Toxicity screening of Diclofenac, Propranolol, Sertraline and Simvastatin using *Danio rerio* and *Paracentrotus lividus* embryo bioassays

Sílvia Ribeiro[a, 1], Tiago Torres[a, 1], Rosário Martins[a, b], Miguel M. Santos[a, c,]

[a] CIMAR/CIIMAR – Interdisciplinary Centre of Marine and Environmental Research, University of Porto, Rua dos Bragas 177, 4050-123 Porto, Portugal

[b] Escola Superior de Tecnologia de Saúde do Porto, Instituto Politécnico do Porto, Porto, Portugal

[c] FCUP – Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, 4169-007 Porto, Portugal

Abstract

Early life-stage bioassays have been used as an alternative to short-term adult toxicity tests since they are cost-effective. A single couple can produce hundreds or thousands of embryos and hence can be used as a simple high-throughput approach in toxicity studies. In the present study, zebrafish and sea urchin embryo bioassays were used to test the toxicity of four pharmaceuticals belonging to different therapeutic classes: diclofenac, propranolol, simvastatin and sertraline. Simvastatin was the most toxic tested compound for zebrafish embryo, followed by diclofenac. Sertraline was the most toxic drug to sea urchin embryos, inducing development abnormalities at the ng/L range. Overall, our results highlight the potential of sea urchin embryo bioassay as a promising and sensitive approach for the high-throughput methods to test the toxicity of new chemicals, including pharmaceuticals, and identify several drugs that should go through more detailed toxicity assays.

Abbreviations

DCF, Diclofenac; DMSO, Dimethylsulfoxide; EE2, ethynilestradiol; NSAID, Non-Steroidal Anti-Inflamatory Drug; hpf, hours post fertilization; PROP, Propranolol; SER, Sertraline; SIMV, Sinvastatin; SSRIs, Selective Serotonin Reuptake Inhibitors; WWTPs, ., Wastewater Treatment Plants

Keywords

Emerging contaminants; Risk assessment; sea urchin; zebrafish; embryo bioassays; high-throughput methods

1. Introduction

In the past decade, the presence of emerging contaminants in the aquatic environment has raised considerable concern (Bossus et al., 2014). Pharmaceuticals are a group of emerging contaminants that have been recently detected in the water compartment at low concentrations in the ng/L or µg/L range (Lapworth et al., 2012). Their occurrence raises concern not only for human health but also for wildlife. In fact, although human exposure to contaminated water occurs in discontinuous process, aquatic ecosystems are continually exposed to this type of chemicals and several studies have already identified different pharmaceuticals in concentrations that are likely to induce adverse effects in aquatic organisms (Fent et al., 2006). Pharmaceuticals can enter into the aquatic environment through point and non-point sources and for several classes the Wastewater Treatments Plants (WWTPs) cannot ensure complete removal, and therefore WWTPs effluents may still have significant concentrations of some pharmaceuticals (Fent et al., 2006). One of the best characterized examples of the toxic potential of pharmaceuticals is the synthetic estrogen ethynilestradiol (EE2). At the low ng/L range (0.1–5 ng/L), EE2 impacts ecologically relevant endpoints in fish such as behavior, embryonic development, reproduction and sex ratio (Micael et al., 2007, Soares et al., 2009, Santos et al., 2010 and Soares et al., 2012). Therefore, this example calls our attention to other bioactive compounds that are ubiquitously present at low concentrations, and for which no detailed ecotoxicological evaluation has been carried out.

Due to restrict regulatory approval processes to evaluate the pharmaceutical effects and efficacy, these compounds have a substantial margin of safety for humans and are better characterized than many others environmental contaminants (World Health Organization, 2011). However, there is still a serious lack of information about the effects in non-target species, particularly considering chronic exposure, sensitive development stages, or possible effects resulting from chemical mixtures (Lapworth et al., 2012). In fact, even if pharmaceuticals have been designed to be bioactive in humans, it is possible that aquatic organisms that share conserved signaling pathways experience the same pharmacodynamic effects (Fent et al., 2006).

Hence, we aimed here at improving our knowledge on the ecotoxicological effects of selected pharmaceutical. Chemical selection in the present study was based on data from literature, taking into account their relevance, either by prescription amount, environmental concentrations and effects already reported or existing gaps in their ecotoxicity. Thus, four compounds were chosen, belonging to different classes: non-steroidal anti-inflamatory drugs (Diclofenac), β-blockers (Propranolol), antidepressants (Sertraline) and hypolimidemic (Simvastatin). Diclofenac (DCF) is a common prescribed non-steroidal anti-inflamatory drug (NSAID) used in treatments of inflammation, high temperature and for some rheumatologic diseases. Propranolol (PROP) is a commonly used β-blocker and inhibits β-1 and β-2 adrenergic receptors. β-blockers act by inhibiting β-adrenergic receptors present in heart, which leads to a decrease of heart beating and contractility (Santos et al., 2010 and INFARMED – Prontuário terapêutico online. 2010. Retrieved on 14.07.14, from http://www.infarmed.pt/prontuario/prontuario_terapeutico.pdf.). Sertraline (SER) is a widely

prescribed antidepressant belonging to the selective serotonin reuptake inhibitors (SSRIs) (Santos et al., 2010 and Park et al., 2012). Simvastatin (SIMV) is a statin with hypolipidemic properties; it is used to decrease cholesterol concentration in blood plasma, reducing mortality and morbidity from coronary heart diseases (Fent et al., 2006 and Santos et al., 2010).

