004). 50 μL of the S_{15} was added to 50 μM dibenzylfluorescein and 100 μM reduced NADPH in 125 mM NaCl, and buffered with 10 mM Hepes–NaOH, pH 7.4. Samples were incubated at 30 °C; with the release of fluorescein measured at 0, 15, 30 and 60 min. Fluorescein was determined by fluorometry using 485 nm (excitation) and 532 nm (emission) filters. Fluorescein was measured using a standard calibration curve developed with concentrations of a standard solution of 5 μM fluorescein.

Glutathione S-transferase (GST) activity (expressed as OD GST/min/mg TP) was determined following the procedure from Boryslawskyj et al. (1998). 50 μL of the S_{15} was added to 200 μL of 1 mM GSH and 1 mM 1-chloro-2, 4-dinitrobenzene in a buffer of 10 mM Hepes–NaOH, pH 6.5 containing 125 mM NaCl. Absorbance, expressed as optical density (OD), was measured at 340 nm for 30 min (5 min interval).

Glutathione peroxidase (GPX) activity (expressed as pmol/min/mg TP) was measured following the methodology from Mcfarland et al. (1999). 20 μL of the S_{15} was added to the reaction mixture (substrate 1 mM cumene hydroperoxide in 50 mM potassium phosphate buffer with a pH of 6). Absorbance was measured at 340 nm, for 10 min (2 min intervals) at 30 °C. The decrease in NADPH absorbance during the oxidation of NADPH to NADP was indicative of GPX activity.

Glutathione reductase (GR) activity (expressed as nmol/min/mg TP) was determined following the procedure from Martín-Díaz et al. (2007). 20 μL of the S_{15} was added to the reaction mixture (substrates 10 mM oxidized glutathione and 1 mM NADPH in 200 mM sodium phosphate buffer with a pH of 7.6). Absorbance was measured at 340 nm, for 10 min (2 min intervals) at 30 °C. GR together with the co-factor NADPH, catalyses the reduction of oxidized glutathione (GSSG) to GSH. The consumption of NADPH produces a decrease in absorbance, which is directly proportional to the GR activity in the sample.

Acetylcholinesterase (AChE) activity (expressed as nmol DTNB/min/mg TP) was evaluated following the methodology from Guilhermino et al (1996). 166.6 μL of the reaction solution [19.23 mL of phosphate buffer, 0.64 mL of reagent dithiobisnitrobenzoate (DTNB) 10 mM (acid dithiobisnitobenzoate and sodium hydrogen carbonate in phosphate buffer) and 0.128 mL of acetylthiocholine 0.075 M] were added to 33.5 μL of the S_3. Absorbance was measured for 20 min (5 min intervals) at room temperature.

Lipid peroxidation (LPO) levels (expressed as nmol TBARS/mg TP) were determined using an adaptation of the thiobarbituric acid reactive substances (TBARS) methodology for marine invertebrates by Martín-Díaz et al., 2007. Oxidative stress leads to malondialdehyde (MDA) production coming from the degradation of initial products of free radical attacks on fatty acids (Janero, 1990). MDA reacts with 2-thiobarbituric acid producing tetramethoxypropane (TMP) which can be measured spectrophotometrically allowing the indirect determination of MDA. Standard solutions and homogenate samples were prepared separately in 1.5 mL eppendorfs. Firstly 150 μL of standard solutions (0, 0.6, 1.5, 3, 4, 6, 10 and 15 μM TMP 0.0001%), 300 μL of trichloroacetic acid (TCA) 10%, 1 mM $FeSO_4$, 150 μL of thiobarbituric acid (TBA) 0.67%; then 150 μL of the homogenate, 300 μL of trichloroacetic acid (TCA) 10%, 1 mM $FeSO_4$, 150 μL of thiobarbituric acid (TBA) 0.67%. Standards and samples were incubated in a Unitronic 320 OR P Selecta Heater® at 70 °C for 10 min. Absorbance of 200 μL of the standards and homogenate samples were measured at 540 nm.

DNA damage (expressed as μg DNA/mg TP) was measured by a 'DNA precipitation' assay described by Olive (1988); this methodology is based on the K-SDS precipitation of DNA–protein crosslink, which uses fluorescence to quantify the DNA strands (Gagné et al., 1995). Denatured single-stranded DNA is released from a physical matrix (cellular proteins). The physical separation during denaturation process allows quantifying the amount of single stranded DNA and double stranded DNA at the end of the assay (Shugart, 2000). The homogenate (25 μL) was mixed with 200 μL of 2% SDS containing 10 mM EDTA, 10 mM Tris-base and 40 mM NaOH. After mixing for 1 min, 200 μL of 0.12 M KCl was added and the solution heated at 60 °C for 10 min, then mixed by inversion, and cooled at 4 °C for 30 min to precipitate the genomic DNA linked to SDS-associated nucleoproteins. This mixture was then centrifuged at 8.000*g* for 5 min (4 °C). Then 50 μL of the supernatant was added to 150 μL of Hoescht dye (0.1 μg mL^{-1}). Fluorescence was measured using 360 nm (excitation) and 450 nm (emission) filters against blanks containing identical constituents, without the homogenate. Salmon sperm genomic DNA standard (Sigma-Aldrich) was added for DNA calibration.

2.4. Statistical analysis

Statistical analysis was performed by SPSS/PC+statistical package® (15.0). Significant differences between pharmaceutical treatments respect to controls were determined using a one-way ANOVA, and using Dunnett's multiple comparison test. The significance level was set at 0.05. For bacterial bioluminescence test

the IC_{50} was calculated using Microtox® Omni Software Version 1.18 available from the manufacturer (Azur Environmental, 1998). For the microalgae growth inhibition test, IC_{50} was estimated by means of the interpolation method with the software ICPin provided by the USEPA (Norberg-King, 1988). The normality of the absorbance values was determined by the Shapiro–Wilk test and the homogeneity of the variance by the Bartlett test (applied as a quality control for the results as recommended by USEPA (2002)). Both statistical conditions normality and homogeneity of the variance were met in all of the tests. For sea urchin EC_{50} was calculated using statistical package TOXSTAT® (Gulley et al., 1989) Probit analysis. Toxic Units (TU) were calculated based on the EC_{50} values from each assay by $TU=(1/EC_{50})\ 100$. For biomarker responses correlations were obtained through Pearson's rank order correlation test.

