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Contribution to the toxicological risk assessment of Simvastatin, Sertraline, 4-MBC, Propylparaben and Triclocarban, under single and combined exposure, using zebrafish and sea urchin embryo bioassays

TIAGO ANDRÉ PEREIRA TORRES

TESE DE MESTRADO APRESENTADA

AO INSTITUTO DE CIÊNCIAS BIOMÉDICAS ABEL SALAZAR

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Supervisor – Miguel Santos
Categoria – Investigador Auxiliar/Professor Auxiliar Convidado
Afiliação – Centro Interdisciplinar de Investigação Marinha e Ambiental (CIIMAR)/Departamento de Biologia, Faculdade de Ciências da Universidade do Porto
Co-supervisor – Maria do Rosário Martins
Categoria – Professor Adjunto
Afiliação – Escola Superior de Tecnologia da Saúde do Porto (ESTSP), Instituto Politécnico do Porto

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Abstract

In the past decade, many emergent organic compounds, including Pharmaceuticals and Personal Care Products (PPCPs) have been detected in water at levels that can negatively impact aquatic ecosystems. The recent knowledge of their occurrence has raised concerns about human health effects and ecosystem risks. Although these compounds are frequently detected at concentrations that are not likely to induce adverse effects in humans and may be too low to cause acute effects in other organisms, there is still a serious lack of information about the effects in non-target species, particularly considering chronic exposure or effects resulting from interactions between them.

Pharmaceuticals and Personal Care Products enter in aquatic environment from different point and non-point sources and for many compounds Wastewater treatments plants cannot ensures complete removal, and therefore they may be present at significant concentrations in effluents. Hence, it is essential to understand the effects of these substances on aquatic organisms. Owing to the large number of new chemicals that must go though toxicity testing, short-term early-life-stages have been frequently used as an alternative to long-term exposures due to its high sensitivity and logistic advantages.

In addition to single exposures, if we aim at improving risk assessment of emergent contaminants under real field conditions, it becomes now important to understand the effects resulting from an actual environmental exposure and the mechanisms of toxic action involved, considering the diversity of contaminants present in mixture and their possible interactions.

Thus, the main aim of the present work was to assess the toxicological risk of five emergent chemicals, both under single and combined exposure: simvastatin, sertraline, triclocarban, propylparaben and 4-MBC during the embryonic development of zebrafish (*Danio rerio*) and the sea urchin (*Paracentrotus lividus*).

All selected compounds induced significant effects on the embryonic development of both test species after individual exposure. However our results show that sea urchin embryos were more sensitive than zebrafish embryos. Regarding the relative toxicity, simvastatin showed the highest toxicity in zebrafish assay, while triclocarban was the compound that induced significant effects in sea urchin assay at lower concentrations. Furthermore, significant effects were also reported in sea urchin and zebrafish embryos exposed to combinations of these compounds.

In conclusion, this study highlights the risk of these compounds to aquatic ecosystems. Hence, it is important to conduct more comprehensive studies about possible chemical interactions in environment and mechanisms involved in order to perform more

reliable risk assessment and to implement guidelines for the protection of the aquatic environment.

Resumo

Na última década, vários compostos orgânicos emergentes, entre os quais fármacos e produtos e de uso pessoal, têm sido detetados na água em concentrações potencialmente perigosas para os organismos aquáticos. A sua presença levanta sérias preocupações sobre os potenciais efeitos na saúde humana e os riscos para os ecossistemas afetados. Embora estes compostos sejam detetados em concentrações abaixo das que induzem efeitos agudos nos humanos e, possivelmente, para a maioria dos organismos expostos, pouco se conhece ainda sobre potenciais efeitos em organismos não-alvo, particularmente após uma exposição crónica ou como resultado de possíveis interações entre os vários compostos presentes em mistura no ambiente.

São diversas as vias de entrada no ambiente e para muitos destes compostos não existem tratamentos nas estações de tratamento de águas residuais que garantam a sua completa remoção, pelo que podem ainda ser detetados nos efluentes em concentrações significativas. Torna-se, portanto, essencial compreender o impacto destes compostos nos organismos aquáticos. Considerando o elevado número de compostos presentes no ambiente, e a consequente necessidade de avaliar a sua toxicidade, os ensaios de toxicidade aguda com organismos em fases iniciais do desenvolvimento embrionário têm sido frequentemente usados em alternativa aos ensaios de toxicidade crónica, como resultado das vantagens logísticas e da sua elevada sensibilidade.

Tendo por objetivo avaliar o risco toxicológico de uma exposição ambiental, torna-se, portanto, necessário compreender os efeitos resultantes de uma exposição em condições reais e os mecanismos de ação envolvidos, considerando a diversidade de contaminantes presentes em mistura e possíveis interações.

Assim, este estudo teve como objetivo principal avaliar o risco toxicológico de cinco compostos emergentes, tanto em exposições individuais como em mistura (sinvastatina, sertralina, propilparabeno, triclocarban e o filtro UV 4-MBC), utilizando bioensaios baseados no desenvolvimento embrionário de peixe-zebra (*Danio rerio*) e do ouriço-do-mar (*Paracentrotus lividus*).

A exposição individual aos compostos selecionados para este estudo induziu efeitos significativos no desenvolvimento embrionário de ambas as espécies. Porém, de acordo com os resultados obtidos, os embriões de ouriço-do-mar mostraram-se mais sensíveis aos compostos testados que os embriões de peixe-zebra. Atendendo à toxicidade relativa de cada composto, a sinvastatina foi o mais tóxico para os embriões de peixe-zebra, enquanto que os embriões de ouriço-do-mar mostraram-se mais sensíveis à exposição a triclocarban, que induziu efeitos significativos no comprimento larvar para concentrações mais baixas. Por outro lado, a exposição de embriões de

peixe-zebra e de ouriço-do-mar a misturas dos compostos selecionados resultou igualmente em efeitos significativos no seu desenvolvimento embrionário.

Este estudo evidencia o risco destes compostos para os ecossistemas aquáticos. Deste modo, torna-se necessário conduzir estudos mais detalhados que contribuam para melhorar a compreensão de possíveis interações entre os compostos e os mecanismos envolvidos, de forma a permitir avaliações de risco mais fundamentadas e a implementação de medidas que garantam a proteção dos organismos mais sensíveis.

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List of Abbreviations and acronyms

µg	Microgram
4-MBC	4-Methylbenzylidene Camphor
bpm	beats per minute
CA	Concentration Addition
d	day
DMSO	Dimethyl sulfoxide
dr	Zebrafish (*Danio rerio*)
EC	Effective Concentration
EDC	Endocrine Disrupting Chemical
EE2	Ethinylestradiol
EOC	Emerging Organic Compound
hpf	hours post fertilization
IC	Inhibitory Concentration
IUPAC	International Union of Pure and Applied Chemistry
L	Liter
LC	Lethal Concentration
LOEC	Lowest Observed Effect Concentration
mg	Milligram
MIX	Mixture
ng	Nanogram
NOEC	No Observed Effect Concentration
OECD	Organization for Economic and Co-operation and Development
PCP	Personal Care Products
pl	Sea urchin (*Paracentrotus lividus*)
PP	Propylparaben
PPCP	Pharmaceutical and Personal Care Products
SER	Sertraline
SIMV	Simvastatin
SSRI	Selective Serotonin Reuptake Inhibitor
TCC	Triclocarban
TCS	Triclosan
VTG	Vitellogenin
WWTP	Wastewater Treatment Plant

Introduction and Objectives

Chapter I

1. CHAPTER I – Introduction and Objectives

1.1. State of knowledge of pharmaceuticals and personal care products in environment

1.1.1. Background and history

Due to analytical chemistry theory and technology advances, many emergent organic compounds (EOCs) have been detected in water at low concentrations in the last few years (Lapworth et al., 2012; Stuart et al., 2012; Jiang et al., 2013). This EOCs designation is applied to compounds that have been reported in the environment but also to new compounds that have recently been developed or categorized as contaminants, including Pharmaceuticals and Personal Care Products (PPCPs) (Lapworth et al., 2012). Although their presence in water has been reported since the 80s, the number of studies that investigated the occurrence of PPCPs in the environment has increased since the 90s, because of the continuous improvement in chemical analysis methodologies (Daughton, 2003). Most of these compounds have been detected in the ng/L or µg/L range (Kim et al., 2009; Chen et al., 2012; Jiang et al., 2013).

The recent knowledge of their occurrence has raised concerns about human health effects and ecosystem risks. Therefore, an increasing number of studies has been focusing on the environmental fate and impact in non-target organisms (Brausch and Rand, 2011). Environmental monitoring studies have identified compounds that are present in some ecosystems at levels that can induce negative effects for organisms (Ferrari et al., 2003). In many cases, negligible effects may occur from a continuous exposure during the entire life of organisms or a multi-generational exposure to low concentrations of PPCPs. These effects might be cumulative thus affecting the population and the ecosystem (Santos et al., 2010).

1.1.2. The importance of ecotoxicological studies with individual compounds and mixture exposures

Although the state of knowledge of some contaminants is sufficient to establish safety values, most EOC are not yet well characterized with respect to their toxicity, behavior, impact and actual environmental occurrence. Compounds selection in many studies take into account consumption, predicted environmental concentrations, pharmacological and physicochemical compound properties as well as ecotoxicological data from previous studies (Lapworth et al., 2012).

Most of the studies related to toxic effects of PPCPs focus on representative organisms of the food chain and were performed with standard ecotoxicological methods

defined by guidelines of Organization for Economic Co-operation and Development (OECD) for testing chemicals (Hernando et al., 2006).

Environmental concentrations of pharmaceuticals are much lower than therapeutic doses and Personal Care Products (PCPs) are also found at low concentrations. Although these concentrations are not likely to induce adverse effects in human and may be too low to cause acute effects in other organisms, there is still a serious lack of information about the effects in non-target species particularly considering chronic exposure. Moreover, even if these compounds are present below the No Observed Effect Concentration (NOEC), toxic effects due to a long-term exposure or to a combination of contaminants cannot be disregarded (Lapworth et al., 2012). Combination of compounds that share the same mechanism of action or that can act at the same target site can result in additive or synergetic effects (Hernando et al., 2004). However, some interactions between compounds belonging to different classes can induce also antagonistic effects, whereby it is not recommended to predict mixture effects directly from results of single compound exposures (Chung et al., 2011).

Taking into account data from several toxicological studies on the effects from a single exposure of different compounds, it becomes now important to understand the effects resulting from an actual environmental exposure and the mechanisms of toxic action involved, considering the diversity of contaminants present in mixture and their possible interactions. Some studies have reported significant effects resulting from an exposure to multi-component mixtures in a concentration range at which single compound exposure do not shows effects (Baas et al., 2010).

There are two models-based methods frequently used for assessing the effects of complex mixtures of a known composition from its individual components: Concentration Addition (CA) and Independent Action (IA). Both methods are based in dose-response curves of all the individual chemicals present in the mixture to calculate the dose-response curve of the mixture. IA model is frequently used for mixtures which compounds have a presumed dissimilar mode of action. CA model is used for mixtures with chemicals that share the same mode of action or the same target site and so components in the mixture can be regarded as dilutions of one another. However, some studies that compared these two models show similar predicted results when applied in mixtures of compounds with different modes of action and the CA model provided conservatively high estimates of toxicity (Belden et al., 2007; Cedergreen et al., 2008). Statistically significant deviations from the model predictions can be interpreted as chemical interactions (Baas et al., 2010).

There is a need to investigate possible multi-generational life-cycle effects or at different life stages or at long-term exposure to assess specific modes of action of PPCPs

in order to better evaluate the implications of these compounds mixtures in ecosystems and perform more reliable risk assessments (Fent et al., 2006).

1.1.3. Sources and pathways

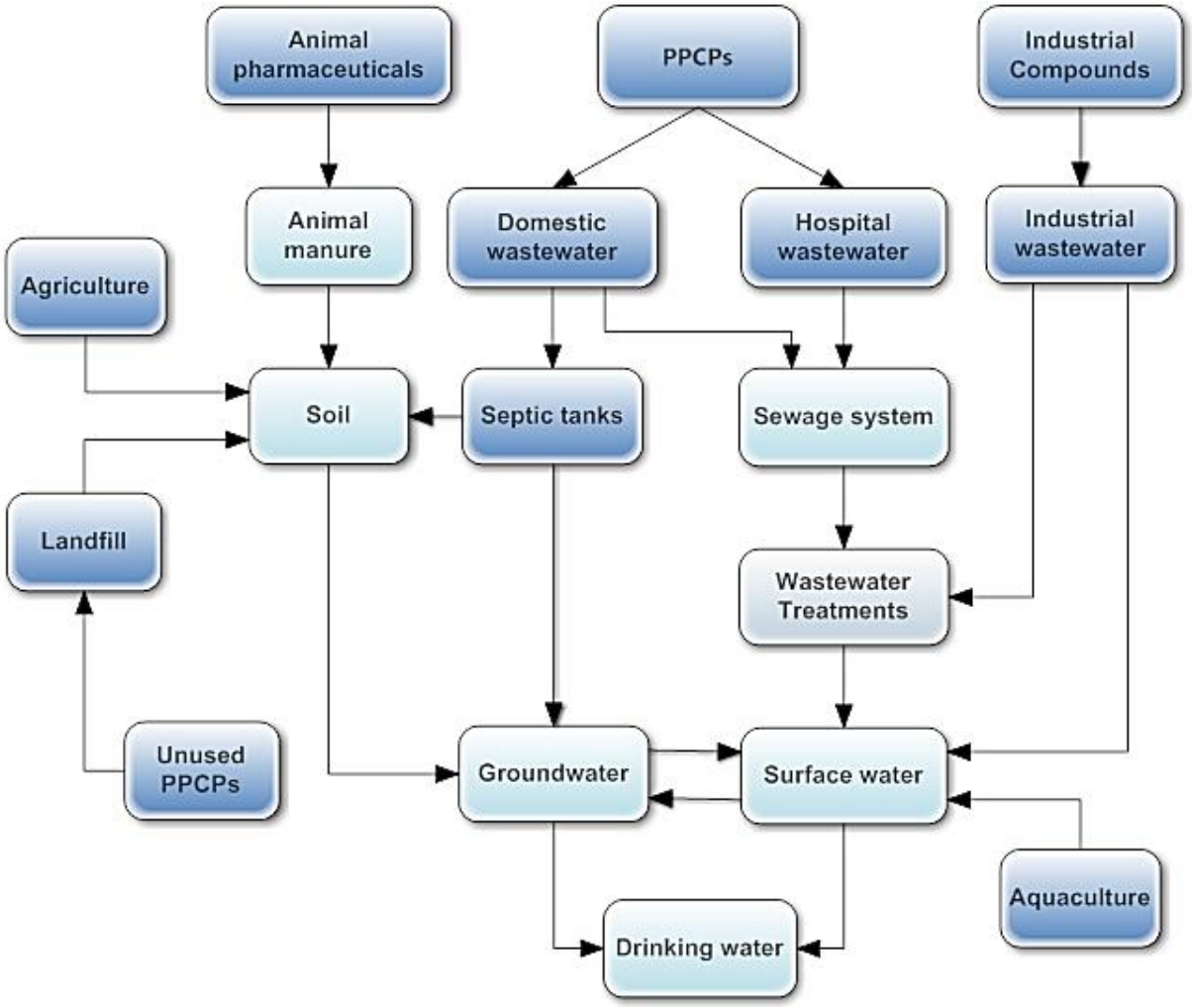


Figure 1. Schematic diagram of sources and pathways of PPCPs in environment
Sources: Lapworth et al., 2012; Stuart el al., 2012

PPCPs enter in the environment from a different number of sources and pathways (Figure 1). Some compounds and their metabolites cannot be entirely used by organisms and are released into the water by excretion (Jiang et al., 2013). Excretion of the compound or products resulting of its biotransformation, improper disposal of unused PPCPs in landfills or discharge into the collection system of wastewater, hospital effluents and septic tanks are some of possible pathways. On the other hand, waste resulting from pharmaceutical production, agricultural activities and industry effluents, as well as hospital, veterinary and aquaculture stations waste, contribute to their occurrence in

wastewater, surface, groundwater and, at a lesser extent, in drinking water (Santos et al., 2010; Snyder and Benotti, 2010; Lapworth et al., 2012; Jiang et al., 2013). There is no treatment in Wastewater Treatments Plants (WWTPs) that ensures complete removal of compounds, and so WWTPs effluents may still have significant concentrations of some PPCPs (Jiang et al., 2013).

In comparison to groundwater, the occurrence of EOC in surface water and wastewater has been much better characterized and they are known to contain a great diversity of EOC. However, this situation may occur due to the reduced number of groundwater studies and the limited capacity of analytical methods than actual environmental groundwater occurrence (Lapworth et al., 2012).

1.1.4. Wastewater treatments

WWTPs do not present specific processes to remove these compounds from wastewaters (Jiang et al., 2013). For this reason effluents from WWTPs are considered one of the most important sources of PPCPs in the aquatic environment (Chen et al., 2012; Lapworth et al., 2012). Treatments like activation sludge and secondary sedimentation applied in many WWTPs seem to not completely eliminate some compounds and the average elimination is very variable and depends on the different physicochemical properties of each compounds. Moreover, even for the same compound it has been reported variability between different treatment plants (Table 1). This variation is expected in part due to differences in equipment and treatment steps of WWTPs and factors such as temperature and weather (Fent et al., 2006). Some compounds are not completely degraded and cross through water treatment system with only small reductions in their concentration, while other are transformed in new compounds or may be completely degraded during the process.

Thus, a large number of studies have been carried out to explore new technologies and methods to treat PPCPs residuals from water and wastewaters (Jiang et al., 2013). To evaluate wastewater treatments it is important to understand the processes of degradation, transport and fate of the different compounds as well as their physicochemical properties. As a result, some techniques have been reported to be effective in removing a large percentage of some compounds, namely ozonation, oxidation, nanofiltration and reverse osmosis membrane filtration and also activated carbon adsorption (Jiang et al., 2013). Some studies have demonstrated that combination of different treatments can result in a higher removal rate, by covering a wider range of compounds properties (WHO, 2011).

Table 1. Removal efficiency of selected pharmaceuticals in WWTWs (%).

Compound	Pharmaceutical group	WWTP removal (%)	Treatment process
Propanolol	β-Blocker	96	Activated sludge
Metoprolol	β-Blocker	83	Activated sludge
Sulfamethoxazole	Antibiotic	67	Activated sludge
Carbamazepine	Antiepileptic	7 – 10	Activated sludge
Ibuprofen	Analgesic	60 – 100	Activated sludge
		14 – 65	Biologic filter
Naproxen	Anti-inflammatory	40 – 78	Activated sludge
		15 – 45	Biologic Filter
Diclofenac	Anti-inflammatory	69 – 75	Activated sludge
		9	Biologic filter
Ketoprofen	Anti-inflammatory	69	Activated sludge
		48	Biologic filter
Gemfibrozil	Hypolipidemic	46 – 69	Activated sludge
		16	Biologic filter
Bezafibrate	Hypolipidemic	50 – 99,5	Activated sludge
		27	Biologic filter

Source: Bound and Voulvoulis, 2005

1.2. Pharmaceuticals and Personal Care Products

1.2.1. Characteristics and Effects

Although PCPs are more often detected in aquatic environment and in higher concentrations, most studies have been conducted examining occurrence and effects of pharmaceuticals and little is known about PCPs toxicity (Brausch and Rand, 2011).

Different classes of pharmaceuticals have been detected in several ecosystems, including analgesic and anti-inflammatory drugs, β-blockers, steroids and related hormones, antibiotics, hypolipidemics and antiepileptics (Hernando et al., 2006). These compounds are subject to restrict regulatory approval processes to evaluate the efficacy and safety, and studies are performed at doses close to the therapeutic dose. For this reason, pharmaceuticals have a substantial margin of safety and are better characterized than many others environmental contaminants (WHO, 2011). A major concern is focused on antibiotics and steroids compounds because they may cause resistance among natural

bacterial populations or induce estrogenic responses and alterations in reproduction, growth or development of exposed species (Hernando et al., 2006).

Although pharmaceuticals have been designed to be bioactive in humans, aquatic organisms that present conserved signaling pathways can experience the same pharmacodynamics effects (Bound and Voulvoulis, 2004). Moreover, secondary effects that are less frequent in human treatments may be more relevant in aquatic organisms (Seiler, 2002).

As many PCPs are designed for external use, they are not subjected to biotransformation in organisms and thus a large quantity of these unaltered compounds is released into the environment through regular usage (Brausch and Rand, 2011).

According to some recent studies, several PPCPs are used in large quantities and could be environmentally persistent, ubiquitous, bioactive and can induce bioaccumulation and disrupting of endocrine functions (Fent et al., 2006; Chen et al., 2012; Brausch and Rand, 2011). Once in the environment, the concentration of PPCPs can be attenuated by different process such as dilution, adsorption, microbial degradation, oxidation, photolysis or by abiotic processes (Stuart et al., 2012).

The rate of metabolism of different compounds in organisms depends on their physic and chemical properties, resulting in different excretion rates of unmetabolized active ingredient (Table 2). On the other hand, some metabolites can be more toxic than the parental compound and represent a threat when excreted into the environment (Lin and Lu, 1997).

Many compounds are considered to be persistent in aqueous ecosystem. However, some compounds that seem to be more easily degraded are also frequently detected in water due to their continuous introduction into the environment and can induce negative effects (Fent et al., 2006). High polarity and low volatility of many compounds contribute to their presence in water but their concentration depends also on other factors such as their consumption rate in a specific population, metabolism in organism, toxicokinetic, its behavior in the environment, natural and anthropogenic processes and the effectiveness of treatments applied in WWTPs.

To humans, consumption of water containing traces of several PPCPs has been identified as one of the main sources of exposure (Kolpin et al., 2002), and the most vulnerable groups are believed to be more susceptible to any possible negative effects.

While human exposure to contaminated water occurs in a discontinuous process, organisms in aquatic ecosystems are continually exposed to this type of contamination and several studies have identified different PPCPs present in water in concentrations that can induce adverse effects in organisms (Henschel et al., 1997; Jones et al., 2002; Ferrari et al., 2003; Fent et al., 2006). Based on existing information on the activity and potential

toxicity of PPCPs, some possible effects resulting from chronic exposure to contaminated water are endocrine disruptor activity, resistance to antibiotics, genotoxicity, carcinogeniticy, allergic reactions, and also effects on reproduction, embryo development and growth (Daughton and Ternes, 1999).

Table 2. Urinary excretion rates of unchanged active ingredient for selected pharmaceuticals (%).

Compound	Pharmaceutical group	Parent compound excreted (%)
Amoxicillin	Antibiotic	60
Atenolol	β-Blocker	90
Metoprolol	β-Blocker	10
Bezafibrate	Hypolipidemic	50
Cetirizine	Antihistamine	50
Sulfamethoxazole	Antibiotic	15
Amoxycillin	Antibiotic	60
Erythromycin	Antibiotic	25
Carbamazepine	Antiepileptic	3
Felbamate	Antiepileptic	40-50
Ibuprofen	Analgesic	10
Paracetamol	Analgesic	4

Source: Bound and Voulvoulis, 2005

Some PPCPs have been classified as Endocrine Disrupting Chemicals (EDCs) given their capacity to affect directly or indirectly endocrine systems in humans and wildlife, even at low concentrations (Roepke et al., 2005; Wang et al., 2011). EDCs are an exogenous substance or mixture that typically exerts effects through receptor-mediate process, mimicking endogenous hormones or inhibiting normal hormone activities of endocrine and neuroendocrine systems as well as organism metabolism (Roepke et al., 2005). Some of these chemicals act as estrogens and induce feminization in organisms, which limit the reproductive capacity of exposed species and can result in serious ecological risks to ecosystems (Roepke at al., 2005).

The effects of Ethinylestradiol (EE2) in non-target organisms are a good example of the risks that low doses of some pharmaceuticals may pose to wildlife (Versonnen and Janssen, 2004). EE2 is a synthetic estrogen present in many oral contraceptives pills and is reported to show estrogenic activity and induce adverse estrogenic effects in fish even

at low levels (0,1 – 5,0 ng/L) (Rodgers-Gray et al., 2000; Kidd et al., 2007; Soares et al., 2009; Santos et al., 2010). Thus, similarly to other EDC, EE2 can cause behavioral and embryonic development changes as well as a significant reduction in fertilization success, an increased egg production and decreased expression of secondary male sex characteristics. It can also induce higher vitellogenin plasma levels in both males and females, which is a common used endpoint for detecting endocrine disruption in fish (Versonnen and Janssen, 2004; Santos et al., 2010). In some studies EE2 was detected in superficial and drinking water at concentrations closely to those reported to induce estrogenic effects (Kolpin et al., 2002) which is an alert to the risks of PPCPs and to emphasize the importance of adopting new strategies to reduce their presence.

1.2.2. Pharmaceutical and Personal Care Products used in this study

The PPCPs selected for the present study were chosen based on data from literature, taking into account their relevance either by environmental concentrations (Table 3) and effects already reported (Table 4) or existing gaps about their ecotoxicological effects as well as their relevance in therapeutics. Thus, five compounds were chosen, belonging to different categories: hypolimidemic (Simvastatin), antidepressants (Sertraline), preservatives (Propylparaben), disinfectants (Triclocarban) and an UV filter (4-Methylbenzylidene Camphor).

Table 3. Environmental concentrations of PPCPs selected for this study (ng/L).

PPCP	Concentration	Local	Reference
Triclocarban	> 10000	WWTPs effluents	1
	6750	Surface water	1
	19 – 1425	Surface water	1
	250	Surface water	2
	100 – 6000	WWTPs effluents	3
	400 – 50000	WWTPs influents	4
	388	Surface water	5
4-MBC	600 – 6500	WWTPs influents	6
	100 – 2700	WWTPs effluents	6
	< 2 – 28	Surface water	6
	799	Coastal areas beach	7
Propylparaben	2800	WWTPs influents	8
	21	WWTPs effluents	8
	44.1 ng/g	Sewage sludge	9
	207	Surface water	10

Sertraline	1.8 – 19.8	WWTP influents	11
	0.9 – 14.6	WWTP effluents	11
	< 0.52	Seawater	11
	0.84 – 2.4	Surface water	11
	570	Surface water	12
Simvastatin	1.0	WWTPs effluents	11
	4.0	WWTPs influents	11
	0.1	Surface water	13
	0.2	WWTPs effluents	13
	1.0	WWTPs influents	13
	91. 2	WWTPs influents	14

References: (1) Brausch and Rand, 2011; (2) Schultz and Bartell, 2012; (3) Higgins et al., 2009; (4) Chalew and Halden, 2009; (5) Zhao et al., 2013; (6) Balmer et al., 2005; (7) Kaiser et al., 2012; (8) González-Mariño et al., 2011 (9) Albero et al., 2012; (10) Yamamoto et al., 2011; (11) Santos et al., 2010; (12) Richards and Cole, 2006; (13) Miao and Metcalfe, 2003; (14) Kosma et al., 2014.

Table 4. Lethal and sub-lethal doses of PPCPs selected for this study (µg/L).

PPCP	Species	Duration / Endpoint	Parameter	Reference
Triclocarban	*Salmo gairdneri*	48h	LC50 = 120	1
		96h acute toxicity	NOEC = < 49 LC50 = 120	4
	Lepomis macrochirus	96h	LC50 = 97 NOEC = 49	1, 4
	Mysidopsis bahia	48h	LC50 = 15	1
		96h	LC50 = 10	1
		28d Reproduction	LOEC = 0.13 NOEC = 0.06 EC50 = 0.21	1, 4
	Daphnia magna	21d Growth	LOEC = 4.7 NOEC = 2.9	1
	Potamopyrgus antipodarum	4w Reproduction	LOEC = 0.2 NOEC = 0.05 EC10 = 0.5	2
	Ceriodaphnia sp.		NOEC = 1.46	3
		48h	NOEC = 1.9	4
		21d chronic toxicity	NOEC = 2.9 LOEC = 4.7	4
	Pimephales promelas	35d hatch eggs, growth, survival	NOEC = 5	3
	Mercenaria mercenaria		LC50 = 30	3
	Oncorhynchus mykiss	96h acute toxicity	LC50 = 180	4
4-MBC	*D. magna*	48h Immobility	LC50 = 560	1,5
		21d Growth	LOEC = 100 NOEC = 200 EC10 = 460	5

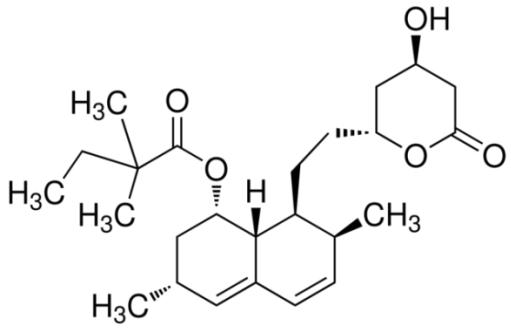
4-MBC	*O. mykiss*	14d Growth	LOEC = 415	1
	Desmodesmus subspicatus	72h Growth	IC10 = 810	5
	Danio rerio	72h	LC50 = 5042 LC100 = 6359 EC50 = 3688	6
Propylparaben	*D. magna*	48h	LC50 = 12300	9
		24h mobility	LC50 = 13000	1
		48h mobility	LC50 = 7000 LOEC = 6000	1
		7d Growth	LOEC = 400	9
		7d Reproduction	LOEC = 6000	9
	P. promelas	48h	LC50 = 9700	1
	Oryzias latipes	7d VTG	LOEC = 9900	1
		96h	LC50 = 4900	8
		96h	LC50 = 9700 LOEC = 2500	9
	Dugesia japonica	48h	LC50 = 12300	7
Sertraline	*O. mykiss*	96h	LC50 = 380 NOEC = 100	10
	D. magna	48h immobilization	EC50 = 1300 LOEC = 180 NOEC = 100	10
		21d Reproduction	LC50 = 120 EC50 = 66 LOEC = 100 NOEC = 32	10
	Xenopus laevis	96h	LC50 = 3900 EC50 = 3300	11
Simvastatin	*Dunnaltella tertiolecta*	96h Growth	EC50 = 22800	
	Palaemonetes purgio	96h Larvae Survival	LC50 = 1180 LOEC = 1250 NOEC = 625	12
		96h Adult Survival	LOEC = 10000 NOEC = 5000	13
	Nitocra spinipes	96h Growth	LC50 = 810 LOEC = 0.16	14

References: (1) Brausch and Rand, 2011; (2) Giudice and Young, 2010; (3) Higgins et al., 2009; (4) Chalew and Halden, 2009; (5) Sieratowicz et al., 2011: (6) Vincent, 2011; (7) Li, 2012; (8) Yamamoto et al., 2011; (9) Dobbins et al., 2009; (10) Santos et al., 2010; (11) Richards and Cole, 2006; (12) DeLorenzo and Fleming, 2008; (13) Key et al., 2008; (14) Dahl et al., 2006.

1.2.2.1. Hypolipidemic drugs: Simvastatin

There are two types of hypolipidemic drugs, namely statins and the group most frequently detected in the environment, fibrates. These compounds are used to decrease cholesterol concentration, and fibrates can also induce a decrease of triglycerides concentration in blood plasma. (Fent et al., 2006; Santos et al., 2010).

Statins interrupt cholesterol synthesis by inhibiting the 3-hydroxymethylglutaril coenzyme A reductase (HMG-CoA), an enzyme that enhances an important step of cholesterol synthesis. This inhibition induces intracellular cholesterol depletion, which results in an increased of low density lipoproteins (LDL) receptors expression, principally in hepatocyte membranes. Therefore, LDL of cholesterol are reabsorbed from blood plasma to LDL receptors (Fent et al., 2006). Inhibiting HMG-CoA conversion to mevalonate, statins interact with mevalonate metabolism, which may result in antioxidative and antiinflamatory effects (Fent et al., 2006).



