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Screening the effects of emerging pollutants using embryo bioassays: triclosan, methyl-triclosan and perfluoroalkyls chemicals.

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SCREENING THE EFFECTS OF EMERGING POLLUTANTS USING EMBRYO BIOASSAYS: TRICLOSAN, METHYL-TRICLOSAN AND PERFLUOROALKYLS CHEMICALS.

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Abstract

The presence of emerging compounds in the environment is a worldwide concern, not only because of the potential negative impact in human health, but also due to the potential toxicity to non-target organisms. The Personal and Care Products (PCPs) are referred as emerging pollutants since they encompass a major class of compounds detected in the waters, with limited available information on their environmental impact. Within the PCPs class, the disinfectant triclosan (TCS) is one of the most concerning compounds. TCS is an antimicrobial used in many products of our daily life such as toothpastes, shampoos, deodorants or skin care products. It is produced to kill bacteria by blocking the fatty acid synthesis, inhibiting the cell growth. It is a photodegradable compound originating several by-products once in the water systems. One of its metabolites, methyl-triclosan (M-TCS), is known to bioaccumulate and to be resistant to photodegradation. M-TCS has been reported in the aquatic environments, although the information on its (eco)toxicity and mode of action is scarce.

Perfluorinated compounds (PFCs) are another class of emerging chemicals and include de perfluoroalkyls acids (PFAAs) which have been extensively used in the chemical industry. Although some of PFAAs have been banned, i.e., perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA), several other homologues have been produced to substitute the formers, i.e., perfluorobutane sulfonate (PFBS) and perfluorobutanoic acid (PFBA). Most of these PFCs are extremely resistant to degradation, accumulating in the organisms and so, there is the need to improve ecotoxicological data.

In this work we aimed to improve the ecotoxicological data of TCS and its metabolite M-TCS and also of several selected PFAAs, i.e, PFOS, PFOA, PFBS, PFBS, PFDA and PFUnA using sea urchin (*Paracentrotus lividus*) and zebrafish (*Danio rerio*) embryos as models for ecotoxicity assessment. We performed bioassays with embryos for 144 hpf (hours-post-fertilization) for zebrafish (early larva) and 48h for sea urchin (larva *pluteus* stage).

Our results point to an impact of both chemicals TCS and M-TCS, i.e., an increase in the abnormalities rates in zebrafish embryos and impact in the development of sea urchin larvae with a NOEC of 40 µg/L and <1.024 µg/L, respectively. PFAAs revealed low toxicity for zebrafish, however, apart from PFBA, all of the PFAAs tested delayed sea urchin larvae development at 1000 µg/L, and PFOS and PFOA affected the larvae development at concentrations of environmental relevance. Given the almost absence of ecotoxicological data on M-TCS and several PFAAs to marine invertebrates, the results present here are

key to improve risk assessment of these chemicals. Further investigation should focus on the effects of chronic exposures and impacted molecular and biochemical pathways.

Resumo

A presença de compostos emergentes no ambiente é um problema a nível mundial, não só por causa do impacto negativo na saúde humana, mas também devido à potencial toxicidade em organismos não-alvo. Os produtos de uso pessoal são referidos como poluentes emergentes já que englobam uma enorme classe de compostos detetados nas águas, para os quais a informação sobre o possível impacto ambiental ainda é limitada. Dentro da classe dos produtos de uso pessoal, o desinfetante triclosan é um dos compostos mais alarmantes. O triclosan (TCS) é um antimicrobiano usado nos mais variados produtos do quotidiano, como as pastas de dentes, shampoos, desodorizantes ou cremes para cuidados da pele. Este composto é produzido para eliminar bactérias, e a sua função é bloquear a síntese de ácidos gordos, inibindo assim o crescimento da célula. Este composto é fotodegradável e uma vez na água pode originar vários subprodutos. Um desses metabolitos, metil-triclosan (M-TCS), é conhecido pela sua capacidade de bioacumulação e resistência à fotodegradação e embora tenha sido detetado nas águas, a informação sobre a sua toxicidade e modo de ação é escassa.

Os compostos perfluorados (PFCs) são outra classe de compostos emergentes, que incluem os ácidos perfluoroalquilos (PFAAs) que têm sido vastamente usados na indústria química. Embora alguns destes compostos tenham sido banidos, como é o caso do ácido perfluorooctano sulfónico (PFOS) e do ácido perfluorooctanoico (PFOA), outros compostos homólogos têm sido produzidos com o intuito de substituir os anteriores, como o ácido perfluorobutano sulfónico (PFBS) e o ácido perfluorobutanoico (PFBA). Grande parte destes compostos perfluorados são extremamente resistentes à degradação, acumulando-se nos organismos e por esse motivo, tem-se verificado uma grande necessidade em melhorar a informação ecotoxicológica destes compostos.

Neste trabalho teve-se como principal objetivo contribuir para a avaliação ecotoxicológica do triclosan, do seu metabolito, metil-triclosan e ainda de vários compostos perfluorados que foram selecionados, como o PFOS, PFOA, PFBS, PFBA, PFDA e o PFUnA, usando embriões de ouriço-do-mar (*Paracentrotus lividus*) e de peixe-zebra (*Danio rerio*) como modelos de avaliação ecotoxicológica. Realizaram-se ensaios com embriões de peixe-zebra até 144 horas pós fertilização (estádio larvar) e até 48 horas pós fertilização para o ouriço-do-mar (estádio de larva *pluteus*).

Os resultados apontam para um impacto do triclosan e do metil-triclosan na percentagem de anomalias do peixe-zebra e no desenvolvimento da larva de ouriço-do-mar, obtendo um NOEC de 40 μg/L e <1.024 μg/L, respetivamente. Os compostos perfluorados revelaram ser pouco tóxicos para o peixe-zebra, no entanto, excetuando o

PFBA, todos os PFAAs atrasaram o desenvolvimento da larva de ouriço-do-mar para a concentração de 1000 µg/L e tanto o PFOS como o PFOA, afetaram o desenvolvimento larvar a concentrações ambientalmente relevantes. Dado que a informação ecotoxicológica do metil-triclosan e de muitos PFAAs para invertebrados marinhos, é praticamente inexistente, os resultados obtidos neste estudo são chave para melhorar a avaliação de risco destes químicos. Posterior investigação deverá focar-se nos efeitos de exposições crónicas e o seu impacto nas vias de sinalização moleculares e bioquímicas.

Contents

Abstract……..1
Resumo…………………………………………………………………………………......3
Contents………………………………………………………………………………..5
List of Figures……………………………………………………………………………10
List of Tables…………………………………………………………………………16
List of Abbreviations and Acronyms……………………………………………………....18

Index

1. Introduction 19

1.1. Emergent Chemicals of concern in the environment 21
1.1.1. Personal Care Products: triclosan (TCS) and methyl-Triclosan (M-TCS)......... 21
1.1.1.1. State of knowledge on triclosan and methyl-triclosan toxicity 23
1.1.2. Synthetic chemicals: perfluorinated compounds (PFCs)................................. 23
1.1.2.1. Background and applications 23
1.1.2.1.1. Perfluorinated compounds properties 25
1.1.2.1.2. State of knowledge on the PFAAs selected to the present study........... 26
1.1.2.1.2.1. Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA) 26
1.1.2.1.2.2. Perfluorobutane sulfonate (PFBS) and perfluorobutanoic acid (PFBA) 29
1.1.2.1.2.3. Perfluorodecanoic acid (PFDA) and perfluoroundecanoic acid (PFUnA) 30
1.2. Aim of the study 31

2. Materials and Methods 33

2.1. Tested chemicals 35
2.2. Artificial salt water 35
2.3. Test organisms 36
2.3.1. Sea urchin (*Paracentrotus lividus*).. 36
2.3.1.1. Experimental design 37
2.3.2. Zebrafish (*Danio rerio*) 38
2.3.2.1. Experimental design 40
2.4. Statistical Analysis 42

3. Results 43

3.1. Disinfectant Triclosan 45
3.1.1. Zebrafish embryos bioassay 45
3.1.1.1. Cumulative mortality...45
3.1.1.2. Abnormal cell growth...45
3.1.1.3. Embryo development delay..46
3.1.1.4. Hatching rate 46
3.1.1.5. Head and eyes abnormalities...47
3.1.1.6. Tail abnormalities..48
3.1.1.7. Yolk-sac abnormalities..48
3.1.1.8. Pericardial edema 49
3.1.1.9. Hemorrhages 50
3.1.1.10. Muscular involuntary contractions 50
3.1.1.11. Total abnormalities..51
3.1.1.12. Heart rate 52
3.1.2. Sea urchin embryos bioassay...52
3.1.2.1. Larval length and abnormalities 52
3.2. Methyl-triclosan 54
3.2.1. Zebrafish embryos bioassay 54
3.2.1.1. Cumulative mortality...54
3.2.1.2. Abnormal cell growth...55
3.2.1.3. Embryo development delay..55
3.2.1.4. Hatching rate 56
3.2.1.5. Head and eyes abnormalities...56
3.2.1.6. Tail abnormalities..57
3.2.1.7. Yolk-sac abnormalities..57
3.2.1.8. Pericardial edema and hemorrhages..58
3.2.1.9. Muscular involuntary contractions 59
3.2.1.10. Total abnormalities..59
3.2.1.11. Heart rate 60

3.2.2. Sea urchin embryos bioassay 61
3.2.2.1. Larval length and abnormalities 61
3.3. Perfluorooctane sulfonate (PFOS) 62
3.3.1. Zebrafish embryos bioassay.. 62
3.3.1.1. Cumulative mortality 62
3.3.1.2. Abnormal cell growth and embryo development delay 62
3.3.1.3. Hatching rate 63
3.3.1.4. Head abnormalities.. 63
3.3.1.5. Eyes abnormalities 63
3.3.1.6. Tail and yolk-sac abnormalities.. 64
3.3.1.7. Pericardial edema and hemorrhages 64
3.3.1.8. Muscular involuntary contractions.. 64
3.3.1.9. Total abnormalities and heart rate 65
3.3.2. Sea urchin embryos bioassay 65
3.3.2.1. Larval length and abnormalities 65
3.4. Perfluorooctanoic acid (PFOA) 67
3.4.1. Zebrafish embryos bioassay.. 67
3.4.2. Sea urchin embryos bioassay 67
3.4.2.1. Larval length and abnormalities 67
3.5. Perfluorobutane sulfonate (PFBS).. 69
3.5.1. Zebrafish embryos bioassay.. 69
3.5.2. Sea urchin embryos bioassay 69
3.5.2.1. Larval length and abnormalities 69
3.6. Perfluorobutanoic acid (PFBA)... 71
3.6.1. Zebrafish embryos bioassay.. 71
3.6.2. Sea urchin embryos bioassay 71
3.6.2.1. Larval length and abnormalities 71
3.7. Perfluorodecanoic acid (PFDA)... 72
3.7.1. Zebrafish embryos bioassay.. 72

3.7.1.1. Cumulative mortality ... 72

3.7.1.2. Abnormal cell growth and embryo development delay ... 72

3.7.1.3. Hatching rate ... 73

3.7.1.4. Head and eyes abnormalities ... 73

3.7.1.5. Tail and yolk-sac abnormalities ... 73

3.7.1.6. Pericardial edema and hemorrhages ... 74

3.7.1.7. Muscular involuntary contractions ... 75

3.7.1.8. Total abnormalities and heart rate ... 75

3.7.2. Sea urchin embryos bioassay ... 76

3.7.2.1. Larval length and abnormalities ... 76

3.8. Perfluoroundecanoic acid (PFUnA) ... 77

3.8.1. Zebrafish embryos bioassay ... 77

3.8.1.1. Cumulative mortality ... 77

3.8.1.2. Abnormal cell growth and embryo development delay ... 77

3.8.1.3. Early hatching and hatching rate ... 78

3.8.1.4. Head and eyes abnormalities ... 78

3.8.1.5. Tail and yolk-sac abnormalities ... 79

3.8.1.6. Pericardial edema and hemorrhages ... 79

3.8.1.7. Muscular involuntary contractions ... 80

3.8.1.8. Total abnormalities and heart rate ... 80

3.8.2. Sea urchin embryos bioassay ... 81

3.8.2.1. Larval length and abnormalities ... 81

4. Discussion ... 83

5. Conclusion and future perspectives ... 93

6. References ... 97

List of Figures

Figure 1 - Chemical structure of TCS and its methylation by-product M-TCS. 22

Figure 2 - Environmental fate of Perfluorinated compounds. 24

Figure 3 - Some examples of Perfluorinated compounds. In bold are the Perfluoroalkyls acids selected for the present work. 25

Figure 4 - Perfluorooctane sulfonate (PFOS) chemical structure. 26

Figure 5 - Chemical formula of PFAAs.. 27

Figure 6 - Perfluorooctanoic acid (PFOA) chemical structure... 27

Figure 7 - Perfluorobutane sulfonate (PFBS) and Perfluorobutanoic acid (PFBA) chemical structures. 29

Figure 8 - Perfluorodecanoic acid (PFDA) chemical structure.. 30

Figure 9 – Perfluoroundecanoic acid (PFUnA) chemical structure. 31

Figure 10 - Sea urchin (*Paracentrotus lividus*). 36

Figure 11 - (A) Sea urchin adults dissected (male and female, respectively). (B) Sea urchin eggs *post* fertilization.. 37

Figure 12 - Experimental design for sea urchin bioassay.. 38

Figure 13 – Zebrafish (*Danio rerio*).. 39

Figure 14 – Representation of zebrafish breading box.. 40

Figure 15 – Experimental design for zebrafish bioassay. 41

Figure 16 – Zebrafish embryonic development.. 41

Figure 17- Cumulative mortality rates (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B)............... 45

Figure 18 - Abnormal cell growth at 8 hpf (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B)........... 46

Figure 19 - Embryo development delay at 32 hpf (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h..46

Figure 20 - Hatching rate at 80 hpf (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B).. 47

Figure 21 - Head abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). 47

Figure 22 - Eyes abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). 48

Figure 23 - Tail abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). 48

Figure 24 - Yolk-sac abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). 49

Figure 25 - Pericardial edema abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B)...49

Figure 26 - Hemorrhages (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). 50

Figure 27 - Muscular involuntary contractions at 144 hpf (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B)...50

Figure 28 - Total abnormal embryos (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). 51

Figure 29 - *D. rerio* at 144 hpf in the control group (A) and exposed to 400 µg/L of the disinfectant Triclosan (B). The black arrows point the malformations on embryos' yolk-sac and pericardial edemas...51

Figure 30 - Heart rate (bpm) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). 52

Figure 31 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of the disinfectant triclosan for 48h. 53

Figure 32 – *P. lividus* at 48 hpf in the control group (A), and exposed to 400 µg/L (B) and 1000 µg/L of triclosan. 53

Figure 33 - Cumulative mortality rates (%) of *D. rerio* exposed to different concentrations of methyl –triclosan for 144 h in the first assay (A) and second assay (B). 54

Figure 34 - Abnormal cell growth at 8 hpf (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). 55

Figure 35 - Embryo development delay at 32 hpf (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B)..55

Figure 36 - Hatching rate at 80 hpf (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). 56

Figure 37 - Head and eyes abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). 56

Figure 38 - Tail abnormalities (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). 57

Figure 39 - Yolk-sac abnormalities (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). 57

Figure 40 – Pericardial edema (%) of *D. rerio* exposed to diferent concentrations of methyl-triclosan for 144 hpf in the first assay (A) and second assay (B). 58

Figure 41 - Hemorrhages (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 hpf in the first assay (A) and second assay (B). 58

Figure 42 - Muscular involuntary contractions (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). 59

Figure 43 - Total abnormal embryos (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). 59

Figure 44 - *D. rerio* at 144 hpf in the control group (A), exposed to 400 µg/L (B) and 1000 µg/L (C) of methyl-triclosan. The black arrows point the embryos malformations. 60

Figure 45 - Heart rate (bpm) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). 60

Figure 46 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of methyl-triclosan for 48h. 61

Figure 47 – *P. lividus* at 48 hpf in the control group (A) and exposed to 1000 µg/L of methyl-triclosan (B). 61

Figure 48 - Cumulative mortality rates (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h. 62

Figure 49 - Abnormal cell growth at 8 hpf and embryo development delay at 32 hpf (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h (A and B, respectively). ..62

Figure 50 - Hatching rate at 80 hpf (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h 63

Figure 51 - Head abnormalities (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h. 63

Figure 52 -Tail and yolk-sac abnormalities (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h (A and B, respectively). 64

Figure 53 - Pericardial edema and hemorrhages (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h (A and B, respectively)..64

Figure 54 - Total abnormal embryos (%) and heart rate (bpm) of *D. rerio* exposed to different concentrations of PFOS for 144 h.. 65

Figure 55 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFOS for 48h. 65

Figure 56 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFOA for 48h. 66

Figure 57 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFBS for 48h..................................68

Figure 58 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFBA for 48h..................................70

Figure 59 - Cumulative mortality rates (%) of *D. rerio* exposed to different concentrations of PFDA for 144 h. 72

Figure 60 - Abnormal cell growth at 8 hpf and embryo development delay at 32 hpf (%) of *D. rerio* exposed to different concentrations of PFDA for 144 h (A and B, respectively)...72

Figure 61 - Hatching rate at 80 hpf (%) of *D. rerio* exposed to different concentrations of PFDA for 144 h (A and B, respectively). 73

Figure 62 - Tail and yolk-sac abnormalities (%) of *D. rerio* exposed to different concentrations of PFDA for 144 h (A and B, respectively). 74

Figure 63 – *D. rerio* embryos at 144 hpf exposed to 10000 µg/L of PFDA. The black arrow points the abnormal embryo, exhibiting spinal curvature. 74

Figure 64 - Pericardial edema and hemorrhages (%) of *D. rerio* exposed to different concentrations of PFDA for 144 h (A and B, respectively). 75

Figure 65 – Total abnormal embryos (%) and heart rate (bpm) of *D. rerio* exposed to different concentrations of PFDA for 144 h (A and B, respectively)..................................75

Figure 66 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFDA for 48h. 76

Figure 67 - Cumulative mortality rates (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h...77

Figure 68 - Abnormal cell growth at 8 hpf and embryo development delay at 32 hpf (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h (A and B, respectively). 77

Figure 69 – Early hatching at 24 hpf and hatching rate at 80 hpf (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h (A and B, respectively)...............................78

Figure 70 – *D. rerio* embryos at 32 hpf exposed to 10000 µg/L of PFUnA. The black arrow points the early hatched embryo...78

Figure 71 - Head abnormalities (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h...79

Figure 72 - Tail and yolk-sac abnormalities (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h (A and B, respectively). 79

Figure 73 - Pericardial edema and hemorrhages (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h (A and B, respectively). 80

Figure 74 - Total abnormal embryos (%) and heart rate (bpm) of *D. rerio* exposed to different concentrations of PFUnA for 144 h (A and B, respectively). 80

Figure 75 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFUnA for 48h. 81

Figure 76 - *P.lividus* at 48 hpf in the control group (A) and exposed to 1000 µg/L of PFUnA (B)..81

List of Tables

Table 1 – References of the chemicals tested. 35

Table 2 - Artificial seawater composition. 35

Table 3 - Effects of PFOA exposure in zebrafish embryos at 144 hpf. Data are expressed as mean ± se (n=8). 67

Table 4 - Effects of PFBS exposure in zebrafish embryos at 144 hpf. Data are expressed as mean ± SE (n=8). 69

Table 5 - Effects of PFBA exposure in zebrafish embryos at 144 hpf. Data are expressed as mean ± SE (n=8). 71

Table 6 – Overview of the overall results, highlighting the significant effects ($p<0.05$) on both model embryo bioassays, for each compound. 82

Table 7 - Overview of the NOECs and LOECs reported in this study (µg/L). 82

Table 8 – Comparison of the LOEC values reported on this study and maximal concentrations on surface waters reported on literature. 91

List of Abbreviations and acronyms

µg Microgram

bpm Beats Per Minute

CECs Contaminants of Emerging Concern

DMSO Dimethylsulfoxide

EC_{50} Effect Concentration for 50% of the population

ECF Electrochemical fluorination

g Gram

hpf Hours Post Fertilization

LC_{50} Lethal Concentration for 50% of the population

LOEC Lowest Observed Effect Concentration

log K_{oc} Soil Organic Carbon-Water Partition Coefficient

log K_{ow} Octanol-Water Partition Coefficient

mg Milligram

M-TCS Methyl-triclosan

ng Nanogram

NOEC No Observed Effect Concentration

OECD Organization for Economic and Co-operation and Development

PCP Personal Care Products

PFAAs Perfluoroalkyls Acids

PFBA Perfluorobutanoic acid

PFBS Perfluorobutanesulfonic acid

PFCAs Perlfuoroalkyls carboxyls Acids

PFCs Perfluorinated Compounds

PFDA Perfluorodecanoic acid

PFOA Perfluorooctanoic acid

PFOS Perfluorooctane Sulfonate

PFPAs Perfluoroalkyls Phosphonates Acids

PFSAs Perfluoroalkyl Sulfonates Acids

PFUnA Perfluoroundecanoic acid

TCS Triclosan

U.S. EPA United States Environmental Protection Agency

VP Vapor Pressure

WWTP Wastewater Treatment Plants

INTRODUCTION AND OBJECTIVES
CHAPTER I

1. Introduction

1.1. Contaminants of emerging concern (CECs) in the environment

Nowadays it is acknowledged that the Wastewater Treatment Plants (WWTP) stations are not fully efficient when it comes to the removal of compounds present in the waters. Hence, a large group of chemicals will ultimately reach the aquatic environment. Although most of these chemicals are present at low concentrations, there is a paucity of data on the effects of low exposure doses and mixture effects, thus sorting them as emerging contaminants.

The term “emerging” is applied to compounds present in the waters on which very little is known about potential impact in the environment (Deblonde *et al.*, 2011). Moreover, data on its toxicity and potential risk is scarce or still unknown. Such chemicals have been a worldwide concern, not only for human health but also for the ecosystems.

1.1.1. Personal and care products: triclosan (TCS) and methyl-mriclosan (M-TCS) metabolite

The so called Personal Care Products (PCPs) are one of the groups that raise major concerns since it encompasses a large number of compounds that are produced for external use. The increase use of PCPs together with the inefficiency of the WWTP stations to complete removal of some of these chemicals, has been rising the levels of these compounds in the environment. Hence, an increase number of studies focus on their occurrence in the water systems. Yet, there is a lack of knowledge about its toxicity to non-target organisms (Brausch and Rand, 2011).

This is the case for the disinfectant triclosan (TCS) (Figure 1) which has been in use for over 40 years (Dann and Hontela, 2011; Pintado-Herrera *et al.*, 2014) in the most varied toiletries such as toothpaste, soaps, skin care products and also in many other industries as textile and plastic industries (Bedoux *et al.*, 2012; Rüdel *et al.*, 2013).

As reviewed in 2011 by Brauch and Rand, this disinfectant in one of the most detected compounds in the WWTP, although there is still a lack of data on possible effects in non-targeted organisms and the underlying mechanism(s) of action. Hence, there is a need to improve risk assessment of this compound.

Triclosan is an antimicrobial agent, more specifically a bactericide, which has the ability of inhibiting the fatty acids synthesis in the lipid membrane, preventing the cell from growing (Russel, 2004). As it is produced to be released in its parental form, this compound usually do not suffers any metabolic alterations thus entering the environment, increasing the levels in the water stations and surface waters. Although the removal rate of TCS in the WWTPs is around 80% (Deblonde *et al.*, 2011), it still has been detected in more than half

of the surface waters analyzed (Brausch and Rand, 2011) including in Portugal (Lygina *et al.*, 2013).

Triclosan is an organochlorine compound and due to its chemical properties, bioacumulates (Figure 1). Once entering the WWTP stations, triclosan can be chemically transformed, resulting mostly chlorophenols (Bedoux *et al.*, 2012) or biologically transformed, being metabolized into a more persistent, lipophilic and non-photodegradable byproduct known as methyl-triclosan (M-TCS) (Figure 1) (Balmer *et al.*, 2004; Heidler and Halden, 2007; Bedoux *et al.*, 2012). As reviewed by Bedoux in 2012, M-TCS can be produced in major quantities when biodegradation of TCS occurs in soil.