3. Results

3.1. Phase I

Measured environmental concentration (MEC) of MTX and TMX to obtain the estimation of environmental exposure are indicated in Table 1. All data was obtained from the literature.

3.2. Phase II

For all the toxicity tests performed in Phase II, no significant differences between control (sea water) and solvent control (DMSO) were observed. Tests performed with DMSO showed that this solvent applied to prepare MTX and TMX stocks at 0.001% v/v had no negative effect on the organisms analyzed.

3.2.1. Bacteria bioluminescence inhibition

Results of the bioluminescence inhibition of bacteria *A. fischeri* exposed to 9 serial dilutions of MTX and TMX are indicated in Fig. 2. Tested microorganisms indicated adequate physiological state, according to the *A. fischeri* protocol (Azur-Environmental, 1998). Control samples indicated 100% of bioluminescence, indicating the good condition of the bacteria during the test. Environmental concentration of MTX and TMX tested were not toxic for the bacteria. Both drugs inhibited bacterial bioluminescence in a concentration-dependent manner with bioluminescence decreasing with increasing concentrations. Bioluminescence results after 5, 15 and 30 min respectively expressed as EC_{50} values are shown in Table 2. The time of exposure seemed to affect the EC_{50} response. After 30 min of exposure, the estimated EC_{50} for TMX was 330,000 μg L^{-1} being lower than for MTX EC_{50}=900,000 μg L^{-1}, and based on TU calculated, it is shown

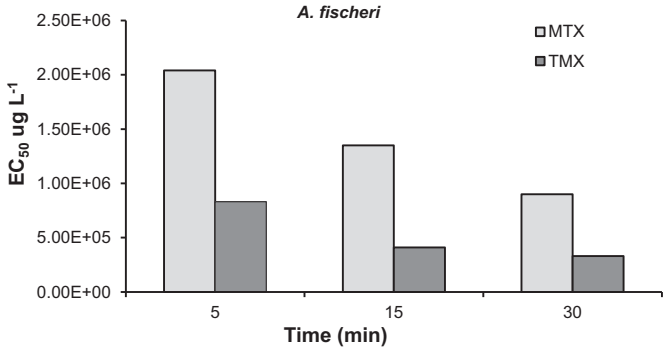


Fig. 2. Bioluminescence of *A. fischeri* after exposure to different concentrations of methotrexate (MTX) and tamoxifen (TMX) during 5, 15 and 30 min (one way ANOVA, $p<0.05$). Data from $n=1$ experiment.

Table 2
Phase II risk assessment of methotrexate [a] and tamoxifen [b] represented by EC_{50} values (μg L^{-1}) (effect concentration for 50% of the test population) and TU (toxic units).

Organism	Phylum	Species	Trophic level	Habitat	Indicator	5 min	15 min	30 min	1 h	48 h	96 h	TU EC_{50}
Bacteria	Proteobacteria	*A fischeri*	Decomposer	Marine	Bioluminescence	2,040,000[a] 830,000[b]	1,350,000[a] 410,000[b]	900,000[a] 330,000[b]	–	–	–	0.1[a] 0.3[b]
Micro algae	Haptophyta	*I. galbana*	Producer	Marine	Growth inhibition	–	–	–	–	–	84,000[a] 35,000[b]	1.2[a] 2.9[b]
Sea urchin	Echinodermata	*P. lividus*	Herbivore	Marine	Fertilization	–	–	–	> 1,000,000[a] 15,000[b]	–	–	0.1[a] 6.7[b]
					Larval development	–	–	–	–	1500[a] 1.5[b]	–	66.7[a] 66666.7[b]

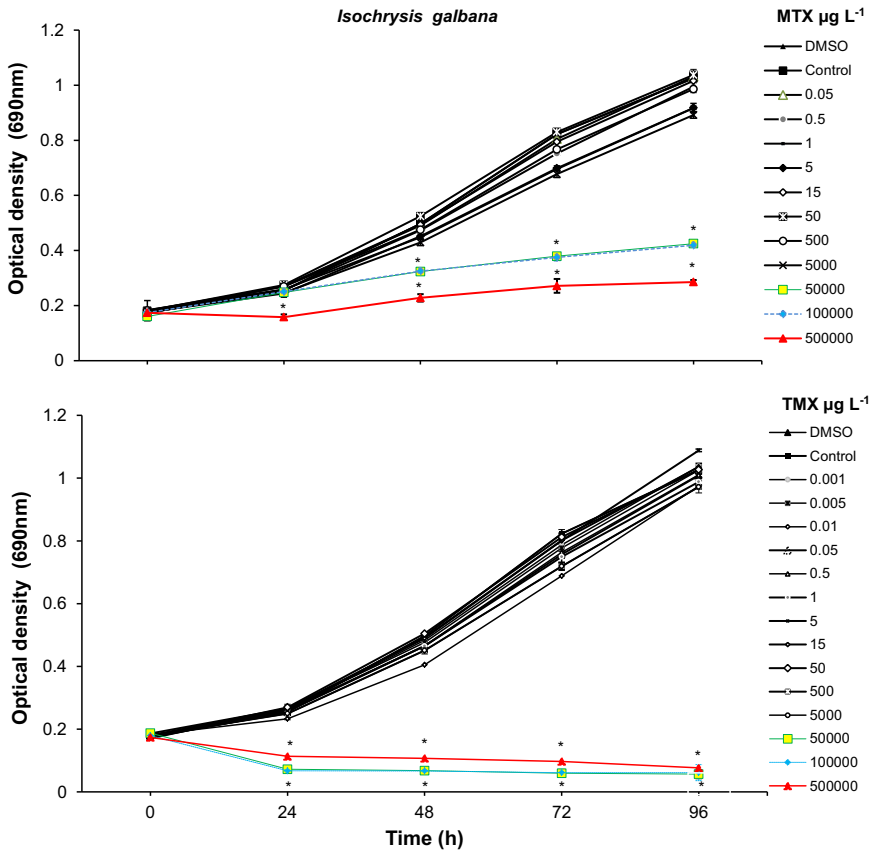


Fig. 3. Growth inhibition of *I. galbana* exposed to increasing concentrations of methotrexate (MTX) and tamoxifen (TMX) during 96 h. Asterisks indicate significant differences from control (one way ANOVA, $p < 0.05$). Data are means ± SE, $n = 3$ vials per concentration.

that TMX is three times more toxic for *A. fischeri* than MTX. Nevertheless, concentrations of MTX and TMX that produced an effect are unlikely to occur in the environment.