The overall aim of the present work was to contribute for the ecotoxicological risk assessment of the pharmaceuticals diclofenac, propranolol, sertraline and simvastatin in aquatic environments. This was approached using as model bioassays the embryonic development of representatives of a vertebrate and an invertebrate group, i.e., the teleost fish zebrafish (*Danio rerio*) and the equinoderm, sea urchin (*Paracentrotus lividus*). Both bioassays have been extensively validated for the screening of priority pollutants, display a high sensitivity, are cost-effective and allow a high-throughput screening approach.

2. Material and methods

2.1. Chemicals

Diclofenac, Propranolol, Sertraline and Simvastatin were purchased from Sigma-Aldrich®, Potassium chloride (CAS 7447-40-7, 99.0%), Calcium chloride (CAS 10043-52-4, 93.0%), Magnesium chloride hexahydrate (CAS 7791-18-6, 99.9%), Magnesium sulfate (CAS 7487-88-9, 99.5%) were purchased from Sigma-Aldrich. Sodium chloride (CAS 7647-14-5, 99.5%), Sodium bicarbonate (CAS 144-55-8) and dimethylsulfoxide (DMSO) were purchased from Merck.

2.2. Species selection

Zebrafish (*Danio rerio*) is a species increasingly used in several fields of research due to its small size, short life-cycle, ease of maintenance and reproduction in laboratory conditions, and full genome sequence available. Furthermore, eggs are translucent which makes easier the monitoring of embryo development (Soares et al., 2009).

The sea-urchin (*Paracentrotus lividus*) is an invertebrate used in ecotoxicological studies due to their ease of obtaining, ecological relevance and reduced development time (48 h) (Bellas et al., 2005).

2.3. Fertilization and embryos collection

2.3.1. Zebrafish

Adult zebrafish were kept at 28 °C and 14:10 h (light/dark) cycle as described by Soares et al. (2009). All experiments conducted in this study were carried out at Biotério de Organismos Aquáticos (BOGA, CIIMAR) aquatic animal facilities, in accordance to the guidelines of the Directorate-General of

Veterinary of Portugal (decree law 113/2013), based on the European directive of animal welfare 2010/63/EU.

For spawning, adult males and females (2:1) were placed in breeding tanks overnight. At the following day, ovulation and fertilization were stimulated by the beginning of light period (Soares et al., 2009).

2.3.2. Sea urchin

Sea urchin were collected in a clean area in the north of Portugal, Granja, Vila Nova de Gaia (N41° 2′ 26,18″, W -8° 39′ 2,24″), and transported to the laboratory in a portable icebox containing seawater.