Regarding TCS and M-TCS occurrence in the water systems it is known that the metabolite is much less prevalent than the parental compound. While TCS concentrations in surface waters where detected up to 22 µg/L in treated water in Spain (Agüera *et al.*, 2003), or even more recently up to 5,16 µg/L in Vellar, India (Ramaswamy *et al.*, 2011), M-TCS has been detected up to 190 ng/L in Cadiz, Spain (Pintado-Herrera *et* al., 2014). Although its occurrence is much less noticed, its hydrophobic characteristics and persistence in the environment along with the scarcity of data, highlights the need for additional research on its ecotoxicity.

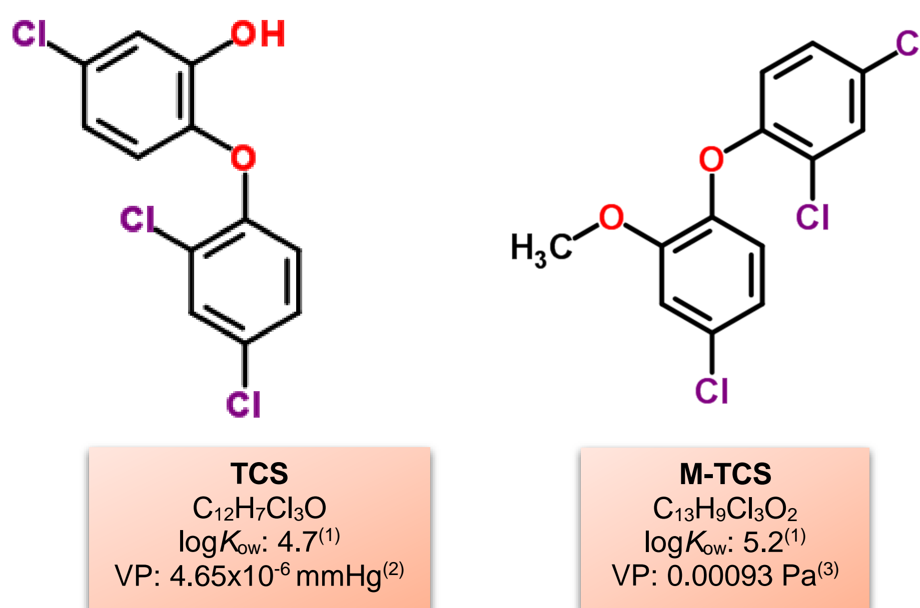

Figure 1 - Chemical structure of TCS and its methylation by-product M-TCS.
Source: CSID:5363, http://www.chemspider.com/Chemical-Structure.5363.html (accessed 21:58, May 11, 2015); CSID:545009, http://www.chemspider.com/Chemical-Structure.545009.html (accessed 21:57, May 11, 2015).[1]Boehmer *et al.*, 2004; [2]Ying *et al.*, 2007; [3]Chen *et al.*, 2011.

1.1.1.1. State of knowledge on triclosan and methyl-triclosan toxicity

Many studies have been conducted in order to better understand the mechanism of action and the No Observed Effect Concentration (NOEC) of this bactericide. In *Scenedesmus vacuolatus*, triclosan inhibited cell reproduction at 1.9 µg/L (Franz *et al.*, 2008). A recent study with the sea urchin *Strongylocentrotus nudus*, showed that triclosan affected reproduction and embryonic development of *pluteus* larva at 113 µg/L (Hwang *et al.*, 2014). On zebrafish, triclosan was lethal to 50% of the embryos at 420 µg/L (Oliveira *et al.*, 2009) and significantly decreased heart rate of embryos at 20 ng/L and at 100 µg/L (Schmidt *et al.*, 2013). Also in Japanese medaka, an 8 days exposure to triclosan at 0.17mg/L, decreased the swimming velocity of the fish (Nassef *et al.*, 2010).

Gaume *et al.* (2012), in an *in vivo* study showed that not only TCS had an inhibiting effect on cells but also M-TCS reveled to be toxic to hemocytes of *Haliotis tuberculata*, at low concentration range. Other studies have been conducted on M-TCS toxicity on algae (Batscher, 2006b) and bacteria (Farré *et al.*, 2008; Villa *et al.*, 2014) and only one conducted on the invertebrate *Daphnia magna (*Batscher, 2006a). To our knowledge, no other studies have been reported on aquatic organisms.

Taking all these data into account, there is the need for research on this subject, especially on M-TCS toxicity. Given the sensitivity of the embryonic development of model fish zebrafish (*Danio rerio*) and the invertebrate sea urchin (*Paracentrotus lividus*) to a range of contaminants, including TCS, make them ideal models to investigate the toxicity of TCS and M-TCS.

1.1.2. Synthetic chemicals: perfluorinated compounds (PFCs)

1.1.2.1. Background and applications

Perfluorinated compounds (PFCs) are synthetic chemicals produced by electrochemical fluorination (ECF) or telomerization and have been used for over 60 years (Simcik, 2005; Lindstrom *et al.*, 2011; Lau, 2012). They encompass a large number of chemicals useful in the most varied industries, mainly in textile industries where they are used as repellants for carpets or clothes (Ulhaq *et al.*, 2013a). Moreover they can be used as surfactants and lubricants, in paints, fire-fighting foams, food packaging, floor polishes, in some products of personal care such as shampoos, cosmetics and also as pesticides, among many others (Lindstrom *et al.*, 2011).

Their great applicability has led to environment contamination of several aquatic ecosystems (Figure 2). Hence, in 2009 the Stockholm convention has listed some of these compounds such as Perfluorooctanesulfonic acid (PFOS) (Figure 4) and other derivatives in Annex B for Persistent Organic Pollutants (POPs), restricting the use of these chemicals

by 2010 and complete elimination by 2015 (US EPA, 2009). Given that in 2010 some PFCs were discontinued (Hagenaars *et al.*, 2011), other homologues with shorter carbon chains have been used in large quantities as alternative and have been released to the environment. Although some studies revealed that these new homologues have low adsorption potential, they seem to be very persistent and more mobile than the original ones (Zhou *et al.*, 2013).

Non-target organisms are continuously exposed to these chemicals which are discharged in the waters through WWTPs (Ulhaq *et al.*, 2013) (Figure 2). Very limited data is available concerning the potential impact of some of these new derivatives to the ecosystems and so it is urgent to deepen knowledge on this subject.

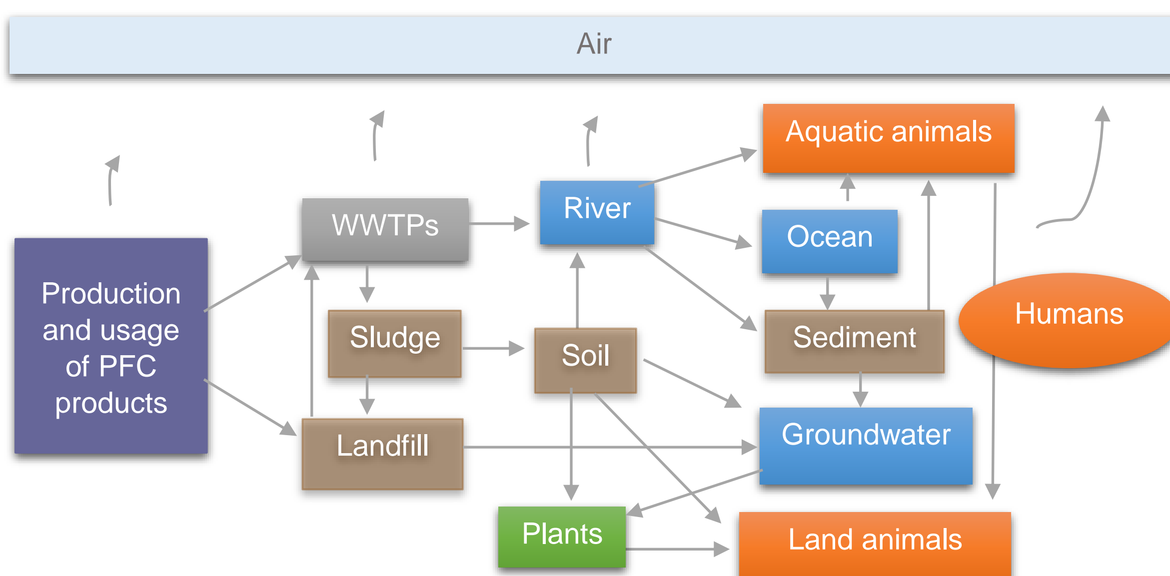

Figure 2 - Environmental fate of Perfluorinated compounds. Adapted from Ahrens, 2011.

The PFCs class encompass a large number of sub-classes. Perfluoroalkyls acids (PFAAs) belong to the PFCs class and include about 30 environmentally relevant chemicals (Lau, 2012). There are 5 different subfamilies within the PFAAs, distinguished by their functional group which can be a carboxylic, sulfonic, phosphonic, sulfinic or phosphinic acid (Schedin, 2013).The main sub-families which have gained more attention are the Perfluoroalkyl sulfonates acids (PFSAs) and Perlfuoroalkyls carboxyls acids (PFCAs) and more recently the Perfluoroalkyls phosphonates acids (PFPAs) (Figure 3) (Lau *et al.*, 2012).

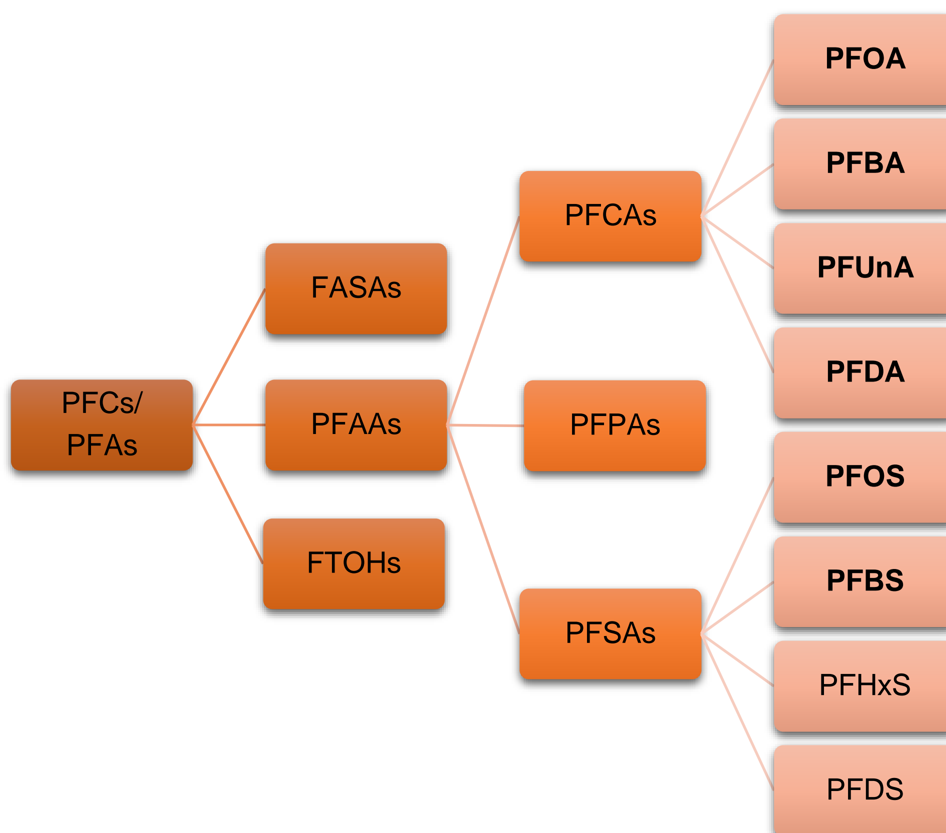

Figure 3 - Some examples of Perfluorinated compounds. In bold are the Perfluoroalkyls acids selected for the present work.

1.1.2.2. PFCs chemical properties

Perfluorinated compounds are organic substances whose structure usually consists in a (4 - 14) carbon chain where all hydrogen molecules in the carbon chain were substituted with fluorines (Figure 4). They are extremely chemically stable due to their strong carbon-fluorine bonds. Their distinctive hydrophobic and lipophobic properties allow them to repel oil and water (Lau *et al.*, 2007). Also, they are nonflammable, non-reactive, and hardly degraded, possibly bioaccumulating and consequently persisting in the environment (Lindstrom *et al.*, 2011). Furthermore, some of these substances have long-range transport in the waters due to their ionic nature (Sinclair and Kannan, 2006), and so, after discharges, the PFCs can easily reach rivers, soils, ground waters, oceans, and consequently affect the aquatic and land life, including humans (Figure 2).

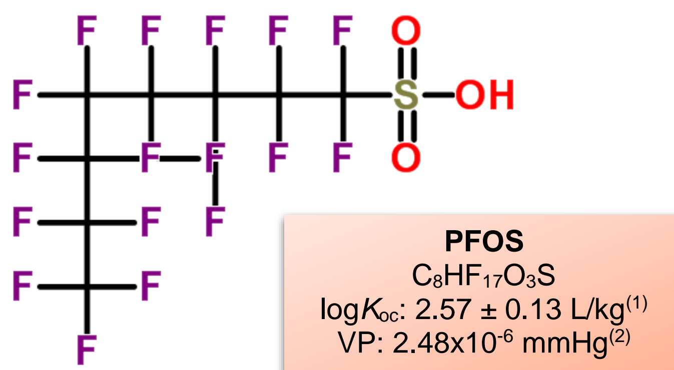

Figure 4 - Perfluorooctane sulfonate (PFOS) chemical structure. Source: CSID:67068, http://www.chemspider.com/Chemical-Structure.67068.html (accessed 12:04, May 20, 2015). (1)Higgins and Luthy, 2006; (2)3M, 2008c.

1.1.2.3. State of knowledge on the PFAAs selected for the present study

Perfluoroalkyls acids (PFAAs) is the main group responsible for studies conducted on Perfluorinated compounds distribution, occurrence or toxicity. They are extensively used, mostly due to their unique chemical properties.

They have been classified as chemicals of concern and after the decreased production of PFOA and PFOS, several other substitutes have been released in large quantities to the environment (Lau, 2012).

One of the main problems is the possibility of increased toxicity of these chemicals when in mixture. In 2002, after an accidental release of fire-fighting foam in Canada, the PFCs concentration in the surface waters reached up to 17mg/L (Moody *et al.*, 2002). Moreover, in west coast of Korea in 2012, concentrations of several PFAAs from the estuarine and coastal area were detected up to 130 ng/L (Hong *et al.*, 2015). In China, the total concentrations of PFAAS was measured, reaching values of 70.4 µg/L (Zhou *et al.*, 2013).

Some long-chained PFAAs (e.g. PFOS) have a high potential for bioaccumulation and biomagnification along the trophic chain (Kannan *et al.*, 2005; Hong *et al.*, 2015). Furthermore, PFAAs are usually extremely resistant to high temperatures, photolysis or even microorganisms, due to their strong fluorine-carbon chain (Schedin, 2013). Figure 5 displays the perlfuoroalkyl compounds chemical formula.

Regarding their distribution, PFAAs are ubiquitous in the environment, being already found in some remote areas such as the Artic or in Antarctica (Butt *et al.*, 2010; Benskin *et al.*, 2012; Cai *et al.*, 2012). Lau (2012) reviewed two possibilities for PFAAs worldwide distribution: atmospheric transport of PFAAs or simply a long-range transport of the PFAAs along the water systems, thus reaching isolated areas.

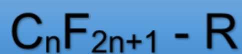

Figure 5 - Chemical formula of PFAAs. **n** – number of carbon atoms; **R**- functional group. Schedin, 2013.

1.1.2.3.1. Perfluorooctane sulfonate (PFOS) and perfluorooctanoic acid (PFOA)

Perfluorooctane sulfonate (PFOS) (Figure 4) and Perfluorooctanoic acid (PFOA) (Figure 6) are an 8 carbon long chained PFAAs, with a sulphonic and carboxylic acid as function group, respectively. In industry they were used mainly as byproducts of other fluorochemicals (Simcik, 2005).

They gained much attention due to their useful characteristics and so, countless studies were conducted on their occurrence and toxicity. Regarding their occurrence in surface waters, before regulation, PFOA concentrations ranged from <25 to 598 ng/L while PFOS ranged from 16.8 to 144 ng/L in Tennessee (Hansen *et al.*, 2002). In Japan, PFOA and PFOS concentrations reached up to 67000 and 526 ng/L, respectively (Nakayama *et al.* 2004).

Although their producing has been reduced after regulation, their bioaccumulative and persistent properties still raise concern on its possible impact in the organisms. Houde *et al.* (2011) reviewed that PFOS was still the major PFC found in animal tissues. Furthermore, PFOS and PFOA were found in artic species tissues, evidencing their worldwide distribution (Butt *et al.*, 2010).

More recently, in Liaoning, China, concentrations up to 31 and 82 ng/l of PFOS and PFOA, respectively, where detected in surface waters (Wang *et al.*, 2012). Also, in Tangxun Lake, China, concentrations of PFOS and PFOA in surface waters of up to 21.3 and 26.3 µg/L, respectively (Zhou *et al.*, 2013).

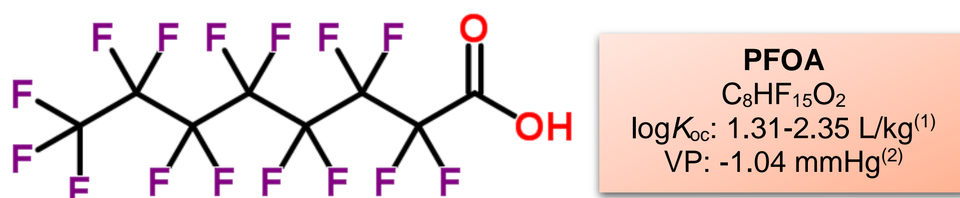

Figure 6 - Perfluorooctanoic acid (PFOA) chemical structure.
Source: CSID:9180, http://www.chemspider.com/Chemical-Structure.9180.html (accessed 12:02, May 20, 2015). [(1)]Dekleva, 2003; [(2)]Bhhatarai and Gramatica, 2010.

Regarding toxicity studies on these two chemicals, several studies have been conducted recently on zebrafish embryos (Shi *et al.*, 2008; Huang *et al.*, 2010; Hagenaars *et al.*, 2011; Zheng *et al.*, 2012; Ding *et al.*, 2013; Ulhaq *et al.*, 2013a,b; Hagenaars *et al.*, 2014). PFOS appears to be more toxic than PFOA possibly due to the presence of a sulphonic functional group (Zheng *et al.*, 2012; Ulhaq *et al.*, 2013a). Hagenaars *et al.* (2011) detected an increase in the fish heart rate above 0.5 mg/L for PFOS and above 75 mg/L for PFOA, with a significant decrease at 250 mg/L. Furthermore, PFOA significantly delayed hatching above 100 mg/L. In Zheng *et al.* (2012) study, PFOS Lowest Observed Effect Concentration (LOEC) for malformations on zebrafish embryos was established at 12.5 mg/L and at 6.5 mg/L for hatching delay at 72 hpf.

Moreover, PFOS along with perfluorobutane sulfonate (PFBS) and Perfluorodecanoic acid (PFDA) seemed to increase swimming speed of zebrafish larvae in comparison with other PFAAs (Ulhaq *et al.*, 2013b). More recently, PFOS significantly reduced the swim bladder, caused spinal curvature and reduced zebrafish larvae length above 2.5 mg/L (Hagenaars *et al.*, 2014).

Concerning studies on invertebrates, PFOA exposure during 48h had an Effect Concentration on 50% of *Daphnia magna* and *Chydorus sphaericus* population (EC_{50}) at 211.6 and 348.7 mg/L, respectively (Ding *et al.*, 2012).

Sea urchin embryos have also been used as ecotoxicological models for studying the effects of some PFCs (Anselmo *et al.*, 2011; Mhadhbi *et al.*, 2012; Gunduz *et al.*, 2013). *Paracentrotus lividus* seems to be relatively sensitive to these particular compounds, showing an increasing number of malformations on the larvae above 0.5 mg/L of PFOS (Gunduz *et al.*, 2013) and a LOEC for PFOS and PFOA of 2 and 20 mg/L, respectively (Mhadhbi *et al.*, 2012). Moreover, Anselmo *et al.*, 2011 detected a slight acceleration on larvae development 9 days *post*-fertilization (dpf) at 371.6 µg/L.

The fact that the potential for bioaccumulation of PFAAs in costal organisms has not been study in detail (Hong *et al.*, 2015) along with the lack of data on the potential toxicity to marine species (Mhadhbi *et al.*, 2012; Gunduz *et al.*, 2013) make this subject of concern.

All these previous studies were conducted with concentrations above environmental relevance. However, since these chemicals are extremely stable and not easily degraded in the environment (Lindstrom *et al.*, 2011), they may bioaccumulate, hence it is urgent to understand their impact. Chronic exposure and biomagnification of these chemicals are of concern as well since they can potentially affect not only the organisms but possibly their offspring (Kannan *et al.*, 2005).

1.1.2.3.2. Perfluorobutane sulfonate (PFBS) and perfluorobutanoic acid (PFBA)

The short-chained perfluorobutane sulfonate (PFBS) and perfluorobutanoic acid (PFBA) have a skeleton constituted by 4 carbons and have been considered the main substitutes of PFOS and PFOA, respectively (Figure 7). Their toxicity seems to be much less noticed due to their short carbon chain length (Shi *et al.*, 2008; Hagenaars *et al.*, 2011; Zheng *et al.*, 2012). Yet, due to their exponential production increase, they are much more prevalent than PFOS and PFOA (Zhou *et al.*, 2013), although the information on their potential toxicity is not well established.

In river Rhine the concentrations of PFCs were measured and PFBA was dominant, reaching concentrations up to 335 ng/L followed by PFBS at 181 ng/L (Möller *et al.*, 2010). In China, concentration of PFBA and PFBS were prevailing, reaching up to 47.8 and 15.3 μg/L, respectively (Zhou *et al.*, 2013).

Concerning toxicity assessment, it is known that these chemicals are not as toxic as the long-chained PFAAs. A study conducted on zebrafish embryos found an EC_{50} of 450 mg/L and 2200 mg/L for PFBS and PFBA, respectively (Ulhaq *et al.*, 2013a). Moreover, PFBS showed reduced zebrafish heart rate at 3000 mg/L (Hagenaars *et al.*, 2011).

In *Daphnia magna* the EC_{50} at 48h for PFBA was reached at a concentration of 181.5 mg/L. As for *Chydorus sphaericus*, the EC_{50} value for PFBA was only reached at 462.3 mg/L (Ding *et al.*, 2012).

PFBA and PFBS growing discharges in the environment along with their great mobility throughout the water systems is concerning. The fact that there is still a massive lack of data on the potential toxicity of these new substitutes on marine organisms makes urgent a detailed toxicity screening.

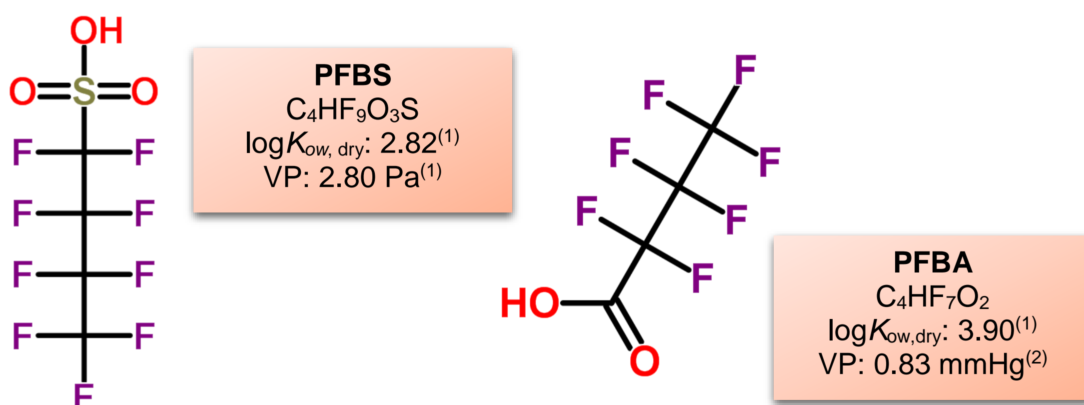

Figure 7 - Perfluorobutane sulfonate (PFBS) and Perfluorobutanoic acid (PFBA) chemical structures. Sources: PFBS: CSID:61132, http://www.chemspider.com/Chemical-Structure.61132.html (accessed 12:15, May 20, 2015). PFBA: http://www.chemspider.com/Chemical-Structure.9394.html (accessed 12:14, May 20, 2015). [1]Wang *et al.*, 2011; [2]Bhhatarai and Gramatica, 2010.