3.2.2. Microalgae growth inhibition

The temporal evolution of the biomass concentrations of the toxicity tests carried out in this work are presented in Fig. 3. Graphics showed an exponential growth of *I. galbana* exposed to treatments (control, DMSO and selected drugs) during experiments. The growth rate of controls was within the range required by the ISO 10253 protocol (2006). Significant growth inhibition was measured only at the highest concentrations of MTX and TMX (50,000, 100,000 and 500,000 µg L^{-1}) compared with control ($p < 0.01$). Furthermore, significant decrease of algal density was observed as early as 24 h of the assay when exposed to MTX at 500,000 µg L^{-1} and to TMX at 50,000, 100,000 and 500,000 µg L^{-1}. TU was calculated indicating that TMX is approximately 2.4 times more toxic than MTX (Table 1). Microalgae growth inhibition results expressed as EC_{50} values (95 % confidence interval) are shown in Table 2, which takes into consideration the EC_{50} estimated values for each pharmaceutical. Finally, this test indicates that environmental concentrations of MTX and TMX are not toxic for these species.

3.2.3. Sea urchin toxicity test

3.2.3.1. Fertilization. Results of *P. lividus* gametes exposed to environmental concentrations of MTX and TMX were similar to those exposed to control > 98% of fertilization success (Fig. 4a). MTX produced a significant decrease in fertilization compared with control ($p < 0.01$) at 100,000 µg L^{-1} (17% decrease), 500,000 µg L^{-1} (27 % decrease) and 1,000,000 µg L^{-1} (30% decrease). While TMX tested at 50,000 µg L^{-1} completely affected the fertilization process, it was observed that 100% of the eggs were unfertilized. Based on EC_{50} and the estimated values of TU for fertilization (Table 2), it can be stated that TMX is 67 times more toxic than MTX.

3.2.3.2. Larval development. The results of larval development success after exposing fertilized eggs of *P. lividus* to MTX and TMX are presented in Fig. 4b. Control treatment indicate > 98% of development of pluteus larvae. Both pharmaceuticals inhibited embryogenesis success (defined as the percentage development of 4 arms pluteus larvae), following a typical concentration-dependent gradient ($p < 0.01$; MTX $r = 0.84$ and TMX $r = 0.93$). A significant decrease in larval development was determined in fertilized eggs exposed to MTX from 5 to 5000 µg L^{-1} compared with control ($p < 0.05$). It was observed that higher concentrations of MTX produced adverse effects in eggs, completely reducing (up to 100 %) the possibility of development. Teratogenic effects were also noticed in eggs exposed to TXM due to a significant decline in percentage of development compared to control ($p < 0.05$). It is important to mention that environmental concentrations tested produce a 20% of reduction of the correct development of the larvae to pluteus stage. Furthermore, at 500 µg L^{-1} of TMX, the development of the larvae is null. Based on the calculated EC_{50} for larval development (Table 3), it can be stated that TMX is more toxic that MTX for sea urchin larval development.

The MEC/PNEC values are indicated in Table 3. Based on the calculated EC_{50} from different endpoints, no further evaluation was required for MTX (assessment = no). In the case of TMX only results from larval development tests obtained indicated that TMX at environmental concentrations require deeper evaluation (assessment = yes), or that a Phase III study should be carried out. Thus, other endpoints applied indicated no deeper evaluation.

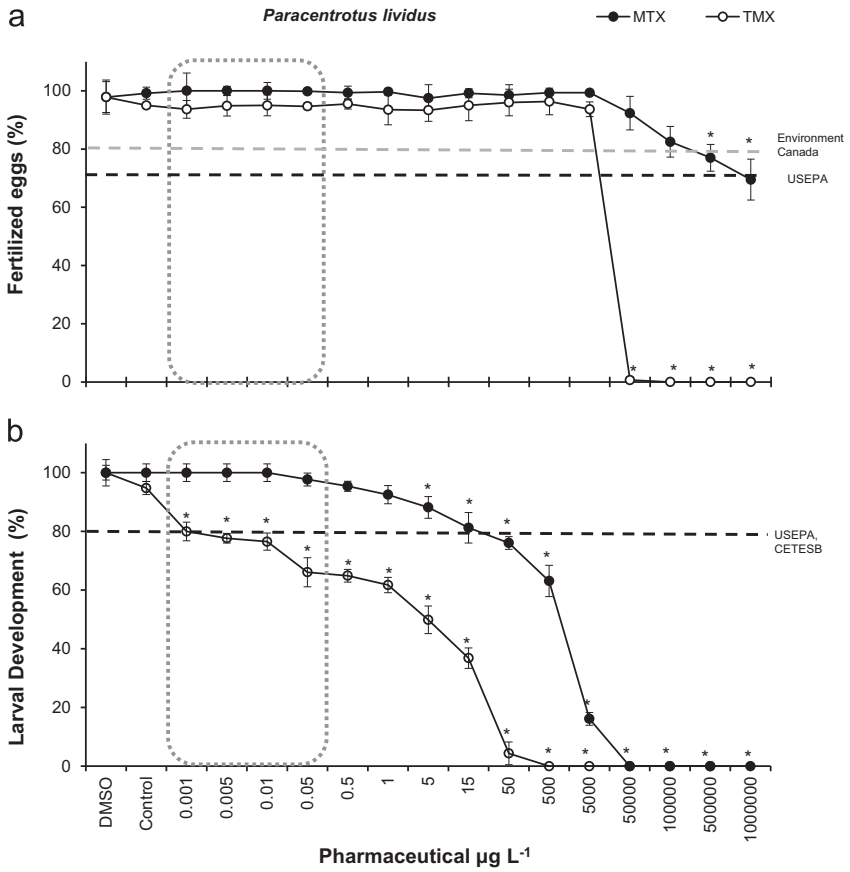


Fig. 4. (a) Percentage of fertilization success after 1 h exposure of *P. lividus* sperm to methotrexate (MTX) and tamoxifen (TMX). Asterisks indicate significant differences from control (one way ANOVA, $p < 0.05$). Data are means ± SE, $n = 4$ vials per concentration, $n = 200$ fertilized eggs per vial (b) percentage of normal pluteus after 48 h exposure of *P. lividus* fertilized eggs to concentrations of MTX and TMX (one way ANOVA, $p < 0.05$). Data are means ± SE, $n = 4$ vials per concentration, $n = 100$ embryo–larva developed per vial. Dotted gray line indicates environmental concentration. Hatched gray line indicates the limits below in which the sample is considered toxic by the Canadian Standards (Environment Canada, 2011) and hatched black line indicates the limits below in which the sample is considered toxic by USEPA (1995,2002) and CETESB (1999).