IUPAC Name	(1*S*,3*R*,7*S*,8a*R*)-8-{2-[(2*R*,4*R*)-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl]ethyl}-3,7-dimethyl-1,2,3,7,8,8ª-hexahydronaphthalen-1-yl2,2-dimethylbutanoate
Molecular formula	$C_{25}H_{38}O_5$
Water solubility	0.03 mg/L
octanol–water partitioning coefficient (log k_{ow})	4.68

Figure 2. Chemical structure and physicochemical properties of Simvastatin.
Sources: http://www.sigmaaldrich.com/catalog/product/sigma/s6196?lang=pt&region=PT; Key et al. 2008

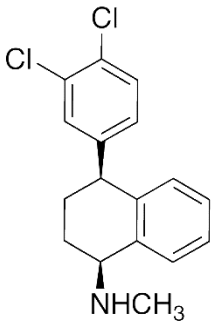
Simvastatin (SIMV) (Figure 2) belongs to statins group and is frequently prescribed, reducing mortality and morbidity from coronary heart diseases (Yang et al., 2011). Measured simvastatin environmental concentrations have been found to be low (Key et al., 2008). However, toxicity data of statins is very limited (Santos et al., 2010).

1.2.2.2. Selective Serotonin Reuptake Inhibitors (SSRIs): Sertraline

Serotonin (5-hydroxytryptamine) is a neurotransmitter involved in important physiological processes and cellular functions modulation, related to neuroendocrine signaling pathways, immune system, food intake, sexual behavior, reproduction and development. This neurotransmitter is released into a synapse space to a pre-synapse, but selective serotonin reuptake inhibit this pump, which lead to an increasing of serotonin level in the synapse space (Fent et al., 2006; Park et al., 2012).

Selective serotonin reuptake inhibitors (SSRIs) are prescribed for clinical depression and compulsive disorders (Park et al., 2012). The SSRIs fluoxetine, fluvoxamine, paroxetine and sertraline (SER) are the most widely used antidepressants (Santos et al., 2010). Fluoxetine is frequently used in ecotoxicological research on SSRI while there are few studies that have investigated sertraline effects (Park et al., 2012).

Serotonin occurs in lower vertebrates and invertebrates. 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). Thus, SSRIs may interfere with functions of nervous, hormonal and immuno systems of these organisms (Fent et al., 2006; Schultz et al., 2011; Park et al., 2012). The effects resulting from SSRIs exposure are different between phyla but several studies have reported alterations in reproduction by affecting endogenous hormone levels and reproductive behaviors (Fent et al., 2006; Park et al., 2012).



IUPAC Name	(1S,4S)-4-(3,4-dichlorophenyl)-N-methyl-1,2,3,4-tetrahydronaphthalen-1-amine
Molecular formula	$C_{17}H_{17}Cl_2N$
Water solubility	3022 mg/L
octanol–water partitioning coefficient (log k_{ow})	1.37 ± 0.1

Figure 3. Chemical structure and physicochemical properties of Sertraline.
Sources: http://www.trc-canada.com/detail.php?CatNum=S280000; Kwon, J., Armbrust, K. (2008)

Despite treatments applied in WWTPs, some SSRIs remain biochemically active and are released into the environment leading to frequent detection of this compounds in surface water (Schultz et al., 2011; Kolpin et al., 2012) and in tissues of some aquatic organisms (Park et al., 2012). Although environmental SSRI concentrations are low, the additive effects of a long term exposure to different SSRI can result in chronic effects to aquatic organisms (Santos et al., 2010).

Sertraline is a common prescribed SSRI (Figure 3). Previous studies have reported the detection of sertraline and its primary metabolite desmethylsertraline in muscle, liver and brain tissues in fish living near an effluent stream (Ramirez et al., 2009).

1.2.2.3. Ultra Violet Filter: 4-Methylbenzylidene Camphor

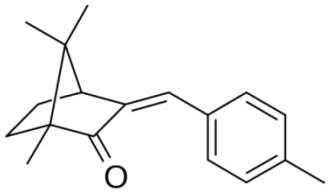
The effects of ultraviolet (UV) radiation exposure have growing concerns in human health and caused an increased usage of UV filters, since the ozone depletion in Australia has been discovered. UV filters are widely used in sunscreen products and cosmetics like beauty creams, body lotions, hair sprays and shampoos to protect from UV radiation (Balmer et al., 2005; Brausch and Rand, 2011). These compounds can also be used as additives in plastics, carpets, clothing and washing powder to protect also products from UV radiation (Kaiser et al., 2012).

The effects caused by over-exposure of sunlight include sunburn and light-induced cell injury and it was estimated that 90 percent of non-melanoma skin cancers and 65 percent of melanomas in humans are caused by sunlight exposure (Vincent, 2011). UV filters can be organic or inorganic micropigments. Organic UV filters (methylbenzylidene camphor) act by absorbing UV radiation while the inorganic (ZnO, TiO_2) can also reflect and scatter UV radiation. Generally, both UV filters types protect against UVB (280 – 315 nm) and some offer protection against UVA (315 – 400 nm) radiation too. Sunscreen products with high sun protect factors generally have higher concentrations of UV filter and often two or more compounds are used in order to cover a larger range of wavelengths (Balmer et al., 2005; Kunz et al., 2006).

Some oxidative damage to DNA may occur due to free radicals generated by the UV filter TiO_2 when absorbing UV radiation, which can result in cancer development in humans (Konaka et al., 1999). Furthermore, sunscreens can enhance the penetration and dermal absorption of certain compounds like pesticides, thereby enhance adverse effects (Pont et al., 2004; Giokas et al., 2007; Vincent, 2011). Due to photostability and high lipophilicity, some UV filters can bioaccumulate and recent in vitro and in vivo studies with rats and fish have reported their potential to induce estrogenic activity and adversely

effects on fecundity and reproduction (Balmer et al., 2005; Kunz et al., 2006; Brausch and Rand, 2011; Zucchi et al., 2011). Considering their physicochemical properties, UV filters are also expected to accumulate in human milk (Schlumpf et al., 2008) but also in sediments and thus affecting benthic organisms (Kaiser et al., 2012).

The UV filter 4-methylbenzylidene Camphor (4-MBC) (Figure 4), an organic UV filter, is one of the most common UV filters used in sunscreens and it is frequently detected in WWTPs effluents (Bachelot et al., 2012). It has been reported as having potential antiestrogenic activity in both *in vivo* and *in vitro* studies as well as estrogenic and antiandrogenic activity (Schreurs et al., 2005; Schlumpf et al., 2008; Brausch and Rand, 2011; Vincent, 2011; Sieratowicz et al., 2011).



IUPAC Name	(3*E*)-1,7,7-Trimethyl-3-[(4-methylphenyl)methylene]-2-nornornanone
Molecular formula	$C_{18}H_{22}O$
Water solubility	Insoluble
octanol–water partitioning coefficient (log k_{ow})	5.1

Figure 4. Chemical structure and physicochemical properties of 4-methylbenzylidene camphor.
Sources: Balmer et al., 2005; Vincent, 2011; http://uniproma.en.b2bfoo.com/wholesale/800928/Sell-4-methylbenzylidene-Camphor.html

1.2.2.4. Preservative: Propylparaben

Parabens are a group of alkyl esters of p-hydrozybenzoic acid. This class of antimicrobial preservatives is widely used in cosmetics such as creams, skin lotions and shampoos, but they are also applied in pharmaceuticals and food products, due to their large antimicrobial spectrum, stability over a wide pH range and high solubility in water (Terasaki et al., 2009; Brausch and Rand, 2011; Yamamoto et al., 2011).

The extensive use of parabens has growing concerns about their potential chronic effects on human health and some recent studies have suggested a possible relationship between parabens and breast cancer, possibly due to prolonged dermal exposure (Darbre

et al., 2004). Although more studies are needed to confirm potential parabens carcinogeniticy, new parabens-free PCPs have been recently available in the market (González-Mariño et al., 2012).

Although advanced treatments in WWTPs revealed effective at removing these compounds, such as membrane filtration, ozonation and powered activated carbon, most of WWTPs still use conventional treatment processes and thus, some parabens present in effluents are released into the aquatic environment (Alberto et al., 2012). There are few studies that have examined parabens occurrence in WWTPs effluents and surface water but existing data reported higher concentrations of parabens in surface water (15 to 400 ng/L) than WWTPs effluents (50 to 85 ng/L), depending on paraben species (Brausch and Rand, 2011). As parabens contain phenolic hydroxyl groups they can easily react with free chlorine when mixed with chlorinated tap water and several chlorinated compounds have been found in aquatic environment, which may result in adversous effects of exposed species (Greenlee et al., 1985; Terasaki et al., 2009; Gozález-Mariño et al., 2011; Sárkány-Kiss et al., 2012).

Some studies have previously indicated that increasing chain length of parabens' substituents can increase paraben acute toxicity and also results in water solubility decreases and in higher periods required for compound biodegradation (Brausch and Rand, 2011; Darbre et al., 2004; González-Mariño et al., 2011; Li, 2012). These compounds can be considered "persistent" contaminants due to their common use and continuous introduction into the environment (Albero et al., 2012).

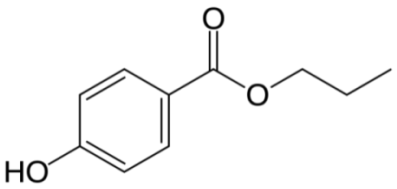
Depending on the physicochemical properties of paraben species, dermal absorption is generally rapid and it can be influenced by the presence of enhancers in the PCP formulation that can assist the process (Dabre et al., 2004; Albero et al., 2012). As other lipophilic compounds, parabens have a high octanol/water partition coefficient, and once enter in organism body are able to accumulate in fatty components of tissues similar as other lipophilic compounds (Darbre et al., 2004; Tavares et al., 2009).

Although most studies have classified parabens as non-mutagenic, there are reports of chromosomal anomalies caused by parabens exposure, particularly in the co-presence of polychlorinated biphenyls, as well as carcinogenic activity. Parabens exposure can cause cellular function disruption through secretion inhibition of lysossomal enzymes and causing also mitochondrial dysfunctions and estrogenic effects (Drabre et al., 2004).

Several in vivo and in vitro studies have reported potential estrogenic activity of parabens (Yamamoto et al., 2011; Alberto et al., 2012). Some parabens can cause VTG synthesis in male fish at low concentrations and it was also reported a significantly

inhibition of spermatogenesis in rats after propylparaben (PP) exposure, without alteration in serum testosterone level (Oishi, 2002; Brausch and Rand, 2011).

Among the different paraben species, methylparaben and propylparaben (Figure 5) are the most commonly used in cosmetics and normally are co-applied to increase preservatives effects (Brausch and Rand, 2011). Furthermore, these two compounds are frequently detected in the environment, at higher concentrations than others parabens (González-Mariño et al., 2012; Albero et al., 2012).



IUPAC Name	proxyl 4-hydroxybenzoate
Molecular formula	$C_{10}H_{12}O_3$
Water solubility	500 mg/L
octanol–water partitioning coefficient (log k_{ow})	2.71

Figure 5. Chemical structure and physicochemical properties of Propylparaben.
Sources: Tavares et al., 2009; Dobbins et al., 2009; http://blog.absurdouee.fr/post/2012/12/15/Parahydroxybenzoate-de-propyle-sors-de-ma-cr%C3%A8me

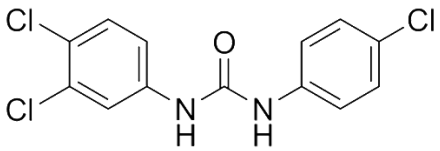
1.2.2.5. Bacteriocide and antifungal agents: Triclocarban

Triclocarban (TCC) is widely used as antimicrobial in soaps, deodorants, skin creams, toothpaste and plastics, since 1957 (Brausch and Rand, 2011). Different studies have reported its bacteriostatic potential against gram positive bacteria (Walsh et al., 2003). However, some studies have reported also efficacy against gram negative bacteria and also fungi, but at higher concentrations.

The Triclocarban mode of action as an antimicrobial compound is not well understood. It presents a $C_6H_5NH_2$ group, belonging to anilide group compounds (Figure 6). These compounds are characterized to induce cell death by adsorbing to the cytoplasmic membrane of organisms and destroying its semipermeable nature. As a result of anilide action, the proton motive force across the bacterial surface is disrupted and the active transport and energy metabolism could also be interrupted (Walsh et al., 2003).

Triclocarban exhibits significance persistence in the environment and has been frequently detected in WWTP effluents and surface water over the last years at concentrations higher than Triclosan (TCS), another disinfectant compound, and its methyl derivate Methyl-Triclosan (Chalew and Halden, 2009; Brausch and Rand, 2011).

This compound has potential to accumulate in organisms and sediments and some recent studies reported TCC endocrine disruption activity (Chen et al., 2008; Chalew and Halden, 2009; Giudice and Young, 2010; Schebb et al., 2011).



IUPAC Name	3-(4-Chlorophenyl)-1-(3,4-dichlorophenyl)urea
Molecular formula	$C_{13}H_9Cl_3N_2O$
Water solubility	< 0.045 mg/L
octanol–water partitioning coefficient (log k_{ow})	3.5 ± 0.06

Figure 6. Chemical structure and physicochemical properties of Triclocarban.
Sources: http://finesseofsimpleliving.blogspot.pt/2012/06/beauty-detox-truth-about-triclocarban.html; Snyder et al., 2010

1.3. The use of embryos in ecotoxicological studies with pharmaceuticals and personal care products

Chronic life-cycle exposure tests give important information of the toxicity of chemicals. However, short-term early-life-stage has been frequently used as an alternative to these long-term in face of its simplicity, low costs, sensitivity and the potential to be used as a high-throughput approach. Furthermore, early life stages are often more susceptible to chemicals action than adults because they are exposed during critical stages of their development, and many chemicals can act at specific developing process (Versonnen and Janssen, 2004). Thus, this toxicological information is needed to perform more reliable risk assessment and to implement protective standards for organisms and ecosystems (Bellas et al., 2005).

A number of different fish early-life-stage toxicity tests have been established being the freshwater zebrafish (*Danio rerio*) the most commonly and well-recognized

aquatic vertebrate model species used (Carlsson and Norrgren, 2004) due to advantages such as egg transparency, rapid development and well-studied embryogenesis (Versonnen and Janssen, 2004).

Similarly, the embryonic and larval stages of marine invertebrates haven been used for assessing quality of marine water and sediments and to understand toxic effects of several compounds. Sea urchin embryos are frequently selected as models for toxicity testing due to their abundance, ecological and commercial relevance as well as embryo sensitivity and extensive knowledge of molecular physiology (Bellas et al., 2005; Roepke et al., 2005).

1.4. Objectives

The overall aim of the present work was to assess the toxicological risk of five emergent chemicals, simvastatin, sertraline, triclocarban, propylparaben and 4-MBC, both under single and combined exposure. Embryonic development of zebrafish (*Danio* rerio) and sea urchin (*Paracentrotus lividus*) were used as models for evaluating toxic effects resulting from PPCPs exposures.

Material and Methods

Chapter II

2. CHAPTER II – Material and Methods

2.1. Chemicals

The selected PPCPs Simvastatin (CAS 79902-63-9, 97.0%), Sertraline hydrochloride (CAS 79559-97-0, 98.0%), Propyl 4-hydroxybenzoate (CAS 94-13-3, 99.0%), 3,4,4'-Trichlorocarbanilide (CAS 101-20-2, 99.0%) and 3-(4-Methylbenzylidene) camphor (CAS 36861-47-9, 98.5%) were purchased from Sigma-Aldrich. All stock solutions were stored in the dark at 4ºC and were prepared in Dimethylsulfoxide (DMSO), purchased from Merck.

Artificial seawater compounds 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 while Sodium chloride (CAS 7647-14-5, 99.5%) and Sodium bicarbonate (CAS 144-55-8) were purchased from Merck.

2.2. Species selection

2.2.1. Zebrafish (*Danio rerio)*

Zebrafish (*Danio rerio*) is a tropical, teleost and cypriniforme fish, native to the rivers of India and south Asia (Jiang et al., 2013). Male and female zebrafish can be easily distinguished under spawning conditions (Figure 7). Male show a slender body shape, whereas females can be recognized by their swollen bellies due to the large number of eggs produced (Lammer et al., 2009).

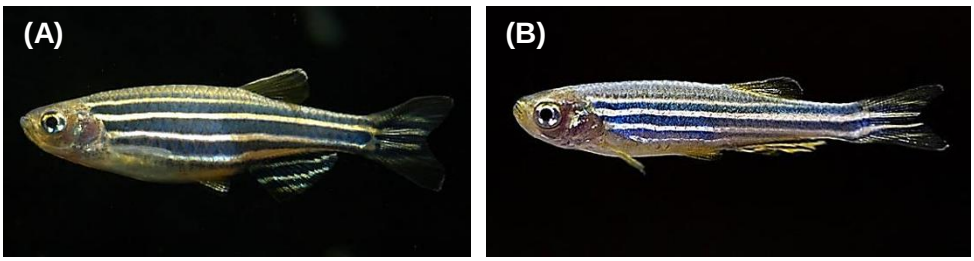


Figure 7. Zebrafish (*Danio rerio*) female **(A)** and male **(B)**.
Sources: http://www.seymourfish.com/zebra-danio-care/
http://www.practicalfishkeeping.co.uk/content.php?sid=4342

These organisms have been largely used as model species for toxicological purposes because of several properties that make this species simple to use. Zebrafish are easily maintained in stock at the laboratory, due to their dimensions, thus reducing costs. Further aspect that supports its use is the available information for this species, including full genome sequence, regulatory sequences and expression profile (Chow et

al., 2012), which is essential for genomic studies and for understanding the toxicological mode of action.

The advantages of their use, associated with the evolutionary relationship to higher vertebrates, makes zebrafish an important model not only for toxicological purposes but also as an ecotoxicological test species. In fact, it provides insights into many aspects of vertebrate biology, genetics, toxicology and disease, being representative for a larger group of organisms.

Under laboratory conditions, zebrafish can be induced to breed all year round with a high fecundity and also large number of offspring, with easy observation and manipulation (Segner, 2009). It has been suggested that embryos might be more susceptible to develop adverse effects as they are exposed during critical stages in their development (Versonnen and Janssen, 2004), which make them a helpful model to developmental toxicology research. In comparison with other biological models, embryonic development is rapid which reduces the duration of the tests and enables to obtain results in a short time (Kimmel et al., 1995). The small size of embryos has the advantage that they can be incubated in microplates and be easily manipulated either by water exposure or by eggs microinjection (Segner, 2009). Other advantage is the optical transparency of the zebrafish eggs which allow the monitoring of possible phenotypic changes in the live organism during the assay and enable gene expression analysis, for instance, in transgenic zebrafish lines (Versonnen and Janssen, 2004; Segner, 2009; Soares et al., 2009).

At the OECD level, the zebrafish embryo toxic assay (FET) has been proposed as an alternative test guideline to classical acute fish toxicity testing with live fish, required for regulatory activities (Lammer et al., 2009; Kaiser et al., 2012).

2.2.2. Sea urchin (*Paracentrotus lividus*)

Paracentrotus lividus (Figure 8) is an herbivorous echinoderm present on rocky bottoms from intertidal and subtidal zone. It is the most common sea urchin species of the Mediterranean Sea infralittoral and along the North-eastern Atlantic coast (from Scotland and Ireland to southern Morocco) (Byrne, 1990; Hereu et al., 2005; Pais et al., 2007; Sugni et al., 2010), and also very abundant in the Portuguese coastline (Cunha et al., 2005).

Sea urchin exhibits an annual cycle of gonadal growth and maturation, with temperature and photoperiod stimulus. In general, spawning began in May/June and extends to August/September. The end of the breeding season is followed by gonadal

growth from October to April (period of decreasing sea temperature) and oocyte accumulation in the ovary in March (Byrne, 1990).

Many compounds that enter in the marine compartment show low seawater solubility. For this reason, they may become associated with organic and inorganic particles present in suspension and, subsequently, they can be rapidly deposited in sediments, where they may persist for a long time (Rojo-Nieto et al., 2013). Animals that live in contact with sediments are more susceptible to possible adverse effects.

Figure 8. Sea urchin (*Paracentrotus lividus*) male (left) and female (right).

In general, invertebrates were rather disregarded in spite of their role on aquatic ecosystems (Sugni et al., 2010). With the recognition of its ecological relevance, it becomes important to conduct further studies and define new experimental models to understand the effects of PPCPs exposure on this group (Sugni et al., 2010).

Echinoderms, being deuterostomes, are phylogenetic more related to chordates than any other invertebrate group. In fact, these organisms exhibit a developmental pattern that is similar to chordates, which means that they may possess similar mechanisms of action or share similar signaling pathways to those of vertebrates (Roepke et al., 2005; Sugni et al., 2010) and contribute to understand conserved metabolic and signalizing pathways.

As a result of extensive knowledge of its molecular physiology and genome characterization, sea urchin becomes an important experimental model to developmental toxicology research. The physiology and biochemistry of fertilization and early embryo development are well understood (Roepke et al., 2005), with a production of a large quantity of gametes and rapid fertilization and embryonic development processes. The sea urchin embryo test (SET) has been frequently used for evaluation of seawater and marine sediment quality, revealing itself a rapid, sensitive and cost-effective bioassay

(Saco-Álvarez et al., 2010). Thus, together with other factors like their abundance, ecological and commercial relevance, sea urchin embryo provides a useful developmental model (Bellas et al., 2005; Roepke et al., 2005).

2.3. Fertilization and embryo collection

2.3.1. Zebrafish (*Danio rerio)*

Adult zebrafish were obtained from local suppliers in Singapore, with no history of an environmental exposure to contaminants. The stock of female and male zebrafish was kept in 250L aquarium with dechlorinated and aerated water in a recirculation system with both mechanical and biological filters, at a water temperature of 28.0±1 ºC and under a photoperiod of 14:10h (light:dark). The fish were fed four times per day by an automatic feeder with a commercial fish diet Tetramin (Tetra, Melle, Germany) supplemented with live brine shrimp (*Artemia* spp.).

On the day before the assays, a group of males and females in a ratio of 2:1 was housed in a breeding box, inside a 30L aquarium and under the same water and photoperiod conditions as the stock. The breeding box had a mesh bottom covered with marbles to allow the passage of eggs to aquarium and prevent acts of cannibalism by parents. At the following day, ovulation and fertilization were stimulated after the onset of light period and take place for one hour. Breeding fish were removed and the eggs were collected, cleaned and observed at a magnifying glass to select the fertilized eggs for the experiments. Fertilized eggs were randomly allocated to 24 wells plate, at most 3 hours after fertilization.

2.3.2. Sea urchin (*Paracentrotus lividus*)

Sea urchin were collected in Granja, Vila Nova de Gaia (N41º 2’ 26,18’’, W -8º 39’ 2,24’’), and transported to the laboratory in a portable icebox containing seawater . *P. lividus* gametes were obtained by dissection from a single pair of mature adults. Gamete quality was checked for the criteria of motility of the sperm and round form of the eggs under a Nikon eclipse 50i microscope. Thus, one male and one female showing optimal conditions were selected. Eggs were transferred into a 100 mL measuring cylinder containing artificial seawater until a dense suspense was formed. A few µL of undiluted sperm were added and the contents were gently stirred to allow fertilization. After a few minutes, three aliquots of 10µL were taken by a pipette and observed under the microscope in order to record the number of eggs present and fertilization success, which

was indicated by the presence of a fertilization membrane. The assay was conducted when fertilization rate was greater than 97%.

2.4. Experimental design and embryo bioassays

2.4.1. Experimental solutions

Stock solutions of each compound were prepared by dissolving Simvastatin, Sertraline, Triclocarban, Propylparaben and 4-MBC in the organic dissolvent Dimethylsulfoxide (DMSO) to obtain the appropriate concentration for experimental use. Similarly, stock solutions of tested mixtures were prepared in DMSO, adding each compound in a specific concentration, based on results from individual exposures, for both organisms tested. 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 volume of 0.01%. Artificial seawater was prepared according to Zaroogian et al. (1969) as in Table 5.

Table 5. Artificial seawater composition.

Compound	Weight (g/L)
NaCl	24.6
KCl	0.67
$CaCl_2$	1.36
$NaHCO_3$	0.39
$MgSO_4$	2.04
$MgCl_2.6H_2O$	4.66

Source: Zaroogian et al., 1969

Experimental concentrations of selected PPCPs were chosen based on the data from the literature, with 10x dilutions, in order to cover a wide range of concentrations. If necessary, a new set of concentrations in 2,5x dilutions or intermediate concentrations were tested in order to estimate the No Observed Effect Concentration (NOEC) (Table 6).

Table 6. Compound concentrations tested in zebrafish and sea urchin assays (µg/L).

Organism	SIMV	SER	4-MBC	PP	TCC
Zebrafish	5000.0	10000.0	5000.0	10000.0	10000.0
	500.0	8500.0	500.0	8500.0	1000.0
	50.0	6000.0	50.0	6000.0	850.0
	5.0	3500.0	5.0	3500.0	600.0
		1000.0		1000.0	350.0
		100.0		100.0	100.0
		10.0		10.0	10.0
Sea urchin	5000.0	10000.0	5000.0	10000.0	10000.0
	500.0	1000.0	500.0	1000.0	1000.0
	50.0	100.0	50.0	400.0	100.0
	5.0	10.0	5.0	160.0	10.0
	2.0	4.0	2.0	100.0	4.0
	0.8	1.6	0.8	64.0	1.6
	0.32	0.8	0.32	10.0	0.64
					0.256
					0.1024
					0.041

Based on results from individual exposures, embryos from both species were exposed to a mixture with these five compounds present at their respective NOEC values. Sea urchin embryos were also exposed to two other mixtures, according the respective LOEC and EC20 values from individual exposures (Table 7).

Considering the results of individual exposures, the expected concentration-response relationship of mixture was calculated using the CA model equation:

$$ECx_{mix} = \left(\sum_{i=1}^{n} \frac{p_i}{ECx_i} \right)^{-1}$$

where ECx_{mix} is the concentration of the mixture that induces an overall effect *x*, ECx_i is the concentration of the component *i* in a *n*-component mixture that induce the same magnitude effect and p_i is the proportion of *i*-component in the mixture (Belden et al., 2007).

Table 7. Nominal concentrations of mixtures compounds tested in zebrafish and sea urchin assays (µg/L).

Mixture	SIMV	SER	4-MBC	PP	TCC
Zebrafish					
MIX_NOEC_dr	5.0	1000.0	500.0	1000.0	100.0
Sea urchin					
MIX_NOEC_pl	2.0	4.0	0.8	160.0	0.256
MIX_LOEC_pl	5.0	10.0	2.0	400.0	0.64
MIX_EC20_pl	775.0	55.0	494.0	13891.0	20.0

2.4.2. Zebrafish (*Danio rerio)*

The static-water renewal toxicological tests with zebrafish were performed according to OECD guidelines (OECD, 1998). After embryos observation using a magnifying glass, 10 fertilized eggs were selected and transferred to 24-wells plates filled with 2 mL of freshly prepared solutions and controls per plate. For chemical individual exposures, eight replicates for six treatments conditions were set up: an experimental control, a solvent control (DMSO) and four concentrations of each chemical (Lammer et al., 2009). Mixtures exposures were performed using the same methodology.

The 24-wells plates were incubated at 26.5ºC during 80h 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. Embryos were not fed during exposure.

Observations were performed at 8hpf, 32hpf and 80hpf and different parameters were recorded (Table 8). The observation periods were selected based on a set of characteristics present in embryos at these stages of development (Kimmel et al., 1995).

Mortality was assessed by daily recordings during the entire exposure period and coagulated eggs or death embryos were removed.

In order to reduce the observation period, six embryos per replicate were randomly selected and assessed for different endpoints. 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. Heart rate was evaluated in one embryo per replicate using a stop-watch during 15s, restarting the counting if the embryo moved.

All zebrafish embryo/larvae observations were performed with a Leica EZ4 magnifying glass.

Table 8. Endpoints recorded at 8hpf, 32hpf and 80hpf in zebrafish assay.

Endpoint	8hpf	32hpf	80hpf
Mortality rate	*	*	*
75% of epiboly stage	*		
Abnormal cell growth	*		
Head abnormalities		*	*
Tail abnormalities		*	*
Eyes abnormalities		*	*
Yolk-sac abnormalities		*	*
Pericardial edema		*	*
Heart rate		*	*
Hatching rate			*
Muscular involuntary contractions(1)			*

(1) Only for 4-MBC exposure

2.4.3. Sea urchin (*Paracentrotus lividus*)

The toxicity assays were performed in 24-wells plates. Within 30 minutes after fertilization, fertilized eggs were placed in 3 mL solution test in a concentration of 20 eggs/mL per well. As performed in zebrafish assays, eight replicates for each treatment, including the controls, were used for both single and combined exposures. The 24-wells plates were isolated with parafilm and embryos were incubated at 20ºC in dark for 48h. 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 measuring larvae length of individuals in *pluteus* stage, defined as the distance between the apex and the end of the post-oral arm, in the first 15 individuals per well, randomly chosen. Larvae were considered normal by the pyramid shape and four separated arms (Saco-Álvarez et al., 2010).

P. lividus larvae 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

Data were analyzed using SPSS version 21.0 software. All data were tested for homogeneity and normality using Levene's and Kolmogorov-Smirnov 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, the non-parametrical Kruskal-Wallis test, followed by Games-Howell test, were used to multiple comparisons between individual treatments (Bellas et al., 2005).

D. rerio statistical analysis was made to the 80hpf endpoints cumulative mortality and hatching rates, pericardial edema and heart rate, abnormalities in head, eyes, yolk-sac and tail as well as to the 8hpf endpoints abnormal cell growth and 75% of epiboly stage. For *P. lividus* bioassay, control and solvent control were grouped, if no significant differences between them were detected. The EC20 and their 95% confidence intervals were calculated according to the Probit method after normalizing data to the mean control response using Abbott's formula (Bellas et al., 2005).

Data were presented as means ± standard error. The significance level was set at 0.05.

Results

Chapter III

3. CHAPTER III – Results

3.1. Hypolipidemic drugs: Simvastatin

3.1.1. Zebrafish (*Danio rerio)*

3.1.1.1. Cumulative mortality and Hatching rates

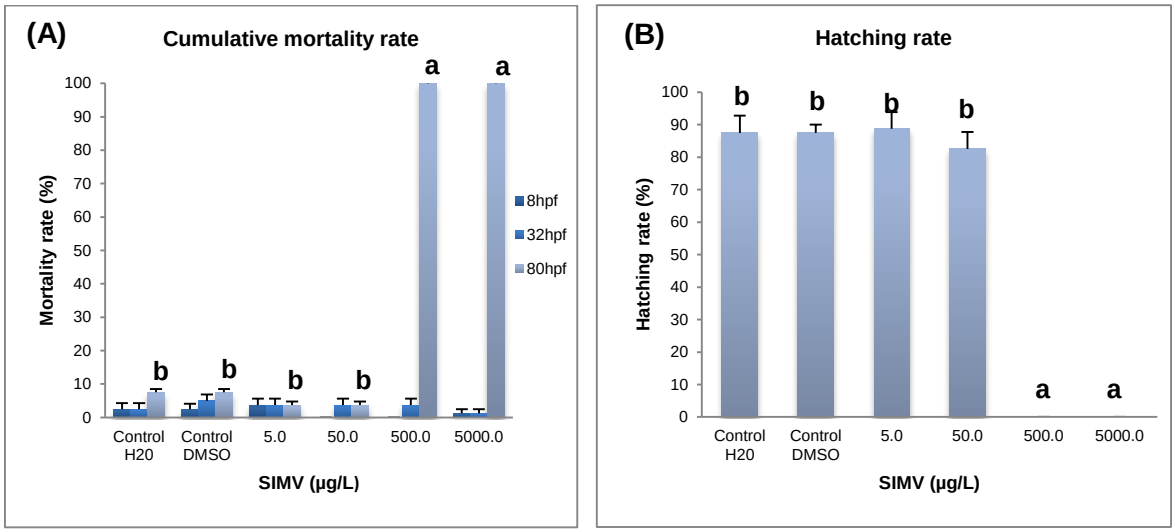


Figure 9. Cumulative mortality **(A)** and hatching **(B)** rates of *D. rerio* exposed to different concentrations of simvastatin for 80h (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

At 8hpf mortality rate ranged from 0 in the 50.0 and 500.0 µg/L groups to 3.75 ± 1.83 in 5.0 µg/L treatment group (Figure 9A). On the following observation (32hpf), mortality ranged from 1.25 ± 1.25 in 5000.0 µg/L group to 5.0 ± 5.35 in the solvent control. Cumulative mortality rate at the end of the assay ranged from 3.75 ± 1.83 in the 5.0 and 50.0 µg/L exposure groups to 100 in the 500.0 and 5000.0 µg/L exposure groups. All embryos from the two highest treatments died before 80hpf. The results obtained in the two highest concentrations are significantly different ($p<0.05$) from those of the other exposure groups and controls.