1.1.2.3.3. Perfluorodecanoic acid (PFDA) and perfluoroundecanoic acid (PFUnA)

Perfluorodecanoic acid (PFDA) (Figure 8) and perfluoroundecanoic acid (PFUnA) (Figure 9) are a 10 and 11-carbon chain perfluorocarboxylates (PFCAs), respectively. They are widely used in manufacturing of fluorinated polymers (Prevedouros *et al.*, 2006). Concerning their occurrence, it is known that these two PFAAs are much less prevalent in the waters in comparison with the previous four PFAAs. In Shenyan, China, PFUnA and PFDA were detected in Donghou River at 1.2 and 0.66 ng/L, respectively (Sun *et al.*, 2011). Also in Touchien, Taiwan, PFDA was found in concentrations of up to 58.2 ng/L (Lin *et al.*, 2009). Furthermore, concentrations of up to 15.4 and 3.52 ng/L were detected in west coast of Korea for PFDA and PFUnA, correspondingly (Naile *et al.*, 2010). Moreover, PFDA was found up to 160 ng/L in Conasauga River, USA (Knowick *et al.*, 2008) and more recently up to 1.2 ng/L in Australia (Thompson *et al.*, 2011).

As mentioned before, Ding *et al.*, 2012 assessed the toxicity of several Perfluorinated compounds on *Dapnia magna* and *Chydorus sphaericus* during 48h. In *D. magna*, a fifty percent inhibition effect at 163.5 mg/L for PFDA and 133.13 mg/L for PFUnA were reported. In *Chydorus sphaericus*, the fifty percent inhibition effect was detected at 45.2 mg/L for PFDA and 19.2 mg/L for PFUnA. This compounds show greater toxicity in comparison with PFBS and PFBA, possibly due to their longer C-F chains. To our knowledge, this is the only study assessing PFUnA toxicity in aquatic organisms.

As long-chained compounds, there is propensity to become more bioaccumulative in the organisms (Lindstrom *et al.*, 2011). However, there is a massive lack when it comes to their potential impact in the environment (Jo *et al.*, 2014). Some studies revealed that PFDA is in the range of PFOS toxicity (Ulhaq *et al.*, 2013a). Yet, toxicity data on these two compounds is very limited.

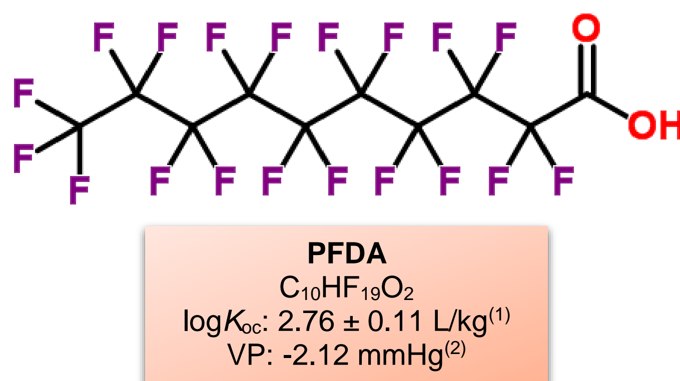

Figure 8 - Perfluorodecanoic acid (PFDA) chemical structure.
Source: CSID:9181, http://www.chemspider.com/Chemical-Structure.9181.html (accessed 12:10, May 20, 2015). [1]Higgins and Luthy, 2006; [2] Bhhatarai and Gramatica, 2010.

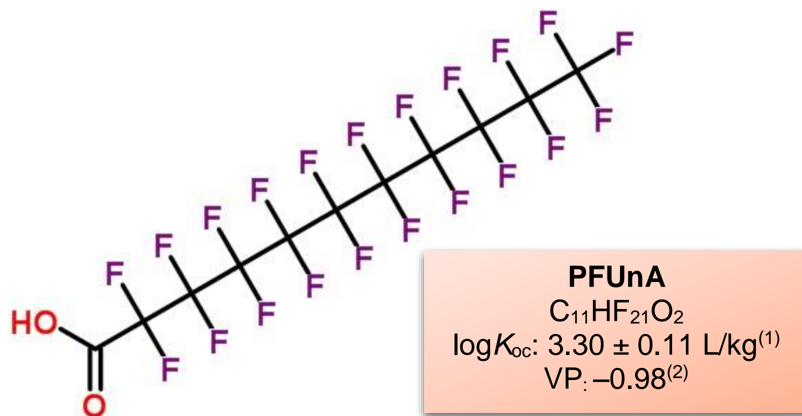

Figure 9 – Perfluoroundecanoic acid (PFUnA) chemical structure.
Source: CSID:69649, http://www.chemspider.com/Chemical-Structure.69649.html (accessed 12:08, May 20, 2015). (1)Higgins and Luthy, 2006; (2)Kaiser *et al*., 2005.

1.2. Aim of the study

In the present study we aimed to improve ecotoxicological information of several emerging contaminants on which available data is scarce. The selected compounds were the disinfectant triclosan and its metabolite methyl-Triclosan and also 6 perfluoroalkyls acids such as perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA), perfluorobutane sulfonate (PFBS), perfluorobutanoic acid (PFBA), perfluorodecanoic acid (PFDA) and perfluoroundecanoic acid (PFUnA). To achieve this goal, the well validated embryo development bioassays with zebrafish (*Danio rerio)* and sea urchin (*Paracentrotus lividus*) embryos were conducted.

MATERIALS AND METHODS

CHAPTER II

2. Materials and Methods

2.1. Tested chemicals

The chemicals tested in this study were obtained from Sigma-Aldrich Company (Table 1). All stock solutions were prepared in Dimethylsulfoxide (DMSO) obtained from Merck.

Table 1 – References of the chemicals tested.

Abbreviation	Name	CAS number
TCS	Triclosan	3380-34-5
M-TCS	Methyl-triclosan	4640-01-1
PFOS	Perfluorooctanesulfonic acid	2795-39-3
PFOA	Perfluorooctanoic acid	335-67-1
PFBS	Perfluorobutanesulfonic acid	375-73-5
PFBA	Perfluorobutanoic acid	375-22-4
PFDA	Perfluorodecanoic acid	335-76-2
PFUnA	Perfluoroundecanoic acid	2058-94-8

2.2. Artificial seawater

The artificial water was prepared according to Zaroogian *et al.* (1969) (Table 2). Sodium chloride and Sodium bicarbonate were both obtained from Merck, whereas Magnesium sulfate, Magnesium chloride hexahydrate and Calcium chloride were all obtained from Sigma-Aldrich.

Table 2 - Artificial seawater composition. Source: Zaroogian *et al.*, 1969

Molecular formula	Name	CAS number
$NaCl$	Sodium chloride	7647-14-5
$NaHCO_3$	Sodium bicarbonate	144-55-8
$MgSO_4$	Magnesium sulfate	7487-88-9
$MgCl_2 \cdot 6H_2O$	Magnesium chloride hexahydrate	7791-18-6
$CaCl_2$	Calcium chloride	10043-52-4

2.3. Test organisms

2.3.1. Sea urchin (*Paracentrotus lividus*)

Paracentrotus lividus is a benthic invertebrate, very common in the Mediterranean Sea (Tomas *et al*., 2004) and eastern Atlantic (Boudouresque and Verlaque, 2013; Jacinto *et al*., 2013). They usually live in seas ranging temperatures from 10-15ºC in winter to 18-25ºC in the summer (Boudouresque and Verlaque, 2013). In the adult stage they can reach about 7 cm of diameter (Boudouresque and Verlaque, 2013) (Figure 10).

Regarding reproduction, these organisms have a specific timing on gonadal growth, occurring once a year. It is stimulated by the photoperiod and the rising of temperature during the summer, usually between May and September (Byrne, 1990).

There is a growing economical relevance of this species in Europe. Furthermore it plays a very important ecologic role in preserving the balance of the ecosystems (Tomas *et al*., 2004).

Toxicity assessment on sea urchin embryonic development as become very important since these organisms seem to be very sensitive to several classes of chemicals (Pinsino *et al*., 2010; Hwang *et al*., 2014; Ribeiro *et al*., 2015). Furthermore, toxicological assays in this species are advantageous because of their short embryonic development. It takes 48h to reach larva *pluteus* stage (Ribeiro *et al*., 2015), allowing to perform a large number of assays in a short period of time and limited space facilities.

Figure 10 - Sea urchin (*Paracentrotus lividus*).
Source: Pixabay Database.
Available on: https://pixabay.com/pt/ouri%C3%A7o-do-mar-animal-natureza-597313/.

In our study, the organisms were all collected in intertidal areas in the Northem Portugal, Vila Nova de Gaia, Granja (N41º 2' 26,18'', W -8º 39' 2,24'') and transported to the laboratory in a refrigerator container.

Sea urchin reproduction was stimulated *in vitro* following Ribeiro *et al*., 2015 protocol. The organisms were dissected and the gametes evaluation was performed on a Nikon eclipse 50i microscope, selecting the female and male based on their eggs quality and sperm mobility, respectively. A viable couple was selected for each assay (Figure 11 A).

A considerable concentration of eggs was collected from the female and placed on 100 mL of artificial salt water and some sperm was added to the mixture. After slowly shaking to help fertilization success, it was determined the fertilization rate calculating the number of fertilized eggs in three drops of 10 µL of solution which is recognized by the appearance of an external membrane (Figure 11 B). The eggs were randomly distributed in the plate.

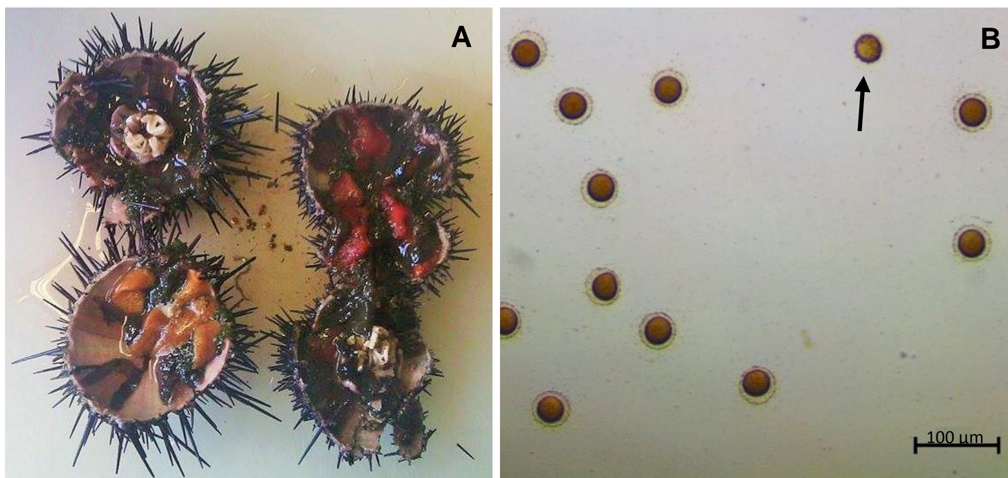

Figure 11 - (A) Sea urchin adults dissected (male and female, respectively). (B) Sea urchin eggs *post* fertilization (the arrow points to a non-fertilized egg).

2.3.1.1. Experimental design

Initially, two pairs of concentrations with a 2.5x dilution factor were tested (1000, 400 and 100, 40 µg/L) in order to cover a wide range of concentrations. Based on the results of the first assay, four additional concentrations (2.5x diluted from the 40 µg/L concentration) were tested in order to test environmentally relevant concentrations. Hence, we exposed sea urchin to eight different concentrations for each chemical: 1000; 400; 100; 40; 16; 6.4; 2.56 and 1.024 µg/L. All stock solutions were dissolved in Dimethylsulfoxide (DMSO) CAS number 2206-27-1 in order to obtain a final DMSO concentration of 0.01%. Finally the solutions were stored at 4°C.

The fertilized eggs were incubated for 48h in the dark at 20ºC in 24-well plates with a concentration of 20 eggs/mL/well. Overall, four independent replicate plates were run for each chemical, i.e., 16 replicates for control and solvent control and 8 replicates for each treatment (Figure 12). LOEC (Lowest Observed Effect Concentration) and NOEC (No Observed Effect Concentration) were determined.

Embryo observations were conducted in a Nikon Eclipse 5100T inverted microscope equipped with a Nikon D5-Fi2 digital camera. Randomly, fifteen larvae per well were observed two days after fertilization in the *pluteus* phase following previous protocols (Ribeiro *et al.,* 2015). The endpoints determined were larval abnormalities and the maximum larvae length which was measured by NIS-Elements version 4.13 image acquisition software.

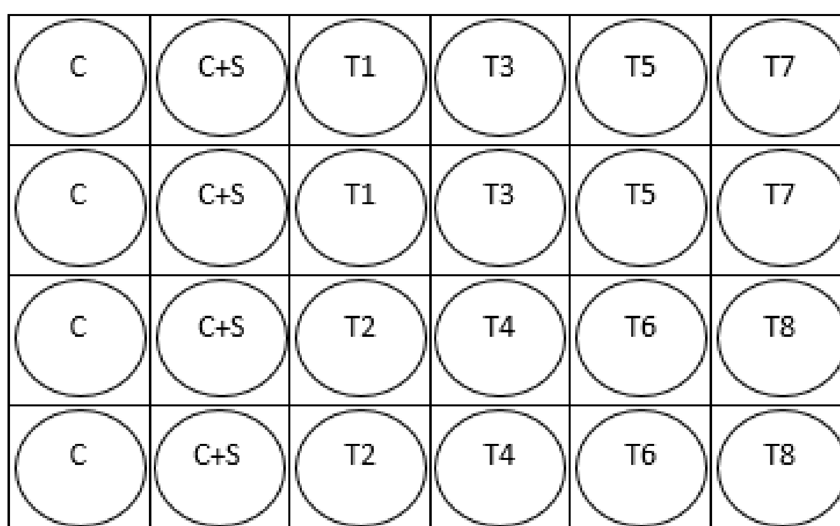

Figure 12 - Experimental design for sea urchin bioassay. C – Control; C+S – Solvent Control; T – Treatment.

2.3.2. Zebrafish (*Danio rerio*)

Zebrafish (*Danio rerio*) is a well-known vertebrate tropical fish. It is native from Asia and usually dwells in streams (Engeszer *et al*., 2007) (Figure 13). In spawning conditions, the adults move into muddy and vegetated areas with stagnant waters to release the eggs (Engeszer *et al*., 2007). When mature, the female and male are easily distinguished by the prominent abdomen of the female.

It has been used for the past two decades as model species in different areas of research. Apart from being cost-effective, the adults are easily obtained and maintained in laboratory conditions. Furthermore, the adults can breed all year around, producing a large number of eggs per spawning, 200-300 per couple (Hill *et al*., 2005). The short embryonic development is also useful, 72h to reach early larva stage (Kimmel *et al*., 1995), allowing to

perform several toxicity assays in a short period of time. In addition, the small size and transparency of the eggs enables the incubation in microplates and the observations of possible phenotypic changes through the embryos chorion, being easily manipulated (Segner *et al.*, 2009). All these benefits make this species ideal for an acute toxicity assay.

Figure 13 – Zebrafish (*Danio rerio*).
Source: Wikimedia Commons repository.
Available on: https://commons.wikimedia.org/wiki/File:Zebrafisch.jpg.

The zebrafish stock was obtained from local suppliers in Singapure and kept in laboratory in a 160L aquarium with dechlorinated water at 28ºC ± 1, pH 8.0 ± 0.5 with a system of recirculation and water renewal passing through mechanical and biological filters. The fish were maintained with a photoperiod of 14/10h (light/dark).The stock was fed with TetraMin® feed four times daily by an automatic feeder and supplemented with *Artemia*.

For the zebrafish reproduction, 5 females and 10 males, were isolated in a breeding box the day before reproduction. It has been described that zebrafish females prefer gravel substrates to spawn, producing higher quality eggs (Spence *et al.*, 2007). Therefore, the breading box contained marbles with a net bellow, thus mimicking the gravel substrate (Figure 14). Furthermore, the marbles with the net allowed the eggs to pass and settle in the bottom of the aquarium, thus preventing the adults to reach the eggs, avoiding acts of cannibalism typical of this species.

In the following day the eggs were collected and cleaned one hour and a half after the light switch on. The eggs' quality was verified through a magnifier and fertilized eggs were selected for the assay and distributed in the microplates within three hours after fertilization.

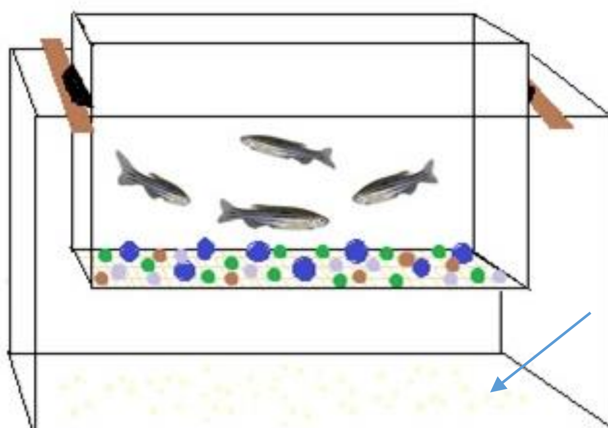

Figure 14 – Representation of zebrafish breading box. The blue arrow points the location of the eggs.

2.3.2.1. Experimental design

For zebrafish exposure assays to PFAAs we selected four concentrations, 10000, 1000, 100 and 10 µg/L. These values were chosen based in the literature (Ulhaq *et al.*, 2013a) following a similar rational to that previously described for sea urchin assay. Only for triclosan the concentrations tested were different: 1000,100, 10 and 1 µg/L and then dilutions of 2.5 times were also performed to refine NOEC and LOEC (Oliveira *et al.*, 2009). Finally, due to the limited number of toxicity studies performed on methyl-triclosan we chose to perform 10x dilutions as in the PFAAs assays and then dilutions of 2.5 times as in TCS assay to refine NOEC.

The eggs were distributed into 24-well plate (10 eggs per well) and 16 replicates for each treatment were performed (Figure 15). The embryos were incubated at 26.5ºC ± 1 for 144h (6 days) and the mediums were renewed every day in order to maintain the oxygen conditions, ensuring the compounds' presence and mortality assessment. Observations were conducted at 8h, 32h, 80h and 144h (Figure 16), following previous protocols (Ulhaq *et al.*, 2013a; Hagenaars *et al* ., 2011; Zheng *et al.*, 2012; Ribeiro *et al.*, 2015). Several endpoints were determined, i.e., abnormal cell growth at 8hpf, embryo development delay at 32 hpf, abnormalities in the eyes, head, tail and yolk-sac, edemas and heart rate at 32, 80 and 144hpf, hatching rate at 80hpf and mortality rate at 8hpf, 32, 80 and 144hpf.

C	C+S	T1	T2	T3	T4
C	C+S	T1	T2	T3	T4
C	C+S	T1	T2	T3	T4
C	C+S	T1	T2	T3	T4

Figure 15 – Experimental design for zebrafish bioassay. C – Control; C+S – Solvent Control; T – Treatment.

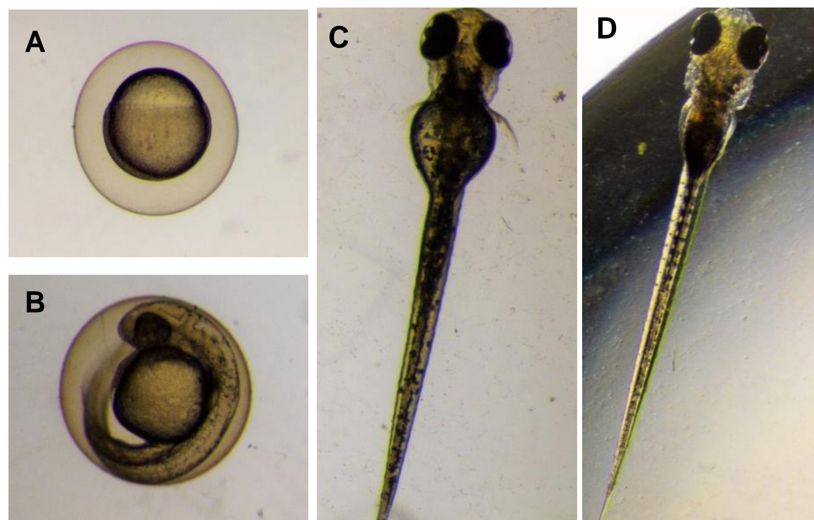

Figure 16 – Zebrafish embryonic development. A – 8 hpf (75% epiboly stage); B – 32 hpf (pharyngula stage); C, D – 80, 144 hpf (early larva stage).

2.4. Statistical Analysis

All data was analyzed in SPSS Statistics software version 22.0. Homogeneity of variances and normality of data were performed using Levene's and Kolmogorov-Smirnov test, respectively. Significant differences among treatments were tested at the end of each assay (at 144 hpf for *D. rerio* assay, and 48 hpf for *P. lividus* assay) by means of One-Way ANOVA., considering significant differences when $p<0.05$. Then, comparisons between control groups and treatments were done using Student-Newman-Keuls multiple comparison test. Moreover, non-parametrical Krustal-Wallis test were also performed to multiple comparisons among individual treatments when homogeneity and normality were not achieved, even after data transformation.

RESULTS
CHAPTER III

3. Results

3.1. Triclosan (TCS)

3.1.1. Zebrafish embryos bioassay

3.1.1.1. Cumulative mortality

Two different embryo bioassays with zebrafish were performed upon exposure to TCS (Figure 1 A, B). At 8 hpf the mortality rate did not differ significantly among treatments. At 32 hpf the mortality rate ranged from 3.75 ± 3.75 in the 1 µg/L treatment to 12.50 ± 3.66 in the first assay solvent control and 12.50 ± 2.50 in the 100 µg/L treatment. At 80 hpf, the mortality rate varied between 7.50 ± 3.66 in the 10 µg/L concentration to 100 in the 1000 µg/L concentration of both assays. At the end of the assays, the mortality rate ranged from 8.75 ± 3.50 in the 64 µg/L treatment (Figure 17 B) to 100 in the higher concentration (Figure 17 A, B). For both assays, the results show a significant increase in the mortality rate ($p<0.05$) for the embryos exposed to the highest concentration (1000 µg/L) in comparison with all the other treatments except for the concentration immediately below (100 and 400 µg/L) (Figure 17 A, B, respectively).

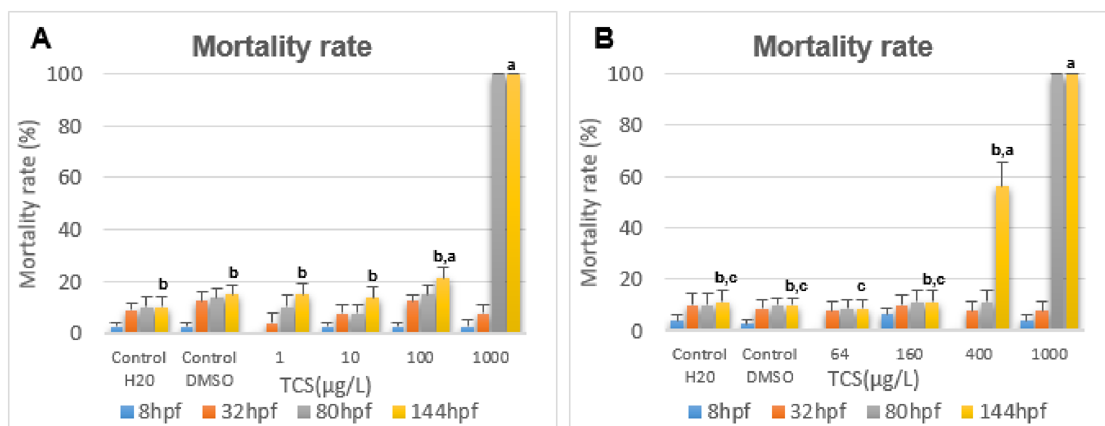

Figure 17- Cumulative mortality rates (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis ($p<0.05$), followed by multiple comparisons between groups for both A and B. Bars with different letters are statistically different from each other.

3.1.1.2. Abnormal cell growth

The percentage of embryos exhibiting abnormal cell growth at 8 hpf was similar between treatments and no significant differences ($p>0.05$) were reported for both assays (Figure 18 A, B).