Further assessment of TMX was carried out with the species *R. philippinarum* via the application of a screening biomarker (LMS), and biochemical biomarkers (EROD, DBF, GST, GPX, GR and AChE activity, LPO levels and DNA damage).

It is noteworthy to mention that based on EC_{50} results obtained in Phase II of the risk assessment (Table 4), and following the EU Directive 93/67/EEC, intended to classify substances according to their measured effective concentration (EC) (CEC, 1996), the toxicity of both drugs were dependent on the species sensitivity and on the assay employed. In this sense, MTX was classified as "toxic"

Table 3
Assessment of methotrexate (MTX) and tamoxifen (TMX) based on measured environmental concentration (MEC)/predicted no effect concentration (PNEC) index. EC_{50} (effect concentration for 50% of the test population).

	Pharmaceutical	EC_{50} (µg L^{-1})	MEC (µg L^{-1})	PNEC	MEC/PNEC Index	Assessment
V. fishery	TMX	330000	0.004[a]	330	0.00001	No
(Bioluminescence)	TMX	330,000	0.37[b]	330	0.00112	No
	MTX	900,000	0.0021[c]	900	0.000002	No
	MTX	900,000	0.245[d]	900	0.00027	No
I. galbana	TMX	35,000	0.004[a]	35	0.00011	No
(Growth inhibition)	TMX	35,000	0.37[b]	35	0.010571	No
	MTX	84,000	0.0021[c]	84	0.00003	No
	MTX	84,000	0.245[d]	84	0.00292	No
P. lividus	TMX	15,000	0.004[a]	15	0.00027	No
(Fertilization)	TMX	15,000	0.37[b]	15	0.02467	No
	MTX	1,000,000	0.0021[c]	1000	0.000002	No
	MTX	1,000,000	0.245[d]	1000	0.00025	No
P. lividus	TMX	1.5	0.004[a]	0.0015	2.66667	Yes
(Larval development)	TMX	1.5	0.37[b]	0.0015	246.66667	Yes
	MTX	1500	0.0021[c]	1.5	0.0014	No
	MTX	1500	0.245[d]	1.5	0.16333	No

[a] Thomas and Hilton (2004).
[b] Roberts and Thomas (2006).
[c] Negreira et al. (2013).
[d] Yin et al. (2010).

Table 4
Toxicity level of pharmaceuticals based on EC_{50} (effect concentration for 50% of the test population) results from bioluminescence[a], growth inhibition[b], fertilization[c], larval development[d] and lysosomal membrane stability[e] tests following classification from E.U. Directive 93/67/EEC.

Pharmaceutical	Extremely toxic	Very toxic	Toxic	Harmful	Non toxic
Ec_{50} ($\mu g\ L^{-1}$)	< 100	< 100–1000	1000–10,000	10,000–100,000	> 100,000
Methotrexate			*P. lividus* [d]	*I. galbana* [b]	*V. fischeri* [a] *P. lividus* [c]
Tamoxifen	*P. lividus* [d] *R. philippinarum* [e]			*P. lividus* [c] *I. galbana*[b]	*V. fischeri* [a]

for *P. lividus* when testing larval development, "harmful" for *I. galbana* and "nontoxic" for *A. fischeri* and *P. lividus* when testing fertilization success. Respecting TMX, it was classified as "Extremely toxic" for *R. philippinarum and P. lividus* when testing percentage of embryogenesis; "Very toxic" for *I. galbana and P. lividus* when assessing fertilization; TMX was considered "not toxic" for the bacteria *A. fischeri*.

3.3. Phase III

During the 14 days of exposure to increasing concentrations to TMX, the mortality rate observed was less than 2% from all treatments (data not shown). It is important to point out that screening and biochemical biomarkers responses observed in *R. philippinarum* at the beginning of the assay (Day 0), clams exposed to control treatment, and clams exposed to the solvent DMSO (0.001 % v/v) showed no significant differences. Consequently these clams were referred as "controls".

3.3.1. Tier 1: screening biomarker

Results of screening biomarker are shown in Fig. 5. Neutral red retention time (NRRT) at the beginning of the assay (day 0) was 134 ± 25 min, whilst the NRRT from control at the end of the experiment (day 14) was 128 ± 18 min. At the end of the assay, evident changes were observed in clams exposed to TMX. The NRRT decreased in clams exposed to environmental concentration of TMX 0.1 $\mu g\ L^{-1}$ (85 ± 18 min); however, this decrease did not differ from control organisms. Significant changes ($p < 0.01$) in the LMS compared with control organisms were recorded in those clams exposed to TMX at 10 and 50 $\mu g\ L^{-1}$ with the retention of the dye reduced by 53 % (NRRT = 60 ± 15 min) and 90% (NRRT = 12 ± 7) respectively. Based on the LMS criteria employed by Aguirre-Martínez et al. (2013c), all clams from day 0, controls and those exposed to DMSO were considered healthy. Clams exposed to 0.1, 1 and 10 $\mu g\ L^{-1}$ of TMX were considered to be stressed but compensating, and those exposed to 50 $\mu g\ L^{-1}$ of TMX were considered to present a diminished health status. Results obtained in this assay showed a concentration-dependent relationship ($p < 0.01$; $r = 0.9$). The EC_{50} (95 % CI) was 19 $\mu g\ L^{-1}$. Based on the chronic EC_{50} results, and following the EU Directive 93/67/EEC intended to classify substances according to their EC (CEC, 1996), TMX can be classified as extremely toxic for *R. philippinarum* when assessing LMS ($EC_{50} < 0.1$ mg L^{-1}) (Table 3).