At 80hpf, hatching rate ranged from 88.75 in the 5.0 µg/L to 0 in the 500.0 and 5000.0 µg/L groups (Figure 9B). The effects of 500.0 and 5000.0 µg/L tested concentrations in embryos hatching rate are significantly different ($p<0.05$) from the other treatments.

3.1.1.2. Abnormal cell growth and 75% epiboly stage of embryos (8hpf)

There were no significant differences ($p>0.05$) among treatments in abnormal cell growth and 75% of epiboly stage at 8hpf (Figure 10A and 10B).

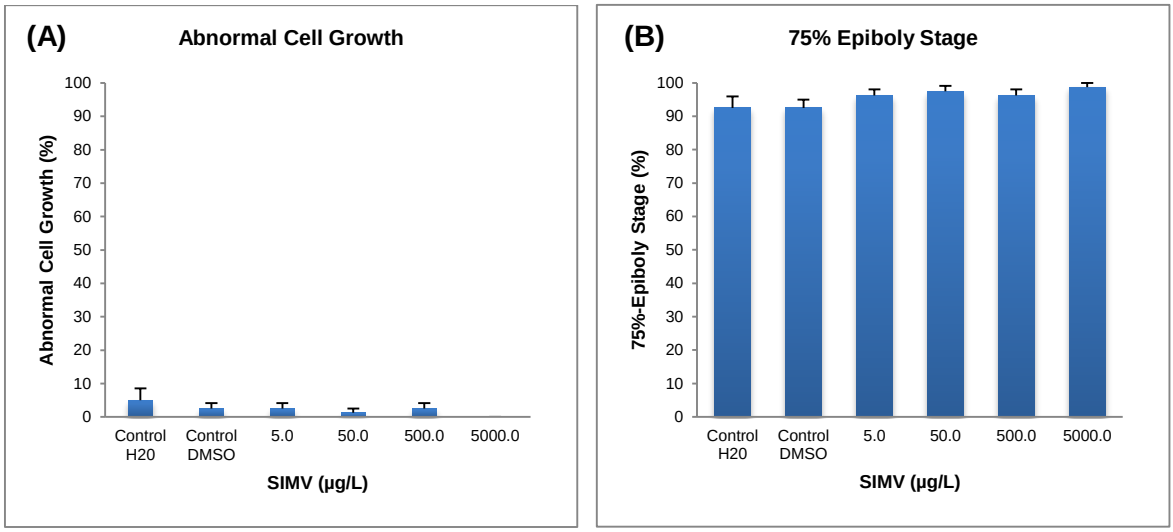


Figure 10. Abnormal cell growth **(A)** and 75% of epiboly stage **(B)** of *D. rerio* embryos exposed to different concentrations of simvastatin at 8hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

3.1.1.3. Head and eyes abnormalities

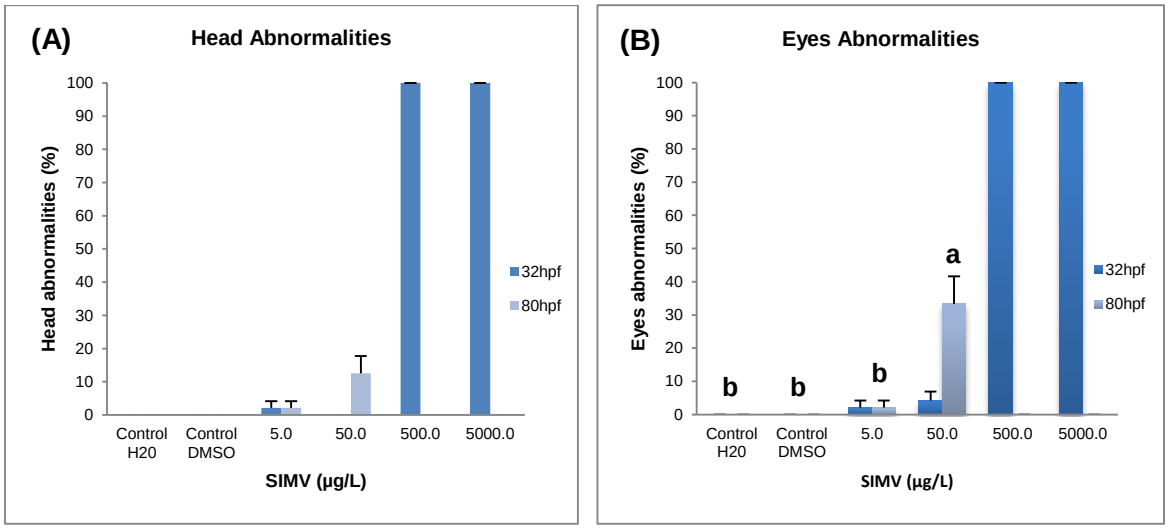


Figure 11. Head **(A)** and eyes **(B)** abnormalities of *D. rerio* exposed to different concentrations of simvastatin at 32hpf and 80hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

At 32hpf the percentage of embryos with head abnormalities ranged from 0 in both controls to 100 at 500.0 and 5000.0 µg/L exposure groups (Figure 11A). There are no significant differences between controls and exposure groups at 80hpf ($p>0.05$).

Similarly, at 32hpf the percentage of eyes abnormalities ranged from 0 in both controls to 100 at the two highest concentrations (Figure 11B). On the next observation

(80hpf) the percentage of eyes abnormalities ranged from 0 in both controls to 33.33 ± 8.33 in the 50.0 µg/L treatment. The results recorded at the end of the assay for the group exposed to 50.0 µg/L were significant different ($p<0.05$) from those obtained in the controls and in the 5.0 µg/L group.

3.1.1.4. Yolk-sac and tail abnormalities

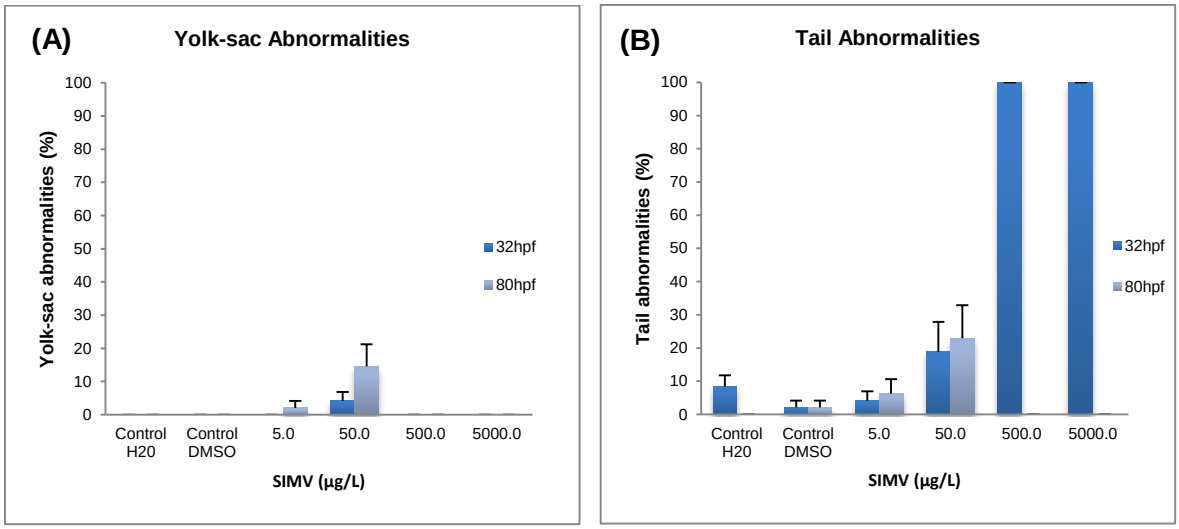


Figure 12. Yolk-sac **(A)** and tail **(B)** abnormalities of *D. rerio* exposed to different concentrations of simvastatin at 32hpf and 80hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

There were no significant differences in the percentage of embryos with abnormalities in yolk-sac between the results obtained from controls and exposure groups (Figure 12A).

All embryos exposed to 500.0 and 5000.0 µg/L of simvastatin reveal tail abnormalities at 32hpf (Figure 12B). No statistically significant differences were recorded at 80hpf in embryo tail abnormalities among controls and 5.0 and 50.0 µg/L groups.

3.1.1.5. Pericardial edema and Cardiac frequency

The percentage of embryos with pericardial edema at 80hpf ranged from 0 in controls to 56.56 ± 8.87 in the 50.0 µg/L group (Figure 13A). On the next observation (80hpf), the percentage of embryos with pericardial edema ranged from 0 in controls to 56.56 ± 8.87 at this concentration. This increase in the percentage of embryos with pericardial edema at 80hpf was statistically significant ($p<0.05$), in comparison to controls and 5.0 and 50.0 µg/L groups.

Embryos heart rate did not differ significantly among groups at 80hpf (Figure 13B).

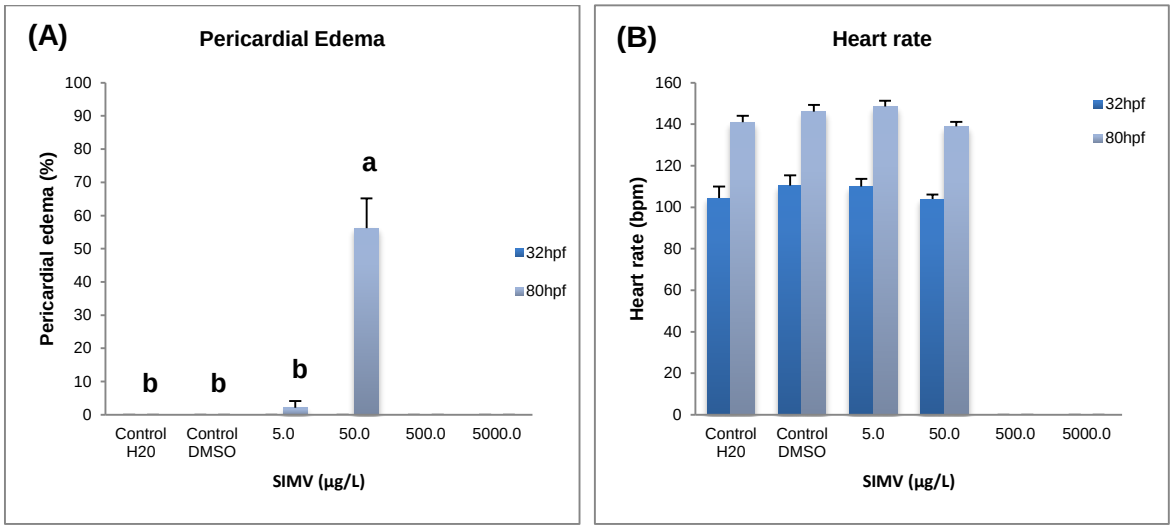


Figure 13. Pericardial edema (%) **(A)** and heart rate (bpm) **(B)** of *D. rerio* embryos exposed to different concentrations of simvastatin at 32hpf and 80hpf. Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A).** One-way-ANOVA **(B)**. Bars with a different letter are significantly different from each other.

3.1.1.6. Total abnormalities in embryos

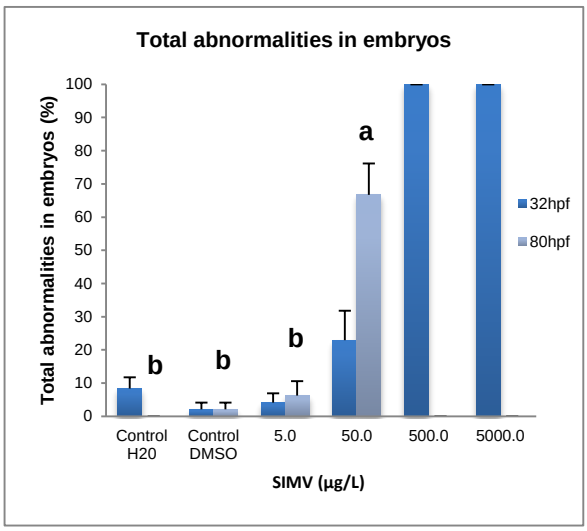


Figure 14. Percentage of total abnormalities in *D. rerio* embryos exposed to different concentrations of simvastatin at 32hpf and 80hpf. Data are expressed as mean ± SE (n=8). One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons between groups. Bars with a different letter are significantly different from each other.

At 32hpf the percentage of embryos with one or more abnormalities ranged from 2.08 ± 2.08 in the control to 100 in the 500.0 and 5000.0 µg/L exposed groups (Figure 14).

At the end of the assay, the percentage of total abnormalities in embryos ranged from 0 in the control to 66.67 ± 9.45 in the 50.0 µg/L group (Figure 14 and 15). There was a significant increase ($p<0.05$) in the percentage of abnormal embryos exposed to 50.0 µg/L of simvastatin.

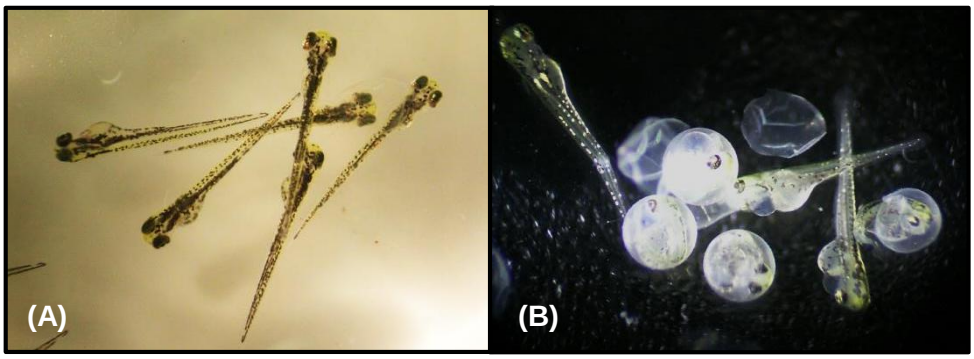


Figure 15. Stage of development of *D. rerio* embryos at 80hpf in the control **(A)** and in the 50.0 µg/L of simvastatin exposure group **(B)**.

3.1.2. Sea urchin (*Paracentrotus lividus*)

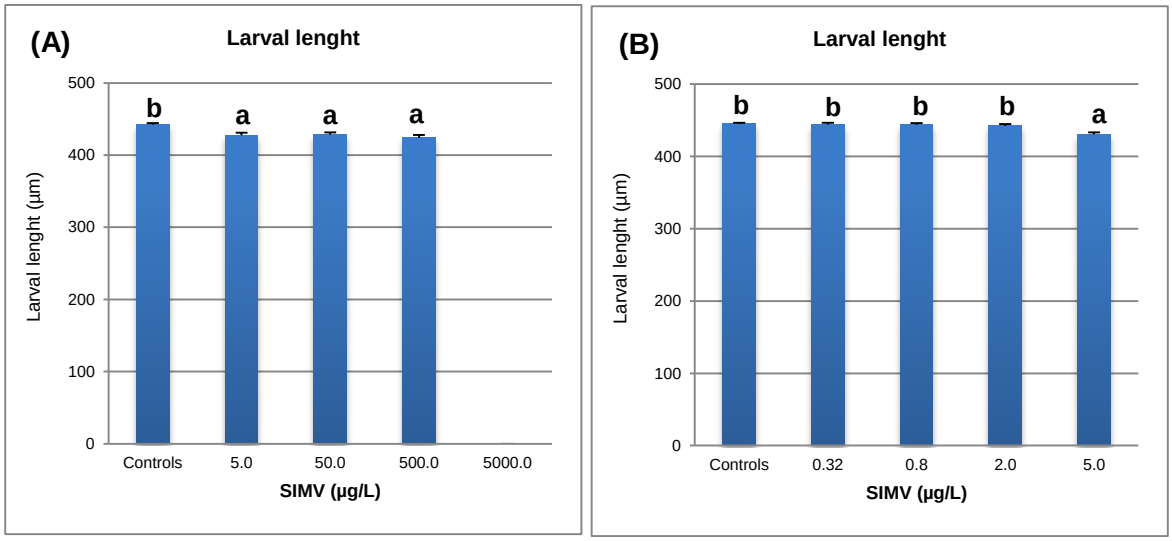


Figure 16. Larval length (µm) of *P. lividus* exposed to different concentrations of simvastatin for 48h (**A** and **B**). Control and solvent control were grouped. Data are expressed as mean ± SE (n=240 for controls; n=120 simvastatin exposed groups). One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons among groups **(A)**. Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(B)**. Bars with a different letter are significantly different from each other.

An exposure of *P. lividus* embryos to simvastatin resulted in significant effects. Simvastatin at 5000.0 µg/L lead to a delay in larvae development so that none reached the four arm stage, and therefore length was not recorded (Figure 17). All simvastatin

treatments induced a significant decrease ($p<0.05$) of larval length, compared to controls (Figure 16A).

Considering the results of the first assay, a second experiment was carried out testing lower concentrations of simvastatin in order to determine the LOEC (Figure 16B). Thus, the LOEC of simvastatin exposure in this study was determined as being 5.0 µg/L (430.33 ± 2.95 µm).

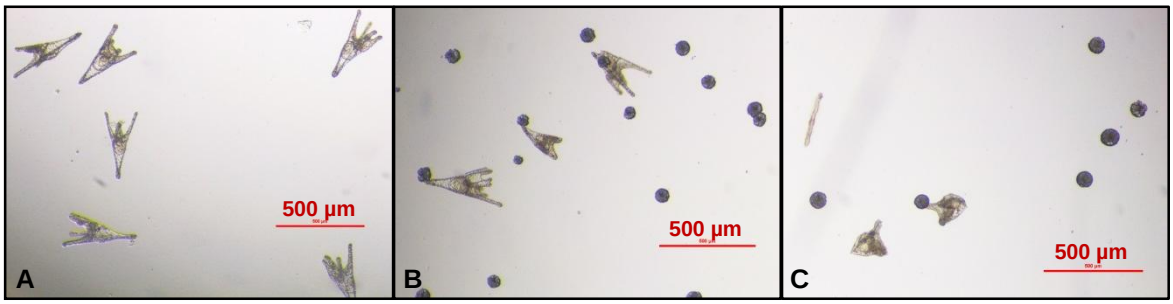


Figure 17. Stage of development of *P. lividus* embryos after exposure to different concentrations of simvastatin for 48h: Solvent control – pluteus larvae **(A)**; 500.0 µg/L – pluteus larvae **(B)**; 5000.0 µg/L – prism larvae **(C)**.

3.2. Selective Serotonin Reuptake Inhibitors (SSRIs): Sertraline

3.2.1. Zebrafish (*Danio rerio)*

3.2.1.1. Cumulative mortality and Hatching rates

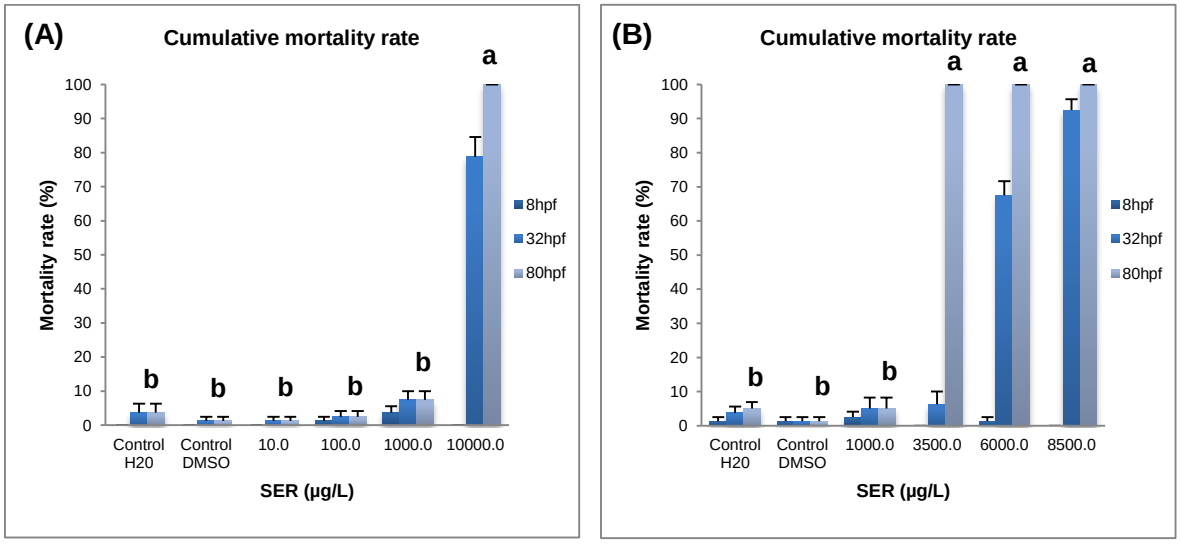


Figure 18. Cumulative mortality rate (**A** and **B**) of *D. rerio* embryos exposed to different concentrations of sertraline for 80h (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

At 8hpf mortality rate ranged from 0 in the controls, 10.0 and 10000.0 µg/L groups to 3.75 ± 1.83 in the 1000.0 µg/L group (Figure 18A). On the next observation (32hpf), an increase in the mortality rate was observed, ranging from 1.25 ± 1.25 in the control and 10.0 µg/L group to 78.75 ± 5.18 in the 10000.0 µg/L group. At 80hpf, all embryos exposed to 10000.0 µg/L of sertraline died. The cumulative mortality rate at this concentration was significantly different ($p<0.05$) from the other exposure groups and controls at the end of the assay.

D. rerio embryos were exposed to concentrations between 1000.0 and 10000.0 µg/L in a second experiment (Figure 18B). At 8hpf mortality rate ranged from 0 in the 8500.0 µg/L to 2.50 ± 1.64 in the 1000.0 µg/L group. On the next observation (32hpf), there was an increase of cumulative mortality, ranging from 1.25 ± 1.25 in the solvent control to 92.50 ± 3.13 in the 8500.0 µg/L group. All embryos exposed to 3500.0, 6000.0 and 8500.0 µg/L of sertraline died before 80hpf. The cumulative mortality rate in these exposure groups is significantly different from the other groups ($p<0.05$).

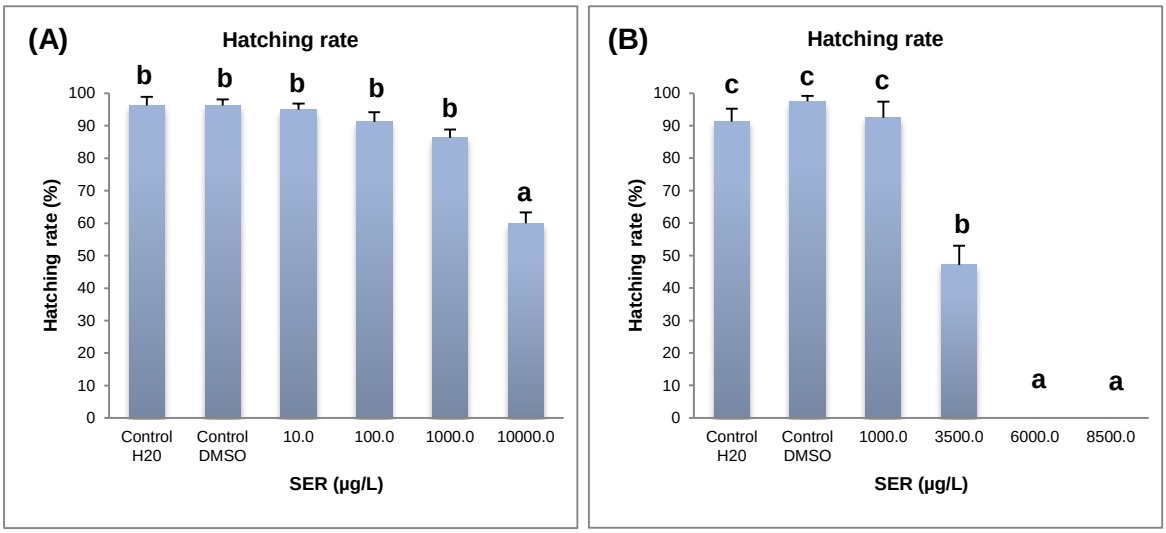


Figure 19. Hatching rate (**A** and **B**) of *D. rerio* embryos exposed to different concentrations of sertraline for 80h (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

Based on results of both experiments, an exposure to sertraline resulted in statistically significant decreases of embryo hatching rate at concentrations equal or higher than 3500.0 µg/L at 80hpf (Figure 19).

3.2.1.2. Abnormal cell growth and 75% epiboly stage of embryos (8hpf)

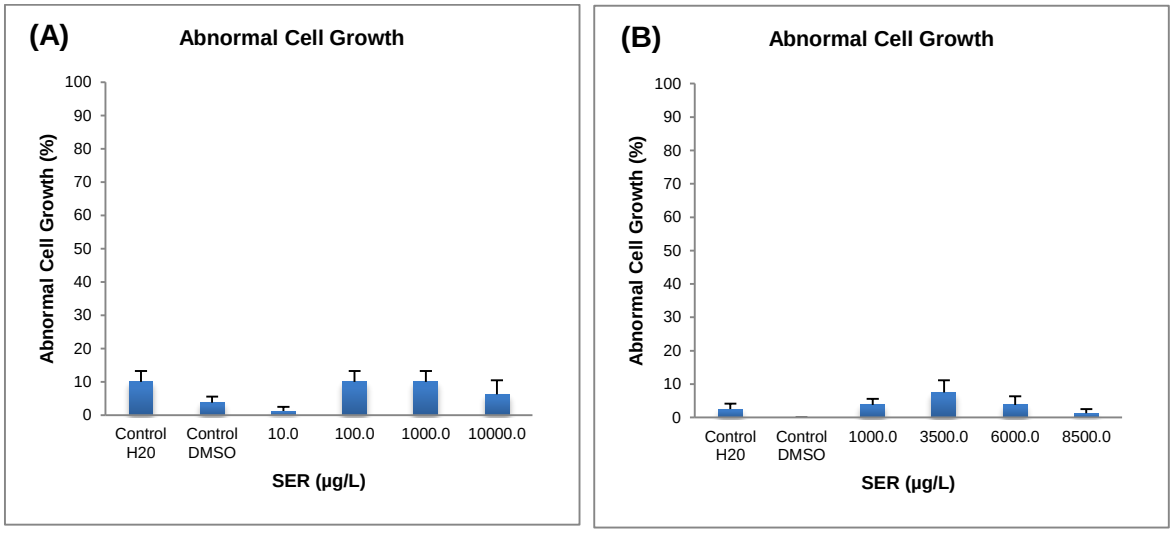


Figure 20. Abnormal cell growth (**A** and **B**) of *D. rerio* embryos exposed to different concentrations of sertraline at 8hpf (%). Data are expressed as mean ± SE (n=8). One-way-ANOVA **(A)**. Non-parametric ANOVA Kruskall-Wallis **(B)**.

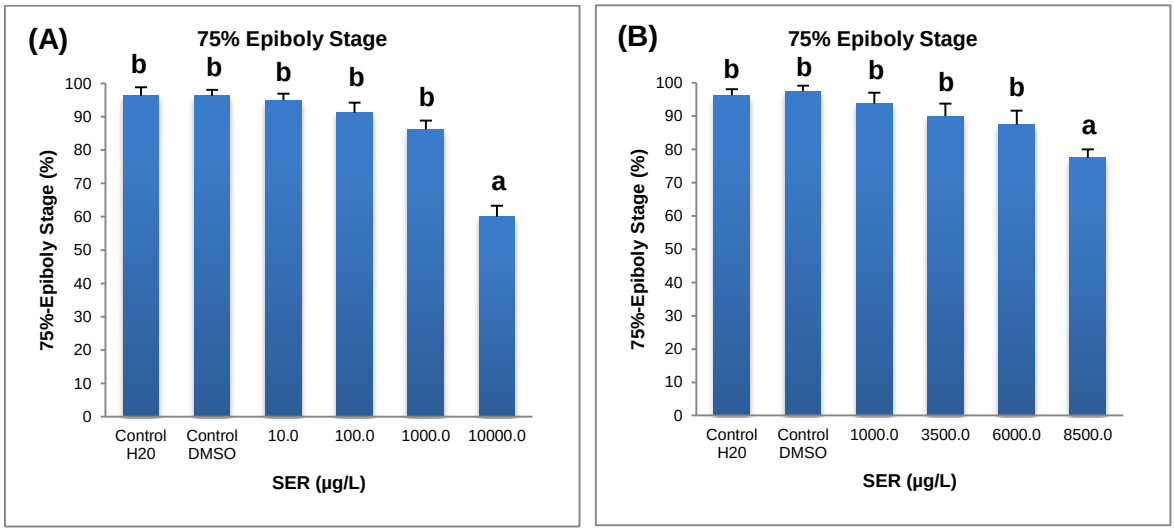


Figure 21. 75% of epiboly stage (**A** and **B**) of *D. rerio* embryos exposed to different concentrations of sertraline at 8hpf (%). Data are expressed as mean ± SE (n=8). One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

An exposure to sertraline did not induce significant differences in the percentage of embryos with abnormal cell growth among all groups ($p>0.05$) in both performed assays, at 8hpf (Figure 20).

The percentage of embryos in 75% of epiboly stage at 8hpf decreased in a dose-dependent manner and ranged from 97.5 ± 1.64 in the solvent control to 60.0 ± 3.27 in the 10000.0 µg/L group (Figure 21A). This decrease was statistically significant in the 8500.0 and 10000.0 µg/L exposure groups ($p<0.05$) (Figure 21).

3.2.1.3. Head and eyes abnormalities

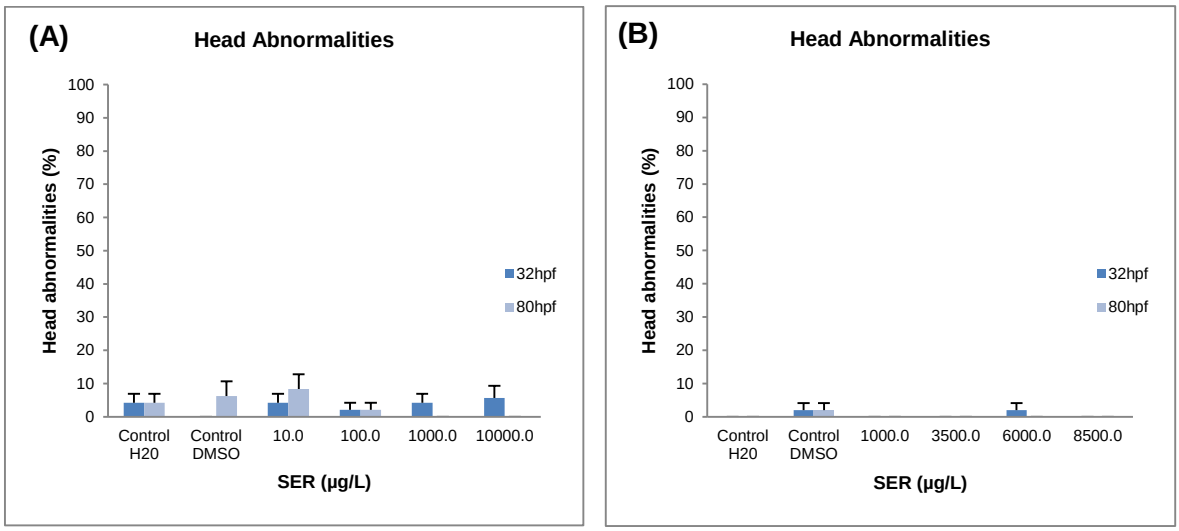


Figure 22. Head abnormalities (**A** and **B**) of *D. rerio* embryos exposed to different concentrations of sertraline at 32hpf and 80hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

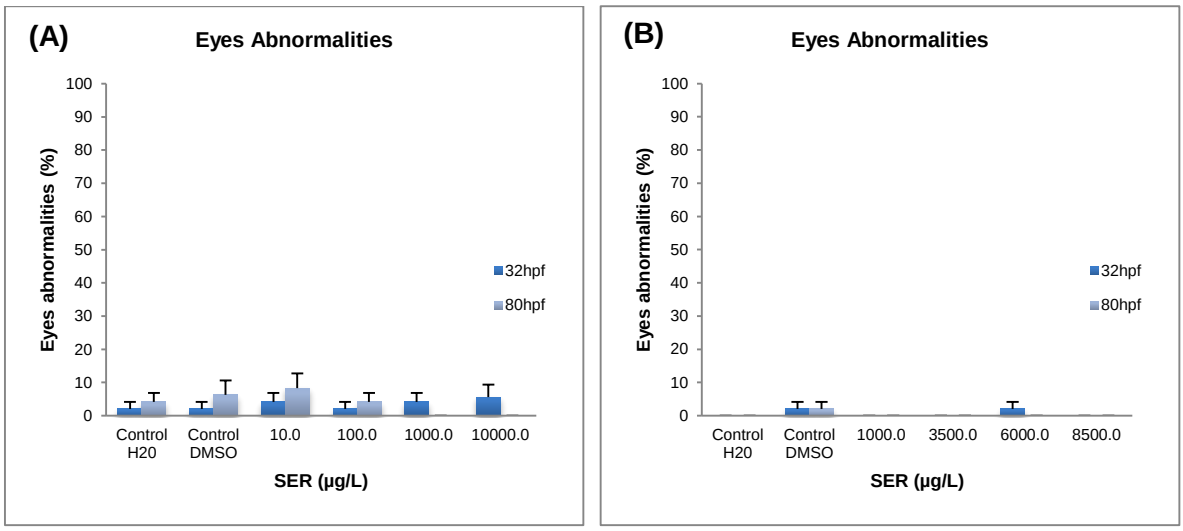


Figure 23. Eyes abnormalities (**A** and **B**) of *D. rerio* embryos exposed to different concentrations of sertraline at 32hpf and 80hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

An exposure to sertraline did not induce significant differences ($p>0.05$) in the percentage of *D. rerio* embryos with head or eyes abnormalities at 80hpf (Figure 22 and 23). All embryos exposed to concentrations equal or higher than 3500.0 µg/L died before 80hpf.