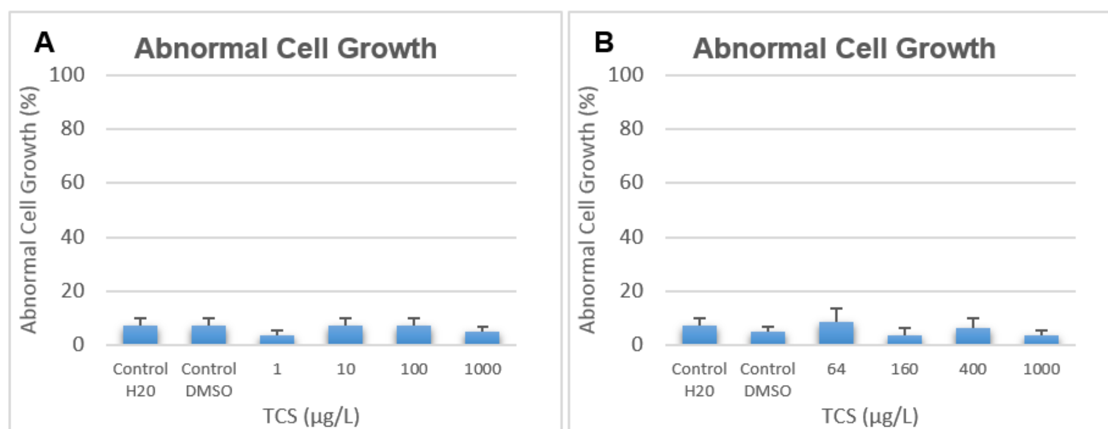

Figure 18 - Abnormal cell growth at 8 hpf (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for A, One-way ANOVA for B.

3.1.1.3. Embryo development delay

In both assays no significant delay (p>0.05) in the embryonic development were observed (Figure 19).

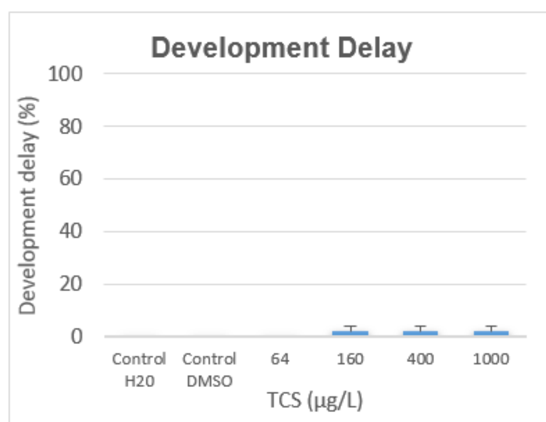

Figure 19 - Embryo development delay at 32 hpf (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h. Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis.

3.1.1.4. Hatching rate

In the first assay, no significant differences (p>0.05) were reported in the hatching rate among treatments (Figure 20 A). In the second assay, the hatching rate ranged from 61.25 ± 9.34 in the 400 µg/L concentration to 86.25 ± 3.75 in the solvent control and 86.25 ± 4.98 in the 64 µg/L treatment, respectively (Figure 20 B). This decrease on hatching was significantly different (p<0.05) from the solvent control and the 10 µg/L concentration (Figure

20 B). In both assays, embryos from the 10000 µg/L concentration were all dead at 80 hpf and so the hatching rate for this treatment was not reported.

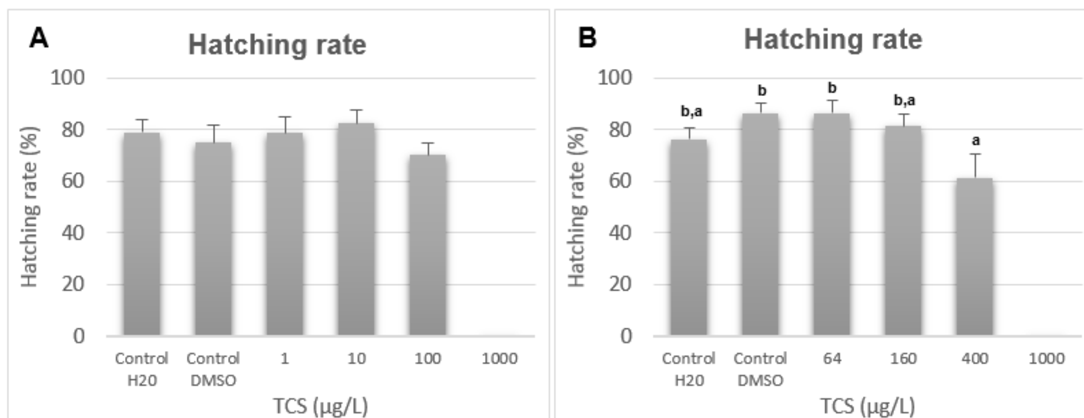

Figure 20 - Hatching rate at 80 hpf (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis (p>0.05) for A and One-way ANOVA (p<0.05) for B. Bars with different letters are statistically different from each other.

3.1.1.5. Head and eyes abnormalities

In both assays, at 144 hpf, the percentage of head (Figure 21 A, B) and eyes abnormalities (Figure 22 A, B) in the embryos were similar among treatments and no significant differences (p>0.05) were detected.

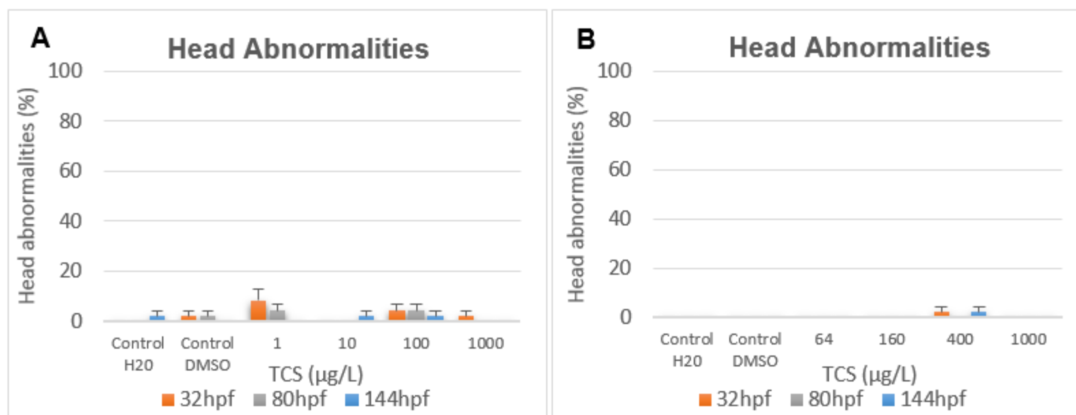

Figure 21 - Head abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis (p<0.05), followed by multiple comparisons between groups for both A and B.

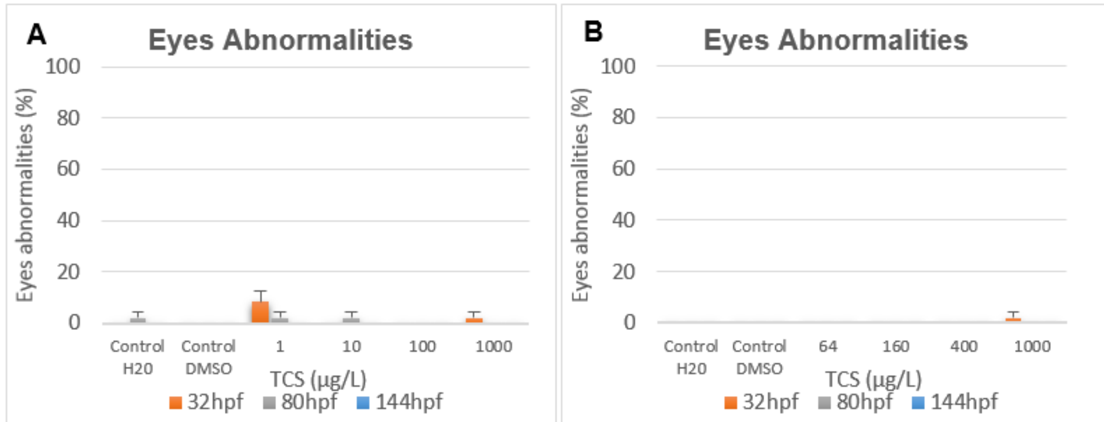

Figure 22 - Eyes abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for both A and B.

3.1.1.6. Tail abnormalities

At 144 hpf, the percentage of tail abnormalities was low and no significant differences (p>0.05) were detected among treatments (Figure 23 A, B).

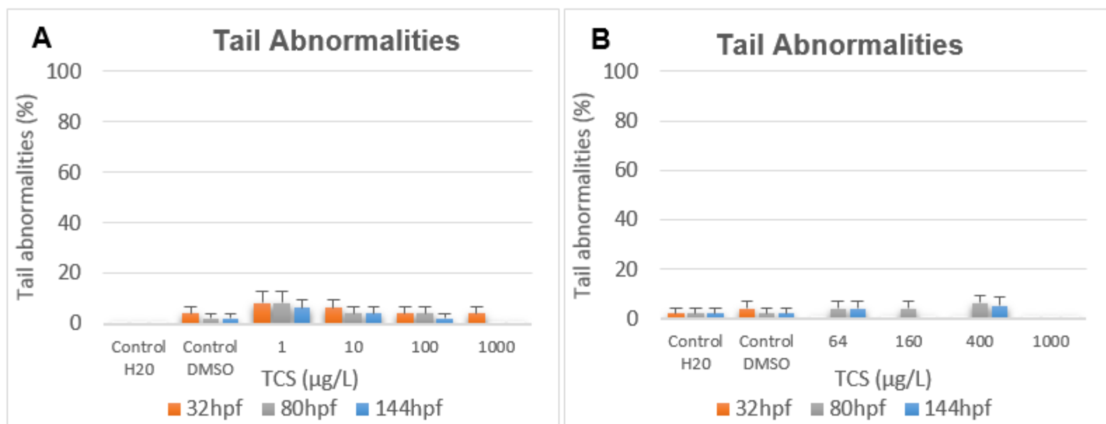

Figure 23 - Tail abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for both A and B.

3.1.1.7. Yolk-sac abnormalities

In the end of the first assay, the percentage of abnormalities on embryos' yolk-sac was similar among treatments and no significant differences (p>0.05) were reported (Figure 24 A). In the second assay, the percentage of yolk-sac abnormalities ranged from 0 in the water control and the 160 µg/L concentration to 17.71 ± 5.55 in the 400 µg/L treatment. This increase was significantly different (p<0.05) in comparison with all treatments (Figure 24 B).

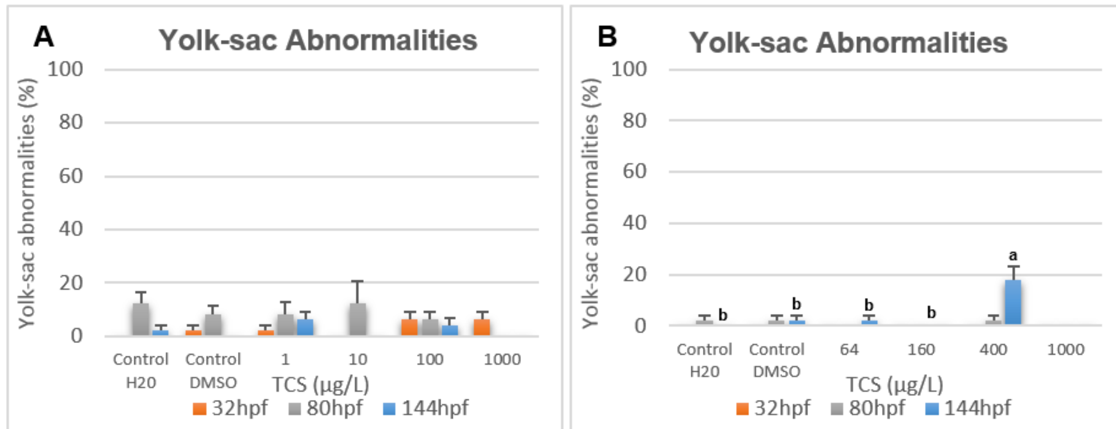

Figure 24 - Yolk-sac abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for A (p>0.05) and B (p<0.05).

3.1.1.8. Pericardial edema

At 144 hpf, the percentage of pericardial edema on embryos from the first assay was not significantly different (p>0.05) among the different groups (Figure 25 A). In the end of the second assay, the percentage of pericardial edemas observed ranged from 0 in all treatments to 18.75 ± 5.84 in the 400 µg/L concentration. This increase was significantly different (p<0.05) in comparison with all treatments (Figure 25 B).

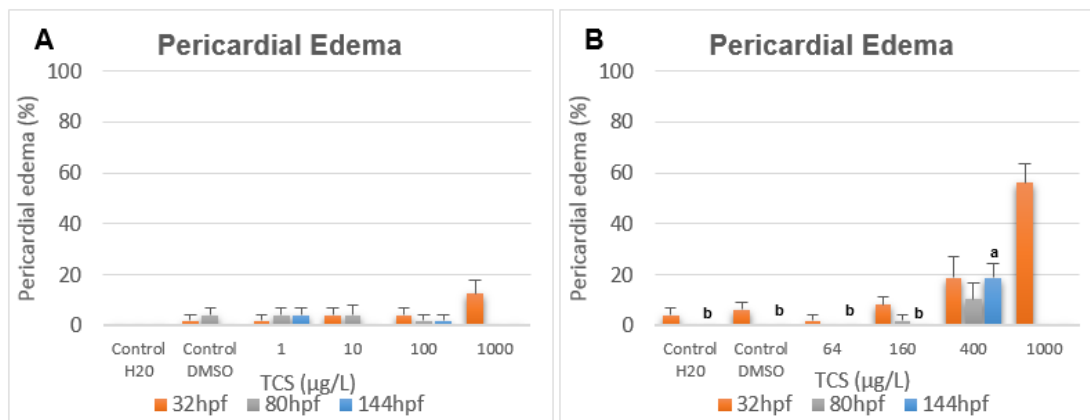

Figure 25 - Pericardial edema abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis, followed by multiple comparisons between groups for both (A) (p>0.05) and (B) (p<0.05). Bars with different letters are statistically

3.1.1.9. Hemorrhages

No significant differences ($p>0.05$) were observed among groups for this endpoint (Figure 26 A, B).

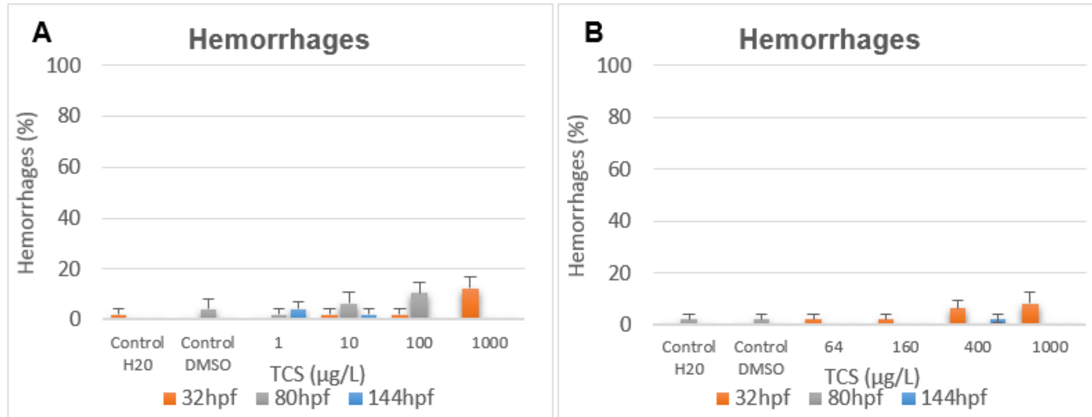

Figure 26 - Hemorrhages (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for A. ANOVA Kruskall-Wallis for B.

3.1.1.10. Muscular involuntary contractions

In the first assay, the rate of muscular involuntary contractions was similar among treatments and no significant differences ($p>0.05$) were reported (Figure 27 A). In the second assay, at the end of the assay the percentage of muscular involuntary contractions ranged from 0 in the water control to 82.29 ± 12.24 in the 400 µg/L treatment. This increase was significantly different ($p<0.05$) in comparison to all groups (Figure 27 B).

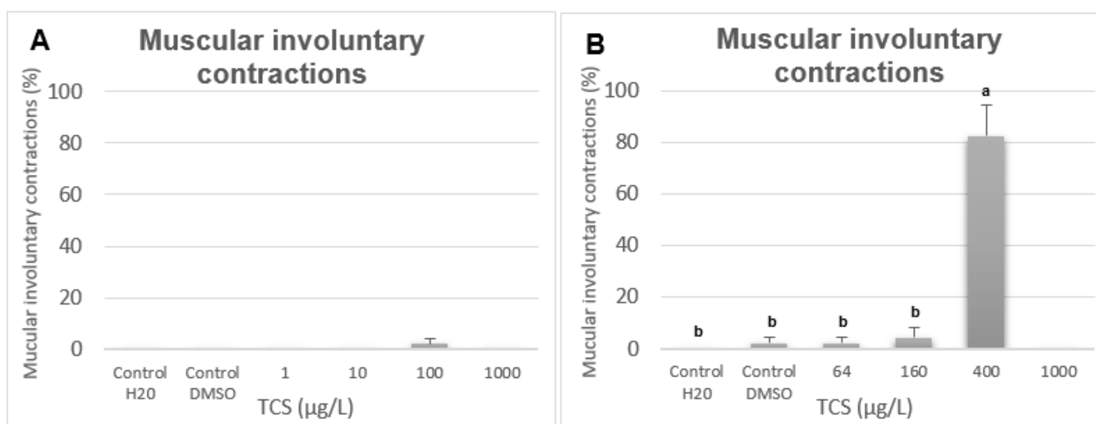

Figure 27 - Muscular involuntary contractions at 144 hpf (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for A ($p>0.05$) and B ($p<0.05$), followed by multiple comparisons between groups for B. Bars with different letters are statistically different from each other.

3.1.1.11. Total abnormalities

For the first assay, no significant differences were detected (p>0.05) for this endpoint at the end of the assay (Figure 28 A). In the second assay, the percentage of total abnormalities ranged from 0 in the 160 µg/L concentration to 100 in the 400 µg/L treatment. This increase was significantly higher (p>0.05) in comparison with all treatments (Figure 28 B).

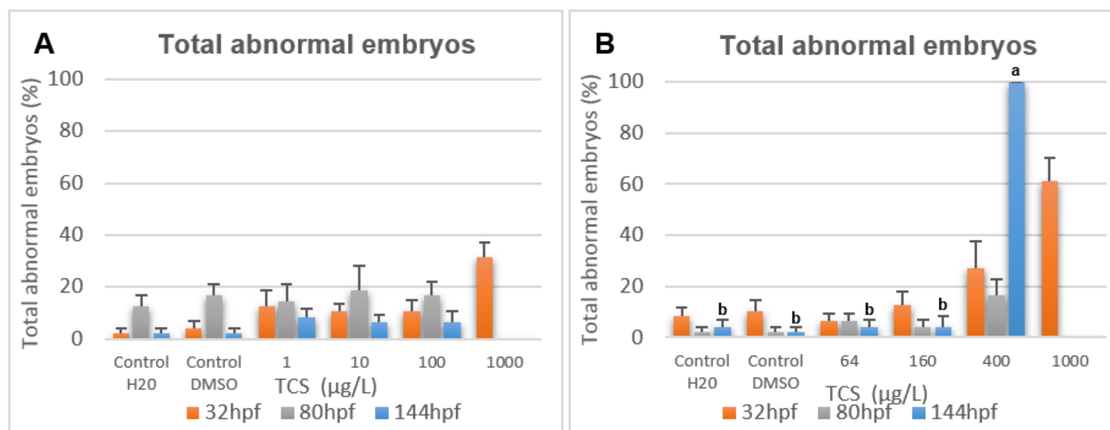

Figure 28 - Total abnormal embryos (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for both A (p>0.05) and B (p<0.05), followed by multiple comparisons between groups for B. Bars with different letters are statistically different from

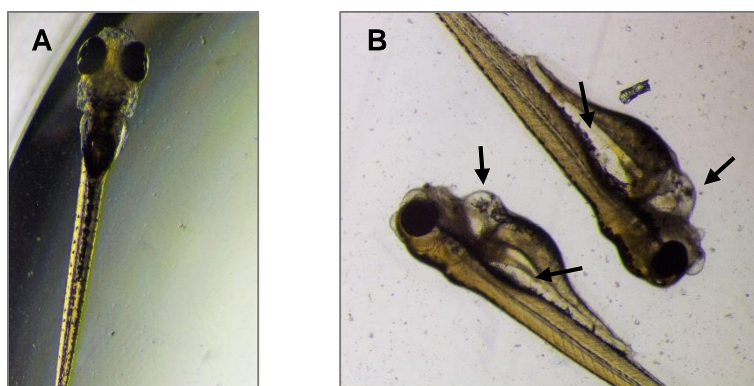

Figure 29 - *D. rerio* at 144 hpf in the control group (A) and exposed to 400 µg/L of the disinfectant Triclosan (B). The black arrows point the malformations on embryos' yolk-sac and pericardial edemas.

3.1.1.12. Heart rate

No significant differences (p>0.05) were found in the embryos heart rate at the end of both assays (Figure 30 A, B).

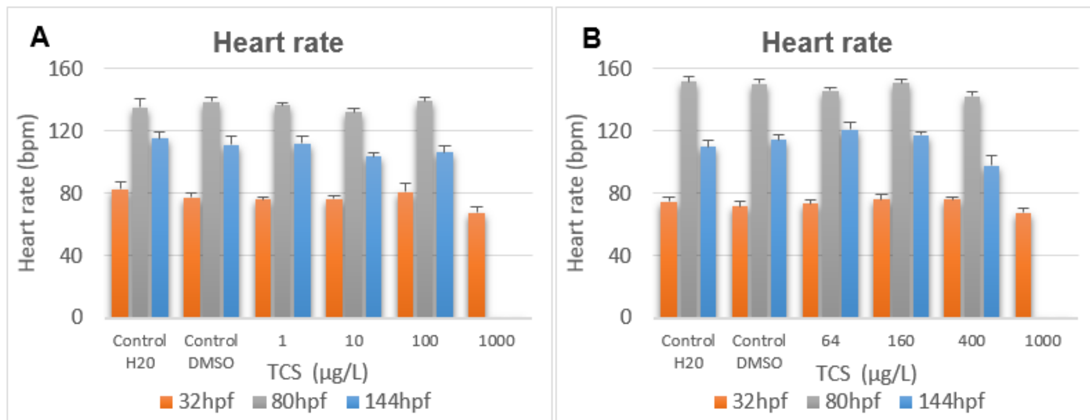

Figure 30 - Heart rate (bpm) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). One-way ANOVA for A. Nonparametric ANOVA Kruskall-Wallis for B.

3.1.2. Sea urchin embryo bioassay

3.1.2.1. Larval length and abnormalities

For the *P. lividus* exposure assay to triclosan, 8 different concentrations were tested. Given that no differences were observed between solvent and water controls, they were grouped. The larval length ranged from 262.9 ± 14.6 in the 400 µg/L concentration to 454.8 ± 6.1 in the controls, exhibiting a significant decrease (p<0.05) on the larval length for the 400 µg/L and 100 µg/L concentration (Figure 31 A). In the end of the assay the larvae exposed to 1000 µg/L of triclosan were all in the 2 or 4-cell stage. The percentage of abnormal larvae ranged from 12.5 ± 1.26 in the 16 µg/L concentration to 100 in the higher concentrations (400, 1000 µg/L), and differed significantly in comparison with other treatments (Figure 31 B).