3.3.2. Tier 2: Biochemical biomarkers

Biochemical biomarker responses are indicated in Fig. 6. Significant induction ($p < 0.05$) of EROD activity compared to control was observed in clams exposed to all concentrations of TMX. Significant concentration-response correlation was observed in digestive gland tissues ($p < 0.01$; $r = 0.78$). The highest induction of EROD activity was noticed in clams exposed to 50 $\mu g\ L^{-1}$ (6.5 fold control). A significant decrease of DBF activity compared with controls was observed in clams exposed to all concentrations of TMX ($p < 0.01$). This decrease followed a concentration-response correlation ($p < 0.01$; $r = 0.80$). Regarding GST activity, the highest concentration of TMX (50 $\mu g\ L^{-1}$) triggered significant ($p < 0.05$) induction in digestive gland tissues (2.6 fold control). Significant differences compared with controls ($p < 0.05$) were observed in clams exposed to 1 $\mu g\ L^{-1}$. At this concentration, the activity recorded was significantly lower than controls. The induction of this activity was directly correlated with TMX concentration following a significant concentration-response ($p < 0.01$; $r = 0.76$). A significant induction of GPX activity compared to control organisms ($p < 0.01$) was observed in clams exposed to 50 $\mu g\ L^{-1}$ (5.9 fold

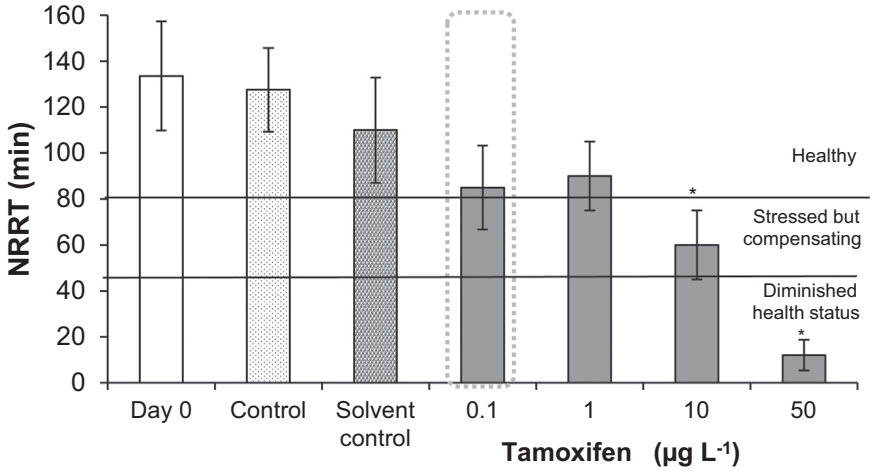


Fig. 5. Screening biomarker (Lysosomal membrane stability) evaluated by the neutral red retention time (NRRT) assay in haemocytes of *R. philippinarum* exposed 14 days to control water, solvent control (DMSO) and tamoxifen. Asterisks indicate significant differences from control (one way ANOVA, $p < 0.05$). Data are means ± SE, $n = 10$. Dashed lines indicate environmental concentration. Black lines indicate health status threshold proposed by Aguirre-Martínez et al. (2013c).