3.2.1.4. Yolk-sac and tail abnormalities

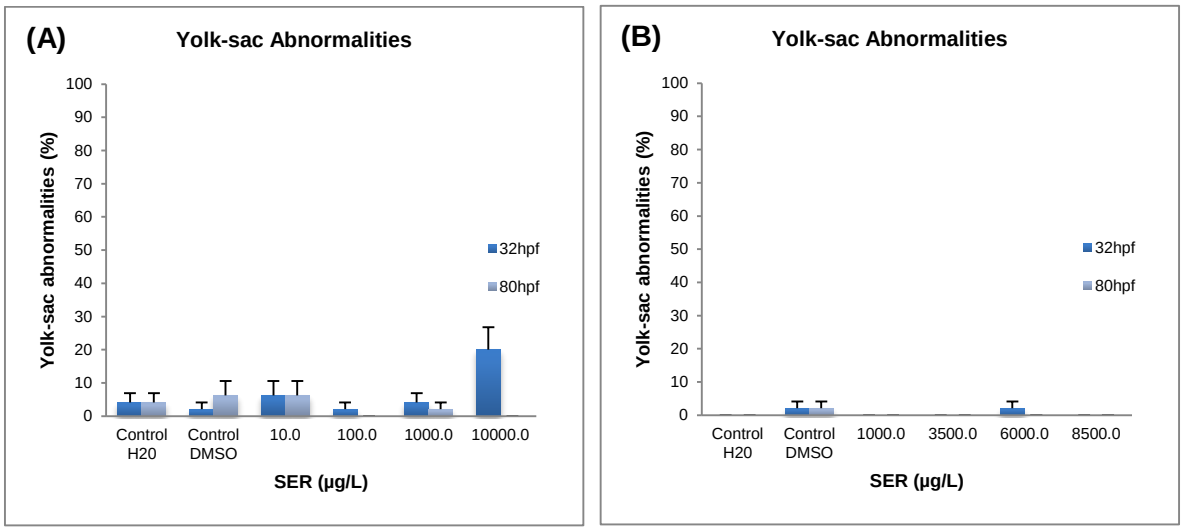


Figure 24. Yolk-sac abnormalities (**A** and **B**) of *D. rerio* embryos exposed to different concentrations of sertraline at 32hpf and 80hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

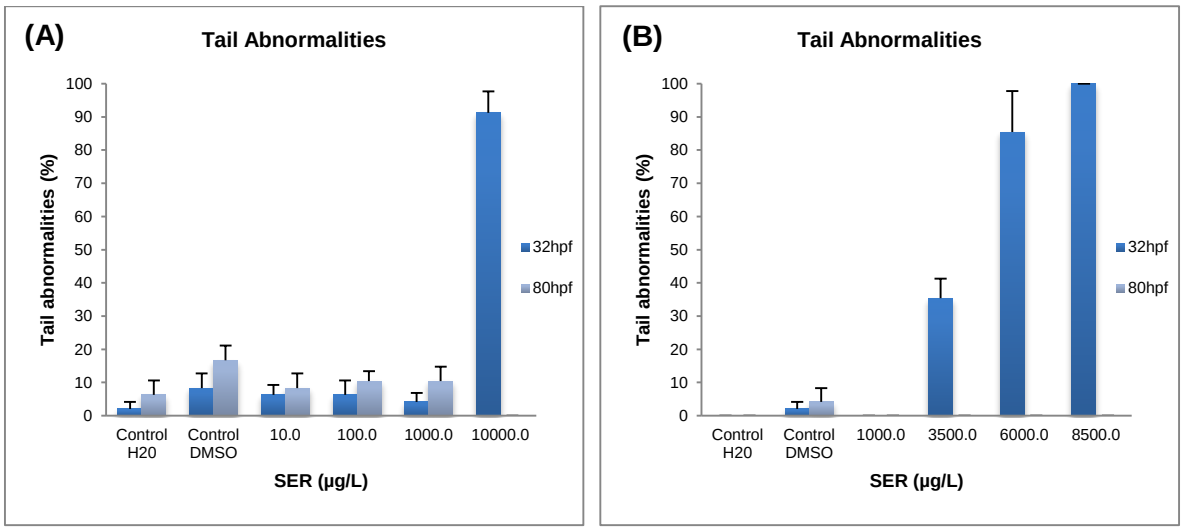


Figure 25. Tail abnormalities (**A** and **B**) of *D. rerio* embryos exposed to different concentrations of sertraline at 32hpf and 80hpf (%). Data are expressed as mean ± SE (n=8). One-way-ANOVA **(A)**. Non-parametric ANOVA Kruskall-Wallis **(B)**.

There was no significant differences ($p>0.05$) in the percentage of embryos with yolk-sac or tail abnormalities at 80hpf among all groups, for both assays performed (Figure 24 and 25).

At 32hpf, there was an increase in the percentage of embryos with tail abnormalities in the 10000.0 µg/L group (Figure 25A). An increase in the percentage of embryos with tail abnormalities was recorded for concentrations equal or higher than 3500.0 µg/L at 32hpf (Figure 25B).

3.2.1.5. Pericardial edema and Cardiac frequency

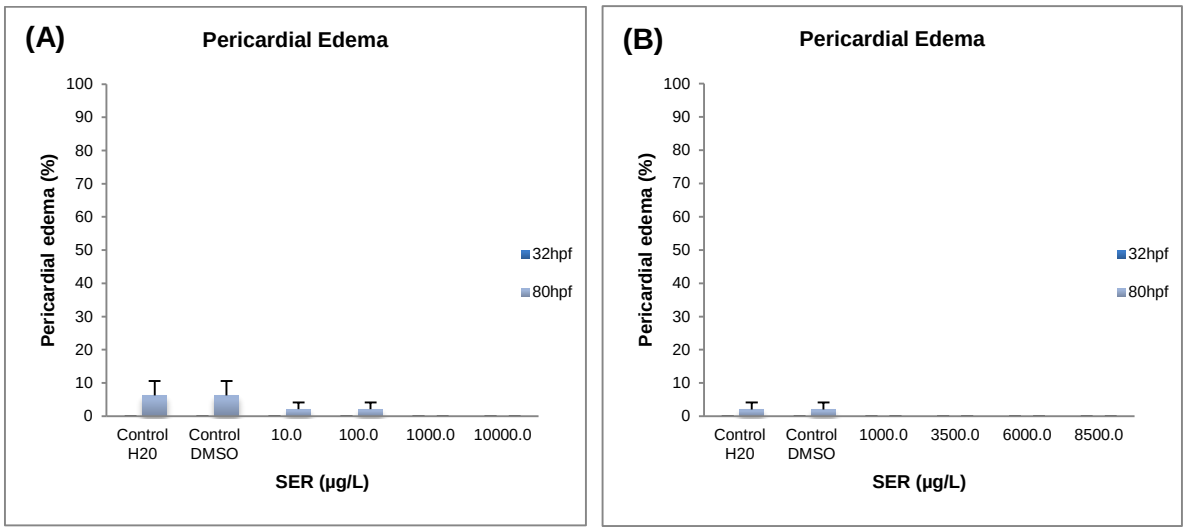


Figure 26. Pericardial edema (**A** and **B**) of *D. rerio* embryos exposed to different concentrations of sertraline at 32hpf and 80hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)**. One-way-ANOVA **(B)**.

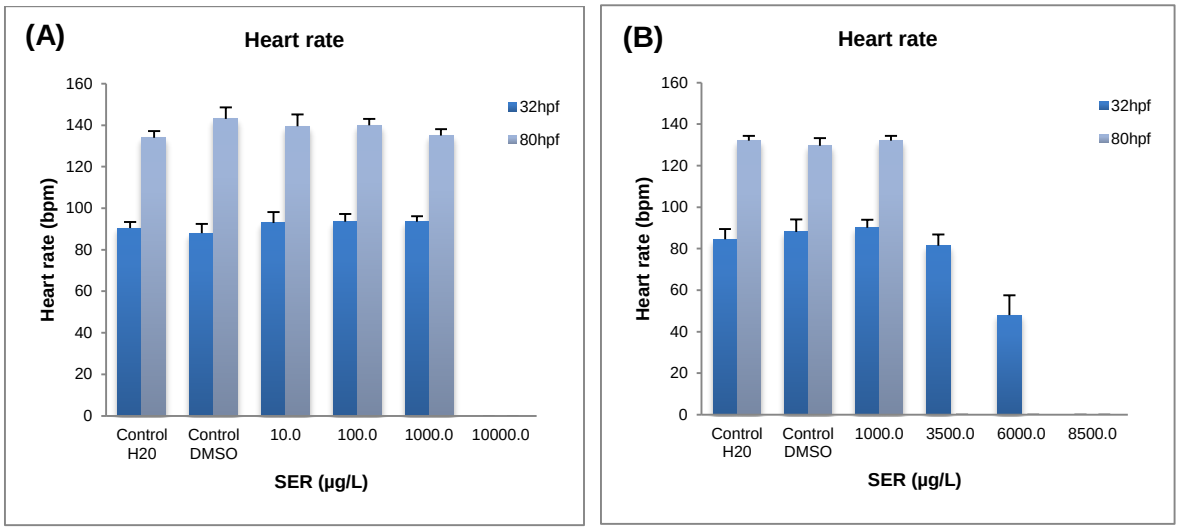


Figure 27. Heart rate (**A** and **B**) of *D. rerio* embryos exposed to different concentrations of sertraline at 8hpf. Data are expressed as mean ± SE (n=8). One-way-ANOVA **(A)** and **(B)**.

An exposure to sertraline did not induce significant differences ($p>0.05$) in the percentage of embryos with pericardial edema at 80hpf, in both assays (Figure 26).

Similarly, differences of embryos heart rate between different groups are not statistically significant at 80hpf ($p>0.05$) (Figure 27).

3.2.1.6. Total abnormalities in embryos

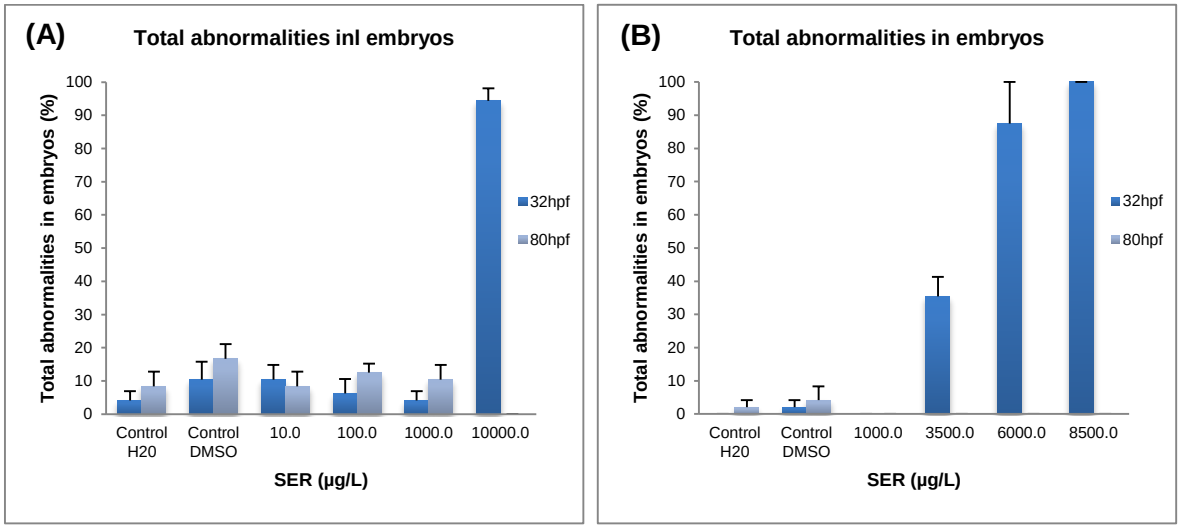


Figure 28. Percentage of total abnormalities (**A** and **B**) in *D. rerio* embryos exposed to different concentrations of sertraline at 32hpf and 80hpf. Data are expressed as mean ± SE (n=8). One-way-ANOVA **(A)** and **(B)**.

An exposure to concentrations equal or higher than 3500.0 µg/L of sertraline induced an increase in the percentage of total abnormalities in embryos at 32hpf (Figure 28). At the end of the assays, the percentage of total abnormalities in embryos was not significant different among controls and all exposure groups ($p>0.05$). All embryos exposed to concentrations equal or higher than 3500.0 µg/L died before 80hpf.

3.2.2. Sea urchin (*Paracentrotus lividus*)

An exposure of *P. lividus* embryos to sertraline lead to a development delay in larvae exposed to 1000.0 and 10000.0 µg/L, so that no larvae reached the four arm stage, and therefore length was not recorded (Figure 29A and 30). On the first assay, larval length in the two other tested concentrations was significantly different from controls ($p<0.05$).

Hence, a follow up experiment was carried out testing lower concentrations of sertraline (Figure 29B). Effects in larval length resulting from an exposure of 4.0 µg/L (445.23 ± 2.68 µm) did not significantly differ from those obtained at low concentrations and from controls (446.05 ± 1.67 µm). Thus, the LOEC of simvastatin exposure in this study was determined as being 10.0 µg/L (434.29 ± 2.69 µm).

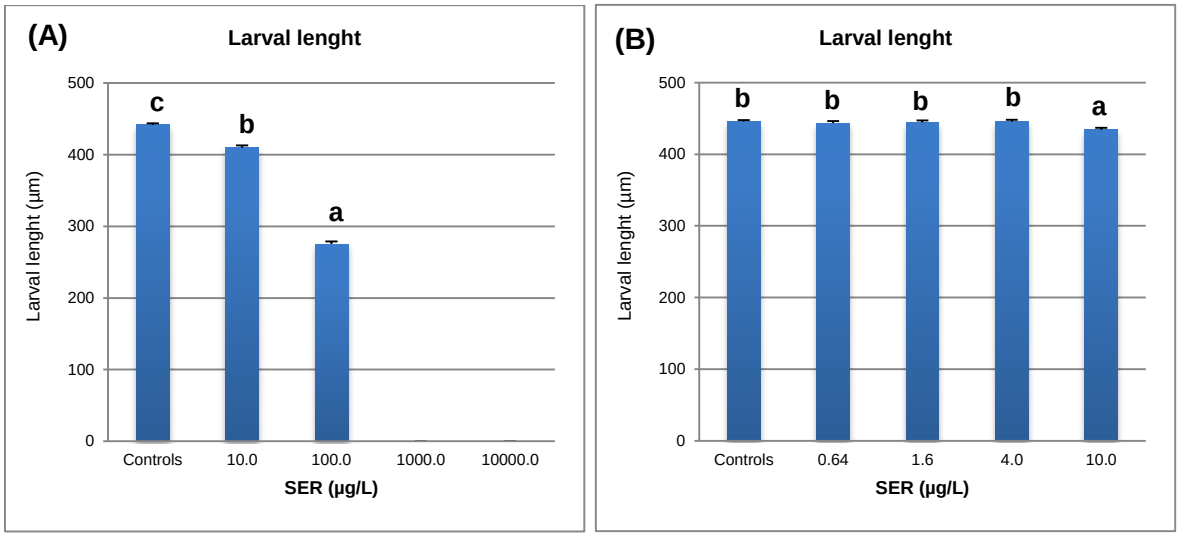


Figure 29. Larval length (µm) of *P. lividus* exposed to different concentrations sertraline for 48h (**A** and **B**). Control and solvent control were grouped. Data are expressed as mean ± SE (n=240 for controls; n=120 sertraline exposed groups). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)**. One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons between groups **(B)**. Bars with a different letter are significantly different from each other.

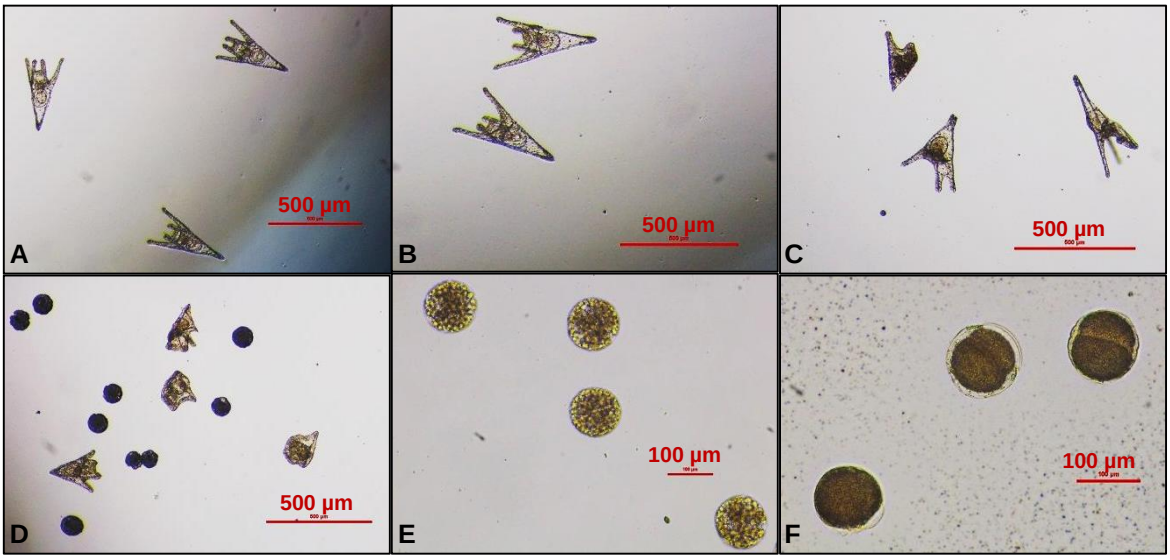


Figure 30. Stage of development of *P. lividus* embryos and larvae after exposure to different concentrations of sertraline for 48h: Control – pluteus larvae **(A)**; Solvent control – pluteus larvae **(B)**; 10.0 µg/L – pluteus and prism larvae **(C)**; 100.0 µg/L – prism larvae**(D)**; 1000.0 µg/L – morula stage **(E)**; 10000.0 µg/L – two cell stage **(F)**.

3.3. Ultra Violet Filter: 4-Methylbenzylidene Camphor

3.3.1. Zebrafish (*Danio rerio)*

3.3.1.1. Cumulative mortality and Hatching rates

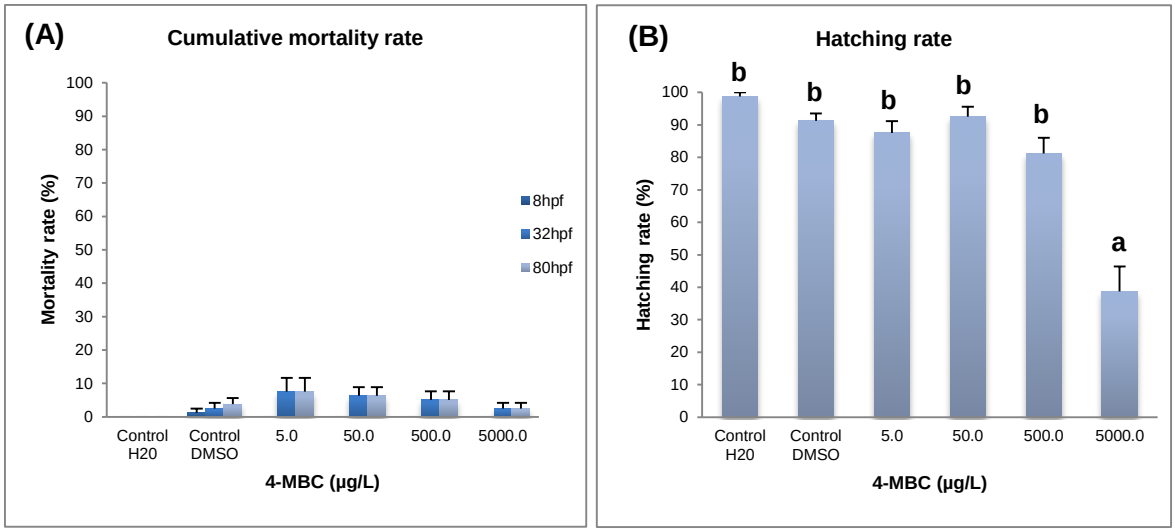


Figure 31. Cumulative mortality **(A)** and hatching **(B)** rates of *D. rerio* embryos exposed to different concentrations of 4-MBC for 80h (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

An exposure of *D. rerio* embryos to UV filter 4-MBC did not induce significant differences ($p>0.05$) in cumulative mortality rate among controls and all exposure groups at 80hpf (Figure 31A).

The hatching rate at the end of the assay ranged from 98.75 ± 1.25 in the control to 38.75 ± 7.66 at the highest tested concentration (Figure 31B). The hatching rate of embryos exposed to 5000.0 µg/L was significant different ($p<0.05$) compared to controls and to the other exposure groups at 80hpf.

3.3.1.2. Abnormal cell growth and 75% epiboly stage of embryos (8hpf)

There was no differences in the percentage of abnormal cell growth and 75% of epiboly stage of embryos exposed to 4-MBC at 8hpf ($p>0.05$) (Figure 32).

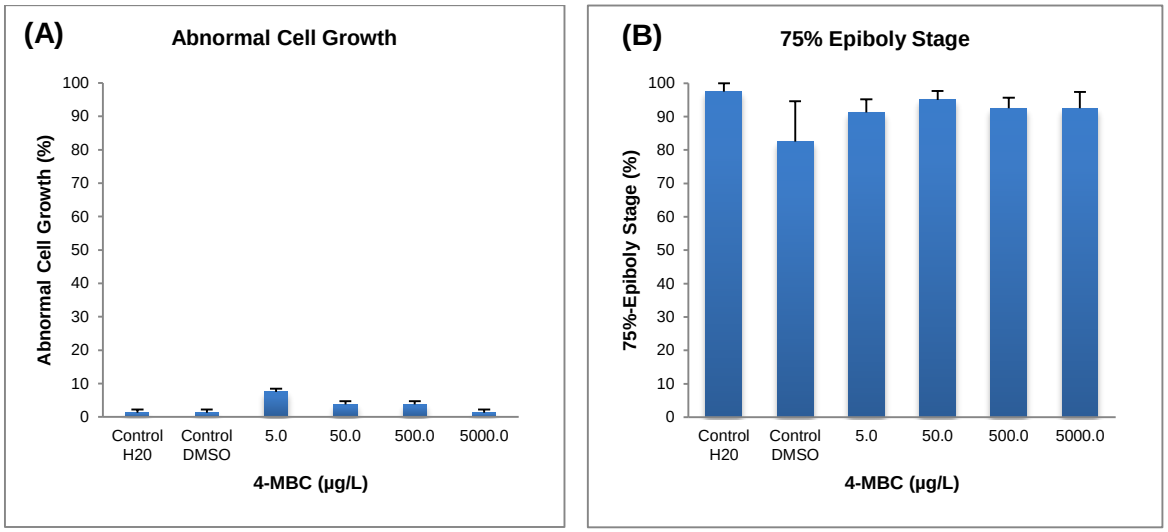


Figure 32. Abnormal cell growth **(A)** and 75% of epiboly stage **(B)** of *D. rerio* embryos exposed to different concentrations of 4-MBC at 8hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)**. One-way-ANOVA **(B)**.

3.3.1.3. Head and eyes abnormalities

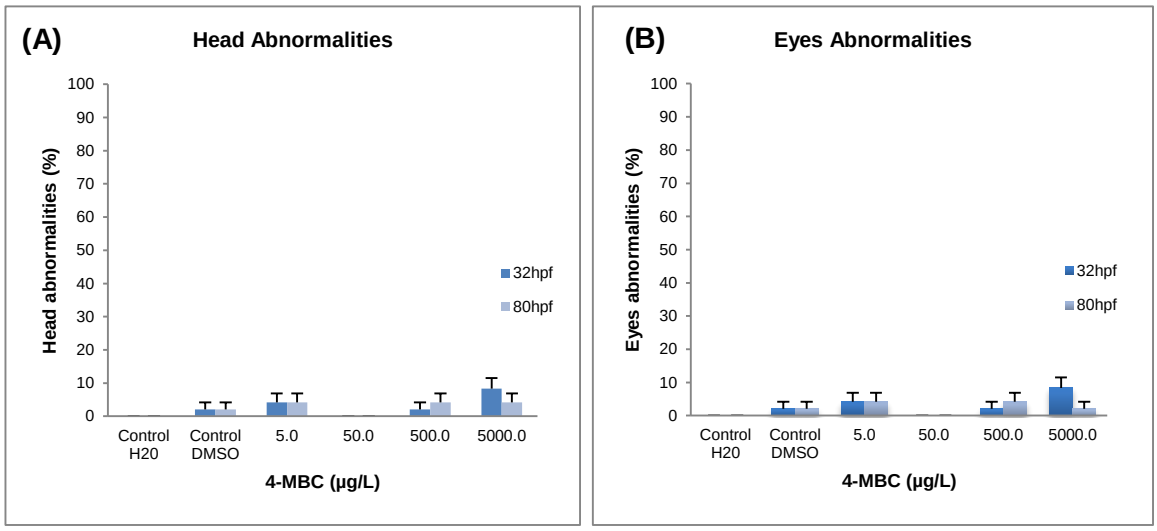


Figure 33. Head **(A)** and eyes **(B)** abnormalities of *D. rerio* embryos exposed to different concentrations of 4-MBC at 32hpf and 80hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

There were no significant differences ($p>0.05$) in the percentage of embryos with head or eyes abnormalities at 80hpf among groups (Figure 33).

3.3.1.4. Yolk-sac and tail abnormalities

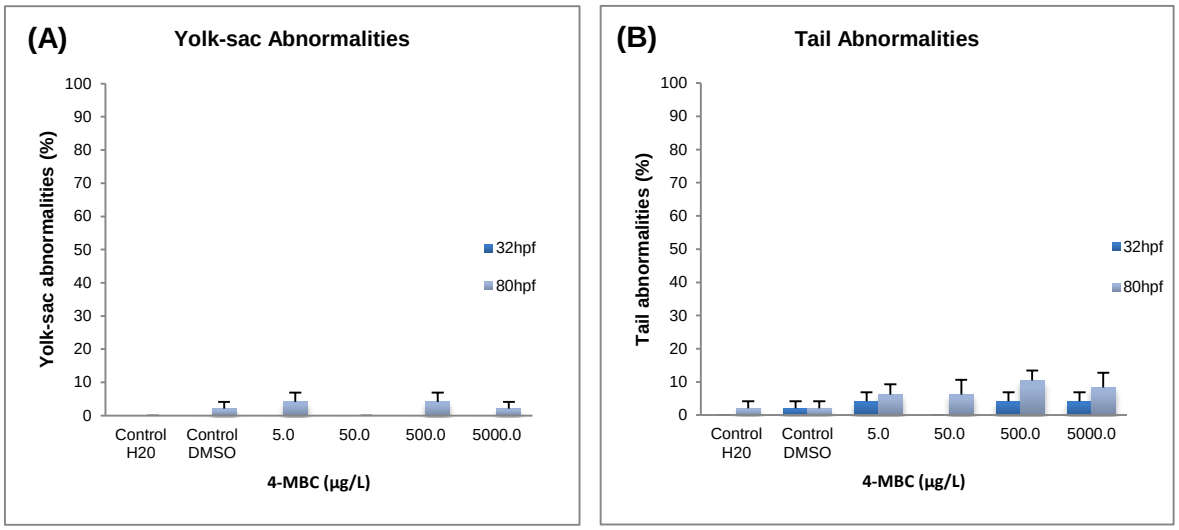


Figure 34. Yolk-sac **(A)** and tail **(B)** abnormalities of *D. rerio* embryos exposed to different concentrations of 4-MBC at 32hpf and 80hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

The effects in the percentage of embryos with yolk-sac or tail abnormalities after exposure to 4-MBC were not significantly different ($p>0.05$) among exposure groups and controls at 80hpf (Figure 34).

3.3.1.5. Pericardial edema and Heart rate

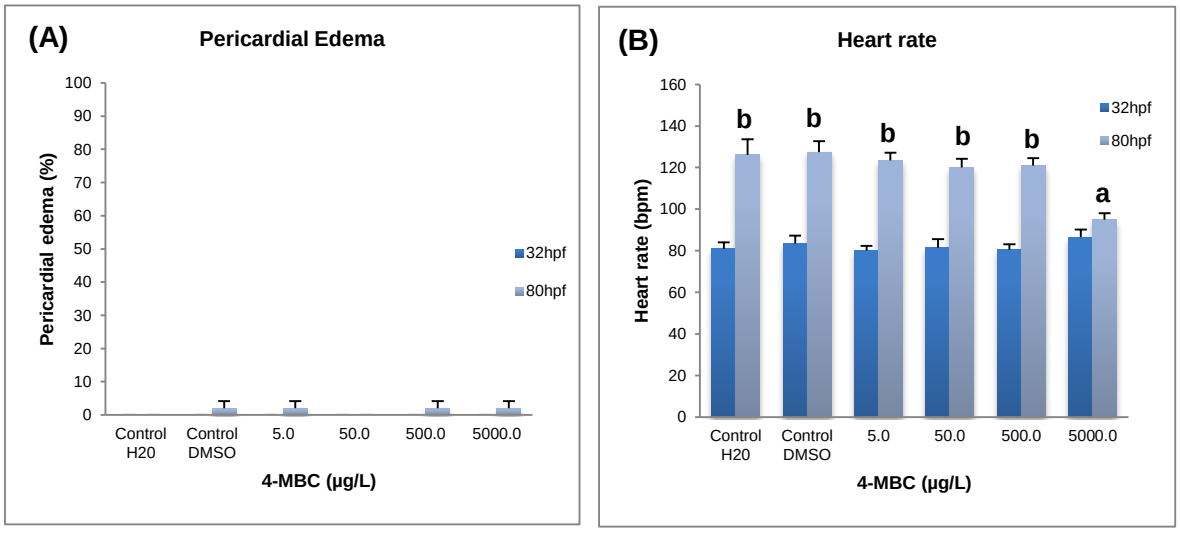


Figure 35. Pericardial edema (%) **(A)** and heart rate (bpm) **(B)** of *D. rerio* embryos exposed to different concentrations of 4-MBC at 32hpf and 80hpf. Data are expressed as mean ± SE (n=8). One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons among groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

At the end of the assay, 4-MBC exposure of *D. rerio* embryos did not results in significant differences ($p>0.05$) in the percentage of embryos with pericardial edema among exposure groups and controls (Figure 35A).

At 32hpf embryo heart rate ranged from 86.50 ± 3.70 in the 5000.0 µg/L group to 80.0 ± 2.39 the 5.0 µg/L group (Figure 35B). At the end of the assay, embryo heart rate ranged from 127.50 ± 5.15 in solvent control to 95.0 ± 3.0 in the 5000.0 µg/L group. An exposure to 4-MBC at the highest tested concentration resulted in a significant decrease ($p<0.05$) of embryo heart rate, at 80hpf.