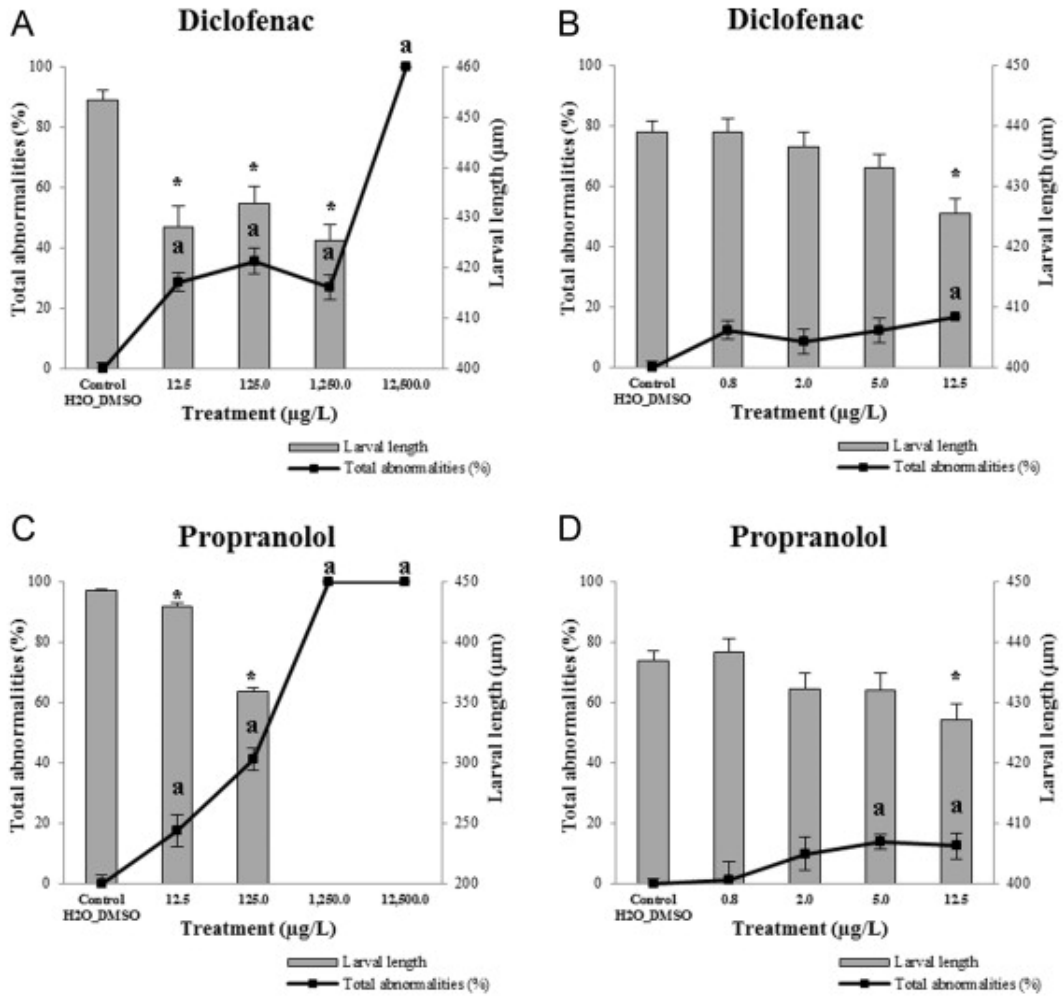
After arrival at the laboratory, animals were dissected, eggs and sperm directly pipetted from the freshly gonads of a single pair of adult individuals and assessed for their quality. Eggs were suspended in artificial sea water (90 ml). Artificial seawater was prepared according to Zaroogian et al. (1999) using Potassium chloride (0.67 g/L), Calcium chloride (1.36 g/L), Magnesium chloride hexahydrate (4.66 g/L), Magnesium sulfate (2.04 g/L), Sodium chloride (24.6 g/L) and Sodium bicarbonate (0.39 g/L).

A few microliters of undiluted sperm were added to the egg suspension and stirred to allow fertilization. The success of fertilization, indicated by the presence of a fertilization membrane, was evaluated under microscope. Toxicity tests were performed when the fertilization rate was above 97%.

2.4. Experimental design and embryo bioassays

2.4.1. Experimental solutions

Experimental concentrations of selected compounds were chosen based on the data from the literature, with 10× dilutions, in order to cover a wide range of concentrations, including pharmacological and environmental relevant concentrations. For the sea urchin embryo bioassay, after a first assay with 10× serial dilutions (Fig. 1; A, C, E, G), a 2.5× serial dilutions, with lower test concentrations (Fig. 1; B, D, F, H) were carried out. In order to assure reproducibility among assays, the lowest tested concentration of the first assays was also run in the second assay.


A
Diclofenac
Total abnormalities (%)
Larval length (µm)
Control H2O_DMSO
12.5
125.0
1,250.0
12,500.0
Treatment (µg/L)
Larval length
Total abnormalities (%)
B
Diclofenac
Control H2O_DMSO
0.8
2.0
5.0
12.5
Treatment (µg/L)
Larval length
Total abnormalities (%)
C
Propranolol
Control H2O_DMSO
12.5
125.0
1,250.0
12,500.0
Treatment (µg/L)
Larval length
Total abnormalities (%)
D
Propranolol
Control H2O_DMSO
0.8
2.0
5.0
12.5
Treatment (µg/L)
Larval length
Total abnormalities (%)