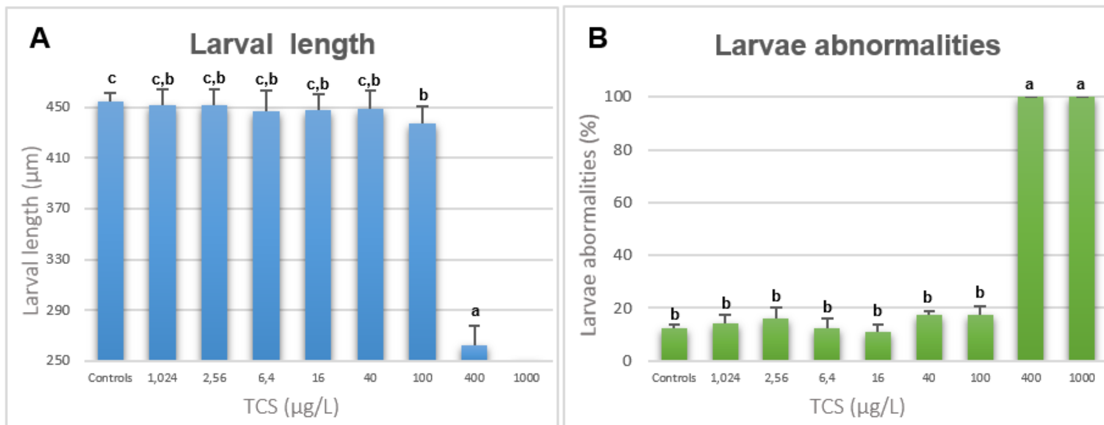

Figure 31 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of the disinfectant triclosan for 48h. Data are expressed as mean ± SEM (n=480 for each control; n=120 for triclosan exposed groups; n=15 for 400 µg/L treatment). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by multiple comparisons between groups for both A and B. Bars with different letters are statistically different from each other.

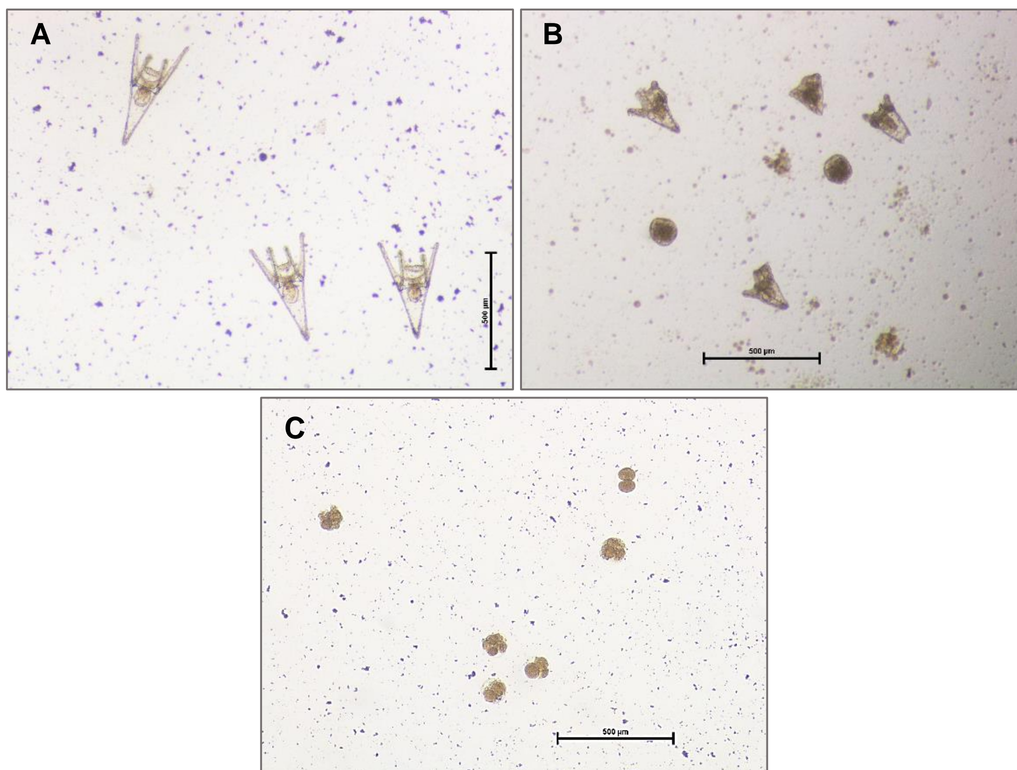

Figure 32 – *P. lividus* at 48 hpf in the control group (A), and exposed to 400 µg/L (B) and 1000 µg/L of triclosan.

3.2. Methyl-triclosan (M-TCS)

3.2.1. Zebrafish embryos bioassay

3.2.1.1. Cumulative mortality

For methyl-triclosan two assays were also conducted. Until 80 hpf, the mortality rate was similar between treatments in both assays. At 144 hpf the mortality ranged from 1.25 ± 1.25 in the second assay water control (Figure 33 B) to 97.5 ± 2.50 in the 10000 µg/L concentration (Figure 33 A). The increase in the mortality rate in the first assay in the 10000 µg/L concentration was significantly different ($p<0.05$) in comparison with the other groups excepting for the 1000 µg/L concentration (Figure 33 A). As in the second assay, for the higher concentration (1000 µg/L concentration) was detected a significant increase ($p<0.05$) comparing to the treatments and controls (Figure 33 B). The mortality rate in the 1000 µg/L concentration was slightly different between assays. In the first assay was 26.25 ± 8.85 as in the second assay reached 41.3 ± 13.42.

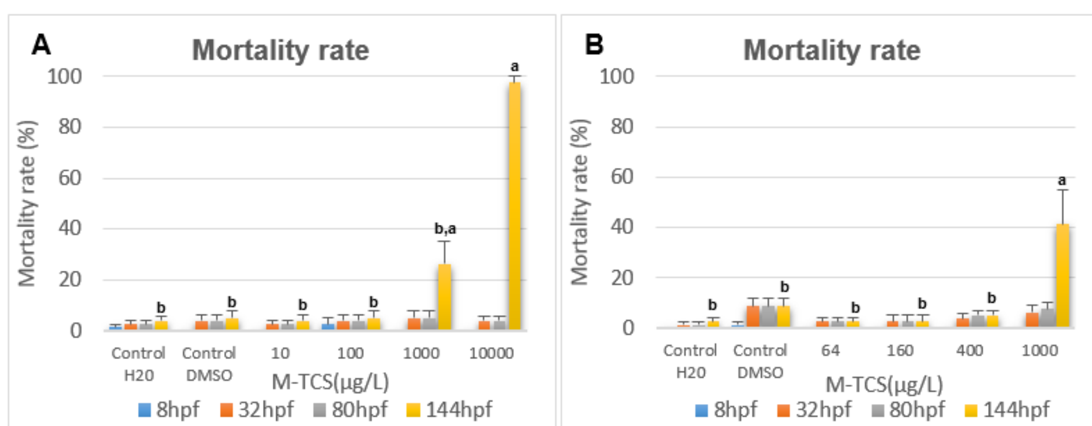

Figure 33 - Cumulative mortality rates (%) of *D. rerio* exposed to different concentrations of methyl –triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis ($p<0.05$), followed by multiple comparisons between groups for A. One-way ANOVA for B ($p<0.05$). Bars with different letters are statistically different from each other.

3.2.1.2. Abnormal cell growth

In both assays no significant differences (p>0.05) were detected in this endpoint among treatments (Figure 34 A, B).

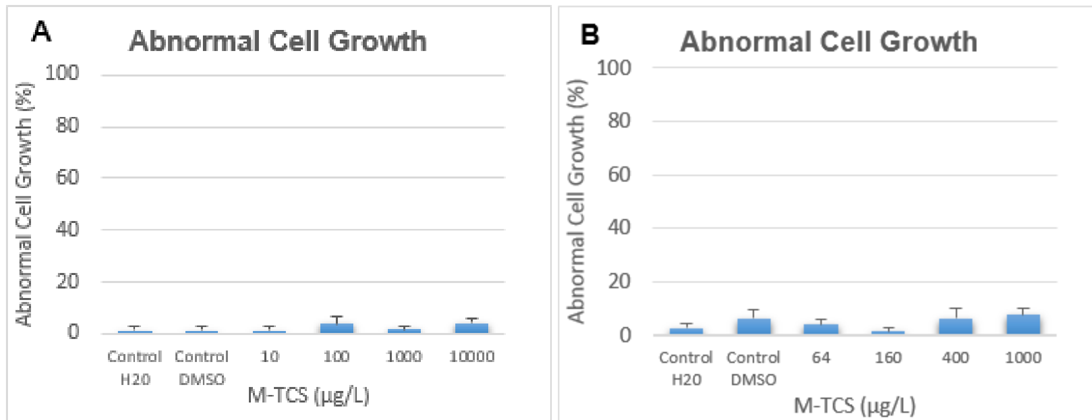

Figure 34 - Abnormal cell growth at 8 hpf (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). One-way ANOVA for A. Nonparametric ANOVA Kruskall-Wallis for B.

3.2.1.3. Embryo development delay

The percentage of embryos with development delay was similar among groups and no significant differences (p>0.05) were detected (Figure 35 A, B).

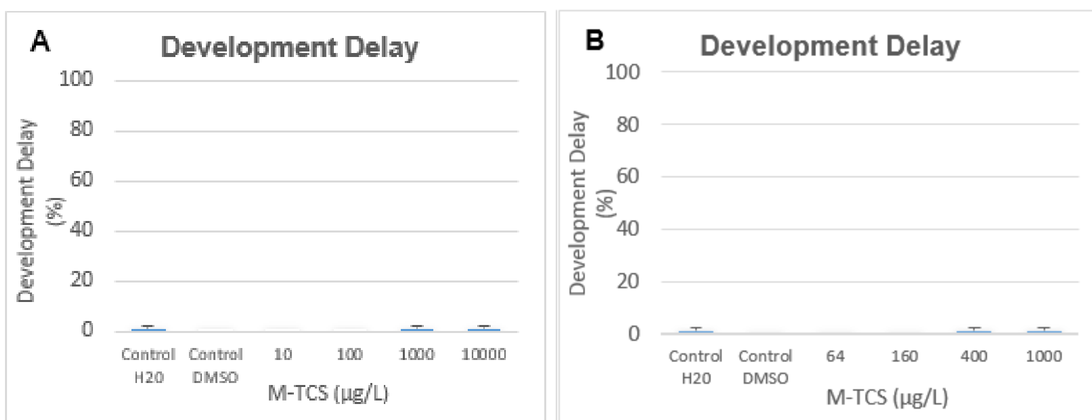

Figure 35 - Embryo development delay at 32 hpf (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B).. Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for both A and B.

3.2.1.4. Hatching rate

The hatching rate did not differ significantly ($p>0.05$) among treatments in both assays (Figure 36 A, B).

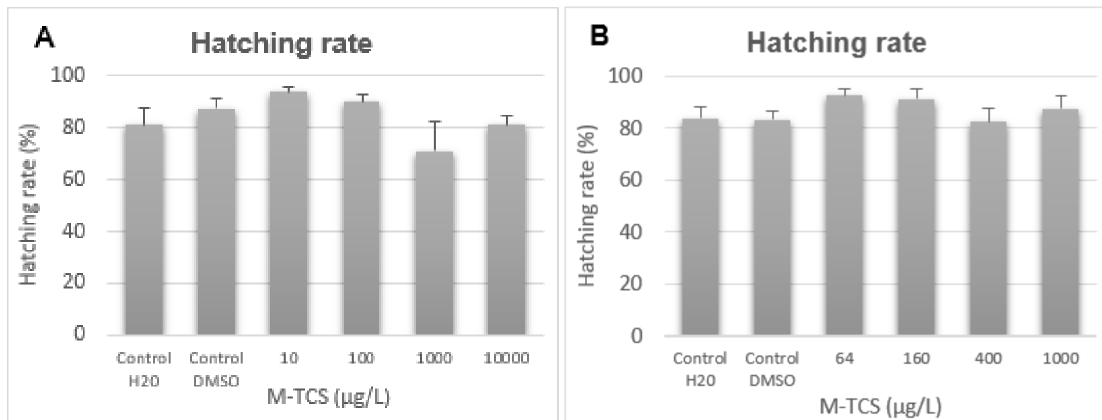

Figure 36 -Hatching rate at 80 hpf (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for A and One-way ANOVA for B.

3.2.1.5. Head and eyes abnormalities

During both assays no significant differences ($p>0.05$) on the percentage of head or eyes abnormalities were detected (Figure 37 A, B).

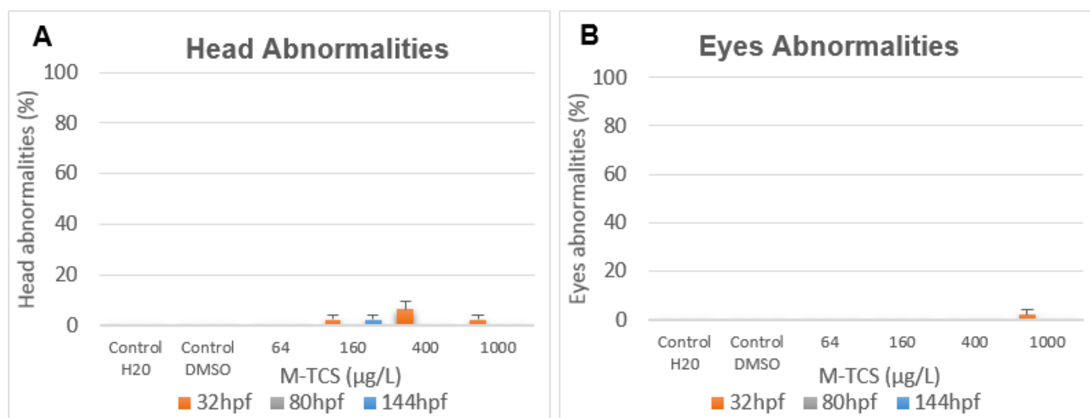

Figure 37 - Head and eyes abnormalities (%) of *D. rerio* exposed to different concentrations of the disinfectant Triclosan for 144 h in the second assay (A and B, respectively). Data are expressed as mean ± SE (n=8; for 1000 µ/L n= 6). Nonparametric ANOVA Kruskall-Wallis ($p<0.05$), for both A and B.

3.2.1.6. Tail abnormalities

Tail abnormalities ranged from 0 to 100% in the 10000 and 1000 µg/L concentrations in both assays (Figure 38 A, B). The increases in the first assay were significantly different ($p<0.05$) in comparison with all groups (Figure 38 A), whereas in the second assay, the increases in the 1000 and 400 µg/L concentrations were only significantly different ($p<0.05$) in comparison with the solvent control and the 64 µg/L concentration (Figure 38 B).

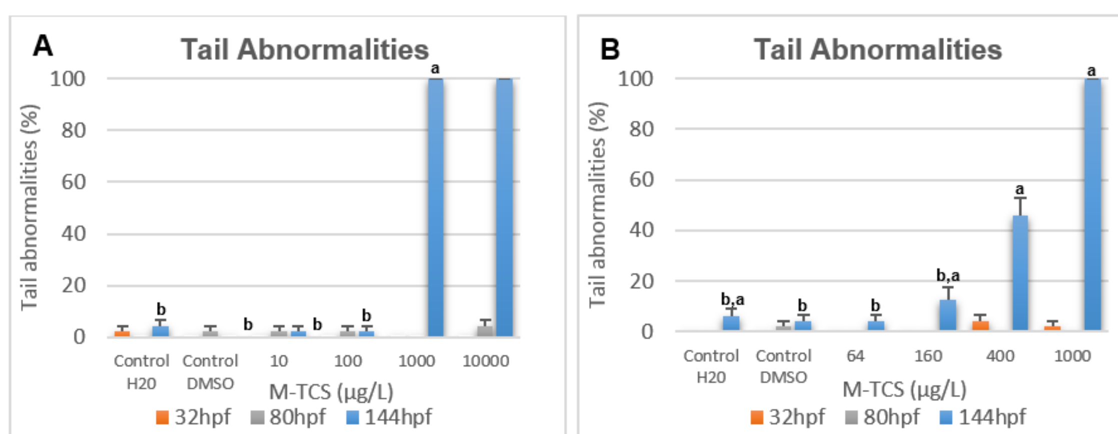

Figure 38 - Tail abnormalities (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8; for 10000µ/L in A n=1; for 1000 µ/L in B n= 6). Nonparametric ANOVA Kruskall-Wallis for both A and B. Bars with different letters are statistically different from each other.

3.2.1.7. Yolk-sac abnormalities

In the end of both assays, the percentage of yolk-sac abnormalities ranged from 0 to 100 in the 1000 and 10000 µg/L concentration. This increase in both concentrations was significantly higher ($p<0.05$) in comparison with all the other treatments (Figure 39 A, B).

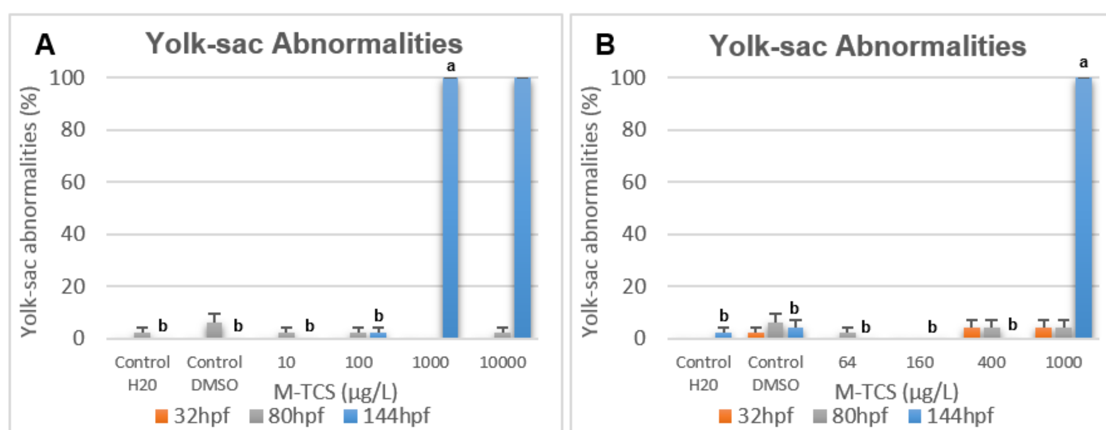

Figure 39 - Yolk-sac abnormalities (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8; for 10000µ/L in A n=1; for 1000 µ/L in B n= 6). Nonparametric ANOVA Kruskall-Wallis for A and B. Barr with different letters are statistically different from each other.

3.2.1.8. Pericardial edema and Hemorrhages

At 144 hpf, in both assays, the percentage of pericardial edema and hemorrhages on embryos was low and no significant differences ($p<0.05$) were detected among groups (Figure 40, 41 A, B).

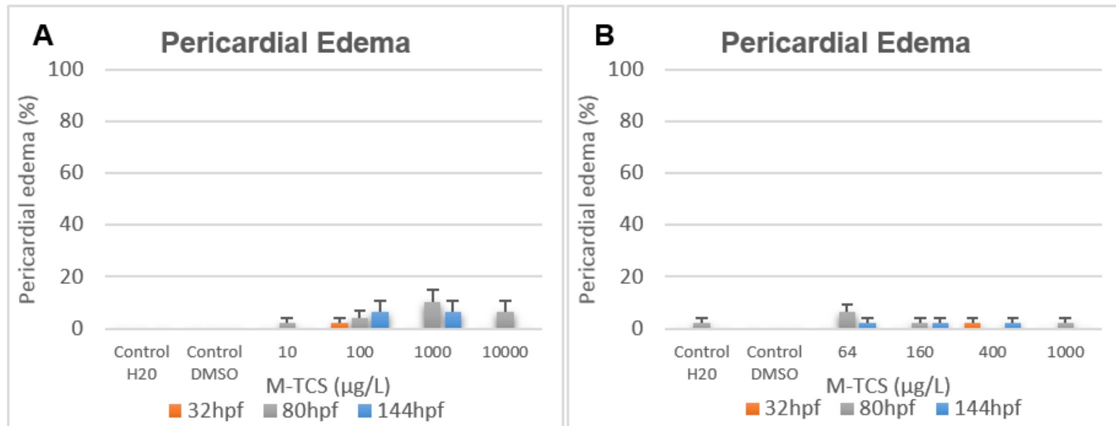

Figure 40 – Pericardial edema (%) of *D. rerio* exposed to diferent concentrations of methyl-triclosan for 144 hpf in the first assay (A) and second assay (B). Data are expressed as mean± SE (n=8; for 10000µ/L in A n=1; for 1000 µ/L in B n= 6). Nonparametric ANOVA Kruskall-Wallis for A and B.

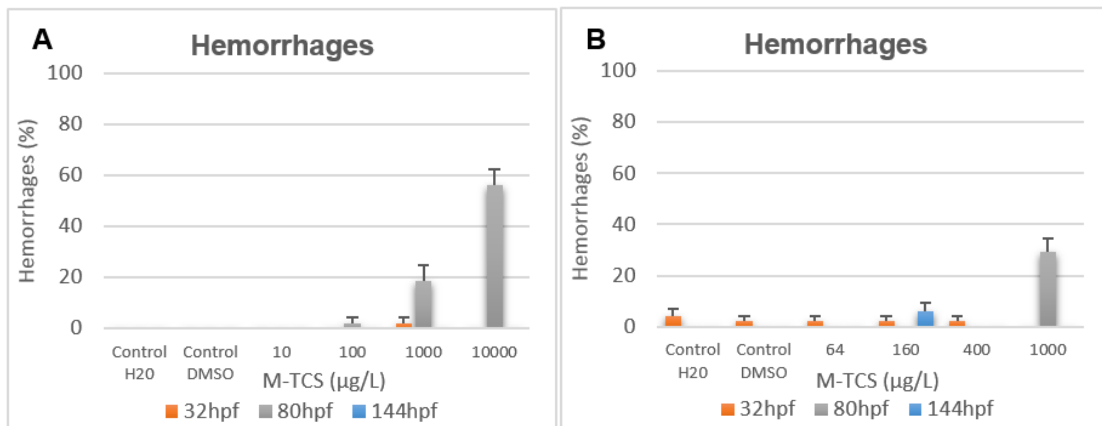

Figure 41 - Hemorrhages (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 hpf in the first assay (A) and second assay (B). Data are expressed as mean± SE (n=8; for 10000µ/L in A n=1; for 1000 µ/L in B n= 6). Nonparametric ANOVA Kruskall-Wallis for A and B.

3.2.1.9. Muscular involuntary contractions

In both assays, at 144 hpf, the percentage of embryos with muscular involuntary contractions ranged from 0 to 100 in the 10000 and 1000 µg/L concentrations. This increase was significantly different ($p<0.05$) in comparison with the other groups (Figure 42 A, B).

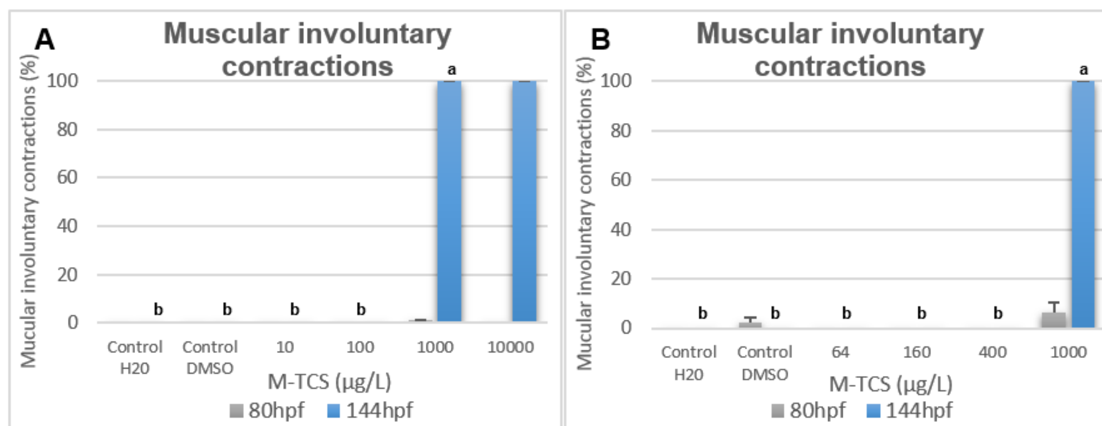

Figure 42 - Muscular involuntary contractions (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h (in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8; for 10000µ/L in A n=1; for 1000 µ/L in B n= 6). Nonparametric ANOVA Kruskall-Wallis for A and B, followed by multiple comparisons between groups. Bars with different letters are statistically different from each other.

3.2.1.10. Total abnormalities

At 144 hpf, the percentage of abnormal embryos ranged from 0 in the first assay solvent control to 100 in the two highest concentrations (10000 and 1000 µg/L). The increases in the 10000, 1000 and 400 µg/L concentrations were significantly different in comparison with all the other treatments apart from the 160 µg/L concentration (Figure 43 A, B).