3.3.1.6. Total abnormalities in embryos

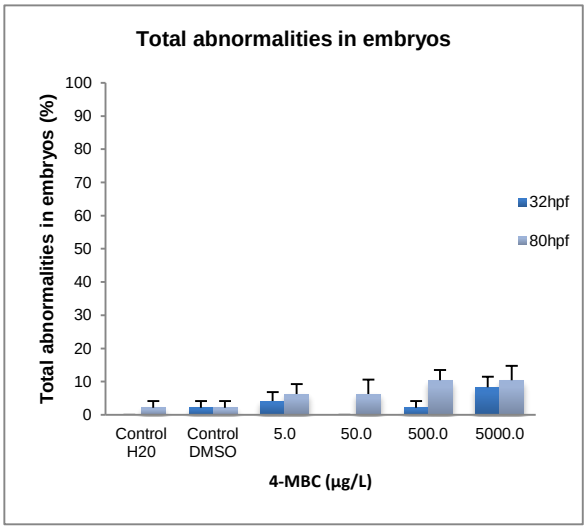


Figure 36. Percentage of total abnormalities in *D. rerio* embryos exposed to different concentrations of 4-MBC at 32hpf and 80 hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis.

The percentage of embryos of controls and exposure groups with one or more abnormalities was similar and no significant differences ($p>0.05$) were reported at 80hpf (Figure 36).

3.3.1.7. Muscular involuntary contractions

At the end of the assay, an exposure to 500.0 and 5000.0 µg/L resulted in a percentage of embryos with muscular involuntary contractions of 35.42 ± 11.55 and 85.42 ± 3.78, respectively (Figure 37). A significant increase ($p<0.05$) in muscular involuntary contractions was reported in embryos exposed to the highest tested concentration of 4-MBC. No effects were recorded in the controls and in the two low 4-MBC concentrations treatments.

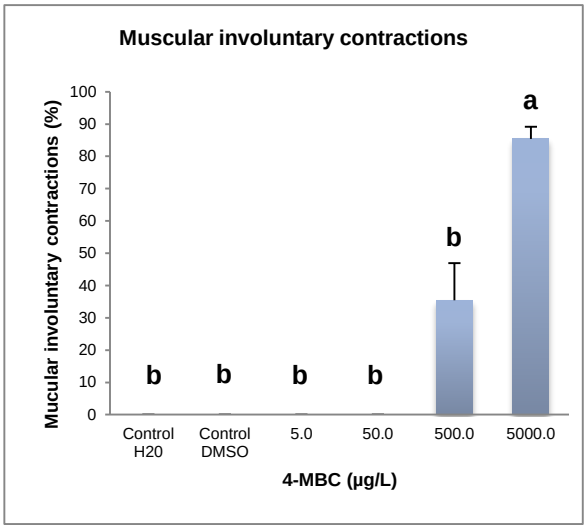


Figure 37. Muscular involuntary contractions of *D. rerio* embryos exposed to different concentrations of 4-MBC at 80hpf (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups. Bars with a different letter are significantly different from each other.

3.3.2. Sea urchin (*Paracentrotus lividus*)

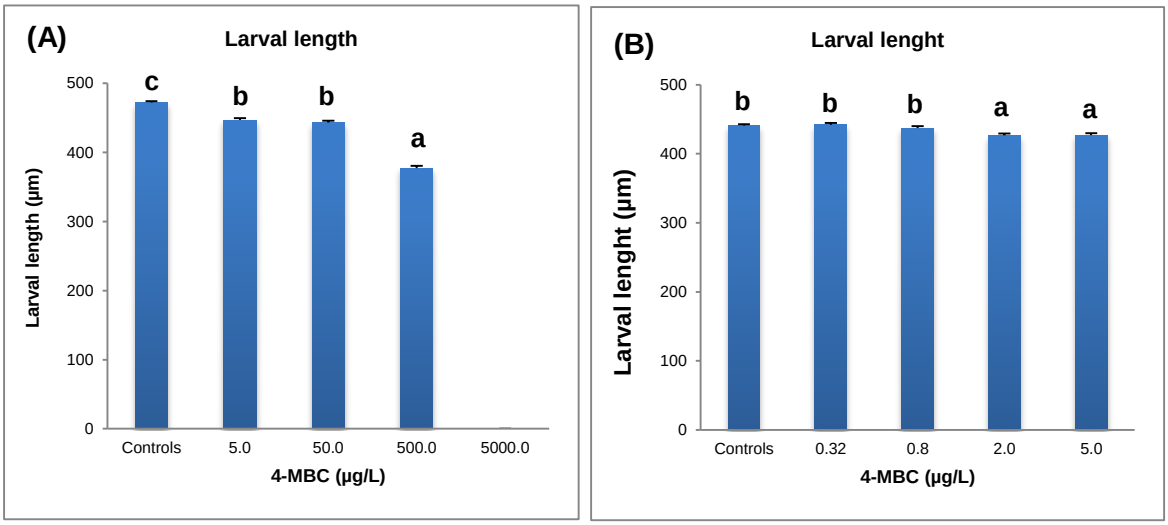


Figure 38. Larval length (µm) of *P. lividus* exposed to different concentrations of 4-MBC for 48h (**A** and **B**). Control and solvent control were grouped. Data are expressed as mean ± SE (n=240 for controls; n=120 4-MBC exposed groups). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)**. One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons between groups **(B)**. Bars with a different letter are significantly different from each other.

An exposure of *P.lividus* embryos to 5000.0 µg/L of 4-MBC revealed lethal for all organisms, and therefore length was not recorded. The other 4-MBC exposure groups

induced a significant ($p<0.05$) decrease of larval length, compared to controls (Figure 38A).

Considering the results of the first assay, a new experiment was carried out testing lower concentrations of 4-MBC (Figure 38B). A significant decrease ($p<0.05$) of larval length was reported for concentrations equal or higher than 2.0 µg/L (426.55 ± 3.09 µm), compared to controls (441.08 ± 1.67 µm). Thus, the LOEC of 4-MBC exposure in this study was determined as being 2.0 µg/L.

3.4. Preservative: Propylparaben

3.4.1. Zebrafish (*Danio rerio)*

3.4.1.1. Cumulative mortality and Hatching rates

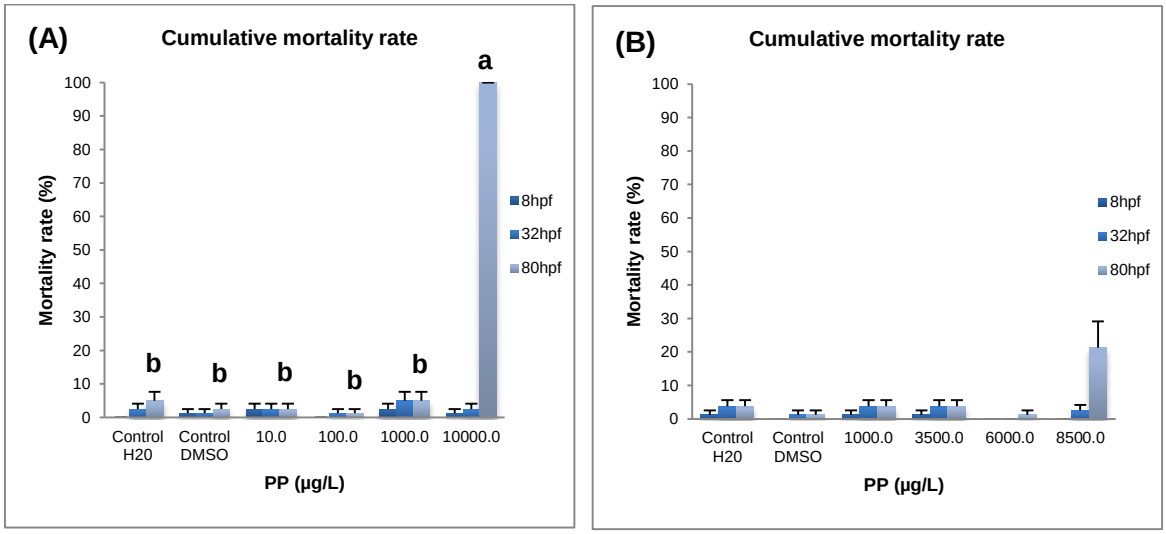


Figure 39. Cumulative mortality rate of *D. rerio* embryos exposed to different concentrations of propylparaben for 80h (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

At 8hpf mortality rate ranged from 0 in the control and 100.0 µg/L group to 2.50 ± 1.64 in the 10.0 and 1000.0 µg/L exposure groups (Figure 39A). On the next observation (32hpf), cumulative mortality rate ranged from 1.25 ± 1.25 in the solvent control and 100.0 µg/L group to 5.0 ± 2.67 in the 1000.0 µg/L group. At the end of this assay, an exposure to 10000.0 µg/L induced lethal effects for all embryos. Thus, cumulative mortality rate was statistically significant ($p<0.05$) for the highest tested concentration, compared to controls and to the other exposure groups at 80hpf.

No significant differences ($p>0.05$) in cumulative mortality rate were recorded for intermediate concentrations tested in a second experiment, between 1000.0 and 10000.0 µg/L of propylparaben (Figure 39B).

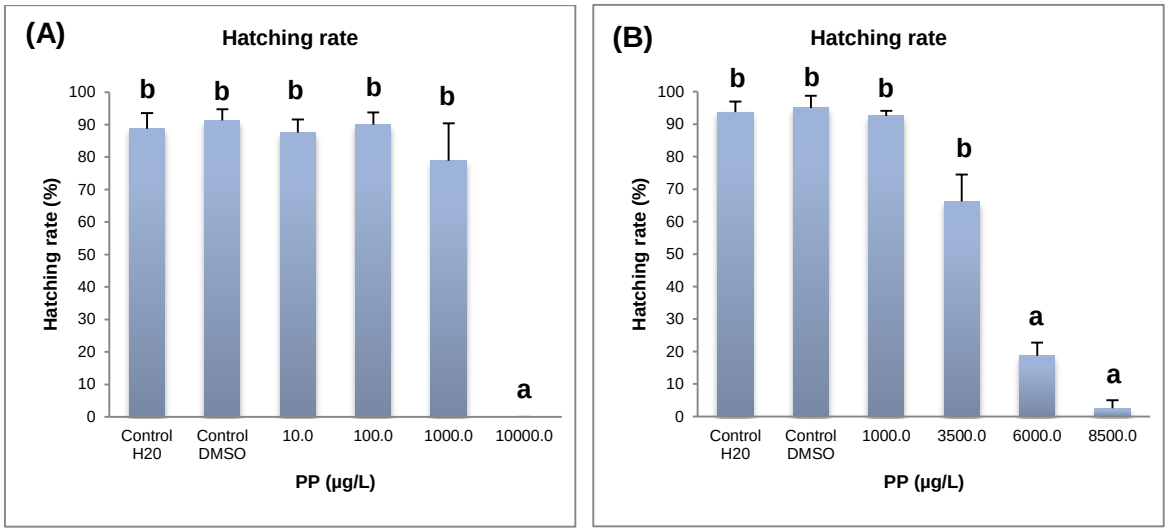


Figure 40. Hatching rate of *D. rerio* embryos exposed to different concentrations of propylparaben for 80h (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

An exposure to propylparaben induced a significant decrease in embryos hatching rate for concentrations equal or higher than 6000.0 µg/L at 80hpf (Figure 40B). All embryos exposed to 10000.0 µg/L of propylparaben died before hatching (Figure 40A).

3.4.1.2. Abnormal cell growth and 75% epiboly stage of embryos (8hpf)

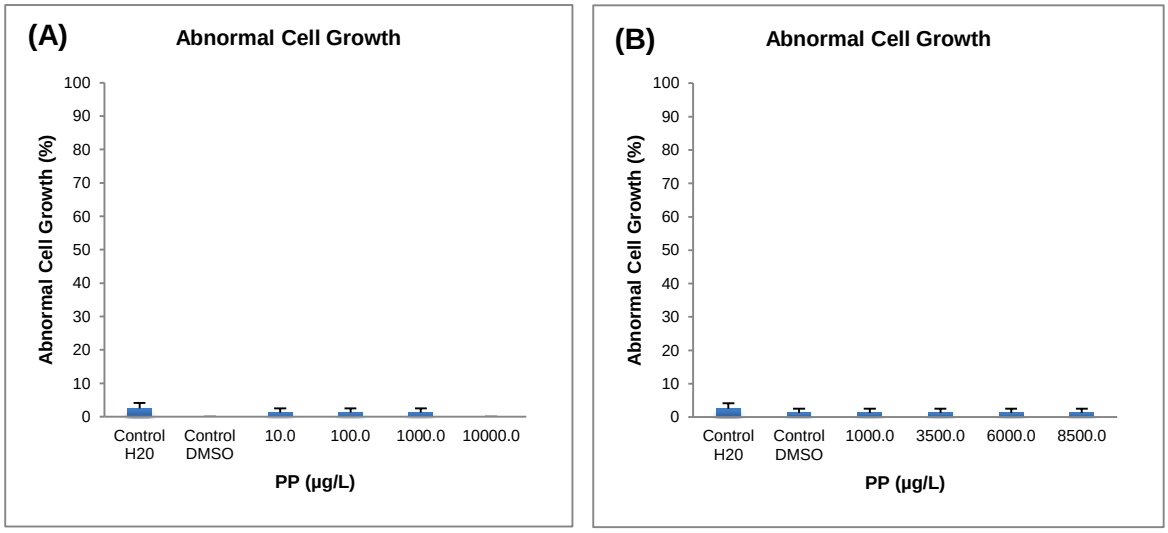


Figure 41. Abnormal cell growth in *D. rerio* embryos exposed to different concentrations of propylparaben at 8hpf (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)**. One-way-ANOVA ($p<0.05$) **(B)**.

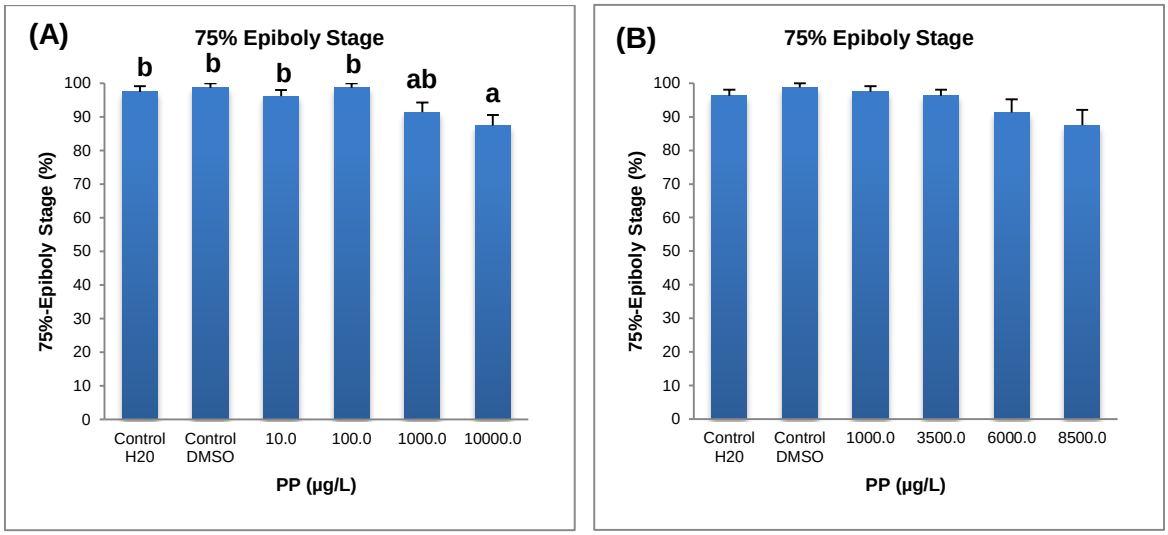


Figure 42. 75% of epiboly stage of *D. rerio* embryos exposed to different concentrations of propylparaben at 8hpf (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons between groups **(A)** and **(B)**.

No significant differences ($p>0.05$) in the percentage of abnormal cell growth were reported at 8hpf among controls and exposure groups, in both assays performed (Figure 41).

An exposure to propylparaben induced a decrease in the percentage of embryos at 75% of epiboly stage at 8hpf in a dose-dependent manner (Figure 42A). The percentage of embryos in 75% of epiboly stage ranged from 98.75 ± 1.25 in the solvent control to 87.50 ± 3.13 in the 10000.0 µg/L exposure group. Embryos exposed to 10000.0 µg/L of propylparaben exhibit a significant delay in their development ($p<0.05$).

A second experiment was carried out testing intermediate concentrations lower than 10000.0 µg/L of propylparaben (Figure 42B). No significant decrease in the percentage of embryos at 75% of epiboly stage was reported at 8hpf.

3.4.1.3. Head and eyes abnormalities

In the initial experiment, no significant differences ($p>0.05$) were recorded in the percentage of head or eyes abnormalities in embryos exposed to propylparaben at 80hpf, among controls and exposure groups (Figure 43A and 44A). All embryos from the highest treatment died before 80hpf.

A second experiment was carried out with a concentration of propylparaben between 1000.0 and 10000.0 µg/L. At 80hpf there was a significant increase ($p<0.05$) in the percentage of embryos with head or eyes abnormalities for concentrations equal and higher than 3500.0 µg/L (Figure 43B and 44B).

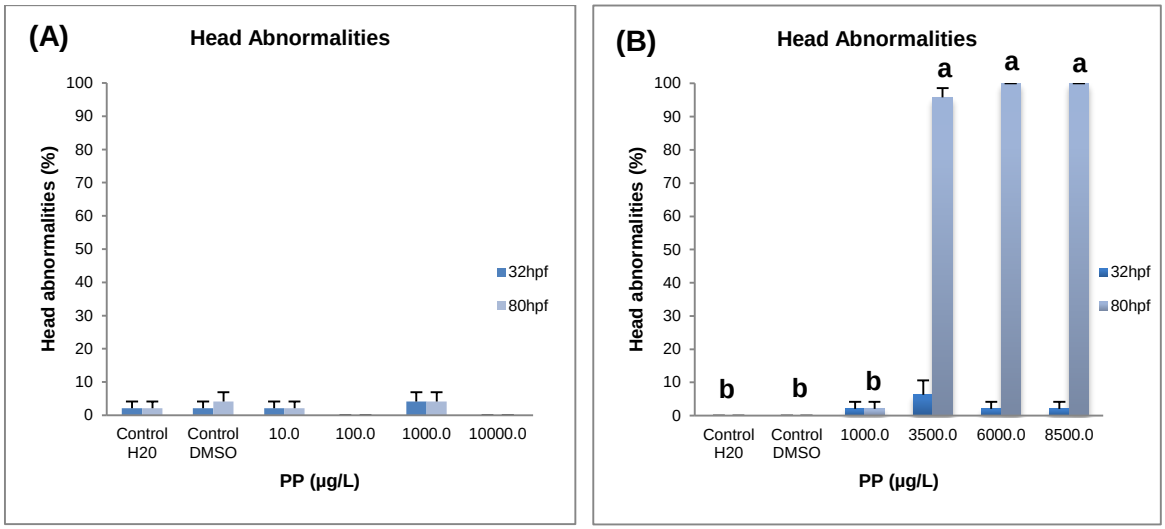


Figure 43. Head abnormalities of *D. rerio* embryos exposed to different concentrations of propylparaben at 32hpf and 80hpf (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

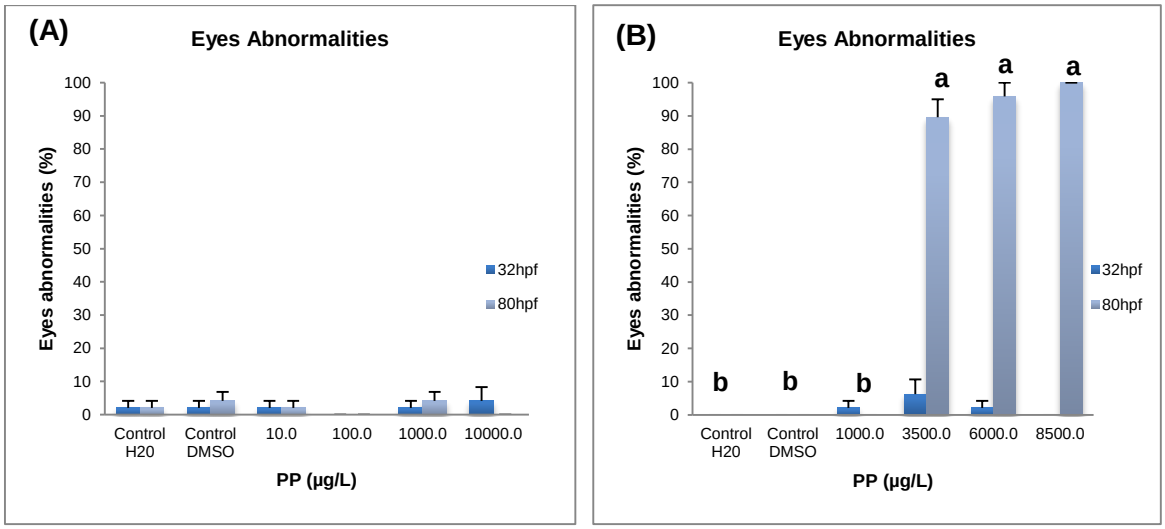


Figure 44. Eyes abnormalities of *D. rerio* embryos exposed to different concentrations of propylparaben at 32hpf and 80hpf (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

3.4.1.4. Yolk-sac and tail abnormalities

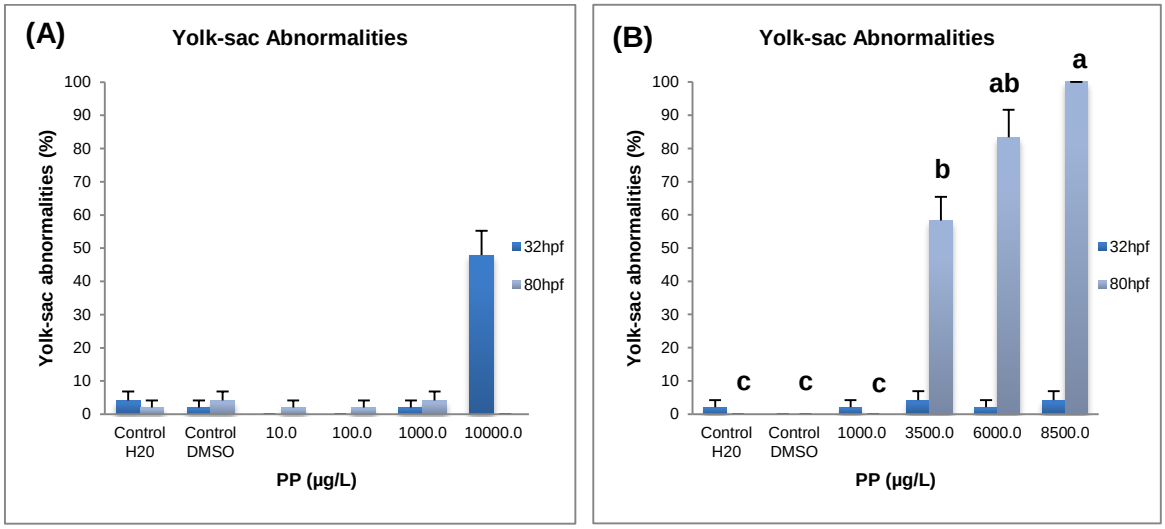


Figure 45. Yolk-sac abnormalities of *D. rerio* embryos exposed to different concentrations of propylparaben at 32hpf and 80hpf (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). One-way-ANOVA ($p<0.05$) **(A)**. Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(B)**. Bars with a different letter are significantly different from each other.

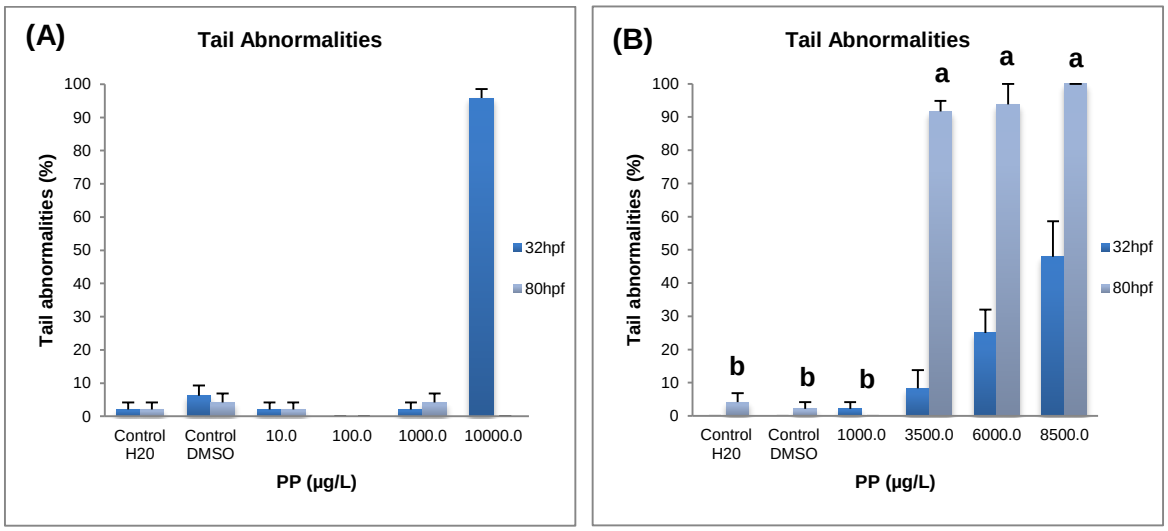


Figure 46. Tail abnormalities of *D. rerio* embryos exposed to different concentrations of propylparaben at 32hpf and 80hpf (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

Similarly to results of head and tail abnormalities, there was no significant differences ($p>0.05$) in percentage of embryos with yolk-sac or tail abnormalities at 80hpf in the initial experiments (Figure 45A and 46A).

However, an exposure to intermediate concentrations of propylparaben induced a significant increase ($p<0.05$) of abnormal tail and yolk-sac in embryos exposed to concentrations equal or higher than 3500.0 µg/L, at 80hpf (Figure 45B and 46B).

3.4.1.5. Pericardial edema and Cardiac frequency

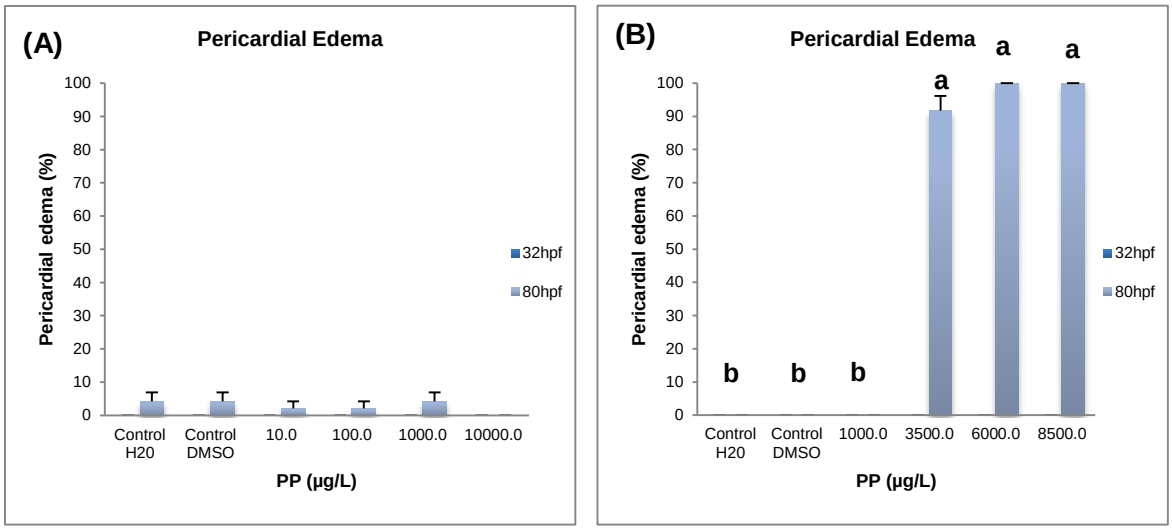


Figure 47. Pericardial edema of *D. rerio* embryos exposed to different concentrations of propylparaben at 32hpf and 80hpf (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). One-way-ANOVA **(A)**. Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(B)**. Bars with a different letter are significantly different from each other.

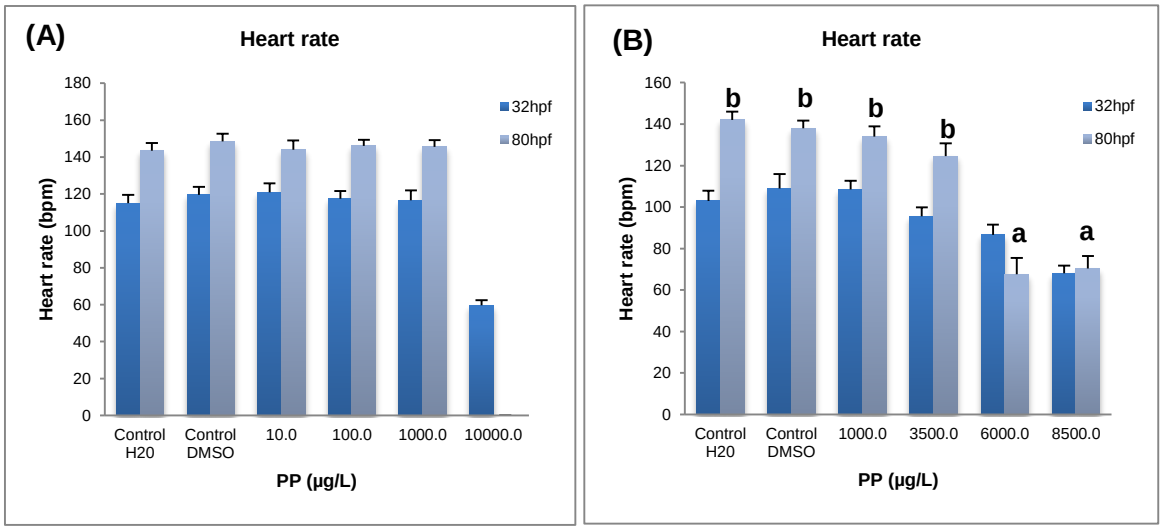


Figure 48. Heart rate of *D. rerio* embryos exposed to different concentrations of propylparaben at 32hpf and 80hpf (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

In the initial assay, the percentage of pericardial edema in embryos was not significantly different ($p>0.05$) among controls and exposure groups at 80hpf (Figure 47A).

Intermediate concentrations tested in the second experiment revealed a significant increase ($p<0.05$) in the percentage of embryos with pericardial edema for concentrations equal or higher than 3500.0 µg/L at the end of the experiment (Figure 47B).

No significant differences ($p>0.05$) were observed in embryos heart rate among controls and exposure groups at 80hpf, in the first experiment (Figure 48A).

In the second experiment heart rate ranged from 109.0 ± 6.83 in the solvent control to 68.0 ± 3.75 in the 8500.0 µg/L group at 32hpf (Figure 48B). At the end of this experiment, heart rate ranged from 142.0 ± 4.07 in the control to 67.5 ± 7.98 in the 6000.0 µg/L group. An exposure to intermediate concentrations induced a significant decrease ($p<0.05$) of embryos heart rate exposed to 6000.0 and 8500.0 µg/L of propylparaben at 80hpf, compared to controls and to other exposure groups.

3.4.1.6. Total abnormalities in embryos

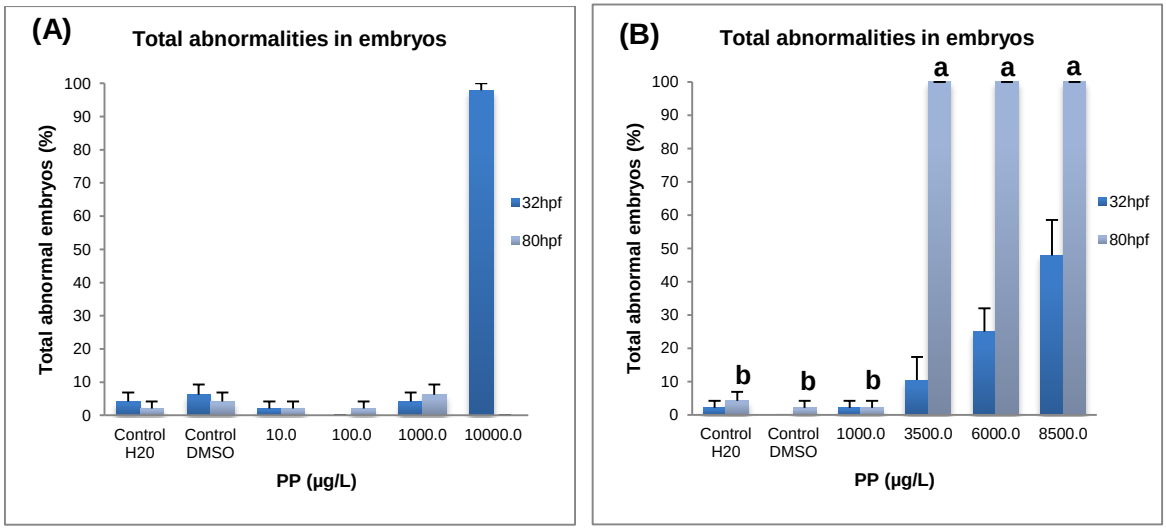


Figure 49. Percentage of total abnormalities in *D. rerio* embryos exposed to different concentrations of propylparaben at 32hpf and 80hpf (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). One-way-ANOVA **(A)**. Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(B)**. Bars with a different letter are significantly different from each other.