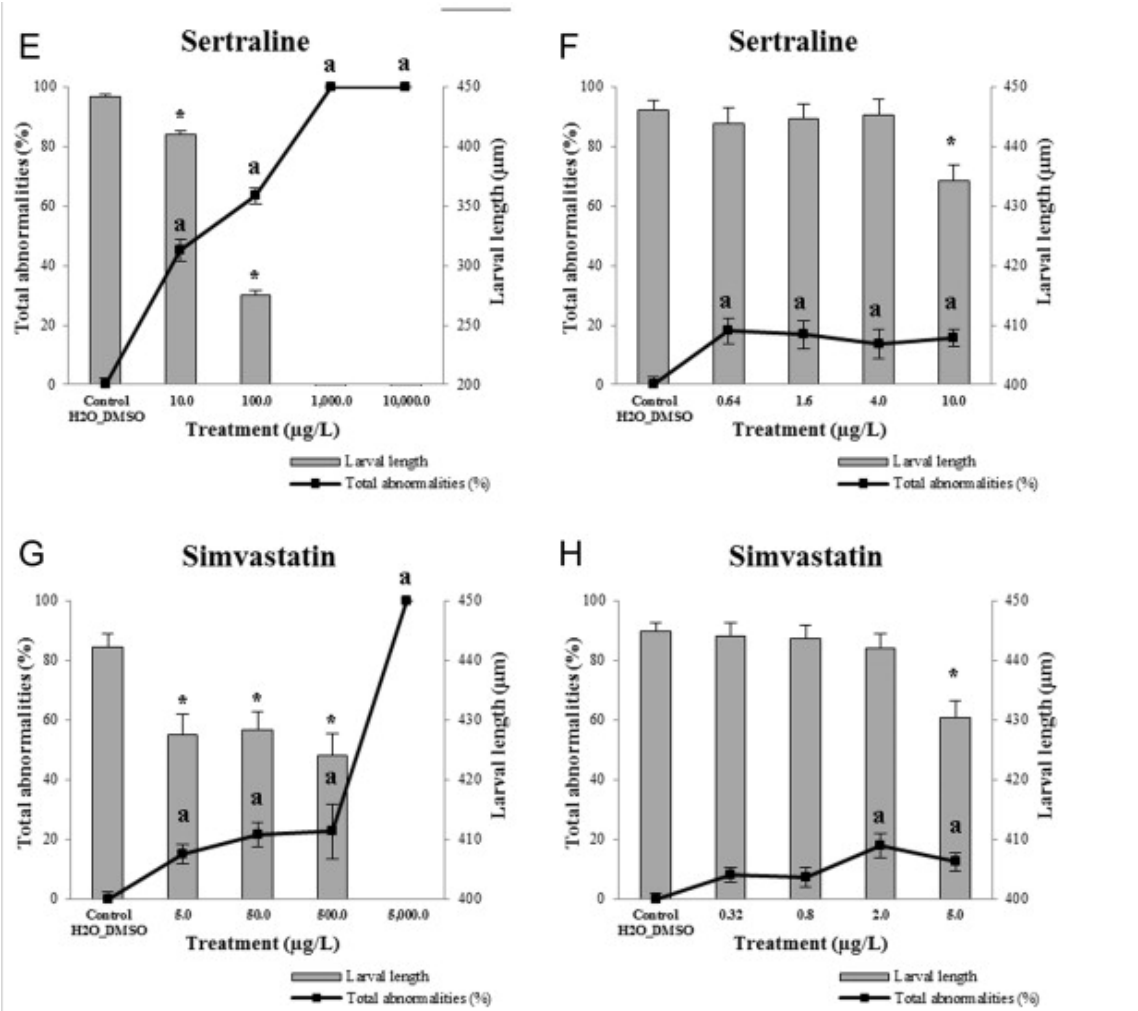


Fig. 1.

Larval length (µm) and percentage of total abnormalities (%) of *P. lividus* exposed to different concentrations of Diclofenac (A, B), Propranolol (C, D), Sertraline (E, F) and Simvastatin (G,H) for 48 h. First assay (A,C,E,G), second assay (B, D, F, H). Control and solvent control were grouped. Bars with the letter (a) or the symbol (*) indicate significant differences in comparison with controls (p<0.05). Larval length data are expressed as mean±SE (n=240 for controls; n=120 for exposed groups). The percentage of total abnormalities data are expressed as mean±SE (n=320 for controls; n=160 for exposed groups).

Eight replicates for a total of six treatments conditions were set up for zebrafish: an experimental control, a solvent control (DMSO) and four concentrations of each chemical. For sea urchin we used a similar experimental design, but serial dilutions with seven concentrations were tested (Lammer et al., 2009).

Stock solutions of each compound were prepared by dissolving Simvastatin, Sertraline, Diclofenac and Propranolol in Dimethylsulfoxide (DMSO). The experimental solutions were obtained by diluting the stock solutions in artificial seawater (Sea urchin assays) or in freshwater (Zebrafish assays). All solutions were prepared in order to have a final DMSO concentration of 0.01%.

2.4.2. Zebrafish

The static-water renewal toxicological tests with zebrafish were performed according to OECD guidelines (Organization for Economic Co-operation and Development (OECD), 1998). After embryos observation using a magnifying glass, 10 fertilized eggs were selected and randomly allocated into 24-wells plates filled with 2 mL of freshly prepared solutions and controls. The 24-wells plates were incubated at 26.5 °C during 80 h and under the same photoperiod conditions as the zebrafish stock. The medium was renewed daily in order to maintain oxygen and toxic nominal concentrations constants during the assay and to remove fungi or other organisms that could develop in the well.