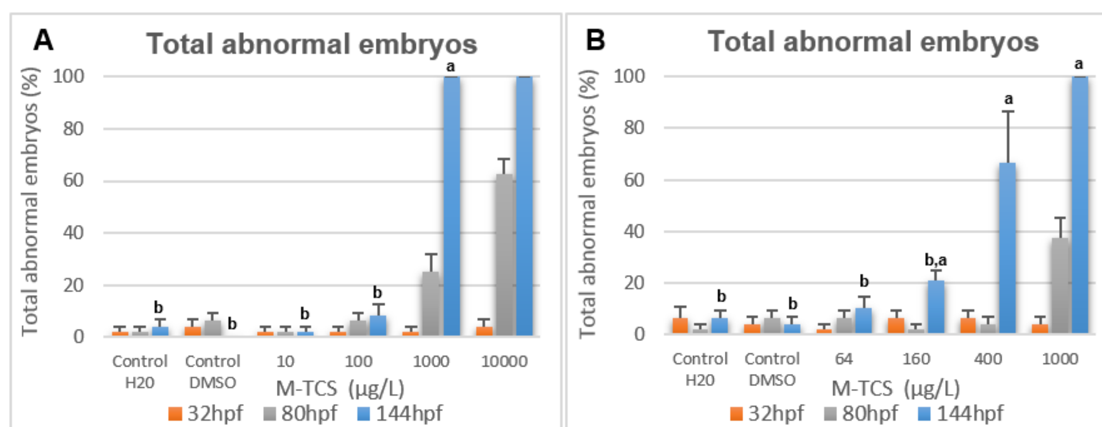

Figure 43 - Total abnormal embryos (%) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8; for 10000µ/L in A n=1; for 1000 µ/L in B n= 6). Nonparametric ANOVA Kruskall-Wallis for both A and B. Followed by multiple comparisons between groups for B. Bars with different letters are statistically different from each other.

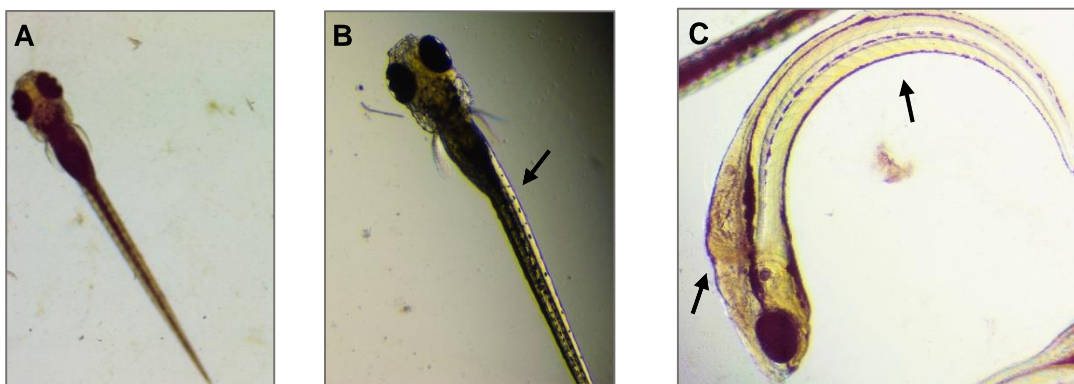

Figure 44 - *D. rerio* at 144 hpf in the control group (A), exposed to 400 µg/L (B) and 1000 µg/L (C) of methyl-triclosan. The black arrows point the embryos malformations.

3.2.1.11. Heart rate

At 144 hpf, the heart rate was similar between groups for both assays and so, no significant differences ($p<0.05$) were detected among groups (Figure 45 A, B).

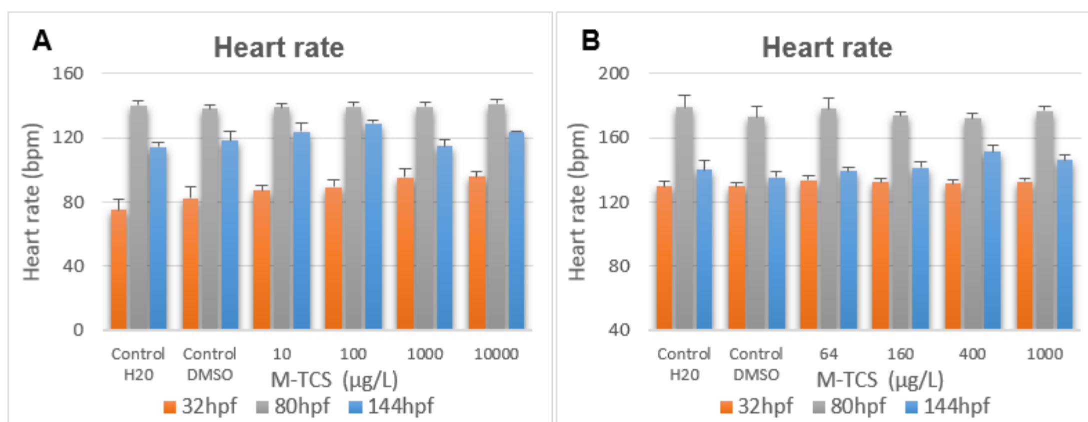

Figure 45 - Heart rate (bpm) of *D. rerio* exposed to different concentrations of methyl-triclosan for 144 h in the first assay (A) and second assay (B). Data are expressed as mean ± SE (n=8; for 10000µ/L in A n=1; for 1000 µ/L in B n= 6). One-way ANOVA for both A and B.

3.2.2. Sea urchin embryo bioassay

3.2.2.1. Larval length and abnormalities

In the methyl-triclosan exposure, the larval length ranged from 265.9 ± 8.96 in the 1000 µg/L concentration to 448.4 ± 5.9 in the controls. All the treatments were significantly different ($p<0.05$) from the controls (Figure 46 A). The percentage of abnormal larvae ranged from 5 ± 1.67 in the 16 µg/L concentration to 31.67 ± 2.75 in the 1000 µg/L concentration. The percentage of abnormalities was significantly higher in the 1000 µg/L treatment ($p<0.05$) in comparison with all the other treatments (Figure 46 B).

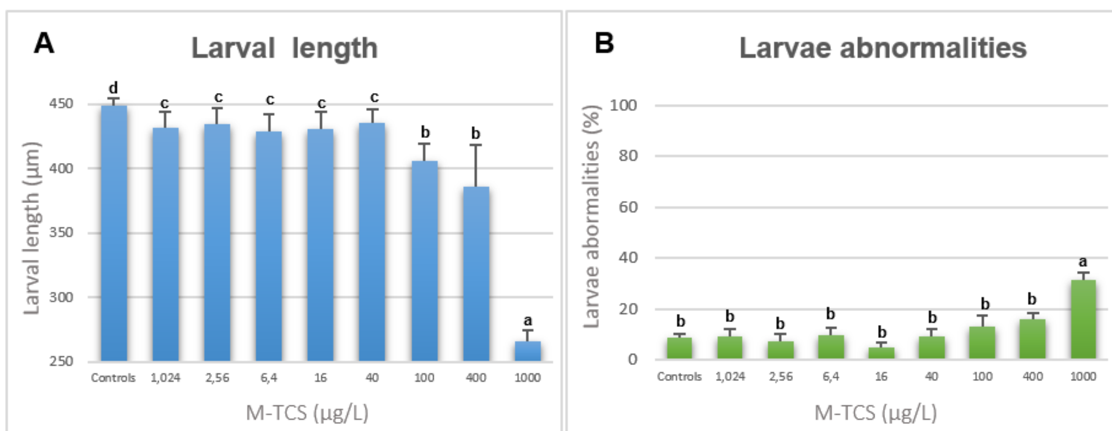

Figure 46 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of methyl-triclosan for 48h. Data are expressed as mean ± SEM (n=480 for each control; n=120 for triclosan exposed groups). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by multiple comparisons between groups for A. One way ANOVA ($p<0.05$) for B. Bars with different letters are statistically different from each other.

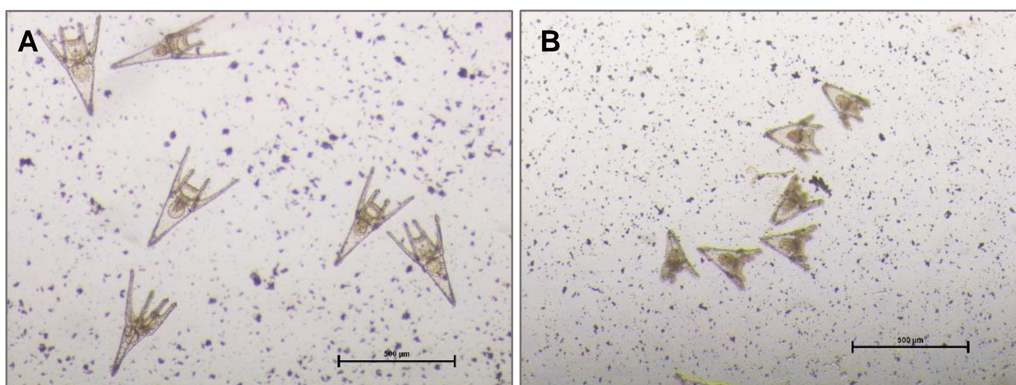

Figure 47 – *P. lividus* at 48 hpf in the control group (A) and exposed to 1000 µg/L of methyl-triclosan (B).

3.3. Perfluorooctane sulfonate (PFOS)

3.3.1. Zebrafish embryos bioassay

3.3.1.1. Cumulative mortality

In the end of PFOS exposure assay, the mortality rate ranged from 13.75 ± 4.6 in the 100 µg/L concentration to 100 in the highest concentration. The increase in the mortality rate in this last treatment was significantly different ($p<0.05$) from all groups, apart from the 10 µg/L concentration (Figure 48).

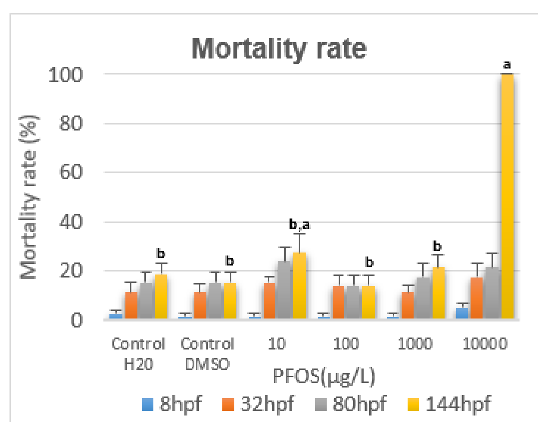

Figure 48 - Cumulative mortality rates (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h. Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis, followed by multiple comparisons between groups. Bars with different letters are statistically different from each other.

3.3.1.2. Abnormal cell growth and embryo development delay

No significant differences ($p>0.05$) were reported between treatments for both endpoints (Figure 49 A, B).

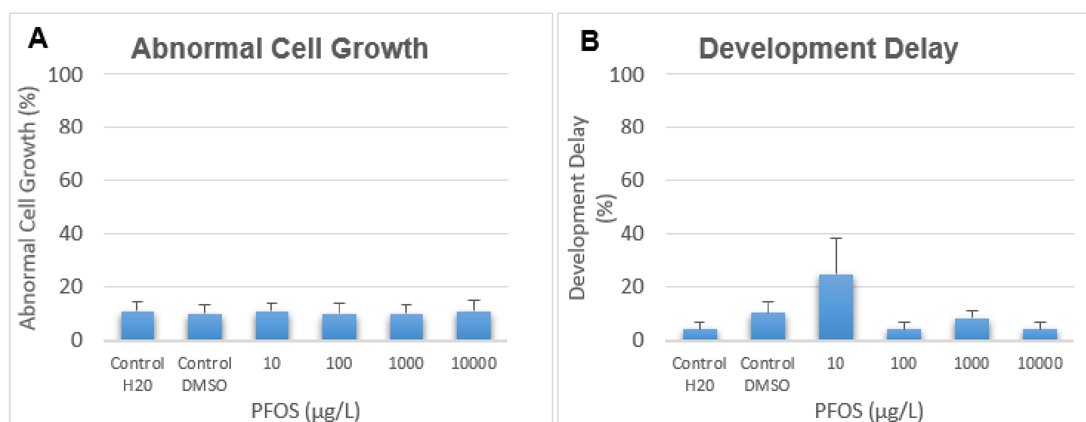

Figure 49 - Abnormal cell growth at 8 hpf and embryo development delay at 32 hpf (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). One-way ANOVA for A. Nonparametric ANOVA Kruskall-Wallis for B.

3.3.1.3. Hatching rate

At 80 hpf, the hatching rate exhibited no significant differences ($p>0.05$) among treatments (Figure 50).

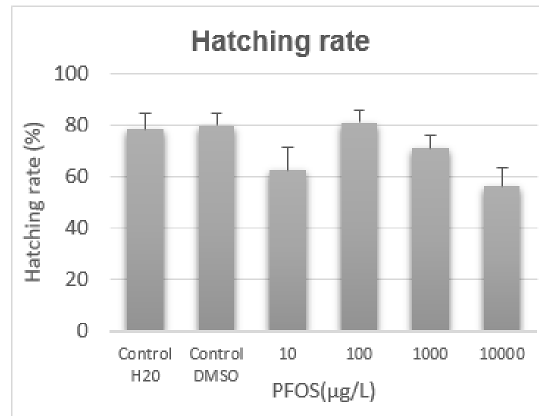

Figure 50 - Hatching rate at 80 hpf (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h Data are expressed as mean ± SE (n=8). One-way ANOVA.

3.3.1.4. Head abnormalities

At the end of the assay, no head abnormalities were detected on embryos (Figure 51).

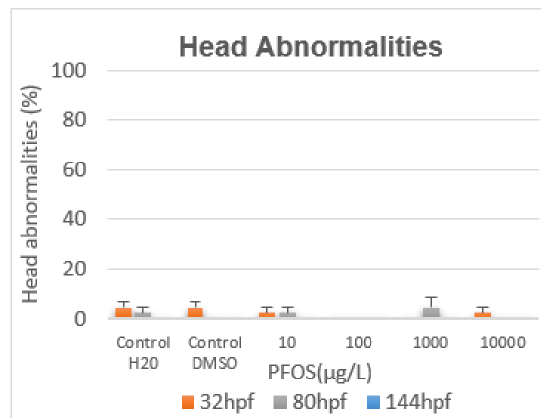

Figure 51 - Head abnormalities (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h. Data are expressed as mean ± SE (n=8).

3.3.1.5. Eyes abnormalities

During the assay no eyes abnormalities were detected.

3.3.1.6. Tail and yolk-sac abnormalities

At 144 hpf the percentage of tail and yolk-sac abnormalities were similar among groups and no significant differences (p>0.05) were reported (Figure 52 A, B).

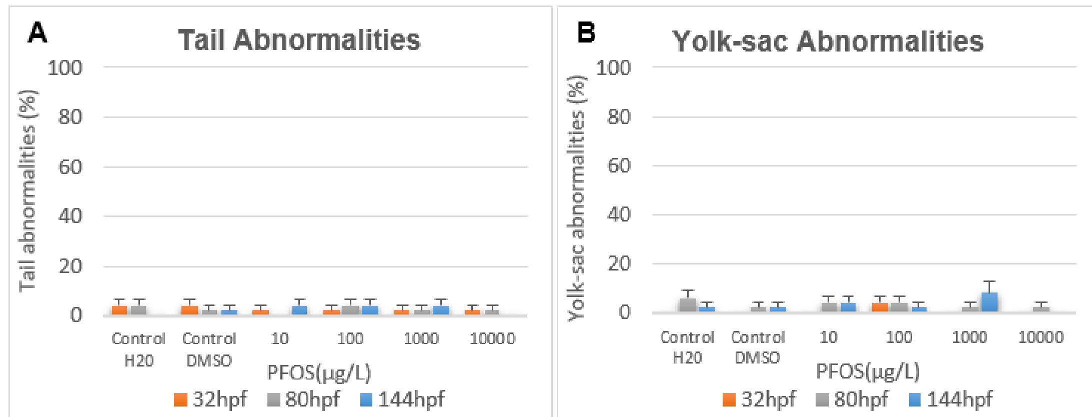

Figure 52 -Tail and yolk-sac abnormalities (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for A. One-way ANOVA for B.

3.3.1.7. Pericardial edema and hemorrhages

At 144 hpf, the percentage of pericardial edema and hemorrhages on embryos was low and no significant differences (p>0.05) were reported on both endpoints (Figure 53 A, B).

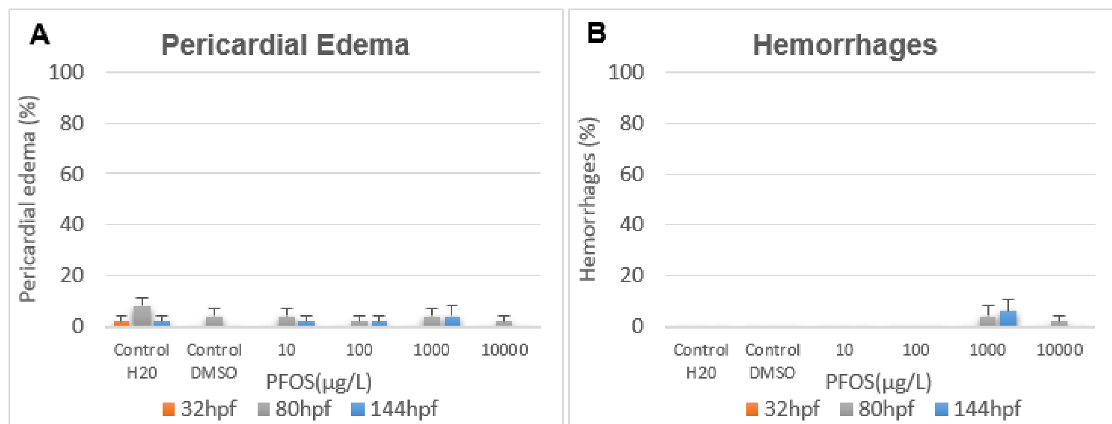

Figure 53 - Pericardial edema and hemorrhages (%) of *D. rerio* exposed to different concentrations of PFOS for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). One-way ANOVA for A. Nonparametric ANOVA Kruskall-Wallis for B.

3.3.1.8. Muscular involuntary contractions

During the assay, no muscular involuntary contractions were detected on embryos.

3.3.1.9. Total abnormalities and heart rate

At 144 hpf the percentage of abnormal embryos and heart rate exhibited no significant differences (p>0.05) among treatments (Figure 54 A, B). In the 10000 µg/L concentration the embryos were all dead.

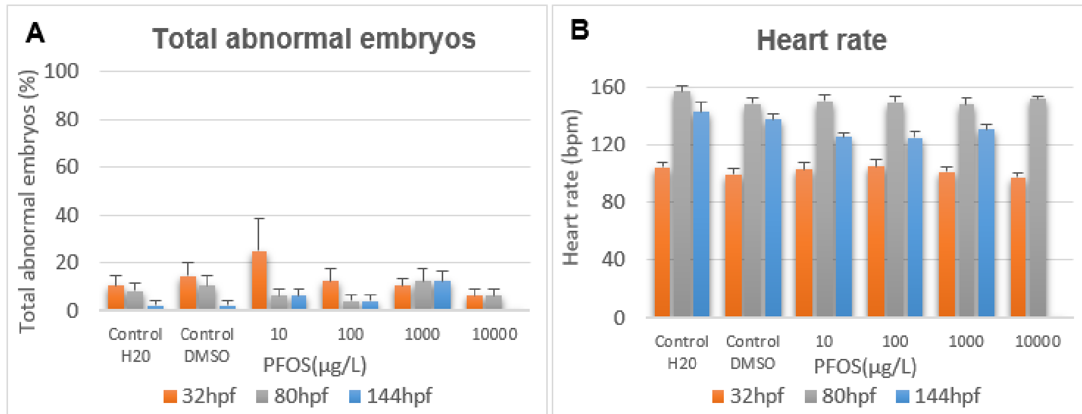

Figure 54 - Total abnormal embryos (%) and heart rate (bpm) of *D. rerio* exposed to different concentrations of PFOS for 144 h. Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for A. One-way ANOVA for B.

3.3.2. Sea urchin embryo bioassay

3.3.2.1. Larval length and abnormalities

At the end of the assay, the larval length ranged from 358.92 ± 15.76 in the highest treatment to 385.61 ± 13.07 in the 6.4 µg/L concentration. The decrease on the 1000 and 2.56 µg/L treatments was significantly different (p<0.05) in comparison with all groups except for the 100 µg/L concentration (Figure 55 A). The percentage of abnormal larvae ranged from 17.1 ± 1.72 in controls to 31.7 ± 5 in the 1000 µg/L concentration. No significant differences (p>0.05) were reported (Figure 55 B).

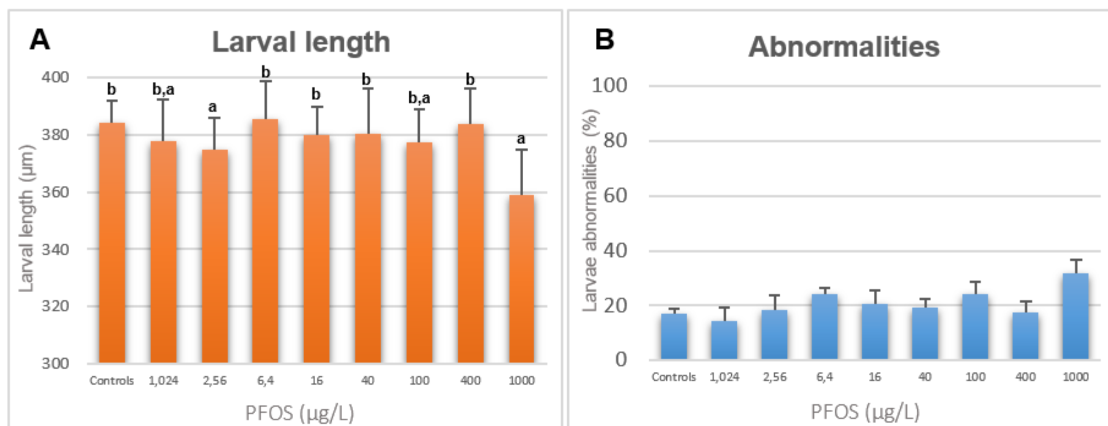

Figure 55 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFOS for 48h. Data are expressed as mean ± SEM (n=480 for controls; n=120 for PFOS exposed groups; n=90 for 1.204 µg/L and n=105 for 400 µg/L treatments). Non-parametric ANOVA Kruskall-Wallis (p<0.05), followed by multiple comparisons between groups for both A. One-way ANOVA for B (p>0.05). Bars with different letters are statistically different from each other.

3.4. Perfluorooctanoic acid (PFOA)

3.4.1. Zebrafish embryos bioassay

For PFOA exposure assay, the same four concentrations were tested. No significant differences ($p>0.05$) were detected for any of the endpoints considered. Table 3 summarizes PFOA exposure effects on zebrafish at the end of the assay.

3.4.2. Sea urchin embryo bioassay

3.4.2.1. Larval length and abnormalities

At the end of the assay, the larval length ranged from 360.33 ± 20.32 in 1000 µg/L concentration to 409.47 ± 14.2 in 2.56 µg/L concentration. The decrease in the higher concentration and the 40 and 100 µg/L concentrations was significantly different ($p>0.05$) in comparison with all the other groups (Figure 56 A). Regarding abnormalities, the percentage of abnormal larvae ranged from 8.3 ± 4.05 in 2.56 µg/L concentration to 24.2 ± 6.03 in 6.4 µg/L concentration. No significant differences ($p>0.05$) were reported (Figure 56 B).