At 80hpf of the initial assay no significant differences ($p>0.05$) were observed in the percentage of total abnormalities among controls and exposed groups (Figure 49A).

An exposure of *D. rerio* embryos to propylparaben in a second experiment resulted in a significant increase ($p<0.05$) of abnormalities in embryos at 80hpf for concentrations equal or higher than 3500.0 µg/L, compared to controls and 1000.0 µg/L group (Figure 49B).

3.4.2. Sea urchin (*Paracentrotus lividus*)

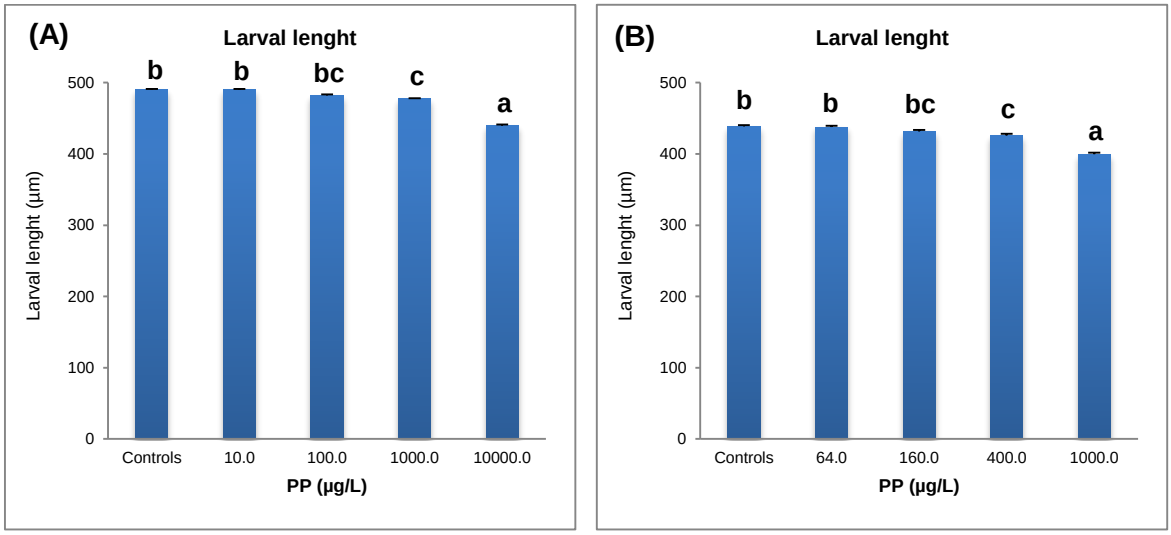


Figure 50. Larval length (µm) of *P. lividus* exposed to different concentrations of propylparaben for 48h (**A** and **B**). Control and solvent control were grouped. Data are expressed as mean ± SEM (n=240 for controls; n=120 propylparaben exposed groups). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

An initial exposure of *P. lividus* embryos to propylparaben induced a significant decrease ($p<0.05$) of larval length for concentrations-exposure of 1000.0 µg/L (477.11 ± 2.79 and 10000.0 µg/L (440.05 ± 2.35 µm), compared to controls (490.23 ± 1.86 µm) (Figure 50A).

Considering the results of the first assay, a second experiment was carried out testing concentrations lower than 1000.0 µg/L (Figure 50B). A significant decrease ($p<0.05$) in larval length was observed at 400.0 µg/L (425.38 ± 2.78 µm), compared to controls (438.72 ± 1.59 µm), corresponding this concentration to the LOEC of propylparaben exposure in this study.

3.5. Bacteriocide and antifungal agents: Triclocarban

3.5.1. Zebrafish (*Danio rerio)*

3.5.1.1. Cumulative mortality and Hatching rates

At 8hpf there was no report of dead embryos in the controls and exposure groups in the first assay (Figure 51A). On the next observation (32hpf), mortality was keept at low levels in all groups. At the end of the assay, embryos exposed to 1000.0 and 10000.0 µg/L were all dead, a significant ($p<0.05$) effect compared to controls and the other exposure groups.

Considering the results of the first assay, a second experiment was carried out testing intermediate concentrations lower than 1000.0 µg/L of triclocarban (Figure 51B). At 8hpf mortality rate ranged from 1.25 ± 1.25 in the controls and 850.0 µg/L group to 6.25 ± 2.63 in the 100.0 µg/L group (Figure A). On the next observation (32hpf), cumulative mortality rate ranged from 1.25 ± 1.25 in the 850.0 µg/L group to 8.75 ± 2.95 in the 100.0 µg/L group. At the end of the assay, cumulative mortality rate ranged from 2.50 ± 1.64 in the solvent control to 100 in the 850.0 µg/L group. There was a significant increase ($p<0.05$) of cumulative mortality rate for all new test-concentrations in a dose-dependent manner at 80hpf.

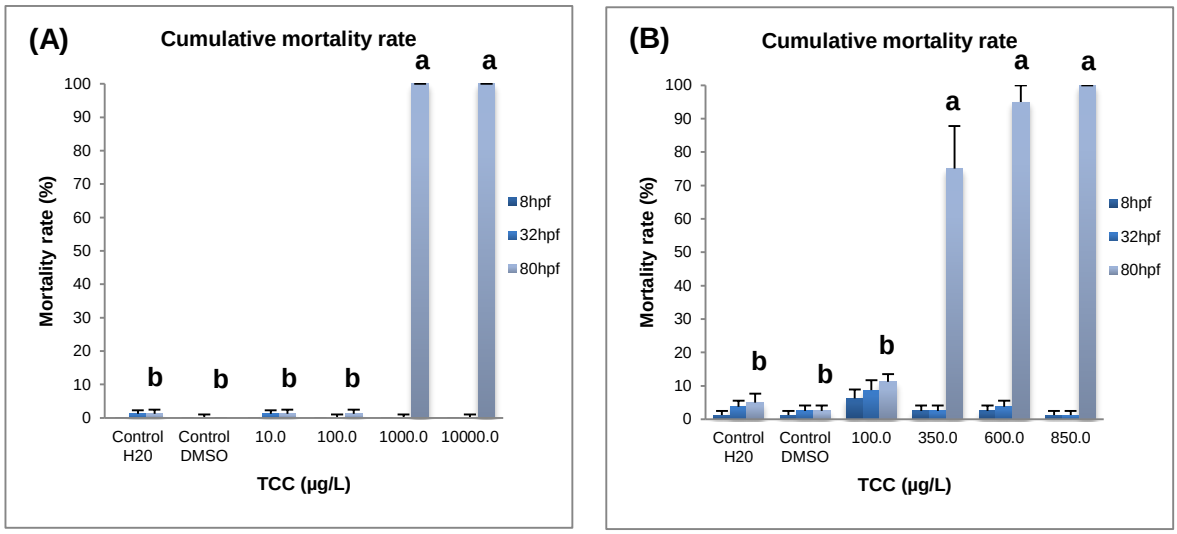


Figure 51. Cumulative mortality rate of *D. rerio* embryos exposed to different concentrations of triclocarban for 80h (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

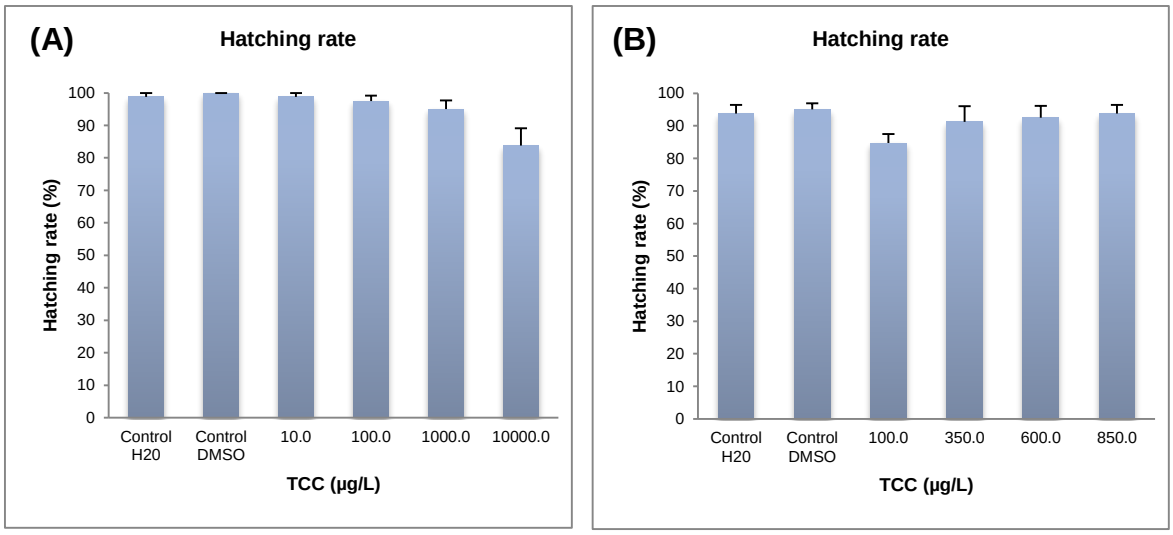


Figure 52. Hatching rate of *D. rerio* embryos exposed to different concentrations of triclocarban for 80h (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A).** One-way-ANOVA **(B)**.

No significant differences (p>0.05) in hatching rate of embryos exposed to triclocarban were reported, in both experiments performed (Figure 52).

3.5.1.2. Abnormal cell growth and 75% epiboly stage of embryos (8hpf)

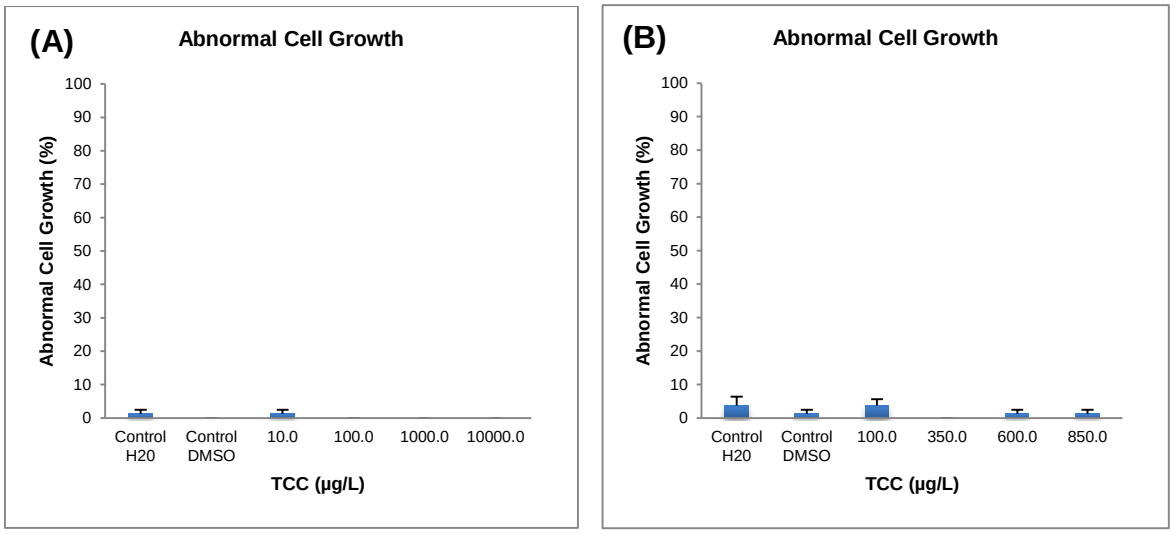


Figure 53. Abnormal cell growth in *D. rerio* embryos exposed to different concentrations of triclocarban at 8hpf (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)**. One-way-ANOVA **(B)**.

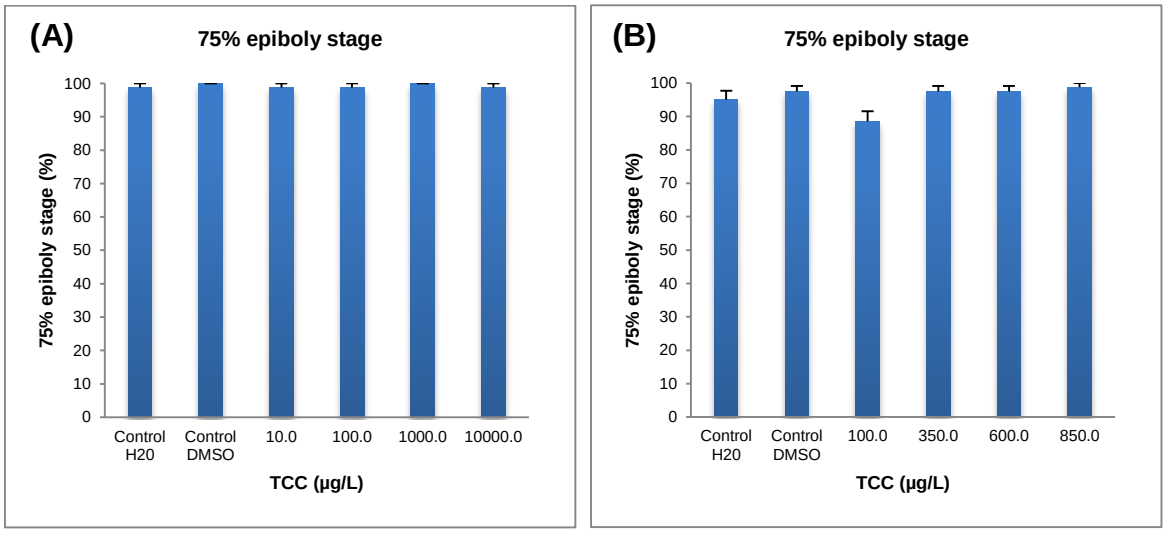


Figure 54. 75% of epiboly stage of *D. rerio* embryos exposed to different concentrations of triclocarban at 8hpf (**A** and **B**) (%). Data are expressed as mean ± SE (n=8). One-way-**(A)**. Non-parametric ANOVA Kruskall-Wallis **(B)**.

An exposure to triclocarban did not induced significant differences in the percentage of embryos with abnormal cell growth at 8hpf, in both performed assays (Figure 53). Similarly, no significant differences were reported in the percentage of embryos in 75% of epiboly stage at 8hpf among controls and exposure groups (Figure 54).

3.5.1.3. Head and eyes abnormalities

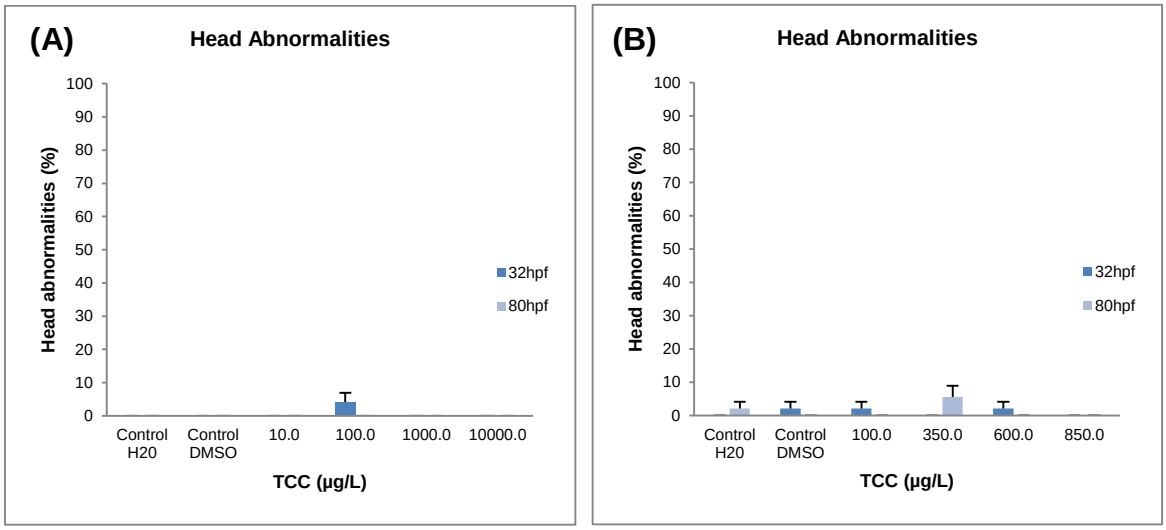


Figure 55. Head abnormalities of *D. rerio* embryos exposed to different concentrations of triclocarban at 32hpf and 80hpf (**A** and **B**) (%). **(A)** Data are expressed as mean ± SE (n=8). **(B)** Data are expressed as mean ± SE (n=8 for control, solvent control and 100.0 µg/L; n=3 for 350.0 µg/L; n=1 for 600.0 µg/L triclocarban exposed groups)**.** Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

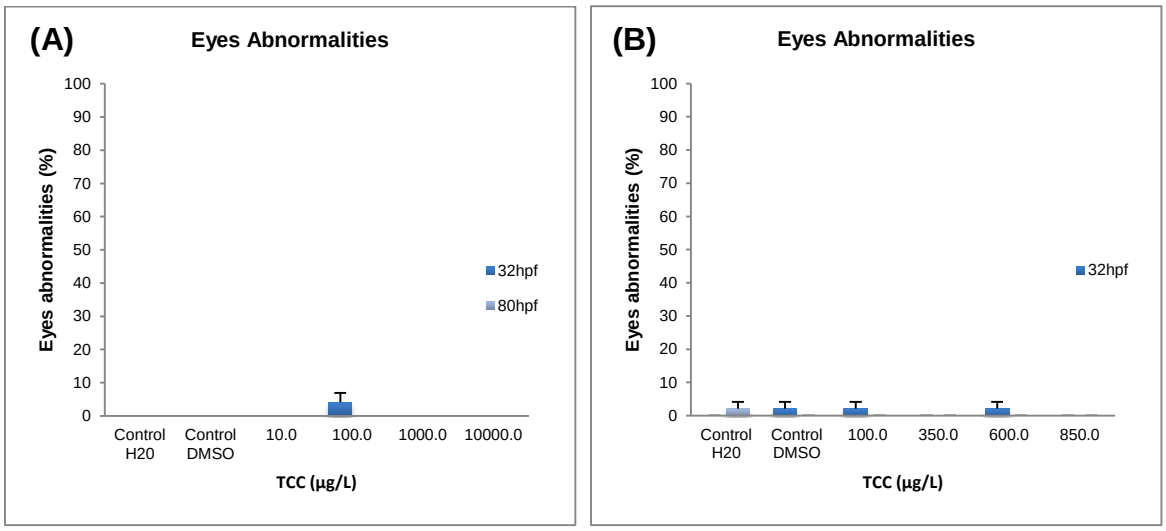


Figure 56. Eyes abnormalities of *D. rerio* embryos exposed to different concentrations of triclocarban at 32hpf and 80hpf (**A** and **B**) (%). **(A)** Data are expressed as mean ± SE (n=8). **(B)** Data are expressed as mean ± SE (n=8 for control, solvent control and 100.0 µg/L; n=3 for 350.0 µg/L; n=1 for 600.0 µg/L triclocarban exposed groups). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

The percentage of embryos with head or eyes abnormalities was not statistically different ($p>0.05$) among controls and exposure groups at 80hpf, in both experiments performed (Figure 55 and 56). All embryos from the two highest test-concentrations of the first assay died before 80hpf.

3.5.1.4. Yolk-sac and tail abnormalities

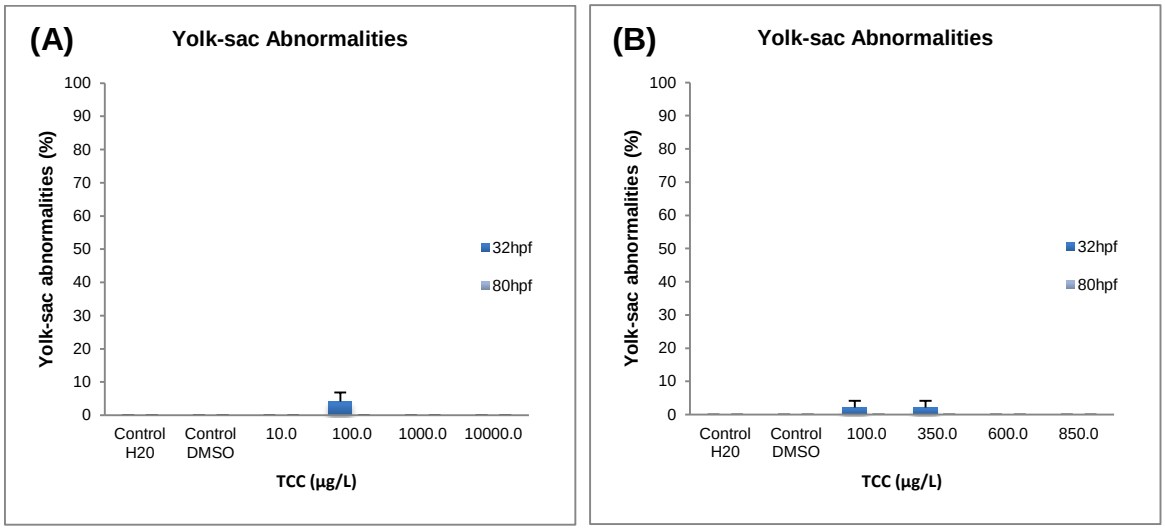


Figure 57. Yolk-sac abnormalities of *D. rerio* embryos exposed to different concentrations of triclocarban at 32hpf and 80hpf (**A** and **B**) (%). **(A)** Data are expressed as mean ± SE (n=8). **(B)** Data are expressed as mean ± SE (n=8 for control, solvent control and 100.0 µg/L; n=3 for 350.0 µg/L; n=1 for 600.0 µg/L triclocarban exposed groups). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

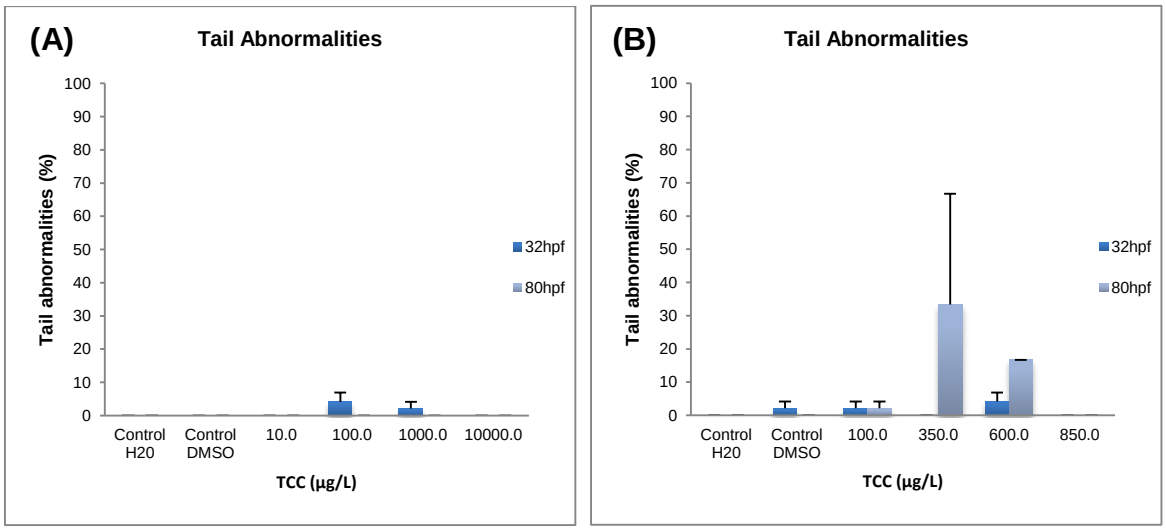


Figure 58. Tail abnormalities of *D. rerio* embryos exposed to different concentrations of triclocarban at 32hpf and 80hpf (**A** and **B**) (%). **(A)** Data are expressed as mean ± SE (n=8). **(B)** Data are expressed as mean ± SE (n=8 for control, solvent control and 100.0 µg/L; n=3 for 350.0 µg/L; n=1 for 600.0 µg/L triclocarban exposed groups). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

Embryos exposed to triclocarban did not showed significant differences ($p>0.05$) in the percentage of yolk-sac or tail abnormalities at 80hpf, in both assays performed (Figure 57 and 58).

3.5.1.5. Pericardial edema and Cardiac frequency

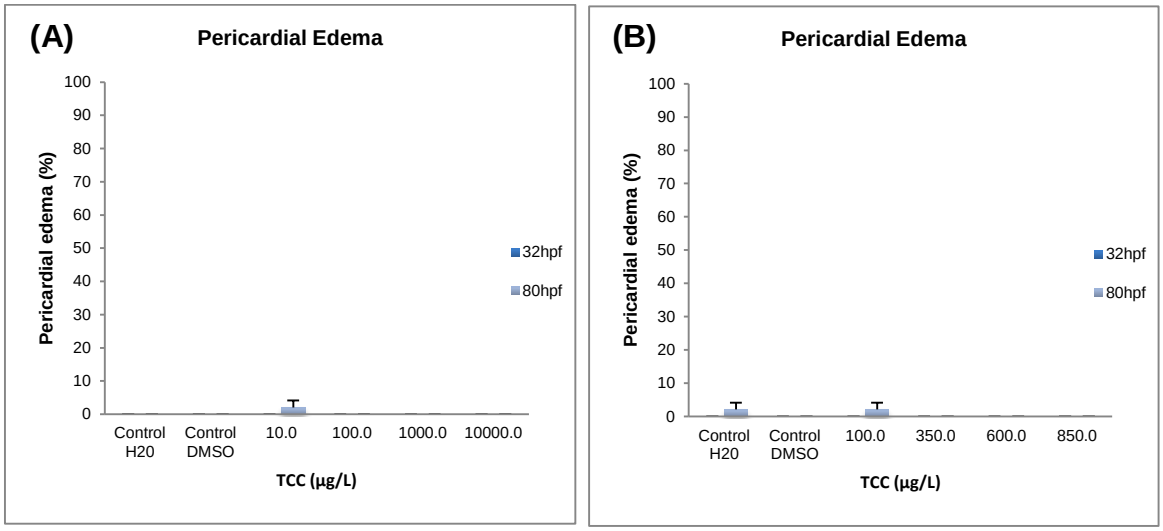


Figure 59. Pericardial edema of *D. rerio* embryos exposed to different concentrations of triclocarban at 32hpf and 80hpf (**A** and **B**) (%). **(A)** Data are expressed as mean ± SE (n=8). Non-parametric ANOVA Kruskall-Wallis **(B)** Data are expressed as mean ± SE (n=8 for control, solvent control and 100.0 µg/L; n=3 for 350.0 µg/L; n=1 for 600.0 µg/L triclocarban exposed groups). One-way-ANOVA.

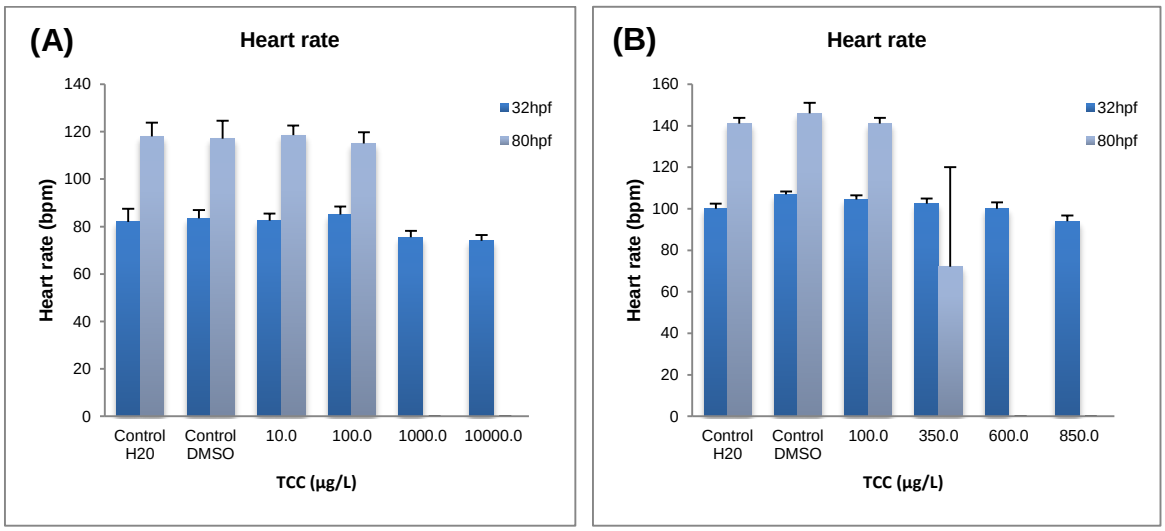


Figure 60. Heart rate of *D. rerio* embryos exposed to different concentrations of triclocarban at 32hpf and 80hpf (**A** and **B**) (%). **(A)** Data are expressed as mean ± SE (n=8). One-way-ANOVA. **(B)** Data are expressed as mean ± SE (n=8 for control, solvent control and 100.0 µg/L; n=2 for 350.0 µg/L triclocarban exposed groups). Non-parametric ANOVA Kruskall-Wallis.

At the end of assays, few embryos developed pericardial edema as a result of triclocarban exposure and no significant differences ($p>0.05$) were reported among controls and exposure groups (Figure 59).

In the initial assay, no significant differences (p>0.05) in embryo heart rate were reported at 80hpf (Figure 60A).

Considering the results obtained in the first assay, a second experiment was carried out with intermediate concentrations lower than 1000.0 µg/L of triclocarban (Figure 60B). There was a decrease in embryos heart rate exposed to 350.0 µg/L, although differences did not reach significance (p>0.05) because of a high individual variability.

3.5.1.6. Total abnormalities in embryos

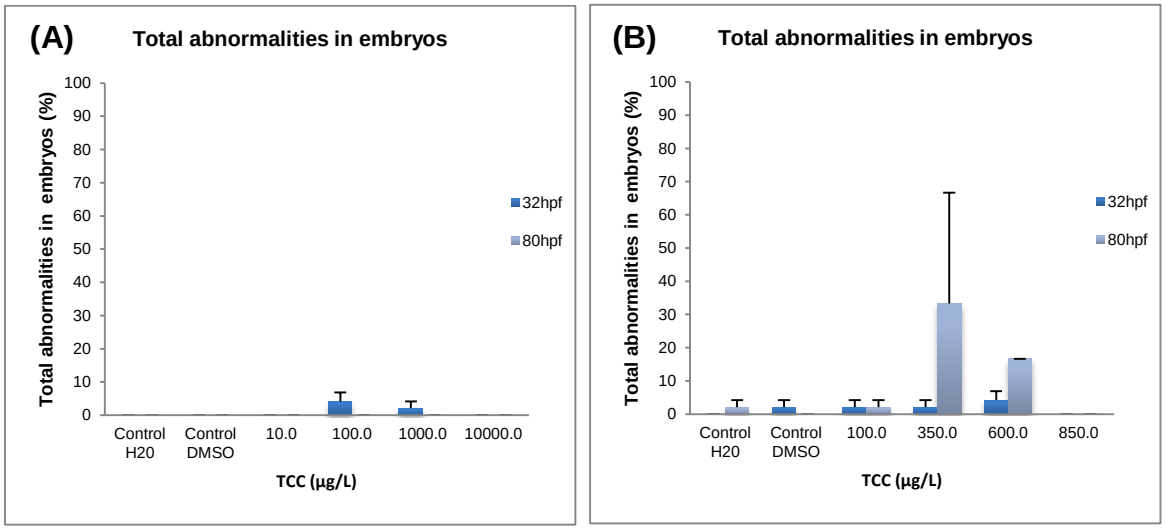


Figure 61. Percentage of total abnormalities in *D. rerio* embryos exposed to different concentrations of triclocarban at 32hpf and 80hpf (**A** and **B**) (%). **(A)** Data are expressed as mean ± SE (n=8). **(B)** Data are expressed as mean ± SE (n=8 for control, solvent control and 100.0 µg/L; n=2 for 350.0 µg/L; n=1 for 600.0 µg/L triclocarban exposed groups). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

Few abnormal embryos were reported in the initial assay and there was no significant differences (p>0.05) among controls and exposure groups at 80hpf (Figure 61A).