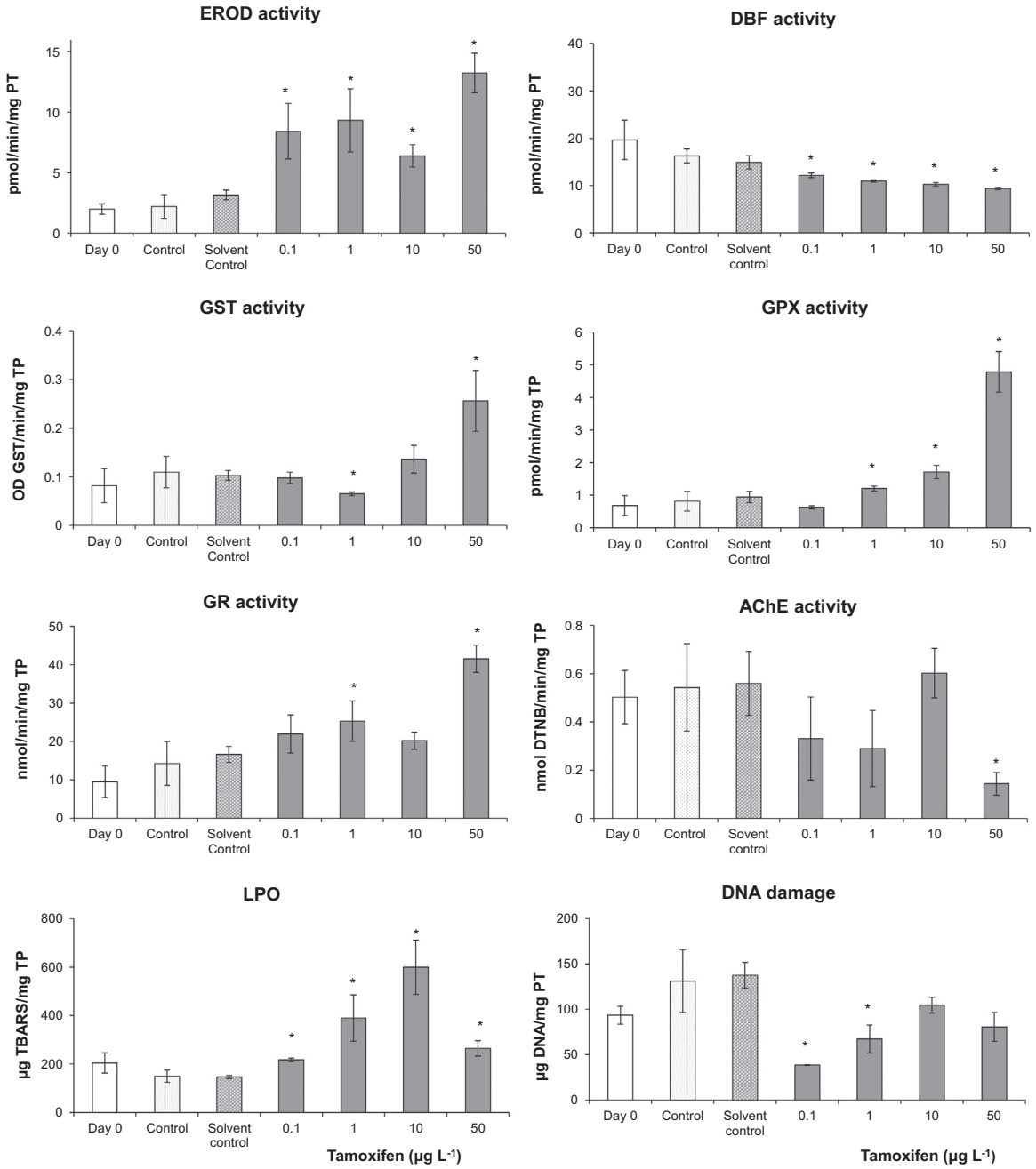


Fig. 6. Biochemical biomarkers [ethoxyresorufin O-deethylase (EROD), dibenzylfluorescein dealkylase (DBF), glutathione S-transferase (GST), glutathione peroxidase (GPX), glutathione reductase (GR), acetylcholinesterase (AChE) lipid peroxidation (LPO) and DNA damage] measured in digestive gland tissues of *R. philippinarum* exposed 14 days to dissolved tamoxifen. Asterisks indicate significant differences from control (one way ANOVA, $p<0.05$). Data are means ± SE, $n=4$ pools.

control), 10 µg L^{-1} (2.9 fold control), and 1 µg L^{-1}. Results indicated a significant concentration-response relationship ($p<0.01$; $r=0.86$). Regarding GR activity, a significant induction was shown ($p<0.01$) in clams exposed to TMX at 1 µg L^{-1} (4.8 fold control) and 50 µg L^{-1} (11.5 fold control), following a strong concentration-response correlation ($p<0.01$; $r=0.81$). AChE activity decreased significantly in clams exposed to the highest concentration tested 50 µg L^{-1} ($p<0.01$). LPO levels increased significantly in clams exposed to all concentrations of TMX compared to controls ($p<0.05$); clams exposed to 10 µg L^{-1} showed the highest values (4 fold control); Results indicate a strong concentration-response correlation ($p<0.01$; $r=0.75$). Increasing concentrations of TMX provoked a decrease of DNA damage in digestive gland tissues following a significant negative concentration-response correlation ($p<0.01$; $r=-0.72$). This decrease was found to be significantly different to controls at 0.1 and 1 µg L^{-1} of TMX ($p<0.01$).

4. Discussion

Risk assessment was applied for MTX and TMX cancer therapeutic drugs following an adapted EMEA guideline. In Phase I, an estimation of environmental exposure was performed, based on measured environmental concentrations (MEC), in order to determine a more realistic ERA for the aquatic environment (Blasco and Del Valls, 2008).

As stated previously, a broad database with chronic toxicity

tests are necessary to carry out the ERA of drugs (Ankley et al., 2007; Blasco and DelValls, 2008). Yet this step has not been fully developed for drugs in marine environments. The literature indicated a MEC $\geq 0.01\ \mu g\ L^{-1}$; therefore, both cancer therapeutic drugs needed to be examined with toxicity tests. In Phase II, acute bioassays were applied using organisms from different trophic levels in order to predict risk. A bacteria bioluminescence toxicity test was applied first. Generally, tests employing bacteria (e.g. the Microtox® assay) are rapid, sensitive and cost effective; thus, they are valuable as preliminary screening tests for determining acute aquatic toxicity (Wong et al., 1995). In the case of chemotherapeutic agents tested herein, it was observed that both MTX and TMX were almost not toxic for the bacteria; similarly to these results Mater et al. (2014) reported that TMX did not affect bioluminescence in *A. fischeri*.

Secondly, a microalgae growth inhibition test was performed using *I. galbana*; marine unicellular algae (phytoplankton, microalgae) are essential to the normal function of marine ecosystems, as they are the main primary producers which form the first link in food webs, as they oxygenate the water, and are important in the cycle of dissolving organic and inorganic substances (Walsh, 1988). Changes produced in phytoplankton may significantly affect the marine ecosystem. As previously mentioned, tests including microalgae are recommended by USEPA (2002), OECD (2011), ECHA (2008) and TSCA (2003) as they are responsive to diverse chemical compounds. Furthermore, it has been reported that when applying EMEA guideline from 2001 for an ERA of pharmaceuticals present in rivers, the microalgae toxicity test appeared to be more sensitive than toxicity tests using fish and *Daphnia* (Ginebreda et al., 2010). From the recommended selection of those three trophic levels, the first trophic level has been indicated as the most sensitive for pharmaceuticals when performing acute toxicity (Blasco and Del Valls 2008). In this regard, the growth inhibition test using *I. galbana* showed that environmental concentrations of MTX and TMX did not negatively affect the growth response. Moreover, it was found that concentrations which produced adverse effects in algal density were too high to occur in environment. Results from this study are in agreement with those indicating that TMX at 1 and $10\ \mu g\ L^{-1}$ do not affect the growth of microalgae (Mater et al., 2014); below these concentrations, Mater and coworkers observed a hormesis effect which was not observed in this experiment. TMX mode of action is complex; it has both estrogenic and anti-estrogenic effects, depending on the target tissue; antiestrogen (inhibiting agent) in mammary tissue and estrogen (stimulating agent) in cholesterol metabolism, bone density, and cell proliferation (Jordan et al., 1980). This study showed that at the environmental concentrations tested the mode of action of TMX did not affect phytoplankton. Thus growth of microalgae was not significantly stimulated or inhibited compared to controls. On the other hand, a significant inhibition was observed when *I.galbana* were exposed to the highest concentrations of TMX (50,000, 100,000 500,000 $\mu g\ L^{-1}$). This inhibition might be due to the antitumor activity of TMX; in this regard there is evidence for one or more additional mechanisms of action that is able to inhibit growth in yeast cells (Wiseman et al., 1990a).