The effects of exposure have been assessed by randomly choosing 6 embryos per well (including controls) at 3 distinct periods, representative of important steps of embryo development: gastrula period (65–75% epiboly stage), pharyngula period (prim15–16) and larval stage (protruding-mouth) at 8, 32 and 80 h post fertilization (hpf), respectively. Non-viable embryos were removed at each observation time. Morphological abnormalities on head, tail, eyes or yolk-sac, pericardial edema, abnormal cell growth and 75% of epiboly stage were rated as present or absent in accordance with previous validated protocols (Lammer et al., 2009, Organization for Economic Co-operation and Development (OECD), 1998 and Pinho et al., 2013). To increase statistical power, all scored abnormalities were grouped to determine the percentage of total abnormalities. The analysis was performed using a stereoscopic microscope (Nikon SMZ-1000) with a maximum magnification of 80 times.

2.4.3. SEA urchin

Within 30 min after fertilization, fertilized eggs of sea urchin were placed in 24-well plates with 3 mL of freshly test solution in a concentration of 20 eggs/mL. The 24-wells plates were isolated with parafilm and embryos were incubated at 20 °C in dark for 48 h. At the end of exposure time, embryos were fixed by adding three drops of 37% formaldehyde and directly observed under an inverted microscope. Embryogenesis success was recorded by applying two toxicity criteria: larvae length and morphological abnormalities of individuals. The number of analyzed individuals for the two criteria was based on Saco-Álvarez et al. (2010). Hence, the maximum larvae length was measured in 15 individuals per well in *pluteus* stage (120 individuals per treatment), defined as the distance between the apex and the end of the post-oral arm. Larvae were considered normal by the pyramid shape and four fully separated arms (Saco-Álvarez et al., 2010). In order to evaluate morphological effects resulting from individual exposures, 20 individuals per well (160 individuals per treatment) were analyzed. Abnormal larvae were counted separately as undeveloped organisms or malformed larvae. Undeveloped organisms included all the embryos which development suffers a delay or a blockage at early stages, i.e., fertilized eggs, 2-cell stage and morula, gastrula and prepluteus stage All changes to the normal development at 48 hpf were recorded and presented as abnormal larvae; this included crossed tip, separated tip, folded tip, fused arms, deformed arms, abnormal arms orientation, absence or asymmetric arms (Fig. 1S, supplementary information).

Observations were performed with a Nikon Eclipse 5100T inverted microscope equipped with a Nikon D5-Fi2 digital cam. Larvae length was measured using NIS-Elements version 4.13 image acquisition software.

2.5. Statistical analysis

Statistical analysis was performed with SPSS software (version 21.0).

All data were first tested for normality and homogeneity of variances using Kolmogorov–Smirnov and Levene's Test. If these assumptions were met, differences between treatments were tested for significance by means of one-way factorial ANOVA followed by Newman–Keuls multiple comparison test to compare the control groups and each of the exposed groups. If the homogeneity and normality were not met even after data transformation, data were analyzed using a non-parametric test – Kruskal–Wallis, followed by a multiple comparison rank test. Differences were considered significant for $p<0.05$. Values were expressed as the mean±standard error (SE).

D. rerio statistical analysis was carried out at 80 hpf as well as to the 8 hpf endpoints abnormal cell growth and 75% of epiboly stage. Control and solvent control were grouped for both species bioassay, when no significant differences between them were detected.

3. Results

3.1. Diclofenac

Zebrafish embryos exposed to diclofenac revealed significant alterations ($p<0.05$) in hatching rate, abnormal cell growth, 75% epiboly-stage and yolk-sac abnormalities (Table 1). Hatching rate significantly decreased ($p<0.05$) in embryos exposed to 12.5 mg/L when compared with other treatments. An exposure to 1.25 and 12.5 mg/L of diclofenac resulted in a significant decrease in the percentage of zebrafish embryos at 75% epiboly stage. A significantly increase ($p<0.05$) in the percentage of embryos with abnormal cell growth at the two highest concentrations was also observed in our study. The percentage of embryos with yolk-sac abnormalities was also significantly increased ($p<0.05$) after exposure to the highest concentration of diclofenac. For the remaining endpoints, exposure to diclofenac did not cause significant alterations in zebrafish embryos ($p>0.05$).

Table 1.

Effects of Diclofenac, Propranolol, Sertraline and Simvastatin exposures in zebrafish embryos at 80 hpf.