An exposure to intermediate test-concentrations lower than 1000.0 µg/L of triclocarban resulted in an increase in the percentage of total abnormalities in embryos in the 350.0 and 600.0 µg/L groups (Figure 61B). However, no significant differences (p>0.05) were reported among controls and exposure groups.

3.5.2. Sea urchin (*Paracentrotus lividus*)

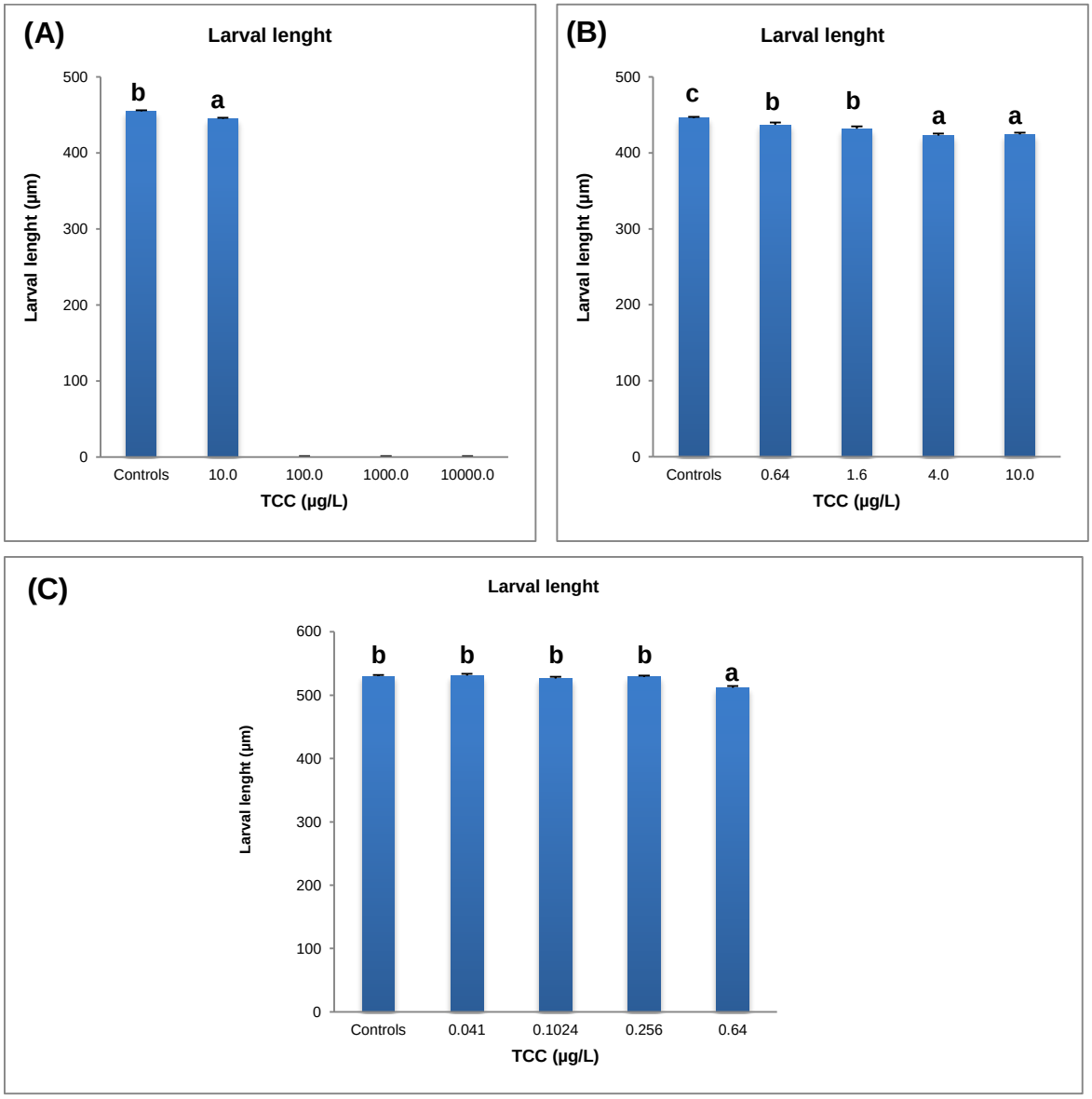


Figure 62. Larval length (µm) of *P. lividus* exposed to different concentrations of triclocarban for 48h (**A**, **B** and **C**). Control and solvent control were grouped. Data are expressed as mean ± SE (n=240 for controls; n=120 triclocarban exposed groups). One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other. Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(C)**.

An exposure of *P. lividus* embryos to triclocarban induced significant effects in larval length at 48h-exposure. An exposure to 100.0, 1000.0 and 10000.0 µg/L of triclocarban resulted in an embryo development delay so that none reached the four arm stage, and therefore length was not recorded (Figure 62A and 63). Embryos exposed to 10.0 µg/L (445.3 ± 2.39 µm) showed a significant decrease ($p<0.05$) of larval length compared to controls (455.01 ± 1.76 µm) (Figure 62A).

Considering the results of the first assay, a new set of concentrations was tested in two another experiments, at concentrations below 10.0 µg/L of triclocarban (Figure 62B and 62C). An exposure to 0.64 µg/L (511.41 ± 2.93 µm) induced a significant decrease ($p<0.05$) of larval length compared to controls (529.98 ± 1.71 µm), corresponding this concentration to the LOEC of triclocarban exposure in our study.

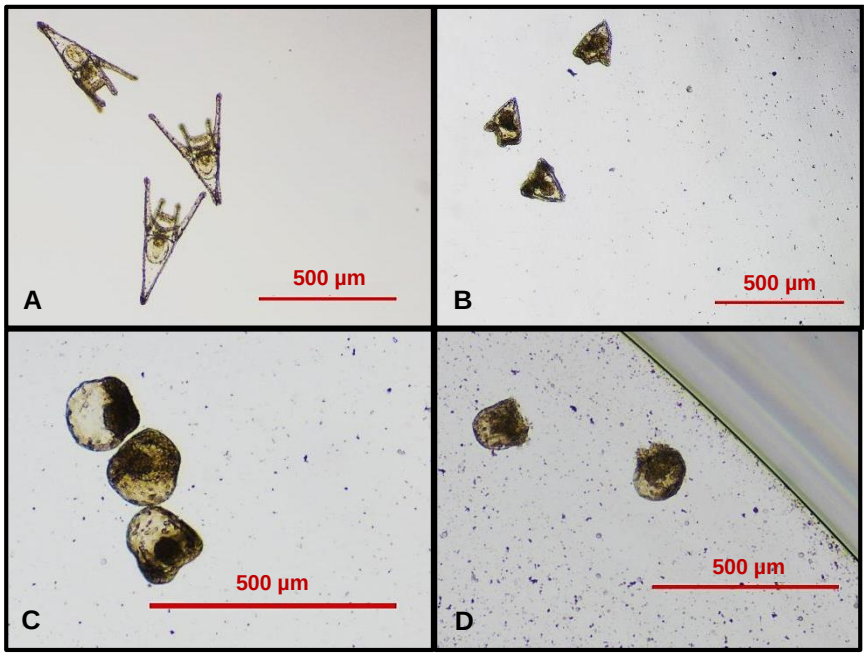


Figure 63. Stage of development of *P. lividus* embryos after exposure to different concentrations of triclocarban for 48h: Solvent control – pluteus larvae **(A)**; 100.0 µg/L – prism larvae **(B)**; 1000.0 µg/L – gastrula stage **(C)**; 10000.0 µg/L – early gastrula stage **(D)** of triclocarban.

3.6. Mixtures

3.6.1. Zebrafish (*Danio rerio)*

3.6.1.1. Cumulative mortality and Hatching rates

An exposure of *D. rerio* embryos to MIX_NOEC_dr did not induce significant differences ($p>0.05$) in cumulative mortality rate at the end of the assay (Figure 64A).

However, hatching rate ranged from 80.0 ± 3.78 in the solvent control to 51.25 ± 4.41 in the exposure group (Figure 64B). Significant differences ($p<0.05$) in hatching rate were recorded in embryos exposed to MIX_NOEC_dr at 80hpf.

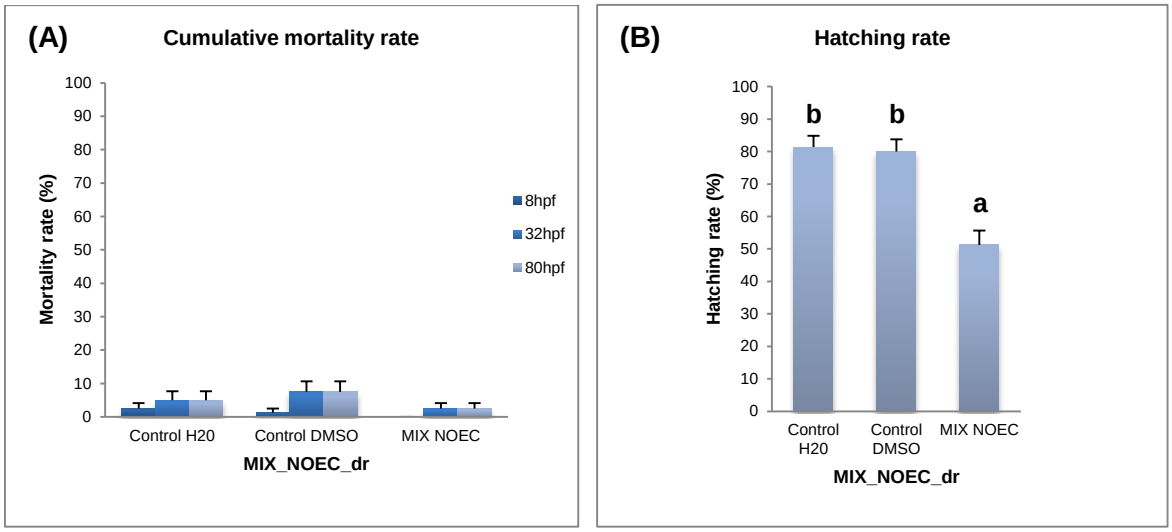


Figure 64. Cumulative mortality **(A)** and hatching **(B)** rates of *D. rerio* embryos exposed to MIX_NOEC_dr for 80h (%). Data are expressed as mean ± SEM (n=8). One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

3.6.1.2. Abnormal cell growth and 75% epiboly stage of embryos (8hpf)

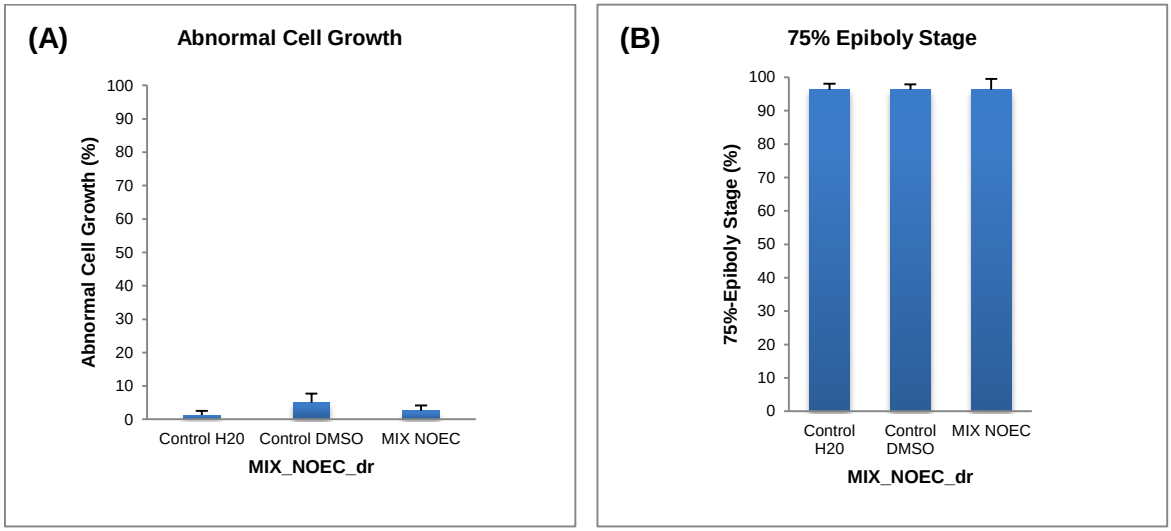


Figure 65. Abnormal cell growth **(A)** and 75% of epiboly stage **(B)** of *D. rerio* embryos exposed to MIX_NOEC_dr at 8hpf (%). Data are expressed as mean ± SEM (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)**. One-way-ANOVA **(B)**.

Similarly to single chemical exposure, no significant differences ($p>0.05$) were reported in the percentage of embryos with abnormal cell growth at 8hpf among controls and exposed group (Figure 65A).

An exposure to MIX_NOEC_dr did not induce significant differences ($p>0.05$) in the percentage of embryos in 75% of epiboly stage at 8hpf among controls and exposed group (Figure 65B).

3.6.1.3. Head and eyes abnormalities

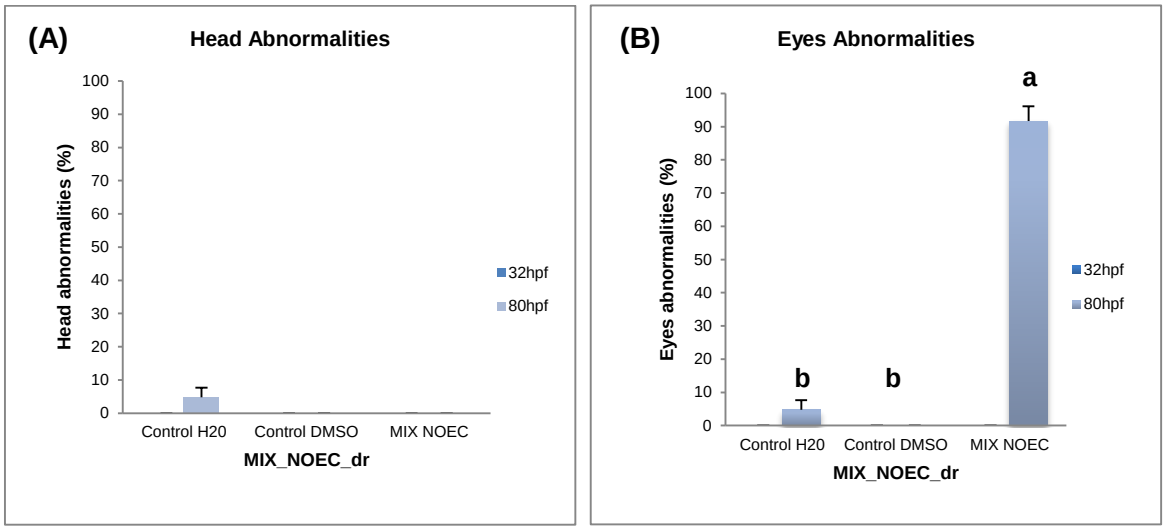


Figure 66. Head **(A)** and eyes **(B)** abnormalities of *D. rerio* embryos exposed to MIX_NOEC_dr at 32hpf and 80 hpf (%). Data are expressed as mean ± SEM (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)** and **(B)**. Bars with a different letter are significantly different from each other.

The percentage of embryos with head abnormalities was not statistically different ($p>0.05$) among controls and exposed group at 80hpf (Figure 66A).

The percentage of embryos with eyes abnormalities at 80 hpf ranged from 0 in the solvent control to 91.67 ± 4.45 in the exposed group (Figure 66B). Although no abnormalities in embryos eyes were reported at 32hpf among all groups, MIX_NOEC_dr exposure induced a high and significant increase ($p<0.05$) in the percentage of embryos with eyes abnormalities at 80hpf.

3.6.1.4. Yolk-sac and tail abnormalities

Embryos exposed to MIX_NOEC_dr did not showed significant differences ($p>0.05$) in the percentage of embryos with yolk-sac or tail abnormalities at 80hpf (Figure 67).

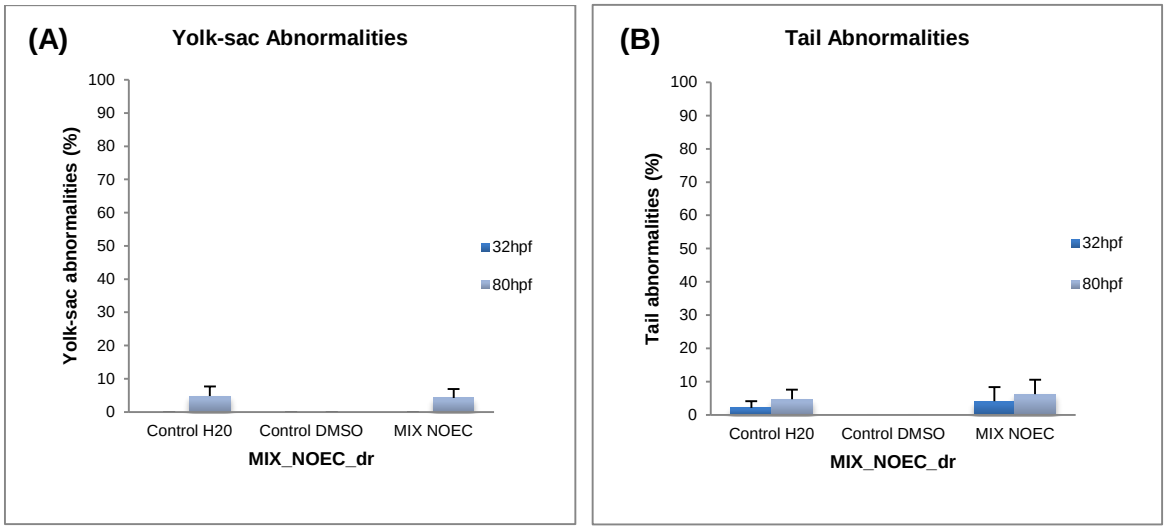


Figure 67. Yolk-sac **(A)** and tail **(B)** abnormalities of *D. rerio* embryos exposed to MIX_NOEC_dr at 32hpf and 80 hpf (%). Data are expressed as mean ± SEM (n=8). Non-parametric ANOVA Kruskall-Wallis **(A)** and **(B)**.

3.6.1.5. Pericardial edema and heart rate

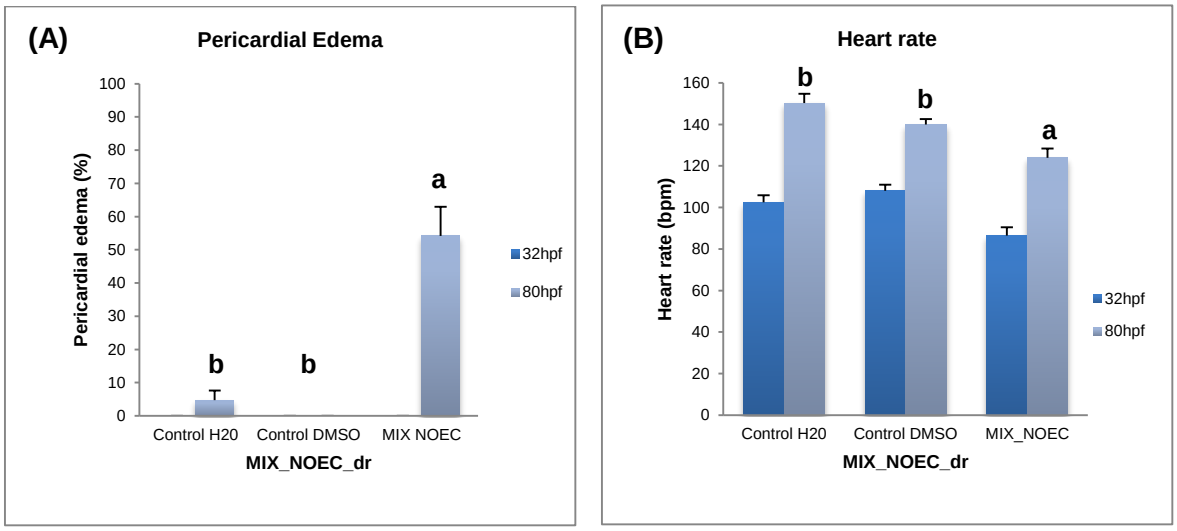


Figure 68. Pericardial edema **(A)** and heart rate **(B)** of *D. rerio* embryos exposed to MIX_NOEC_dr at 32hpf and 80 hpf (%). Data are expressed as mean ± SEM (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups **(A)**. One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons between groups **(B)**. Bars with a different letter are significantly different from each other.

The percentage of embryos with pericardial edema ranged from 0 in the solvent control to 54.17 ± 8.77 in the exposed group, at the end of the assay (Figure 68A). An exposure to MIX_NOEC_dr induced a significant increase ($p<0.05$) in the percentage of embryos with pericardial edema at 80hpf, compared to controls.

Embryo heart rate ranged from 108.0 ± 2.93 in the solvent control to 86.5 ± 3.92 in the exposed group at 32hpf (Figure 68B). At the end of the assay, heart rate ranged from 150.29 ± 4.53 in the control to 124.0 ± 4.42 in the exposed embryos. A significant decrease ($p<0.05$) was reported in heart rate of embryos exposed to MIX_NOEC_dr at 80hpf.

3.6.1.6. Total abnormalities in embryos

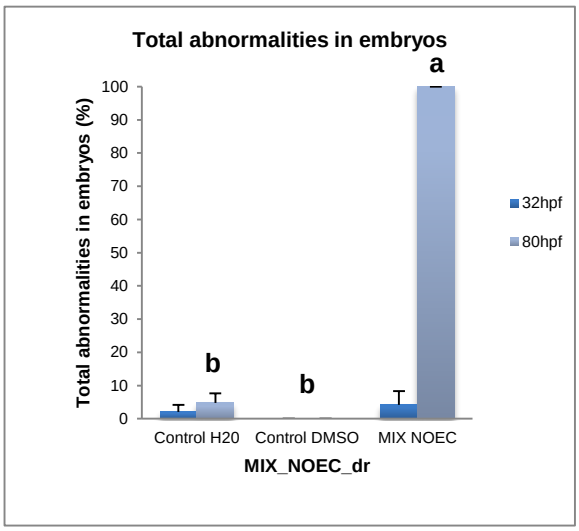


Figure 69. Percentage of total abnormalities in *D. rerio* embryos exposed to MIX_NOEC_dr at 32hpf and 80 hpf (%). Data are expressed as mean ± SEM (n=8). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by Games-Howell test for multiple comparisons between groups. Bars with a different letter are significantly different from each other.

The percentage of total abnormalities in *D. rerio* embryos exposed to MIX_NOEC_dr was statistically significant ($p<0.05$) compared to controls, at 80hpf (Figure 69).

3.6.2. Sea urchin (*Paracentrotus lividus*)

After individual PPCPs exposures, *P. lividus* embryos were exposed to mixtures of PPCPs at different concentrations.

The CA model was used to predict PPCPs mixture effects in larval length and a concentration-response curve was obtained (Figure 70), considering the results from effective concentrations of individual compound exposures (Table 9).

Table 9. EC20 values for Simvastatin, Sertraline, 4-MBC, Propylparaben and Triclocarban from individual exposures of *P. lividus* embryos.

PPCP	EC20 (µg/L)	95% Confidence limits
SIMV	775.0	717.0 – 838.0
SER	55.0	52.0 – 57.0
4-MBC	494.0	464.0 – 527.0
PP	13891.0	12354.0 – 16070.0
TCC	20.0	19.0 – 21.0
MIX_EC20_pl concentration = 15235.0 µg/L		

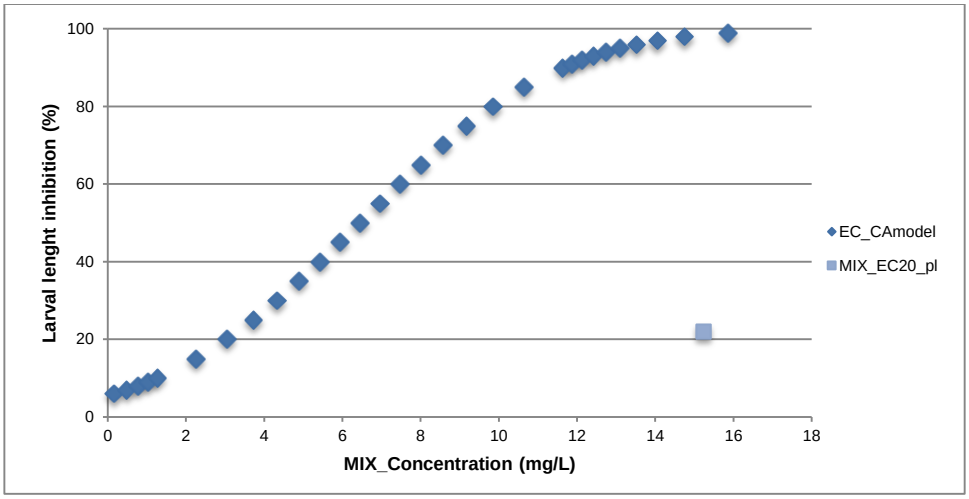


Figure 70. Concentration-response curve according to the CA model in inhibition of larval length (%) by exposure to a mixture of simvastatin, sertraline, triclocarban, propylparaben and 4-MBC, present at the same effective concentration those individual exposures. Effect of MIX_EC20_pl in larval length inhibition (%).

An exposure to MIX_NOEC_pl did not induced significant differences ($p>0.05$) in larval length, compared to controls (Figure 71A).

Significant larval decrease ($p<0.05$) was reported for embryos exposed to MIX_LOEC_pl and MIX_EC20_pl, compared to controls (Figure 71B and 71C). Larval length of MIX_EC20_pl exposure group decrease 22.06% compared to controls (Figure 70 and 71C).

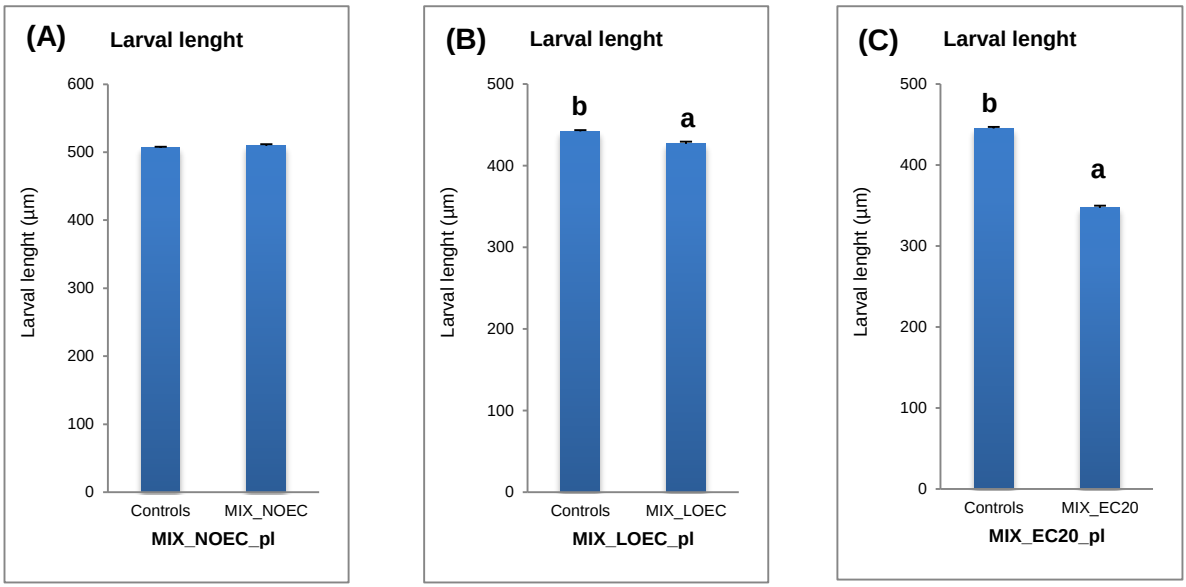


Figure 71. Larval length (µm) of *P. lividus* exposed to MIX_NOEC_pl **(A)**, MIX_LOEC_pl **(B)** and MIX_EC20_pl **(C).** Blank control and solvent control were grouped. Data are expressed as mean ± SEM (n=240 for controls; n=120 mixture exposed group). One-way-ANOVA ($p<0.05$), followed by Student Newman-Keuls test for multiple comparisons between groups **(A)**, **(B)** and **(C)**. Bars with a different letter are significantly different from each other.

Discussion

Chapter 4

4. Discussion

Several PPCPs have been detected in aquatic ecosystems at concentrations that might represent a risk for the natural populations. Additionally, important interactions may occur between different compounds present in mixture in environment, which can significantly increase the individual effects of compounds, even if present at low concentrations. However, available data is still too limited to get a clear picture on the consequences of PPCPs exposure on the ecosystems (Chalew and Halden, 2009). Moreover, some compounds are persistent in the environment due to a continuous input and/or low biodegradable rates, and therefore aquatic organisms are continuously exposed throughout multiple generations (Chalew and Halden, 2009).

Embryos of aquatic organisms have been frequently used in toxicology assays due to its simplicity and low costs. Moreover, early life stages show a high sensitivity to contaminants present in the environment because these active compounds can interfere in specific stages of their development. Setting the effective concentrations of PPCPs is crucial to perform more reliable risk assessments and to implement new standards of protection.

D. rerio is the most commonly and well-recognized aquatic vertebrate model species used in toxicology assays. Egg transparency, rapid development and well-studied species embryogenesis allow monitoring its embryonic development and observing the occurrence of abnormalities in specific development stages during exposure. Thus, it is possible to understand potential mechanisms and pathways affected by PPCPs exposure. Similarly, the embryonic and larval stages of *P. lividus* are frequently chosen to toxicological tests due to its sensitivity and extensive data of its molecular physiology.

When exposing organisms from different taxonomic groups to the same test-conditions, it is possible to compare the effects of the same compound and more effectively anticipate the impact at ecosystem scale. Furthermore, it contributes to elucidate possible conserved mechanisms and pathways.

An exposure of zebrafish embryos to selected PPCPs in this study resulted in abnormalities, development delay, decrease of heart beat and lethal effects. These effects were compounds and concentration specific. Also, sea urchin embryos exposed to the same compounds showed a development delay, body abnormalities, a decrease in larval length or an interruption of embryos development (Table 10). These effects can compromise survival of affected organisms and thus population balance, by affecting locomotion, predation and reproduction of the species. In this study, simvastatin was the most toxic compound for zebrafish embryos, while sea urchin embryos were more susceptible to the toxic effects of triclocarban than the other selected PPCPs (Table 11).

Table 10. Overview of significant effects reported for each endpoint by exposure to individual and combined selected PPCPs.

Endpoint	SINV	SER	4-MBC	PP	TCC	MIX_NOEC_dr	MIX_NOEC_pl	MIX_LOEC_pl	MIX_EC20_pl
Zebrafish									
Mortality rate	✓	✓	x	✓	✓	x	-	-	-
75% of epiboly stage	x	✓	x	✓	x	x	-	-	-
Abnormal cell growth	x	x	x	x	x	x	-	-	-
Head abnormalities	x	x	x	✓	x	x	-	-	-
Tail abnormalities	x	x	x	✓	x	x	-	-	-
Eyes abnormalities	✓	x	x	✓	x	✓	-	-	-
Yolk-sac abnormalities	x	x	x	✓	x	x	-	-	-
Pericardial edema	✓	x	x	✓	x	✓	-	-	-
Heart rate	x	x	✓	✓	x	✓	-	-	-
Hatching rate	✓	✓	✓	✓	x	✓	-	-	-
Muscular involuntary contractions	-	-	✓	-	-	-	-	-	-
Sea urchin									
Larval Length	✓	✓	✓	✓	✓	-	x	✓	✓

References: (✓) significant effects; (x) no significant effects; (-) not evaluated.