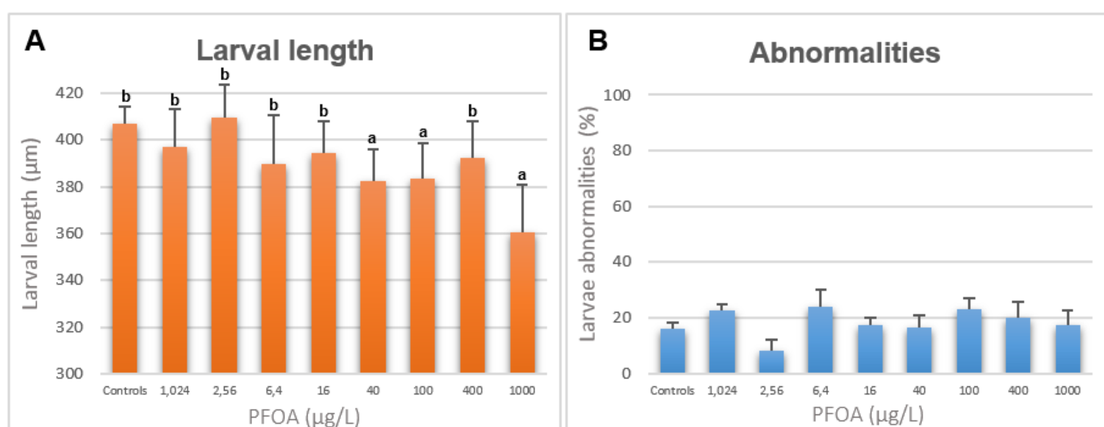

Figure 56 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFOA for 48h. Data are expressed as mean ± SEM (n=480 for controls; n=120 for PFOA exposed groups; n=105 for 100, 400 and 1000 µg/L treatments; n= 90 for 2.56 µg/L treatment). Non-parametric ANOVA Kruskall-Wallis, followed by multiple comparisons between groups for both A. One-way ANOVA for B. Bars with different letters are statistically different from each other.

Table 3 - Effects of PFOA exposure in zebrafish embryos at 144 hpf. Data are expressed as mean ± se (n=8).

ENDPOINT (%)	TREATMENT					
	Water control	Solvent control	10 µg/L	100 µg/L	1000 µg/L	10000 µg/L
Mortality	12.50 ± 4.12	12.50 ± 2.50	10 ± 2.67	8.75 ± 2.27	15 ± 3.27	11.3 ± 2.95
Abnormal cell growth[(a)]	3.75 ± 2.63	1.25 ± 1.25	2.50 ± 1.64	1.25 ± 1.25	3.75 ± 1.83	3.75 ± 2.63
Development delay[(a)]	6.25 ± 3.05	4.17 ± 2.73	0	2.08 ± 2.08	2.08 ± 2.08	0
Hatching[(b)]	86.25 ± 5.65	83.75 ± 3.75	87.50 ± 3.66	87.50 ± 2.50	80 ± 4.23	81.25 ± 2.27
Head abnormalities	0	0	2.08 ± 2.08	0	0	0
Eyes abnormalities	0	0	0	0	0	0
Tail abnormalities	2.08 ± 2.08	6.25 ± 3.05	0	0	0	4.17 ± 2.73
Yolk-sac abnormalities	2.08 ± 2.08	2.08 ± 2.08	2.08 ± 2.08	2.08 ± 2.08	0	0
Pericardial edema	4.17 ± 2.73	2.08 ± 2.08	2.08 ± 2.08	2.08 ± 2.08	0	0
Hemorrhages	0	0	0	0	0	0
Muscular involuntary contractions	0	0	0	0	0	0
Total abnormalities	6.25 ± 4.38	4.17 ± 2.73	4.17 ± 2.73	2.08 ± 2.08	0	4.17 ± 2.73
Heart rate	127.5 ± 5.1	119.5 ± 6.39	124 ± 3.02	120.5 ± 3.06	124 ± 5.18	121 ± 2.24

[(a)]8 hpf [(b)]80 hpf

3.5. Perfluorobutane sulfonate (PFBS)

3.5.1. Zebrafish embryos bioassay

In the PFBS exposure, no significant differences (p>0.05) were detected for any of the endpoints considered. Similarly to PFOA, in Table 4 are summarized the effects of PFBS exposure in the end of the assay.

3.5.2. Sea urchin embryo bioassay

3.5.2.1. Larval length and abnormalities

At 48 hpf, the larval length ranged from 360.37 ± 17.22 in the 1000 µg/L concentration to 398.37 ± 13.13 µg/L in the 6.4 µg/L concentration. The increase in the 6.4 µg/L concentration was significantly different (p<0.05) from the controls, as in the decrease in the higher concentration was significantly different (p<0.05) from the controls (Figure 57 A). No significant differences (p>0.05) were reported for the endpoint larvae abnormalities (Figure 57 B).

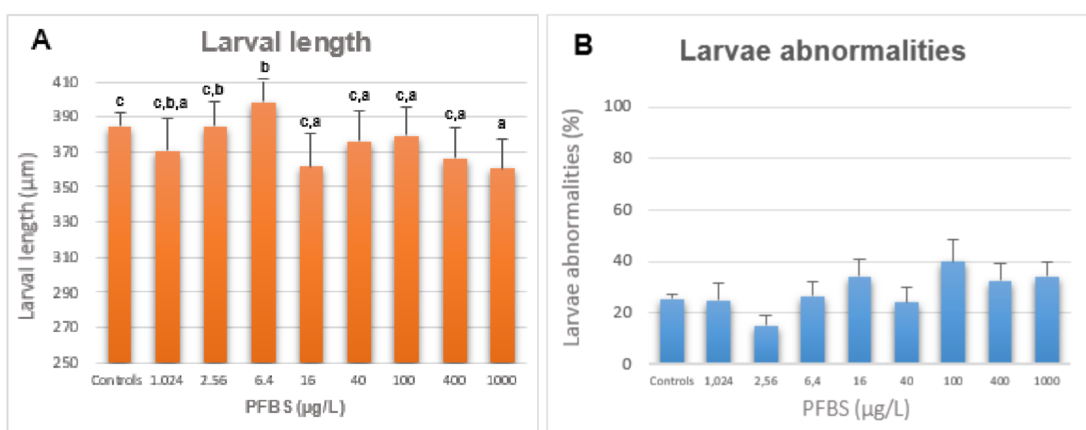

Figure 57 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFBS for 48h. Data are expressed as mean ± SEM (n=450 for each control; n=120 for triclosan exposed groups; n=105 for 2.56 µg/L treatment). Non-parametric ANOVA Kruskall-Wallis (p<0.05), followed by multiple comparisons between groups for both A. One-way ANOVA for B. Bars with different letters are statistically different from each other.

Table 4 - Effects of PFBS exposure in zebrafish embryos at 144 hpf. Data are expressed as mean ± SE (n=8).

ENDPOINT (%)	TREATMENT					
	Water control	Solvent control	10 μg/L	100 μg/L	1000 μg/L	10000 μg/L
Mortality	5 ± 1.89	5 ± 2.67	2.5 ± 1.64	4.03 ± 2.88	3.75 ± 1.83	7.5 ± 4.12
Abnormal cell growth[(a)]	2.5 ± 1.64	0	1.25 ± 1.25	2.5 ± 1.64	2.5 ± 1.64	1.25 ± 1.25
Development delay[(a)]	2.08 ± 2.08	0	0	2.08 ± 2.08	2.08 ± 2.08	0
Hatching[(b)]	87.5 ± 3.66	93.75 ± 2.63	96.25 ± 1.83	84.86 ± 7.06	92.5 ± 2.5	91.25 ± 3.5
Head abnormalities	0	0	0	0	0	0
Eyes abnormalities	0	0	0	0	0	0
Tail abnormalities	2.08 ± 2.08	6.25 ± 4.38	6.25 ± 3.05	4.17 ± 2.73	4.17 ± 2.73	2.08 ± 2.08
Yolk-sac abnormalities	0	0	0	2.08 ± 2.08	2.08 ± 2.08	6.25 ± 3.05
Pericardial edema	0	0	0	0	0	2.08 ± 2.08
Hemorrhages	0	0	0	0	0	0
Muscular involuntary contractions	0	0	0	0	2.08 ± 2.08	0
Total abnormalities	2.08 ± 2.08	6.25 ± 4.38	6.25 ± 3.05	6.25 ± 3.05	4.17 ± 2.73	8.33 ± 3.15
Heart rate	126 ± 3.46	130 ± 3.63	127.5 ± 2.2	130 ± 3.02	127.5 ± 3.5	126 ± 2.93

[(a)]8 hpf [(b)]80 hpf

3.6. Perfluorobutanoic acid (PFBA)

3.6.1. Zebrafish embryos bioassay

At the end of the assay, no significant differences (p>0.05) were detected in any of the endpoints. Table 5 summarizes PFBA exposure to zebrafish in the end of the assay.

3.6.2. Sea urchin embryo bioassay

3.6.2.1. Larval length and abnormalities

In PFBA exposure assay, the larval length ranged from 378.36 ± 16.3 in the 1000 µg/L treatment to 402.73 ± 12.48 in the 100 µg/L concentration. The decrease in the larval length from the 1000, 400 and 2.56 µg/L treatments were significantly different (p<0.05) from the 100 µg/L concentration (Figure 58 A). For the larvae abnormalities percentage, it ranged from 23.54 ± 1.87 in controls to 39.17 ± 4.62 in the 100 µg/L concentration. No significant differences (p>0.05) were detected among groups (Figure 58 B).

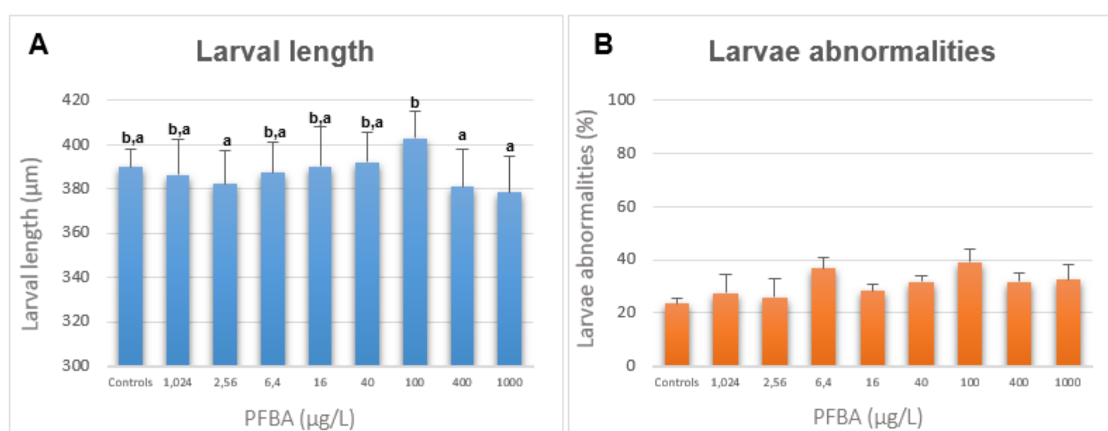

Figure 58 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFBA for 48h. Data are expressed as mean ± SEM (n=480 for controls; n=120 for PFBA exposed groups; n=105 for 2.56 µg/L treatment). Non-parametric ANOVA Kruskall-Wallis (p<0.05), followed by multiple comparisons between groups for both A and B (p>0.05). Bars with different letters are statistically different from each other.

Table 5 - Effects of PFBA exposure in zebrafish embryos at 144 hpf. Data are expressed as mean ± SE (n=8).

ENDPOINT (%)	TREATMENT					
	Water control	Solvent control	10 µg/L	100 µg/L	1000 µg/L	10000 µg/L
Mortality	11.07 ± 4.5	11.25 ± 2.95	8.75 ± 2.27	15 ± 4.63	6.25 ± 2.63	18.8 ± 5.81
Abnormal cell growth[(a)]	9.82 ± 4.71	10 ± 2.67	7.5 ± 1.64	10 ± 2.67	5 ± 1.89	15 ± 4.23
Development delay[(a)]	6.25 ± 4.38	0	0	0	0	0
Hatching[(b)]	81.7 ± 4.9	87.5 ± 3.13	88.75 ± 3.5	82.5 ± 5.26	83.75 ± 4.98	77.5 ± 5.9
Head abnormalities	0	0	0	0	0	0
Eyes abnormalities	0	0	0	0	0	0
Tail abnormalities	2.08 ± 2.08	4.17 ± 2.73	4.17 ± 2.73	4.17 ± 2.73	2.08 ± 2.08	2.08 ± 2.08
Yolk-sac abnormalities	4.17 ± 2.73	2.08 ± 2.08	6.25 ± 3.05	2.08 ± 2.08	2.08 ± 2.08	0
Pericardial edema	0	0	2.08 ± 2.08	0	2.08 ± 2.08	0
Hemorrhages	0	0	0	0	0	0
Muscular involuntary contractions	0	0	0	0	2.08 ± 2.08	0
Total abnormalities	4.17 ± 2.73	4.17 ± 2.73	8.33 ± 4.45	6.25 ± 3.05	4.17 ± 2.73	2.08 ± 2.08
Heart rate	122 ± 5.76	121.5 ± 7.63	122.5 ± 5.07	128.5 ± 3.33	112 ± 3.85	112 ± 4.47

[(a)]8 hpf [(b)]80 hpf

3.7. Perfluorodecanoic acid (PFDA)

3.7.1. Zebrafish embryos bioassay

3.7.1.1. Cumulative mortality

For PFDA exposure assay, the mortality rate was low and at 144 hpf, ranged from 1.25 ± 1.25 to 5 ± 1.89 in water control and 5 ± 2.67 in solvent control and 10 µg/L concentration. No significant differences (p>0.05) were reported between groups (Figure 59).

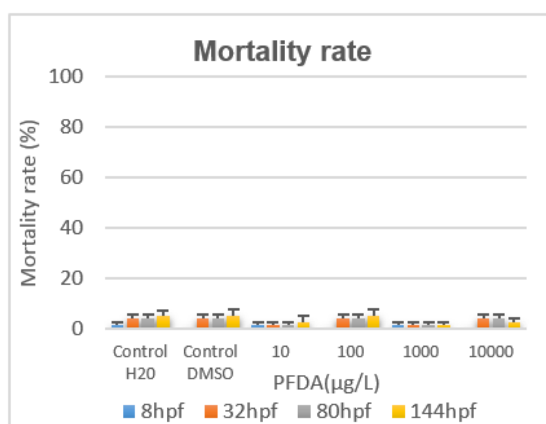

Figure 59 - Cumulative mortality rates (%) of *D. rerio* exposed to different concentrations of PFDA for 144 h. Data are expressed as mean ± SE (n=8). One-way ANOVA.

3.7.1.2. Abnormal cell growth and embryo development delay

The percentage of embryos exhibiting abnormal cell growth at 8 hpf and the embryo development delay at 32 hpf were similar among groups and no significant differences (p>0.05) were observed among treatments (Figure 60 A, B).

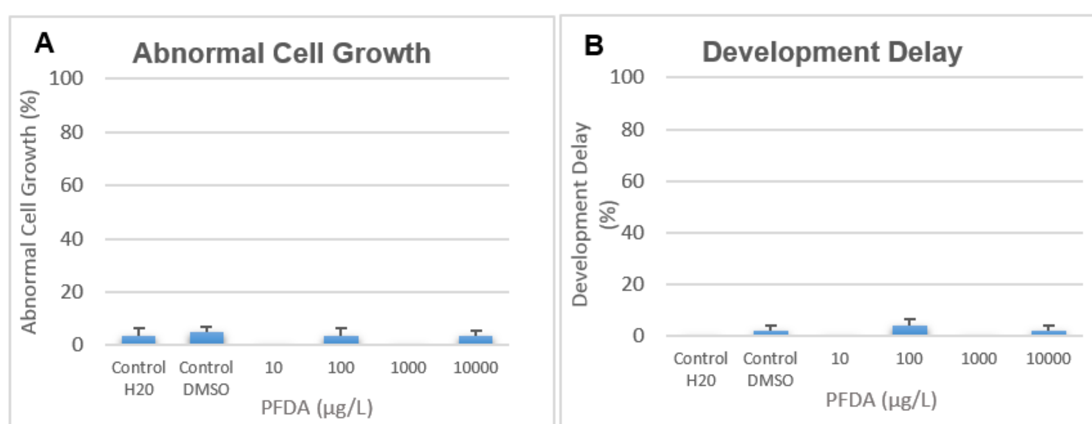

Figure 60 - Abnormal cell growth at 8 hpf and embryo development delay at 32 hpf (%) of *D. rerio* exposed to different concentrations of PFDA for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for both A and B.

3.7.1.3. Hatching rate

At 80 hpf, the percentage of hatched embryos ranged from 86.25 ± 3.24 in the higher concentration to 97.50 ± 1.64 in the 10 μg/L concentration. No significant differences (p>0.05) among groups were reported (Figure 61).

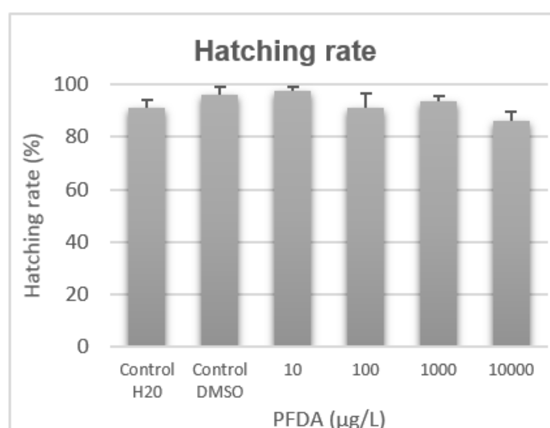

Figure 61 - Hatching rate at 80 hpf (%) of *D. rerio* exposed to different concentrations of PFDA for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). One-way ANOVA.

3.7.1.4. Head and eyes abnormalities

During the assay, no head or eyes abnormalities were detected on embryos during the assay.

3.7.1.5. Tail and yolk-sac abnormalities

At 144 hpf, the percentage of tail abnormalities ranged from 0 in the water control and the first treatment to 12.50 ± 4.17 in the highest concentration. All the tail abnormalities in the higher concentration at 144 hpf represent spinal curvatures (Figure 62 A). This increase was significantly different from the water control and the 10 μg/L concentration. The yolk-sac abnormalities rate was low and no significant differences (p>0.05) were reported (Figure 62 B).

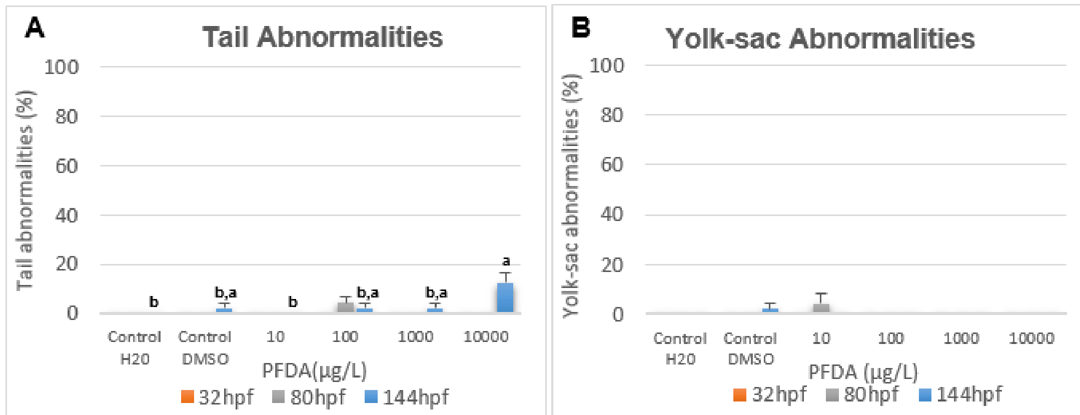

Figure 62 - Tail and yolk-sac abnormalities (%) of *D. rerio* exposed to different concentrations of PFDA for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis followed by multiple comparisons between groups for both A ($p<0.05$) and B ($p>0.05$). Bars with different letters are statistically different from each other.

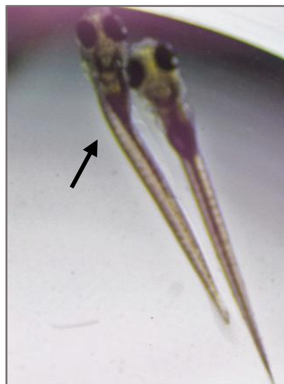

Figure 63 – *D. rerio* embryos at 144 hpf exposed to 10000 µg/L of PFDA. The black arrow points the abnormal embryo, exhibiting spinal curvature.

3.7.1.6. Pericardial edema and hemorrhages

At the end of the assay the percentage of pericardial edemas was low and no significant differences ($p>0.05$) were reported between treatments (Figure 64 A). No hemorrhages were detected at 144 hpf (Figure 64 B).

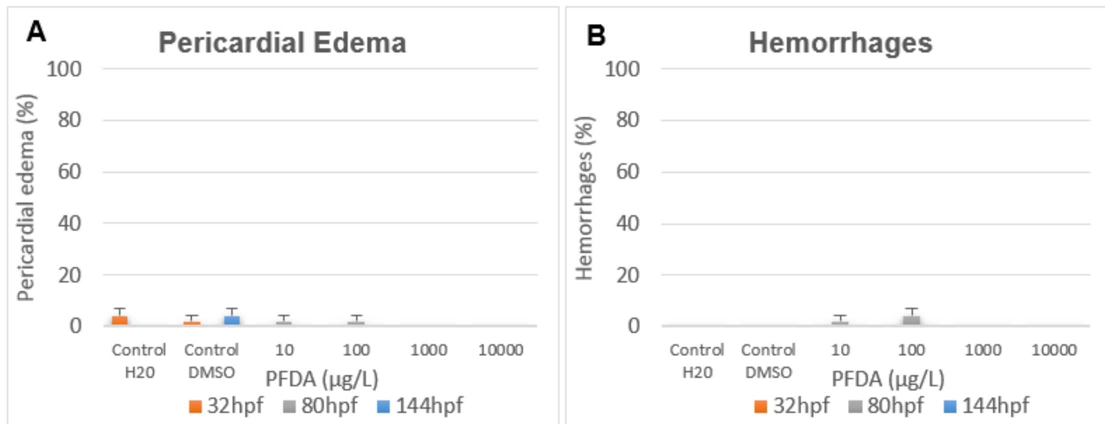

Figure 64 - Pericardial edema and hemorrhages (%) of *D. rerio* exposed to different concentrations of PFDA for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis ($p>0.05$) for A.

3.7.1.7. Muscular involuntary contractions

During the assay, no muscular involuntary contractions were observed.

3.7.1.8. Total abnormalities and heart rate

At 144 hpf the percentage of abnormal embryos ranged from 0 in the water control and the first treatment to 12.50 ± 4.17 in the 10000 µg/L concentration. This increase was significantly different ($p<0.05$) from the water control and the first treatment (Figure 65 A). No significant differences ($p>0.05$) were reported in the embryos heart rate (Figure 65 B).

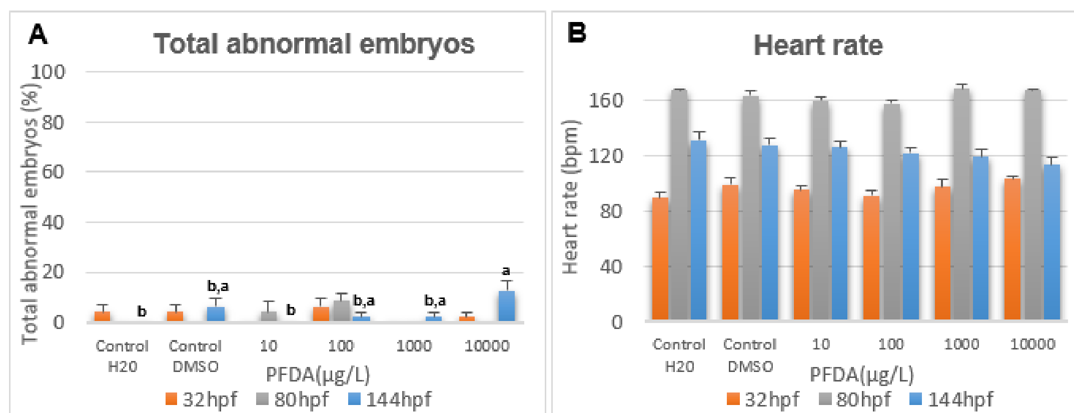

Figure 65 – Total abnormal embryos (%) and heart rate (bpm) of *D. rerio* exposed to different concentrations of PFDA for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for A ($p<0.05$). One-way ANOVA for B ($p>0.05$). Bars with different letters are statistically different from each other.