		ENDPOINTS (%)						
Compound	Treatment (µg/L)	Mortalitiy rate	Hatching rate	Abnormal celular growth	75% -epiboly	Head abnormalities	Eyes abnormalities	Yolk-sac abnormalities
Diclofenac	Controls	19.4±2.3	53.8±5.4	6.3±2.2	91.3±2.5	0.6±0.6	3.4±0.9	3.8±1.3
	12.5	21.3±5.2	45.0±3.8	6.3±2.6	88.8±3.5	2.5±2.5	8.9±3.1	2.5±1.6
	125	17.5±3.5	50.0±7.1	6.3±2.6	92.5±2.5	1.3±1.3	4.6±1.6	7.5±3.7
	1250	21.3±4.4	40.0±4.2	**16.3±2.6 ***	**82.5±2.5 ***	3.8±1.8	5.3±1.5	11.3±2.3
	12500	18.8±4.0	**1.3±1.3 ***	**17.3±4.1 ***	**80.0±4.6 ***	3.8±1.8	9.2±3.2	**40.0±6.5 ***
Propranolol	Controls	11.9±2.1	85.0±2.0	4.4±1.6	93.8±2.2	1.3±0.9	1.3±0.9	0.6±0.6
	12.5	7.5±4.1	90.0±3.8	3.8±2.6	93.8±5.0	1.3±1.3	2.5±0.9	0.0±0.0
	125	18.8±6.7	72.5±7.5	5.0±2.7	93.8±3.2	0.0±0.0	6.3±1.8	5.0±3.3
	1250	12.5±3.1	75.0±5.7	3.8±2.6	91.3±2.3	1.3±1.3	2.5±0.9	3.8±1.8
	12500	**100±0.0 ***	–	3.8±1.8	88.8±3.5	–	–	–
Sertraline	Controls	14.4±2.9	70.6±4.2	10.0±2.6	90.0±2.6	0.6±0.6	1.3±0.9	5.6±1.8
	10	13.8±3.8	60.0±8.5	1.3±3.5	88.8±3.5	1.3±1.3	1.3±1.3	2.5±1.6
	100	15.0±3.3	67.5±5.9	10.0±3.3	88.8±3.0	0.0±0.0	3.8±1.8	15.0±3.8 *
	1000	6.3±3.2	52.5±4.9	5.0±3.3	95.0±3.3	2.5±1.6	7.5±2.5	7.5±1.6
	10000	**100±0.0 ***	–	17.5±5.9	**67.5±5.3 ***	–	–	–
Simvastatin	Controls	6.3±1.5	81.3±4.0	4.4±1.8	93.8±2.7	1.3±0.9	1,9±1.0	2.5±1.1
	5	15.0±3.8	67.5±5.9	5.0±1.9	93.8±2.6	1.3±1.3	2.5±1.6	5.0±2.7
	50	6.3±3.8	76.3±8.0	2.5±2.5	96.3±2.6	6.3±3.2	2.5±2.5	7.5±1.6
	500	35.0±11.8	51.3±9.9	3.8±2.6	95.0±3.3	1.3±5.8	**50.0±9.8 ***	**46.3±10.5 ***
	5000	**100.0±0.0 ***	–	3.8±2.6	95.0±2.7	–	–	–

Control and solvent control were grouped. Significant differences from controls ($p<0.05$) are marked with a symbol (*) and bold. Data are expressed as mean±SE ($n=16$ for controls; $n=8$ for exposed groups).

An exposure of sea urchin embryos to diclofenac resulted in a significant decrease of larval length and an increase in the percentage of abnormal development ($p<0.05$) for concentrations equal or higher than 12.5 µg/L (Fig. 1A and B).

3.2. Propranolol

In this study, zebrafish embryos exposed to the highest tested concentration of propranolol had an increased mortality rate between 32 and 80 hpf and all embryos were dead at the end of the assay (Table 1). For the remaining criteria significant departure from control were not recorded (p>0.05). However, an exposure to 125 µg/L of propranolol induced a significant increase (p<0.05) in the percentage of total abnormalities when compared to controls and 12.5 µg/L exposure group.

Sea urchin embryos exposed to propranolol were significantly affected (Fig. 1C and D). All embryos exposed to 1.25 and 12.5 mg/L of propranolol were in morula stage at the end of the assay, and therefore no measurements were performed for these two exposure groups. An exposure to concentrations equal or higher than 12.5 µg/L of propranolol resulted in a statistically significant decrease of larval length (p<0.05). However, an exposure to propranolol concentrations equal or higher than 5 µg/L resulted in a statistically significant increase in the percentage of abnormal organisms (p<0.05).

3.3. Sertraline

In the present study exposure to 10 mg/L of sertraline led to the mortality of all zebrafish embryos at 80 hpf. No significant differences in cumulative mortality rate (p>0.05) were observed for the other exposure groups in comparison with control groups (Table 1). Considering the sub-lethal parameters, a significant decrease (p<0.05) in the percentage of zebrafish embryos at 75% epiboly-stage was observed in the groups exposed to the highest concentration. At 32 hpf, this exposure group showed a higher percentage of embryos with tail, yolk-sac and head abnormalities. These effects, associated with the development delay observed at 8 hpf, explain the mortality rate in the 10 mg/L exposure group at the end of the assay. We also observed a significantly increase (p<0.05) in the percentage of embryos with yolk-sac abnormalities at 100 µg/L when compared to controls and 10 µg/L exposure group. At the end of the assay, the percentage of total abnormalities in embryos did not differed significantly among groups (p>0.05).