Table 11. Overview of NOEC and LOEC values of selected PPCPs reported in this study. Maximal concentrations of selected PPCPs detected in surface water and in WWTPs influents and effluents. (µg/L)

	Zebrafish		Sea urchin		Maximal concentration		
PPCP	**NOEC**	**LOEC**	**NOEC**	**LOEC**	**WWTPs Influents**	**WWTPs Effluents**	**Surface water**
SIMV	5.0	50.0	2.0	5.0	0.09[(1)]	0.001[(2)]	0.0001[(3)]
SER	1000.0	10000.0	4.0	10.0	0.02[(2)]	0.015[(2)]	0.57[(4)]
4-MBC	500.0	5000.0	0.8	2.0	6.5[(5)]	2.7[(5)]	*0.799[(6)]
PP	1000.0	10000.0	160.0	400.0	2.8[(7)]	0.021[(7)]	0.207[(8)]
TCC	100.0	1000.0	0.256	0.64	50.0[(9)]	> 10.0[(10)]	6.75[(10)]

References: (1) Kosma et al., 2014; (2) Santos et al., 2010; (3) Miao and Metcalfe, 2003; (4) Richard and Cole, 2006; (5) Balmer et al., 2005; (6) Kaiser et al., 2012; (7) González-Mariño et al., 2011; (8) Yamamoto et al., 2011; (9) Chalew and Halden, 2009; (10) Brausch and Rand, 2011
*Coastal areas beach

4.1. Hypolipidemic drugs: Simvastatin

In this study, simvastatin was the most toxic compound for zebrafish embryos, by inducing clearly and statistically significant observed effects at lower concentrations. An exposure to 500.0 and 5000.0 µg/L was lethal to all embryos between 32hpf and 80hpf. Although cumulative mortality rate at 32hpf was similar for controls and exposure-groups, all zebrafish embryos exposed to the two highest test-concentrations of simvastatin showed abnormalities in head, eyes and tail at this stage of development. Furthermore, no heart rate was recorded due to the high development delay of embryos at these two concentrations. These abnormalities and development delay led to death of all zebrafish embryos and no hatches were recorded at 80hpf.

At the end of the assay, an exposure of zebrafish embryos to 50.0 µg/L of simvastatin resulted in a significant increase in the percentage of embryos with eyes abnormalities and pericardial edema, but no significant differences in heart rate were reported for this group at 80hpf. This exposure group showed also a no-significant increase in the percentage of embryos with head, tail and yolk-sac abnormalities compared to controls and to 5.0µg/L. However, these abnormalities contributed to significantly increase the percentage of total abnormalities in zebrafish embryos at 80hpf.

Sea urchin embryos were also affected by exposure to simvastatin. At the end of the assay, an exposure to 5000.0 µg/L of simvastatin results in a significant delay in embryo development and no length recording were performed for this exposure group. A significant decrease in larval length was recorded for concentrations equal or higher than 5.0 µg/L.

In a study performed by Key et al. (2008), after exposure of 96h to different concentrations of simvastatin, larval and adult grass shrimp (*Palaemonetes pugio*) showed a LC50 of 1118.0 µg/L and higher than 10000.0 µg/L, respectively. Simvastatin treatment-exposure did not significantly affected glutathione and acethylcholinesterase biomarkers in larval and adult organisms. However, a significantly lipid peroxidation was reported for larval organisms at the lowest (1.0 µg/L) and highest (1000.0 µg/L) simvastatin exposures, and for adults at a concentration range between 625.0 and 2500.0 µg/L. An exposure at 1000.0 µg/L results in a significant decrease in cholesterol levels in larvae, but no significant differences were reported for all treated-exposure in adults. In our study, all zebrafish embryos exposed to simvastatin at concentrations similar to the highest concentrations tested in key et al., died before the end of the assay. For this reason, zebrafish embryos seem to be more susceptible to simvastatin exposure than adult and larval grass shrimp.

In another study, the adults harpacticoid copepod *Nitocra spinipes* was exposed to simvastatin for 96h, which a LC50 of 810.0 µg/L (Dahal et al.,2006). A significant decrease in development time of *N. spinipes* was reported after exposure to simvastatin in a range of concentrations between 0.16 and 1.6 µg/L. Also, a concentration of 1.6 µg/L lead to a significantly increase in body length and the growth rate was significantly higher at 0.16-5.0 µg/L concentrations, in comparison to control. These results were observed at the same range of effective concentrations that induced a decrease in sea urchin larval length in our study. Simvastatin induced also a delay in zebrafish embryos development but at higher concentrations. These differences in effects observed at low and high concentrations in Dahal and coauthors study, suggest that simvastatin might have different ecotoxicological modes of action. According to the authors, the effects at higher concentrations are most related to energy-mediated processes, while effects of endocrine disruption in several mechanisms may occur at low concentrations of simvastatin.

Although data on sinvastatin presence in the environment is limited (see Table 11) the tested-concentrations that induce statistically significant differences in the development of zebrafish and sea urchin embryos were higher than environmental concentrations reported in some studies. However, we cannot rule out the hypothesis of interactions with other compounds present in the environment that could result in severe effects in long-term exposures even if when present at lower concentrations.

4.2. Selective Serotonin Reuptake Inhibitors (SSRIs): Sertraline

Several studies reported that SSRIs can induce disruption of reproductive functions in fish, but some results are inconsistent among studies. In fish, serotonin can stimulate gonadotropin-releasing hormone and release of luteinizing hormone. These hormones regulate important pathways involved in reproductive physiology, whereby the effects reported include repression of sex hormones, increase plasma vitellogenin, inhibition of egg production and gene expression modulation of estrogenic signaling functions (Park et al., 2012). Although some studies focused on the toxicological effects of SSRIs to aquatic organisms, most of these refer to fluoxetine. In fact, there is a lack of information concerning the occurrence, fate and effects of sertraline in aquatic ecosystems (Park et al., 2012). Furthermore, few studies have investigated sertraline effects in initial stages of development.

In our study, all zebrafish embryos exposed to concentrations of sertraline higher than 1000.0 µg/L died before 80hpf. For this reason, no reports of abnormalities, heart rate and pericardial edema were made for these exposure-groups at the end of the assay.

No significant differences in cumulative mortality rate were observed for the other exposure-groups and controls at 80hpf.

At 8hpf, zebrafish embryos exposed at concentrations equal or higher than 8500.0 µg/L showed a delay in their development in a dose-dependent manner, resulting in a significant decrease in the percentage of embryos at 75% of epiboly stage.

At 32hpf the percentage of zebrafish embryos with tail abnormalities increases in a dose-dependent manner for concentrations equal or higher than 3500.0 µg/L. These effects can explain high mortality of embryos at 32hpf for concentrations equal or higher than 6000.0 µg/L and the sudden mortality of embryos exposed to 3500.0 µg/L between 32hpf and 80hpf. An exposure to 10000.0 µg/L induced an increase in the percentage of zebrafish embryos with yolk-sac abnormalities at 32hpf. There were no significant records of head and eyes abnormalities as well as pericardial edema in embryos of controls and exposure groups at 80hpf. However, it was reported a decrease of heart beat in embryos exposed to 6000.0 µg/L of sertraline at 32hpf and no heart beat were observed in embryos exposed at higher concentrations at the same exposure time.

Although all zebrafish embryos exposed to concentrations equal or higher than 3500.0 µg/L were dead at 80hpf, it was possible to record embryo hatching rate at the end of the assay. There was a significant decrease in percentage of embryo hatching for these exposure groups, but some differences between the two assays performed were also observed, with no hatched embryos at 6000.0 and 8500.0 µg/L and a slightly higher hatching rate at 10000.0 µg/L than 3500.0 µg/L. These differences between the two assays may be related with different sensibilities of embryos from these two spawns. However, results from the same toxicological assay are consistent between treatments.

Sea urchin embryos exposed to sertraline were significantly affected. An observation of 10000.0 µg/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 1000.0 µg/L of sertraline were in morula stage at the end of the assay. For these reasons, no measurements were performed for these two exposure-groups. The percentage of abnormal sea urchin larvae at the end of the assay was higher at 100.0 µg/L than controls and 10.0 µg/L exposure group. Larval length of organisms of these two treatments was significantly lower than controls and different between them. Exposures to concentrations bellow 10.0 µg/L did not induce significant differences in larval length compared to controls.

In a study performed by Minagh et al. (2009), sertraline induced acute effects in *D. magna*, *Psedokirchneriella subcapitata* and *O. mykiss*, although at higher concentrations than those reported in the environment. The most sensitive species tested to sertraline was 21d *D. magna* reproduction (EC50 = 66.0 µg/L) followed by 21d *D. magna* mortality

(LC50 = 120.0 µg/L). An exposure of *O.mykiss* to different concentrations of sertraline for 96h results in a LC50 of 380.0 µg/L. In our study, zebrafish embryos were not affected at this concentrations range, but significant effects were reported in sea urchin embryo exposed to these concentrations.

Schultz et al. (2011), evaluated the effects resulting from an 21d-exposure of male fathead minnows (*P. 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). A sertraline exposure to 1.6 ng/L resulted in a statistically significant decrease in interstitial cell prominence in fish. In our study, no effects were reported in zebrafish and sea urchin embryos at this concentration range.

In another study, *Xenopus laevis* blastulae (Stage 9) were exposed for 96h to different concentrations of sertraline (Richards and Cole, 2006). Sertraline exposure at concentrations higher than 2000.0 µg/L has induced tail abnormalities and some thoracic edemas, with EC10 and EC50 values of 3000.0 µg/L and 3300.0 µg/L, respectively. The authors reported also an LC10, LC50 and LC100 values of 3600.0, 3900.0 and 5000.0 µg/L, respectively. These results are similar to those obtained in our study for cumulative mortality and tail abnormalities in zebrafish, although zebrafish embryos were more sensitive than *X. laevis*. In fact, zebrafish embryos exposed to sertraline exhibit total lethal mortality for concentrations equal or higher than 3500.0g/L, without significant effects at lower tested concentrations. The absence of a dose dependent relationship for sertraline exposure in our study is comparable to previous findings regarding other SSRIs (Schultz et al., 2011). The effective concentrations for sea urchin embryos were in close range to actual concentrations in the environment.

4.3. Ultra Violet Filter: 4-Methylbenzylidene Camphor

During the last decade UV filters use has increased as a result of public concern about the effects of UV radiation. Therefore, UV screens have been widely and extensively used. The consequences of UV filters for aquatic organisms remain unclear. The available ecotoxicological studies for UV filters are limited and fragmentary, which do not allow to perform reliable risk assessment of these compounds on aquatic ecosystems. Thus, it is important to perform new studies in order to understand the toxicokinetics of UV filters and to reassess the beneficial and adverse effects of UV screens usage (Schereus et al., 2002).

Some studies reported a seasonal variation of UV filters in aquatic ecosystems related with high recreational activities periods such as bathing and swimming, which is considered an important direct input of these compounds into the environment, by washing-off from skin (Balmer et al; 2005; Zucchi et al., 2011; Bachelot et al; 2012; Kaiser et al., 2012).

The UV filter 4-MBC is frequently used in sunscreens and some studies have reported its potential to induce endocrine disruption. In fact, 4-MBC has the potential to affect process mediated by signaling mechanisms of estrogen receptors and can compete with estradiol for estrogen binding sites in the uterus, which can disturb the balance of calcium activity in the cell (Vincent, 2001). Some studies reported also adverse effects in puberty, gene expression and weight of reproductive organ after 4-MBC exposure as well as endocrine disruption and depression of thyroid hormones (Schreurs et al., 2002; Schlumpf et al., 2008; Nakata et al., 2009; Vincent, 2011).

Although some studies addressed the impact of 4-MBC in aquatic organisms, there is a lack of information about the effects in initial embryonic development (Vincent, 2011).

In our study, zebrafish embryos mortality was negligible and there were no differences between controls and exposure-groups, and thus no relation to 4-MBC exposure. However, significant decrease in hatching rate was reported in embryos exposed to the highest tested concentration.

No significant abnormalities and pericardial edema were reported at the end of the assay, but a significant decrease in heart rate zebrafish embryos were observed in the 5000.0 µg/L exposure group. It is possible that an exposure to 4-MBC at this concentration can induce a development delay in embryos, affecting heart beat and delaying the hatching time. Although it was not a predicted effect, zebrafish embryos exposed to 500.0 and 5000.0 µg/L of 4-MBC showed an increase in the percentage of embryos with abnormal involuntary muscular contractions, being significantly different from controls in the 5000.0 µg/L exposure group. This effect was not observed in the other selected PPCPs exposures and may be related to 4-MBC effects in specific development stages of zebrafish embryos.

All sea urchin embryos exposed to the highest concentration of 4-MBC died and therefore no measurements were performed at this exposure group. An exposure to concentrations higher than 0.8 µg/L resulted in a significant decrease in larval length, compared with controls and lower tested concentrations. Differences in larvae length of sea urchin between control groups of different assays are expected and are related to maternal nutrition and habitat (Bertram and Strathmann, 1998).

In a study performed by Vincent (2011), dechorinated zebrafish embryos were exposed to different concentrations of 4-MBC. There was a sharp increase in mortality from 3820.0 µg/L to 6360.0 µg/L, which induce total embryo lethality. An exposure to 4-MBC induced also an increased in the percentage of embryos with axial curvature from 2540.0 µg/L and all embryos were affected at 5600.0 µg/L. An exposure of zebrafish embryos to 3820.0 µg/L of 4-MBC during gastrulation and/or segmentation resulted in higher percentage of embryos with altered axial curvature than those obtained for pharyngula-only exposure. In our study, this concentration range did not induce significant differences in cumulative mortality rate and tail abnormalities of zebrafish embryos between controls and exposure-groups. These differences may be related to different experimental design or genetic background of the fish stock. In fact, in Vincent‘s study, zebrafish embryos exposed to 4-MBC did not had chorion protection, and thus they were more susceptible to toxic effects resulting from an exposure, which can explain the high mortality rate and tail curvature reported. These results supports the hypothesis that the lower sensibility of zebrafish embryos compared to sea urchin embryos may be related in part to the embryo protection given by the chorion.

In the same study, 4-MBC-treated embryos showed swimming incapability and no response to tactile stimulations, which might be attributed in part to abnormal axial formation (Vincent, 2011). According to this author, the effects in altered axial curvature and shortened body are not likely to be caused by endocrine disruption but rather are related to failure in notochord differentiation process. Moreover, it was also reported an acethylcholinesterase inhibition after 4-MBC exposure, which leads to an accumulation of the neurotransmissor acetylcholine and inactivation of acetylcholine receptors, resulting in defects in axonogenesis and muscle formation. Thus, the increase in the percentage of zebrafish embryos with abnormal involuntary muscular contractions at the two highest tested concentrations in our study may be related to muscular dysfunctions. These effects in locomotion compromise organisms’ survival and can affect population and ecosystems.

Balmer and collaborators performed a study in Switzerland to verify the occurrence of some UV filters in samples from WWTPs, surface water and in fish tissue. Relative quantities of the evaluated UV filters in WWTPs influents were similar to the patterns of average contents in sunscreens. 4-MBC was the most persistent UV filter, being detected most frequently and at highest concentrations than the other UV filters examined, in wastewater effluent, surface water and in fish tissue (Balmer et al., 2005). In the same study, the authors reported a seasonal variation of UV filters concentration in WWTPs influents, being higher during summer than in spring, which reflects the increased use of UV filters (sunscreens) in summer. Similarly, it is possible that 4-MBC concentration in intertidal zone increases during summer due to usage of greatest amount of sunscreens

and to recreational beach activities. On the other hand, bathing season corresponds with sea urchin spawning period and some studies have reported 4-MBC environmental concentrations similar or higher than those that have induced significant effects in sea urchin embryo development in our study (Balmer et al., 2005; Kaiser et al., 2012). As sea urchins are present on intertidal and subtidal zone, it is possible that actual concentrations of 4-MBC or other UV filters in this area can affect sea urchin embryo development, compromising reproduction success of the species.

4.4. Preservative: Propylparaben

Propylparaben, as well as methylparaben, are widely used in cosmetic formulations and frequently detected in aquatic environment. Few studies were performed in order to understand long-term effects of parabens in aquatic organisms and there is a lack of information about chronic effects resulting from paraben exposure (Brausch and Rand, 2011).

In a studied performed by Albero et al. (2012), methyl- and propylparaben were the only parabens detected in sewage sludge collected in different areas of Madrid (Spain). Similarly, these two compounds were the most frequently detected and the most abundant in raw wastewater, in a study conducted by González-Mariño et al. (2011).

Generally, parabens with higher hydrocarbonated chain length are more persistent in environment and can induce more adverse acute effects in organisms. In fact, methyl- and ethylparaben are rapidly degraded in environment while propyl- and butylparaben required more time for the same biodegradation rate (González-Mariño et al., 2011). Li (2012), exposed *Dugesia japonica* to different concentrations of four parabens and reported an increase of 48h and 96h LC50 values ranked as butilparaben < propilparaben < etilparaben < metilparaben, being the first one the most toxic paraben tested. The magnitude of estrogenic response increase also with the alkyl group size, whereby butyl ester shower great activity (Oishi, 2002). Similarly, in a study performed by Dobbins et al. (2009) the chronic effects of paraben exposure were addressed and the authors reported benzyl- and butylparaben as being most toxic to invertebrates and fish, while methyl- and ethylparaben appeared less toxic to organism.

Although low environmental concentrations of parabens does not seem to have potential to induce estrogenic effects, some parabens, specifically benzyl-, butyl and propylparaben, can induce low-level estrogenic effects in aquatic organisms (Dobbins et al., 2009; Alberto et al., 2012). Propylparaben was found to induce increase of VTG concentration in plasma and up-regulate the expression of VTG genes in male medaka.

Intraperitonial injections of propylparaben in rainbow trout also cause estrogenic responses in organisms, with a significant induction of VTG in male fish (Yamamoto et al., 2012).

In our study, an exposure to propylparaben induced a lethal mortality for all zebrafish embryos in the 10000.0 µg/L exposure group at the end of the assay. For this reason, no reports of abnormalities, heart rate and pericardial edema were made for this group at 80hpf. Although no significant differences in mortality were reported among controls and exposure groups, an exposure to 8500.0 µg/L resulted in a no-significant increase of cumulative mortality rate at 80hpf.

At 8hpf, an exposure to propylparaben resulted in a significant decrease in the percentage of zebrafish embryos at 75% of epiboly stage when exposed to 10000.0 µg/L.

At 32hpf, zebrafish embryos exposed to 10000.0 µg/L of propylparaben showed an increase in yolk-sac and tail abnormalities and also a decrease in heart rate. No differences in pericardial edema and head, eyes and yolk-sac abnormalities among controls and exposure groups were reported at 32hpf. However, the percentage of zebrafish embryos with tail abnormalities increased in a dose dependent manner for concentrations equal or higher than 3500.0 µg/L at 32hpf. A sudden and statistically significant increase in the percentage of zebrafish embryos with pericardial edema and head, eyes, yolk-sac and tail abnormalities were observed at the end of the assay for these exposure groups.

Heart rate was significantly affected by propylparaben exposure. At 32hpf zebrafish embryos exposed to concentrations equal or higher than 6000.0 µg/L showed a high decrease in heart rate, which was statistically significant at 80hpf.

Although all zebrafish embryos exposed to 10000.0 µg/L of propylparaben died before 80hpf, it was possible to record embryos hatching rate at the end of the assay. No hatched embryos were observed in this exposure group and there was a significant decrease in embryo hatching rate in the 6000.0 and 8500.0 µg/L exposure groups at the end of the assay. Thus, although all zebrafish embryos exposed to concentrations equal or higher than 3500 µg/L showed one or more abnormalities at the end of the assay, differences in hatching rate and heart beat among these exposure groups reflect the increasing severalty of effects resulting from an exposure to increasingly propylparaben concentrations. The effects of an exposure to 10000.0 µg/L observed at 32hpf can explain the high mortality of zebrafish embryos in this exposure group and its development delay at the end of the assay.

An exposure of sea urchin embryos to initial concentrations of propylparaben resulted in a significant decrease of larval length for concentrations equal or higher than 1000.0 µg/L. As these compound revealed less toxic than the other selected PPCPs, a

new set of intermediate concentrations was tested in order to precise the NOEC of propylparaben exposure in sea urchin. Results were consistent between these two assays performed and a significant decrease in larval length was recorded for exposures equal or higher than 400.0 µg/L of propylparaben.

To the best of our knowledge, there is a lack of studies reporting the effects of propylparaben in embryonic development of zebrafish and sea urchin.

During 4 weeks, Oishi (2012) exposed male rats to propylparaben present in a modified diet at 0, 0.1, 1.0 and 10.0 mg/g and reported adversely effects in testosterone secretion and in male reproductive system functions. The body weight of exposed organisms was slightly but significantly lower than control organisms, at the highest tested concentration, but no effects were reported in organ weights. Thus, this decrease in body weight may be a toxic effect induced by propylparaben rather than an estrogenic response. The daily sperm production in testes and its efficiency decreased in dose-dependently manner, being statistically significant in all treated-groups, and resulted in a significant decreased of sperm reserves at the two highest tested concentrations. Testosterone concentration also decreased in a dose-dependent manner. However, this decrease was significant only in the group that received the highest dose of propylparaben, which was higher than those that have induced a decrease in sperm reserves. Although the decrease in testosterone concentration may result in sperm reserves decrease, it seems that decrease in sperm reserves are a direct toxic effect on the spermatogenesis or estrogenic action of propylparaben. The propylparaben exposure level that induced these effects is the same as the upper-limit acceptable daily intake (10 mg/kg body weigh/day) of parabens in the European Community and Japan (Oishi, 2012).

Mikula et al. (2009), studied the influence of propylparaben on vitellogenesis and sex ratio in juvenile zebrafish. Experimental juvenile zebrafish with 20 days post hatching were fed with a diet containing 500, 1000 or 2000 mg/kg of propylparaben. After 20 days of exposure, there were no reports of effects in vitellogenin synthesis but it seemed to influence sex differentiation processes in exposed organisms. In fact, after 45 days of exposure the number of females was significantly higher in treated-groups than the control one, in a diet at 500 mg/kg. Although an exposure to propylparaben did not induced estrogenic or antiestrogenic effects in zebrafish, the potential of fish to respond to estrogenic stimulation was confirmed by the authors in the positive control, which was exposed to 17β-estradiol and resulted in a significantly higher vitellogenin concentration than control and all treated-groups.

An exposure to propylparaben resulted in significant effects in zebrafish and sea urchin embryos when exposed to higher concentrations. Actual environmental concentrations of propylparaben are lower than the effective concentrations observed in

our study, and therefore effects resulting from an acute exposure in the environment are not expected to affect embryonic development of these species. However, possible adverse effects may result from a long-term exposure to propylparaben in environment due to its potential to bioaccumulate in organisms.

4.5. Bacteriocide and antifungal agents: Triclocarban

There are only a few studies reporting triclocarban toxicity in aquatic organisms, but recent reports indicate triclocarban is slightly more toxic to aquatic invertebrate and fish than triclosan. Due to disinfectants potential sorption to sediment, it is possible that triclocarban could affect benthic invertebrates (Brausch and Rand, 2011). Some studies show a bioaccumulation of this compound in aquatic organisms, which can promote biomagnification on the food chain (Chalew and Halden, 2009; Schebb et al., 2011). Some metabolic enzymes present in fish are conserved and similar to mammals. Schebb et al. (2011), reported a triclocarban metabolism in *Oryzias latipes* similar to mammals possibly due to similar metabolic pathways to those previously observed in mammals.

Recent studies have reported endocrine disruption activity of triclocarban by amplification of transcriptional activity of steroid hormones or their receptors as well as triclocarban affinity for these receptors (Chen et al., 2008; Giudice and Young, 2010). It was also showed in vivo that a diet containing a mixture of triclocarban and testosterone result in synergetic effects related to testosterone-induced transcription and an increase of the sex organ weight in male castrated rats compared to control diets or single compounds diets (Chen et al., 2008). Furthermore, triclocarban affected the transcription of genes responding to thyroid hormone in frog and rat cells (Schebb et al., 2011). For these reasons, triclocarban should be classified as a new type EDC.

In our study, an exposure of zebrafish embryos to triclocarban resulted in a significant increase in cumulative mortality rate for concentrations equal or higher than 350.0 µg/L at the end of the assay. In fact, an exposure to concentrations equal or higher than 850.0 µg/L was lethal for all zebrafish embryos. For this reason, no reports of body abnormalities, heart rate and pericardial edema were made for these exposure groups at 80hpf. There were no differences in cumulative mortality rate among controls and exposure groups at earlier stages of development.

There were no significant differences in the percentage of zebrafish embryos with pericardial edema and abnormalities in head, eyes and yolk-sac among controls and all exposed groups at 80hpf. The percentage of embryos with tail abnormalities increased in the 350.0 and 600.0 µg/L groups at 80hpf. However, due to high mortality for these

exposure groups at the end of the assay, there was a high standard error associated and no-significant differences were reported.

An exposure to triclocarban did not induced significant differences in heart rate of zebrafish embryos between controls and exposure groups at 80hpf. There was a decrease in heart rate of embryos exposed to 350.0 µg/L but a high standard error associated. No heart beat was observed in embryos exposed to 600.0 µg/L of triclocarban at 80hpf.

Although all zebrafish embryos exposed to concentrations equal or higher than 850.0 µg/L were dead at 80hpf, it was possible to record embryo hatching rate at the end of the assays and no-statistically significant differences in the percentage of embryo hatching were reported among controls and all exposure groups.

Triclocarban was the most toxic compound in this study for sea urchin embryos, inducing significant effects at lower concentrations. An exposure to concentrations of triclocarban equal or higher than 0.64 µg/L resulted in a significant decrease in larval length at the end of the assay. An exposure to concentrations equal or higher than 100.0 µg/L resulted in a significant delay in embryo development and no larvae reached the four-arm stage, whereby length was not recorded for these exposure groups. All embryos exposed to 1000.0 and 10000.0 µg/L of triclocarban were in gastrula stage at the end of the assay. At 100.0 µg/L exposed embryos reached only the prim larvae stage. Exposures to concentrations bellow 0.256 µg/L did not induce significant differences in larval length compared to controls, being this concentration the NOEC of triclocarban exposure in our study.

In the freshwater mudsnail, *Potamopyrus antipodarum*, triclocarban exposure promoted a significantly increase in embryo production to concentrations equal or higher than 0.2 µg/L, with a significantly increase in the number of unshelled embryos comparative to controls (Giudice and Young, 2010). In a study performed by Schultz and Bartell, a decreased in aggression was seen in *Pimephales promelas* adults male exposed to triclocarban (1.6 µg/L) or present in mixtures (560 ng/L TCS + 179 ng/L TCC and 1.6 µg/L TCS + 450 ng/L TCC). This effect seemed to persist until 4 days after the end of the exposures and can result in a decrease of organisms' defense ability and reproduction success (Schultz and Bartell, 2012). These concentration ranges are similar to those that have induced significant effects in development of sea urchin embryos, in our study. Zebrafish embryos were affected by triclocarban exposures only at higher concentrations.

Although actual environmental concentrations of triclocarban are lower than the effective concentrations in zebrafish embryos in our study, they are similar to test-concentrations that have induced significant effects in sea urchin larval length. For this

reason, it is possible that sea urchin embryos are being affected by environmental occurrence of triclocarban, which compromise their development success and organism's survival.

4.6. Mixtures

Zebrafish embryos were exposed to a mixture of selected PPCPs each present at its NOEC (MIX_NOEC_dr). Thus, all compounds were present at a concentration that did not induce any significant effect in all the endpoints evaluated in single exposures.

There were no significant effects in cumulative mortality rate among controls and exposed groups at the end of the assay. However, zebrafish embryos exposed to this PPCPs mixture showed a significant decrease in hatching rate.

Embryos exposed to MIX_NOEC_dr did not showed significant differences in the percentage of embryos in 75% of epiboly stage at 8hpf and in head, yolk-sac and tail abnormalities at 80hpf. A significant increase was reported in the percentage of embryos with eyes abnormalities at the end of the assay. Exposed embryos exhibited also a significant increase in the percentage of pericardial edema and a significant decrease of heart rate at 80hpf. All embryos exposed to MIX_NOEC_dr were abnormal at 80hpf. The regulatory processes of PPCPs in the environment take into account results of studies with single exposures. For this reason, the results obtained in this study are very important since they show that a mixture of compounds, each one present in a concentration that did not induced significant effects in organisms, can induce significant effects due to possible interactions between the compounds.

Considering the high sensitivity of larval length as endpoint in all exposures performed in our study, sea urchin embryos were exposed to different mixtures of selected PPCPs each one present at its NOEC (MIX_NOEC_pl), LOEC (MIX_LOEC_pl) or EC20 (MIX_EC20_pl).

Similarly to those reported for individual exposures at the same PPCPs concentration, an exposure to MIX_NOEC_pl did not induce significant differences in larval length among controls and exposure group.

When exposed to MIX_LOEC_pl, there were significant differences in larval length at the end of the assay. This effect was expected as each compound was present in mixture at concentrations that induced significant effects in individual exposures. However, it is important to note that in this mixture, all compounds were present at a concentration 2.5x higher than in MIX_NOEC_pl, at which no significant effects were reported. Thus, small differences in compounds concentrations can represent a risk to embryonic development of embryos and affect ecosystems. In fact, annual or periodic

variations in concentrations of PPCPs present in environment are expected to occur. Hence, it is important to understand possible effects of combined exposures resulting from an environmental relevant mixture.

After individual exposure of selected compounds, the EC20 values of each PPCP were determined and MIX_EC20_pl was performed. CA model was used to predict the effects of this mixture in larval length. A concentration-response curve was obtained considering the results from effective concentrations of individual compounds exposures and assuming that compounds share the same mode of action and contribute equally to the overall effect of the mixture. An exposure to MIX_EC20_pl resulted in a larval length decrease of 22.06% compared to controls. Although, a significant decrease in larval length for this mixture was expected, as each compound was present in mixture at an effective concentration, the effect was lower than those estimated by CA model for this mixture concentration. Thus, possible antagonistic interactions might have occurred.

Although mixtures of compounds with the same modes of action tend to be best modulated by CA model while mixtures of compounds with different modes of action tend to be best modulated by IA model, the predicted results of these two models in studies with mixtures compounds with different modes of action were generally similar. For this reason, model selection may be mainly based on conservative of its predicted results. In fact, CA model tend to provide best conservatively estimates of mixture toxicity compared to IA model and with similar predictive accuracy (Belden et al., 2007). Overall, the results highlight the importance of testing mixture effects of PPCPs in order to better predict the risk to natural populations.

Conclusions and Future work

Chapter V

5. Conclusions and Future work

In our study, all selected PPCPs induced significant effects in embryonic and larval development of zebrafish and sea urchin, some of them at relevant environmental concentrations. Simvastatin was the most toxic compound in embryonic development of zebrafish, while sea urchin embryos were more susceptible to the toxic action of triclocarban, which induced significant decrease in larval length at lower concentrations. Significant effects were also reported for mixture exposures and, therefore, the present work contributes to demonstrate that compounds present in mixture may interact and induce different and significant effects from those observed in individual exposures.

The endpoints of *D. rerio* and *P. lividus* embryo toxicity tests are well-recognized by OECD and Environmental Protection Agency (EPA) and are also considered the most sensitive endpoints to evaluate the effects resulting from exposures to different compounds for each species. Attending to the results of this study, sea urchin embryos are more sensitive to PPCPs exposure than zebrafish. These different are likely to be due to genetic differences between the two species. Moreover, as zebrafish embryos present chorion during initial stages of development this might work as a barrier that interferes with the uptake of PPCPs.

Larval length seems to be a sensitive and robust endpoint to evaluate the impacts of PPCPs exposures in sea urchin embryos. Conversely, abnormal cell growth revealed a weak endpoint in evaluating toxic impacts of PPCPs exposures in embryonic development of zebrafish, as no significant effects were observed for this endpoint.

Considering the results obtained in this study, it is now important to perform additional studies to understand the action mode of each compound and the biochemical and molecular mechanisms involved in the observed effects. It is also relevant to extend individual and mixture exposures to all organisms' life-cycle and perform new toxicological assays with other relevant species belonging to different taxonomic groups. Thus, it will be possible to understand the consequences at population level as well as identify conserved mechanisms of action across different phyla.

Data from studies of PPCPs effects after individual and mixture exposures are important to perform more reliable risk assessments and to implement protective standards for the environment and for organisms at their most sensitive development stages.

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