Later, two short term bioassays were performed with the sea urchin *P. lividus* (fertilization and larval development). Results indicated that neither MTX nor TMX affected the fertilization success when tested at environmental concentration. Nevertheless, environmental concentrations of TMX had an effect on larval development, being toxic to *P. lividus* at this range as teratogenic effects were observed during the test. Teratogenicity of TMX at environmental concentration for *P. lividus* found in this study is in agreement with previous work, indicating a significant decrease of normal development of sea urchin *Strongylocentrotus purpuratus exposed* at environmental concentrations of TMX (Roepke et al., 2005). Significant decrease and teratogenic effect provoked by environmental concentrations of pharmaceuticals have been described previously in sea urchin species, for example, in *P. lividus* exposed to, caffeine, ibuprofen, carbamazepine and novobiocin (Aguirre-Martínez et al., 2015); in *Lytechinus anamesus* and *S. purpuratus* exposed to natural and synthetic reproductive hormones (Roepke et al., 2005), and in *Arbacia lixula* exposed to beta blocker atenolol (Karaaslan et al., 2012).

Selecting early life stage provides a quick, relatively easy and sensitive toxicity test, with the added advantage of having a low cost and test duration. In fact, many standardized test protocols often use early life stage i.e., US Environmental Protection Agency (EPA-812-R-02-013 (US EPA, 2002) and American Society for Testing and Materials (ASTM-E724-98 (2004); ASTM-E1192-97 (2008) test protocols recommend the use of early life stages (Mohamed, 2013). In this sense, it has been indicated that aquatic invertebrate larvae are more sensitive than adults (Hutchinson et al., 1998). Organisms from different, or even from the same phyla display different degrees of sensitivity towards substances, thus there are no species which are classed as "the most sensitive" (Bakopoulou et al., 2011; Van der Grinten et al., 2010). Nevertheless, in this study and in previous studies, it has been found that tests performed with *P. lividus*, specifically those involving larval development, were the most appropriate for determining any potential negative effects of organic contaminants (Carballeira et al., 2011, 2012; Marin et al., 2007; Pesando et al., 2003).

Bioassays applied in Phase II indicated that an environmental concentration of MTX was not harmful to the three marine species tested, posing no risk to the environment; therefore, no further assessment was required for this drug (Fig. 1). Results that indicated no deeper assessment of MTX (Table 2) support those from Perazzolo et al. (2010), who in order to select those substances that may do the most harm to the environment and propose a priority list for assessment of pharmaceuticals, discarded MTX from the list. This decision was based on a lack of analytical feasibility prior to performing measurements and extensive risk assessment. It is important to indicate that based on the tier approach applied in this study, a deeper evaluation for this drug was not performed; nevertheless MTX has been reported to be teratogen in higher animals (rats, rabbits and mice) (DeSesso and Goeringer, 1992; Pellizzer et al., 2004), and in the fly, *Drosophila melanogaster,* by diminishing severely fecundity in these species (Affleck et al., 2006). In addition, mutagenicity and genetic damage might be assumed for MTX (Deng et al., 2005; Chow and Rubin, 1997). The particular mode of action of MTX could negatively affect cell proliferation, and therefore, interact in the survival of species. For this reason, the substance is highly toxic and is classified as "toxic" in Europe and "very toxic" in the United States. Therefore, it has to be taken into consideration that bioassays applied in Phase II of the EMEA guidelines (even the use of larval development applied in this study) might be unsuitable for evaluation of this drug, as they donot detect the possible negative effects and toxicity of MTX for aquatic biota; therefore the use of more sensitive endpoints would be advisable. For example, those indicated in the 2-tier approach applied in Phase III would be more suitable to detect adverse effects of MTX. Thus if current EMEA guideline are applied, the possible environmental risk of MTX present in the marine environment might be underestimated.

On the other hand, results displayed potential adverse effects for TMX with the requirement of further analysis leading to the next step (Phase III). This phase is important, since it takes into consideration possible chronic effects and also shows a more realistic side of the long term exposure of aquatic biota to a continuous discharge of pharmaceuticals. LMS was evaluated as a Screening biomarker (Tier 1). In this step, changes were demonstrated in membrane stabilization in haemocytes of *R.*

philippinarum after 14 days of exposure to environmental concentrations of TMX. This data confirmed that TMX is capable to produce adverse effects at the environmental range tested. LMS was evaluated by the NRRT reflect stress and damage in haemocyte cells, in clams, following TMX exposure. This damage is of interest and thus indicates the cellular well-being of the organism traduced in health status (Grisham and Smith, 1984). TMX in this study is shown to affect the health status of clams reducing LMS at environmental range and higher concentrations tested. This indicates that clams were in a state of stress but compensating, and that a concentration of 50 $\mu g\ L^{-1}$ of TMX caused deleterious effects in these species, as they showed a significant decrease in LMS indicating diminished health status. The variation in the NRRT found in this research agrees with results from previous studies of *R. philippinarum* and *C. maenas* exposed to similar pharmaceuticals at the environmental range performed by our laboratory, confirming the sensitivity of the LMS test and the responsiveness of these species (Aguirre-Martínez et al., 2013c, 2013d).

Tier 2 of Phase III, involving biochemical biomarkers showed that TMX at environmental relevant concentration was capable to induce EROD activity in *R. philippinarum* after 14 days of exposure, whilst significantly decreasing DBF activity, compared with controls. It has been stated that CYPs play an important role metabolizing (principally in the liver) potentially toxic compounds, such as xenobiotics (including drugs) and products of endogenous metabolism (Guengerich, 2008; Samsel and Seneff, 2013). Induction of EROD activity in this research indicated that TMX drug was metabolized in the digestive glands of clams, involving the cytochrome P450 (CYP). These results are in agreement with statements indicating that tamoxifen can be catalysed in liver from rats and humans by the cytochrome P450 (CYP) (Kim et al., 2003). Moreover, CYP enzymes have been shown to play a major role in TMX metabolisms, being involved in the formation of N-desmethyl-TAM and 4-OH-TAM (Mani et al., 1993; Jacolot et al., 1991). On the other hand, the decrease of DBF activity observed in this study may be the result of inhibition produced by the active compound of TMX.

AChE activity has been recommended as a biomarker of neurotoxicity in aquatic organisms (Cajaraville et al., 2000). Environmental concentration of TMX did not induce or inhibit significantly AChE activity in digestive glands, however, a significant decrease was noticed when clams were exposed to TMX at 50 $\mu g\ L^{-1}$, this inhibition is considered of interest as AChE has an important role in the functioning of the neuromuscular system, preventing continuous muscular contraction. These results are consistent with previous work indicating inhibition of AChE activity in gills tissues of *R. philippinarum* exposed to triclosan (Matozzo et al., 2012) and exposed to ibuprofen (Milan et al., 2013); AChE inhibition has been reported also in gills of *M. galloprovincialis* exposed to propranolol and acetaminophen (Solé et al., 2010).