Sertraline significantly impacted sea urchin embryo development (Fig. 1E and F). An observation of 10 mg/L exposed-embryos revealed fertilized eggs and embryos in the two-cell cleavage stage, and hence the embryonic development was arrested. All embryos exposed to 1 mg/L of sertraline were in morula stage at the end of the assay. For this reason, no measurements were performed for these two exposure groups. Larval length of 100 and 10 µg/L exposure groups was significantly lower than controls. Although no statistically significant changes were reported in larval length for exposures bellow 10 µg/L of sertraline, an exposure to concentrations equal or higher than 0.64 µg/L resulted in a statistically significant increase in the percentage of larvae with morphological abnormalities (p<0.05) compared to controls.

3.4. Simvastatin

In this study, simvastatin was the most toxic compound for zebrafish embryos. An exposure to 5 mg/L of simvastatin was lethal to all zebrafish embryos between 32 hpf and 80 hpf. Zebrafish embryos exposed to the highest tested concentration showed general body abnormalities at 32 hpf. These abnormalities led to

death of all zebrafish embryos and no hatches were recorded at 80 hpf. An exposure of zebrafish embryos to 500 µg/L of simvastatin resulted in a statistically significant increase (p<0.05) in the percentage of embryos with eyes, tail and yolk-sac abnormalities and also pericardial edema at the end of the assay (Table 1).

Sea urchin embryos were also affected by exposure to simvastatin. At the end of the assay, an exposure to 5 mg/L of simvastatin resulted in a significant delay in embryo development and no length recording were performed for this exposure group. A significant decrease (p<0.05) in larval length was recorded for concentrations equal or higher than 5 µg/L (Fig. 1G and H). However, embryos exposed to concentration equal or higher than 2 µg/L showed also a significant increase (p<0.05) in the percentage of total abnormalities at the end of the assay, being this endpoint more sensitive than larval length.

4. Discussion

4.1. Diclofenac

Diclofenac has received much attention on the past decade due to its effects in vulture populations in the Indian subcontinent (Oaks et al., 2004). Although this compound is not persistent, it is frequently detected in the environment due to its continuous released as well as a result of incomplete removal in WWTPs, inducing potential negatives effects in exposed organisms (Heberer et al., 2002 and Lee et al., 2011).

The effects of diclofenac in zebrafish embryos in our study are similar to data reported in the literature (Brandhof and Montforts, 2010). The most sensitive endpoints were hatching rate, 75% epiboly-stage, abnormal cellular growth and yolk-sac abnormalities, which contributed to a significant increase of total abnormalities. Taking together our results for sea urchin embryos (LOEC of 12.5 µg/L) and the reported environmental concentrations of diclofenac of up to 3 µg/L in sewage, possible long-term effects in aquatic organisms cannot be disregarded (Heberer et al., 2002).

4.2. Propranolol

Propranolol is a β-blocker frequently detected in aquatic ecosystem (Ternes, 1998). As β-adrenergic receptors are also present in heart, hepatocytes and reproductive tissues of fish and other vertebrates, it is possible that these organisms can also be affected by β-blockers action (Nickerson et al., 2001).

In a previous study performed by Fraysse et al. (2006), an higher percentage of zebrafish embryos exposed to propranolol showing pericardial edema and tail abnormalities were recorded at the highest tested concentration (108 µM). Fraysse et al. (2006) reported also an absence of hatched embryos at the end of the assay at the highest tested concentration, which is in agreement with our results.

To the best of our knowledge, our study is the first to address the effects of propranolol in the embryonic development of sea urchin. The LOEC reported here for sea urchin (5 µg/L) and zebrafish development (125 µg/L) is in the range of environmental levels of propranolol (304 µg/L in seawage treatment plant outflow) (Ternes, 1998 and Roberts and Thomas, 2006), and hence a negative impact may occur in the most contaminated areas.