Regarding the detoxification enzyme, GST activity was significantly induced at the highest TMX level tested, compared with controls indicating that the biotransformation enzymes of clam's metabolism were activated. This result of induction of GST activity is in agreement with those demonstrating an increase of this activity in digestive gland tissues of *Elliptio complanata* (fresh water mussel) as a result of pharmaceutical exposure, when immersed to a municipal effluent plume in Canada (Gagné et al., 2004). Similarly to these results it has been reported an increase of GST activity in digestive gland tissues of *Mytilus galloprovincialis* (marine mussel) exposed directly to xenobiotics in The South Coast of Portugal (Bebianno et al., 2007). It is noteworthy to indicate that the antioxidant response GPX activity and GR activity was activated by TMX at an environmental concentration. Induction of this activity by TMX indicated oxidative stress *in R. philippinarum* metabolism. In order to protect the organism from this stress, antioxidant systems such as GPX and GR enzyme activity are activated to avoid oxidative damage by eliminating reactive oxygen species (Di Giulio et al., 1989). These results of induction are in agreement with those reporting Increase of the antioxidant response of GPX and GR activity in digestive glands of mussels exposed to organic pollutants (PAHs, PCBs, DDTs and lindane) (Solé et al., 1995). Results of the present research and previous studies from our laboratory (Aguirre-Martínez et al., 2013a, 2013b), together with other mentioned investigations indicate that drugs are able to induce phase I and II biotransformation enzymes, and induce oxidative stress during the biotransformation process in invertebrates. Oxidative stress is a common consequence of xenobiotic presence responsible for biomolecule alteration. Some of these alterations constitute probable links with signaling processes which bring about cell death or, conversely, increase of resistance capacities (Latendre et al., 2012). As soft bodied organisms xenobiotics can enter in molluscs through different ways (digestive tract by ingestion of water and food and respiratory surfaces), and accumulate because of low degradation capacities. Oxidative stress induces biochemical changes in invertebrates, and the extent of these changes depends on the severity of the oxidative stress (Latendre et al., 2012).

LPO levels were measured to evaluate effectiveness of antioxidant defences thus oxidative stress can promote lipid peroxidation (LPO) (Winston and DiGiulio, 1991). Digestive gland tissues of clams exposed to TMX at all concentrations tested, including environmental concentration, showed a significant rise of LPO levels compared with control organisms, indicating oxidative stress resulting from an intensified xenobiotic metabolism (Livingstone, 2001). Our results are in disagreement with previous results indicating that TMX inhibit LPO in humans and rats thus changes in the rate of lipid peroxidation seem to be a general feature of cancerous cells and may be a prerequisite to cell division (Wiseman et al., 1990a). In addition, it has been reported that TMX and its more active metabolite (OHTMX) induce multiple cellular effects, including antioxidant actions. TMX and its metabolite OHTMX are efficient inhibitor of lipid peroxidation in cellular membranes and in rat liver microsomes and ox-brain phospholipid m3 liposomes (Wiseman et al., 1990a, 1990b). Increases of LPO might lead to DNA damage however; results from this research indicate that TMX did not increase DNA damage in *R. philippinarum.* These results are also in agreement with findings of Mater et al. (2014), reporting no DNA strand breaks in HepG2 cell cultures exposed to TMX at 0.01–10 $\mu g\ L^{-1}$. On the other hand, these results are in disagreement with the statement indicating high levels of DNA adducts in liver of rats exposed chronically to TMX (Firozi et al., 2000), nevertheless they tested TMX at concentrations that were orders of magnitude higher that the ones tested herein (200 and 500 $mg\ L^{-1}$). DNA damage has been detected in monkeys, and in human endometrial explants treated with TMX or its metabolites, and also in women administered TMX (Gaikwad and Bodell, 2012). Although the mechanisms which cause cancer have not been determined yet, several laboratories have reported that administration of TMX causes gentoxicity in the liver and, to a lesser extent, in other organs of rats, mice, and hamsters (Han and Liehr, 1992; Moorthy et al., 1997; Parvez et al., 2006). Therefore, it seems that a metabolism of TMX in vertebrates is different to invertebrates.

Finally, induction and inhibition of biomarkers measured in Tier 2 demonstrated the bioavailability of TMX during the experiment. When a xenobiotic is bioavailable, and enters into the organism, it is biotransformed into a more water soluble form to be excreted (Koenig et al., 2012). All the biotransformation reactions have been measured in this approach in order to assess the bioavailability of the tested compounds. The bioavailability of TMX

during experiments can be deduced when comparing results of induction and inhibition of biomarkers with those from controls clams.

To conclude, it has been demonstrated that there is an inadequacy of current EMEA guideline implemented for the risk assessment of MTX and TMX within marine environment. The endpoints applied in Phase II (bioluminescence, microalgae growth inhibition and fertilization) using marine organisms were not adequate enough to indicate the adverse effects of these chemotherapeutic agents; therefore, it would not be recommended that these short term tests are to be applied in ERA of pharmaceuticals. Larval development of sea urchin was show to be the most sensitive endpoint indicating the adverse effect of TMX (but not MTX) at an environmental concentration. The endpoints applied at Phase II dismissed the possible toxicity of MTX in the marine environment. All the biomarkers tested in Phase III (screening biomarker and biochemical biomarkers) were effective and representative from a toxicological point of view. Furthermore, this battery applied herein was capable to indicate stress the case of LMS and indicate effect in the case of the biochemical biomarkers applied; results indicate the suitability of including a biomarker approach in Phase III, as the biomarkers were sensitive and responsive. This demonstrated the cause-effect and concentration-effect produced by the exposure to TMX chemotherapeutic agent, even at environmental concentrations. Induction and inhibition of biomarkers in this phase shows the bioavailability of TMX during the experiment. Results from this study indicate the possible risk of TMX in the marine environment. The potential adverse effects of this compound must not be excluded, and therefore, precautionary and safety measures should be applied.

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