4.3. Sertraline

Several studies report SSRIs as disruptors of reproductive functions in fish, although some contradictory findings were reported and little is known about their effects (Bossus et al., 2014 and Park et al., 2012). As the cellular receptors of SSRIs are evolutionary conserved, aquatic organisms may experience similar responses or side effects to those reported for humans (Schultz et al., 2011). This is particularly important since the use of antidepressants increased over 60% in the past decade. Furthermore, this class of pharmaceuticals has been detected in coastal waters and estuaries, representing about 4% of the therapeutic drugs reported in the environment (Fent et al., 2006). According to IMS Health, sertraline was the second most prescribed psychiatric drug in U.S., during 2013 (Grohol, 2008). Moreover, few studies have investigated sertraline effects in initial stages of development. Nevertheless, based on some preliminary studies, sertraline seems to be the most toxic SSRIs, showing the higher bioconcentration factor and being the compound that contributes the most to the toxicity of SSRIs mixture (Bossus et al., 2014 and Styrishave et al., 2011). Schultz et al. (2011) evaluated the effects resulting from a 21D-exposure of male fathead minnows (*Pimephales promelas*) to several SSRIs at environmentally relevant concentrations. The authors reported a significant decrease of organism's survival in the group exposed to 5.2 ng/L of sertraline. Fish exposed to 5.2 ng/L and 1.6 ng/L showed a sertraline brain concentration higher than suggested by water concentrations (0.06 ng/L and 0.023 ng/L, respectively). In our study, no effects were reported in zebrafish and sea urchin embryos at this concentration range. The most sensitive endpoints for zebrafish embryo exposure were cumulative mortality rate and 75%-epiboly stage and all tested concentrations induced a significant impact in the percentage of total abnormalities in sea urchin assay, which was a more sensitive endpoint than larval length.

In a recent study, the amphipod *Echinogammarus marinus* was exposed to environmentally relevant concentrations of fluoxetine and sertraline from 0.001 to 0.1 µg/L (Bossus et al., 2014). Significant differences were observed on velocity after 1 h-exposure to sertraline at 0.01 µg/L. Predicted Environmental Concentrations (PECs) of sertraline in Norwegian aquatic environments have been estimated at 170 ng/L, which is in the same range of the LOEC for sea urchin in our study (640 ng/L) (Grung et al.). Similar to propranolol exposure, larval length endpoint was less sensitive than the percentage of abnormal sea urchin larvae. Taken together the findings of our study and others, and given the increasing usage of SSRIs, possible long-term effects in the most sensitive species are likely to occur.

4.4. Simvastatin

Only a few studies addressed the effects of simvastatin to aquatic animals and almost no information is available for early life-stages. In a study performed by Key et al. (2008), after exposure of 96 h to different concentrations of simvastatin, larval and adult grass shrimp (*Palaemonetes pugio*) showed a LC50 of 1.118 mg/L and higher than 10 mg/L, respectively. In our study, all zebrafish embryos exposed to simvastatin at concentrations similar to the highest concentrations tested in Key et al. (2008) study died before the end of the assay.

Ellesat et al. (2010) evaluated the effects of simvastatin and atorvastatin cytotoxicity in primary hepatocytes of *O. mykiss* exposed to concentrations ranging from 1 to 200 mg/L. The authors reported toxic effects in a dose–response manner which seemed to be related with the reducing of metabolic activity and membrane stability. In another study, the adults harpacticoid copepod *Nitocra spinipes* was exposed to simvastatin for 96 h, which a LC50 of 810 µg/L (Dahl et al., 2006). A significant decrease in development rate of *N. spinipes* was reported after exposure to simvastatin in a range of concentrations between 0.16 and 1.6 µg/L. These results were observed at the same range of effective concentrations that induced a decrease of larval length and an increase in the percentage of morphological abnormalities in sea urchin larvae in our study. Similar to the other selected compounds, total abnormalities was more sensitive for sea urchin assay than larval length endpoint. Recently Neuparth et al. (2014) observed a significant impact in the amphipod *Gammarus locusta* reproduction at predicted environmental concentrations, further stressing the sensitivity of several invertebrate taxa to simvastatin exposure. Although data on environmental concentration of simvastatin are very limited, recent PECs in Norwegian aquatic environments have been estimated at 630 ng/L, which is in the same range of the LOEC recorded here for sea urchin (2 µg/L) (Grung et al.). Furthermore, given that the generic of simvastatin was recently introduced in European countries, it is likely that environmental levels tend to increase, and hence might increase the risk of this pharmaceutical to the most sensitive taxa.

5. Conclusions

In conclusion, all tested compounds induced significant effects in embryonic and larval development of zebrafish and sea urchin, which indicates that the most sensitive taxa might be at risk. Considering the present findings and given that the toxicity data for sertraline and simvastatin are very limited, additional studies should be carried out involving other taxa and chronic exposures.

In contrast to zebrafish embryo bioassays, there is a paucity of data on the use of sea urchin embryo bioassays in the toxicity screening of emerging pollutants. The results of this study indicates high sensitivity of sea urchin embryos to the tested pharmaceuticals, and suggest the combined use of zebrafish and sea urchin embryo bioassays in toxicological risk assessment of emerging pollutants. Overall, our study shows that early life-stages of aquatic animals are a promising approach for the increasing demand of high-throughput methods to test the toxicity of new chemicals, including pharmaceuticals.

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