3.7.2. Sea urchin embryo bioassay

3.7.2.1. Larval length and abnormalities

At the end of the assay, the larval length ranged from 402.92 ± 17.66 in the 1000 µg/L concentration to 452.33 ± 14.27 in the 400 µg/L concentration. The decrease larvae length in the highest concentration was significantly different ($p<0.05$) in comparison with all the other groups (Figure 66 A). The 16 µg/L concentration was significantly different ($p<0.05$) from the 40 and 400 µg/l concentrations (Figure 66 A). The abnormalities rate ranged from 8.89 ± 4.01 in the 2.56 µg/L treatment to 18.89 ± 3.18 in the highest concentration. No significant differences ($p>0.05$) were reported among groups (Figure 66 B).

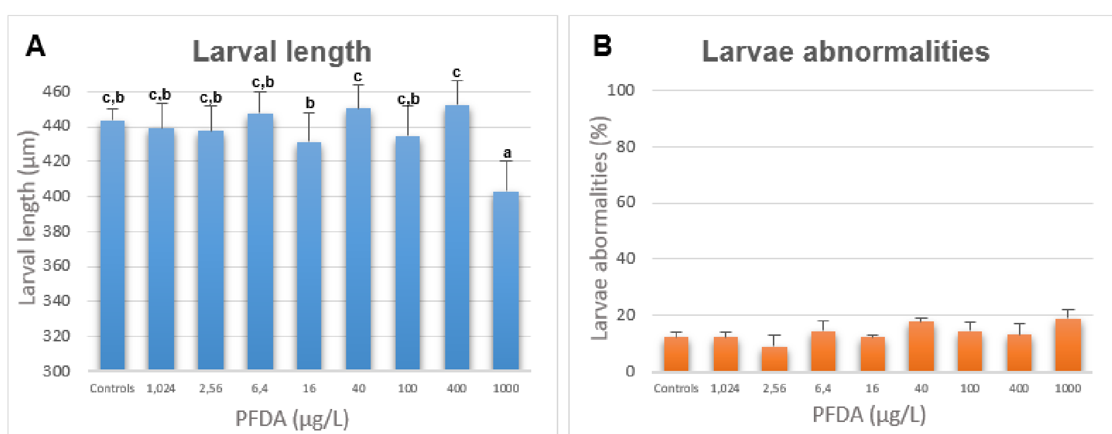

Figure 66 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFDA for 48h. Data are expressed as mean ± SEM (n=360 for controls; n=90 for PFDA exposed group). Non-parametric ANOVA Kruskall-Wallis ($p<0.05$), followed by multiple comparisons between groups for A. One way ANOVA for B. Bars with different letters are statistically different from each other.

3.8. Perfluoroundecanoic acid (PFUnA)

3.8.1. Zebrafish embryos bioassay

3.8.1.1. Cumulative mortality

In this assay, no mortality was observed in all groups at 8 hpf. At the end of the assay, 144 hpf, the mortality ranged from 8.75 ± 2.27 in the water control and 8.75 ± 3.50 in the 1000 µg/L concentration to 81.3 ± 3.5 in 10000 µg/L concentration. At this last observation time-point, the increased mortality in the highest treatment was significantly different ($p<0.05$) in comparison with all the other groups (Figure 67).

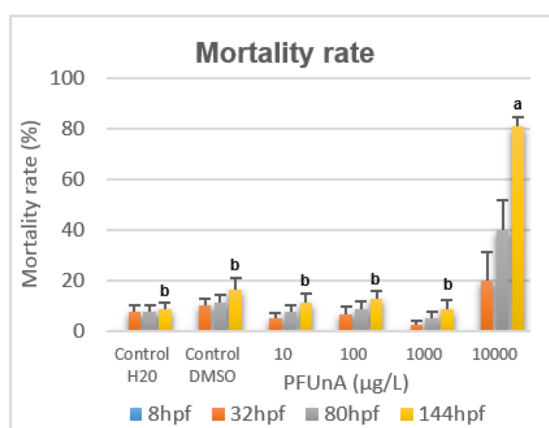

Figure 67 - Cumulative mortality rates (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h. Data are expressed as mean ± SE (n=8). One-way ANOVA. Bars with different letters are statistically different from each other

3.8.1.2. Abnormal cell growth and embryo development delay

No significant differences ($p>0.05$) were detected among groups for both endpoints (Figure 68 A, B).

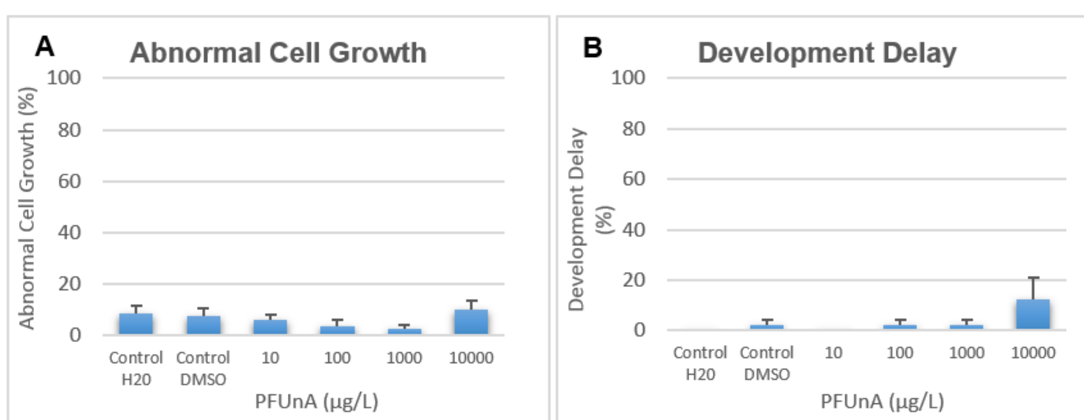

Figure 68 - Abnormal cell growth at 8 hpf and embryo development delay at 32 hpf (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). One-way ANOVA for A. Nonparametric ANOVA Kruskall-Wallis for B.

3.8.1.3. Early hatching and hatching rate

At 24 hpf, the percentage of embryos hatched ranged from 0 in both controls and the lowest exposure group to 58.33 ± 11.79 in the highest concentration. This increase was significantly different (p<0.05) in comparison with all the other treatments (Figure 69 A). The hatching rate at 80 hpf ranged from 35 ± 7.07 in the 10000 µg/L concentration to 95 ± 2.67 in the 10 µg/L concentration. The hatching rate in the higher treatment was significantly different (p<0.05) in comparison to the 10 and 1000 µg/L concentrations (Figure 69 B).

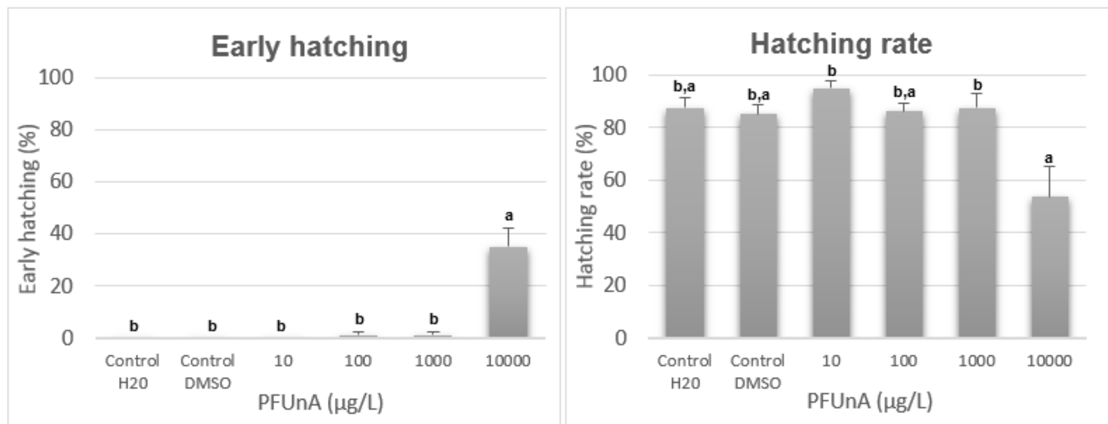

Figure 69 – Early hatching at 24 hpf and Hatching rate at 80 hpf (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for both A and B (p<0.05). Bars with different letters are statistically different from each other.

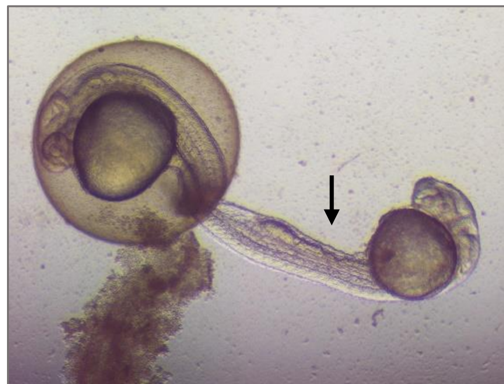

Figure 70 – *D. rerio* embryos at 32 hpf exposed to 10000 µg/L of PFUnA. The black arrow points the early hatched embryo.

3.8.1.4. Head and eyes abnormalities

The percentage of head abnormalities at 144 hpf exhibited no significant differences (p>0.05) among treatments (Figure 71). During this assay, no eyes abnormalities were detected on embryos.

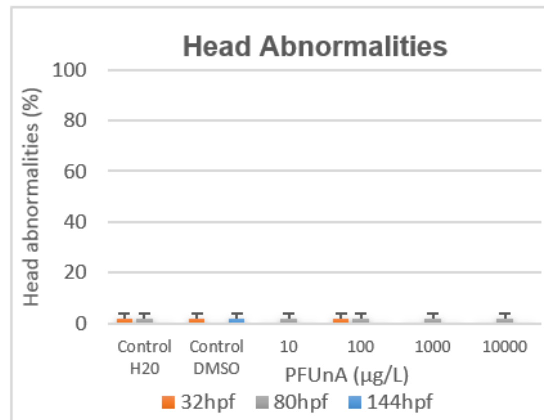

Figure 71 - Head abnormalities (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h . Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis.

3.8.1.5. Tail and yolk-sac abnormalities

The percentage of tail abnormalities at 144 hpf ranged from 4.17 ± 2.73 in the water control and 100 µg/L concentration to 27.78 ± 3.04 in the 10000 µg/L concentration. This increase was significantly different (p<0.05) in comparison with all groups (Figure 72 A). At 144 hpf, the percentage of embryos with yolk-sac abnormalities was low and no significant differences (p>0.05) were detected among groups (Figure 72 B).

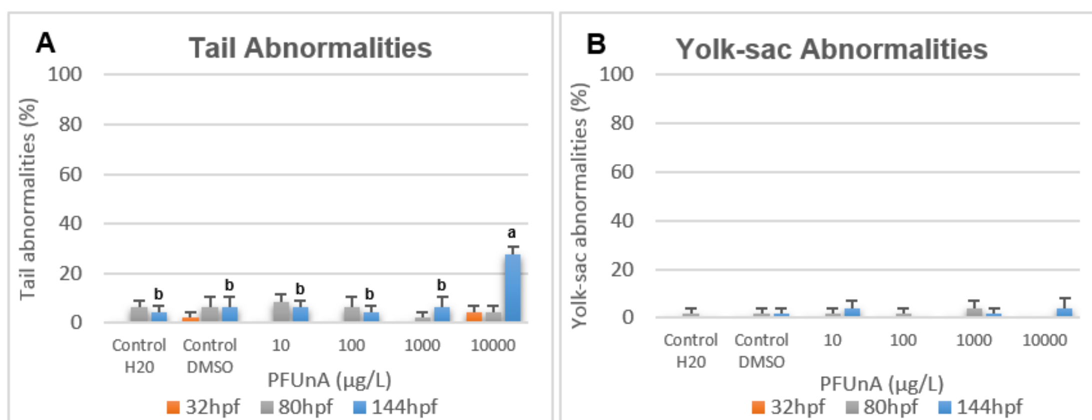

Figure 72 - Tail and yolk-sac abnormalities (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). One-way ANOVA for A (p<0.05). Nonparametric ANOVA Kruskall-Wallis for B (p>0.05). Bars with different letters are statistically different from each other.

3.8.1.6. Pericardial edema and hemorrhages

At 144 hpf the percentage of pericardial edema exhibited no significant differences (p>0.05) among treatments (Figure 73 A). No hemorrhages in embryos were detected at the end of the assay (Figure 73 B).

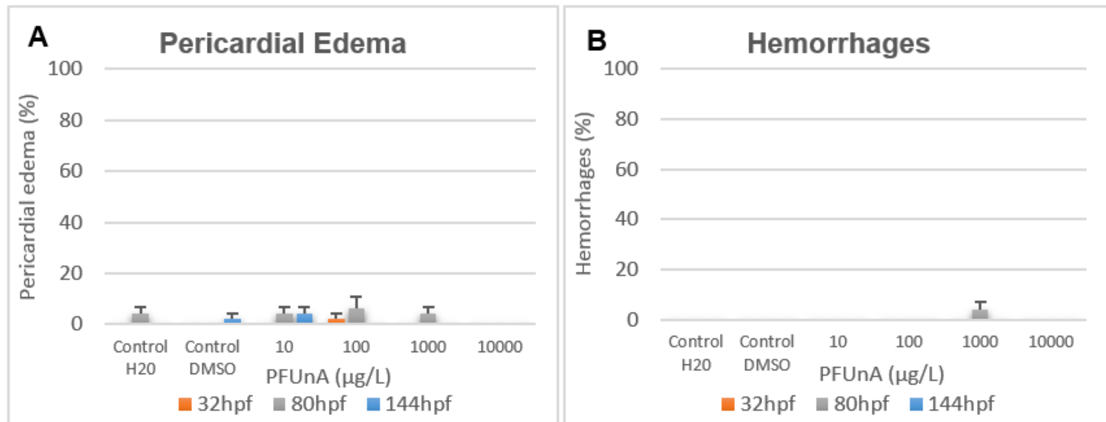

Figure 73 - Pericardial edema and hemorrhages (%) of *D. rerio* exposed to different concentrations of PFUnA for 144 h (A and B, respectively). Data are expressed as mean ± SE (n=8). Nonparametric ANOVA Kruskall-Wallis for A.

3.8.1.7. Muscular involuntary contractions

During the assay, no muscular involuntary contractions were detected.

3.8.1.8. Total abnormalities and heart rate

At the end of the assay, the percentage of total abnormalities ranged from 4.17 ± 2.73 in the water control and the 100 µg/L concentration to 40.48 ± 11.21 in the 10000 µg/L concentration. This increase was significantly different (p<0.05) among groups (Figure 74 A). The heart rate ranged from 104.50 ± 6.57 in the 1000 µg/L concentration to 125.14 ± 8.63 in highest concentration. No significant differences (p>0.05) were detected among groups (Figure 74 B).

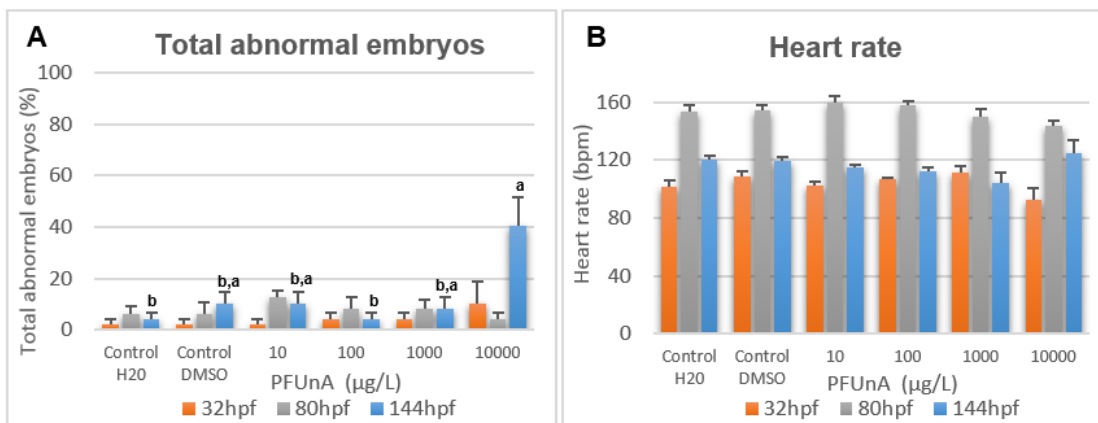

Figure 74 - Total abnormal embryos (%) and heart rate (bpm) of *D. rerio* exposed to different concentrations of PFUnA for 144 h (A and B, respectively. Data are expressed as mean ± SE (n=8). One-way ANOVA for A (p<0.05). Nonparametric ANOVA Kruskall-Wallis for B (p>0.05). Bars with different letters are statistically different from each other.

3.8.2. Sea urchin embryo bioassay

3.8.2.1. Larval length and abnormalities

In the sea urchin exposure to PFUnA, the larval length ranged from 398.86 ± 14.23 in the 1000 µg/L to 452.95 ± 11.8 in the 2.56 µg/L concentration. The increase in the 2.56 µg/L concentration was significantly different ($p<0.05$) from all groups, except for the 100 µg/L concentration, as in the decrease in the higher concentration was significantly different ($p<0.05$) in comparison with all the other groups (Figure 75 A). The percentage of abnormalities ranged from 12.5 ± 4.61 in the 1.024 µg/L concentration to 22.5 ± 2.8 in the 1000 µg/L treatment. No significant differences ($p>0.05$) were reported among treatments (Figure 75 B).

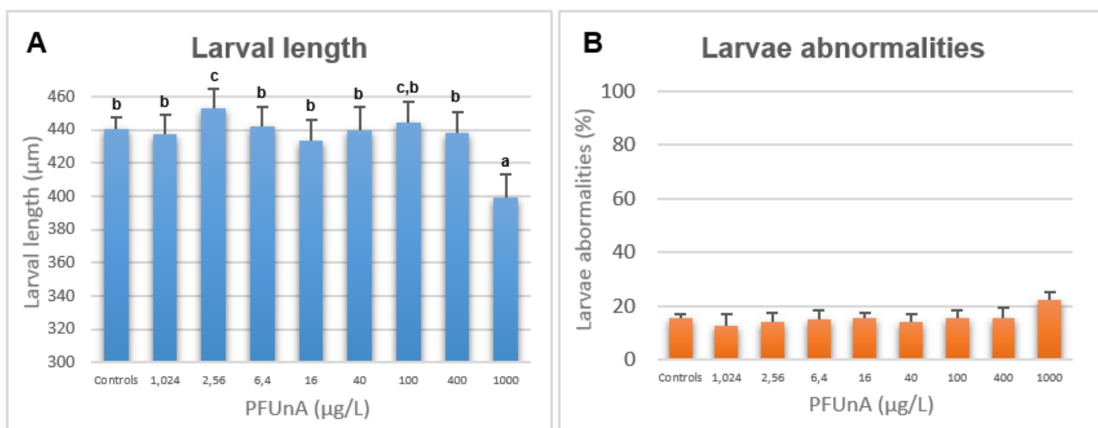

Figure 75 - Larval length (µm) and abnormalities rate (%) of *P. lividus* (A and B, respectively) exposed to different concentrations of PFUnA for 48h. Data are expressed as mean ± SEM (n=480 for controls; n=120 for PFUnA exposed group). One way ANOVA for both A ($p<0.05$) and B ($p>0.05$). Bars with different letters are statistically different from each other.

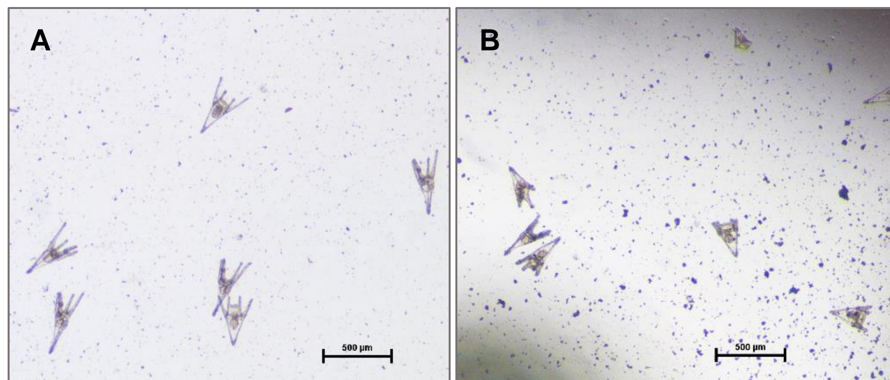

Figure 76 – *P.lividus* at 48 hpf in the control group (A) and exposed to 1000 µg/L of PFUnA (B).

Table 6 – Overview of the overall results, highlighting the significant effects ($p<0.05$) on both model embryo bioassays, for each compound. The [√] marks the significant endpoints that differed significantly from the control treatment.

	ENDPOINT	TCS	M-TCS	PFOS	PFOA	PFBS	PFBA	PFDA	PFUnA
D. rerio	Mortality	√	√	√					√
	Abnormal Cell Growth								
	Development Delay								
	Hatching	√							
	Early hatching								√
	Head abnormalities								
	Eyes abnormalities								
	Tail abnormalities		√						√
	Yolk-sac abnormalities	√	√						
	Pericardial edema	√							
	Hemorrhages		√*						
	Muscular involuntary contractions	√	√						
	Heart rate								
	Total abnormalities	√	√						
P. lividus	Larval length	√	√	√	√	√		√	√
	Abnormalities	√	√						

*at 80 hpf.

Table 7 - Overview of the NOECs and LOECs reported in this study (µg/L).

	D. rerio		***P. lividus***	
	NOEC	**LOEC**	**NOEC**	**LOEC**
TCS	160	400	40	100
M-TCS	160	400	<1.024	1.024
PFOS	1000	10000	1.024	2.56
PFOA	10000	>10000	16	40
PFBS	10000	>10000	2.56	6.4
PFBA	10000	>10000	1000	>1000
PFDA	10000	>10000	400	1000
PFUnA	1000	10000	1.024	2.56

DISCUSSION
CHAPTER IV

4. Discussion

Toxicity assessment of chemicals using organisms' embryonic development is a very sensitive and cost-effective alternative. The increasing use of several chemicals, along with the inefficiency of WWTP stations to complete remove several of these compounds and their metabolites, make this subject of great concern. Many of these compounds are released to the environment and the information about their mechanisms of action, bioaccumulative properties or even possible interactions with other chemicals are still not well understood. This work aimed at improving the toxicological data of two groups of emerging contaminants, i.e., the M-TCS, a metabolite of the disinfectant TCS, and the perfluorinated chemicals PFBS, PFBA, PFDA and PFUnA, in comparison with the well-studied disinfectant TCS and perfluorinated chemicals PFOS and PFOA, respectively. We performed embryo toxicity bioassays using two organisms from different taxonomic groups: the zebrafish (*Danio rerio*) and sea urchin (*Paracentrotus lividus*). The use of different species on chemicals' toxicity assessment allows us to accomplish a more accurate evaluation on their toxicity, since chemicals can affect different biological routes to exert toxicity. Hence, it allow us to better predict chemicals' impact on the aquatic ecosystems. Furthermore, some data extrapolation is possible since in zebrafish, the initial embryonic development stages are relatively well conserved among vertebrates and also, sea urchin being a deuterostomic, allows a better understanding of the mechanisms of action on conserved pathways.

In general, our results revealed that sea urchin larvae were more sensitive to chemicals exposure than zebrafish. The greater sensibility of the sea urchin was also reported by Ribeiro *et al.*, 2015 when exposed to different pharmaceuticals. This fact could be explained by the existence of a chorion in the zebrafish embryos that can work as a barrier to some chemicals, unlike the sea urchin larvae which is directly exposed to water pollutants.

Regarding zebrafish assays, our results show that TCS and M-TCS were both toxic to zebrafish, wherein TCS was apparently more toxic to zebrafish than its metabolite. TCS induced 100 % mortality on embryos at 80 hpf at a concentration of 1000 µg/L (Figure 17 A, B) while M-TCS induced 98 % mortality of embryos but at 144 hpf and at a concentration of 10000 µg/L (Figure 33 A). Comparing both compounds, for a concentration of 1000 µg/L, TCS induced 100% mortality at 80 hpf (Figure 17 A, B) while M-TCS induced mortality to 40% of the embryos only at 144 hpf (Figure 33 B). Our results show that for the same concentration triclosan was more toxic during the first stages of development. In TCS bioassay, 400 µg/L were lethal to 56% of the embryos (Figure 17 B), which is in agreement with previous studies (Oliveira *et al.*, 2009) whereas, in M-TCS bioassay no significant

mortality occurred for the same concentration. Regarding sub-lethal effects, Schmidt *et al.* (2013) observed a significant decrease in the heart rate at 100 µg/L of TCS, as in our assay no significant differences were observed in the embryos heart rate for both TCS and M-TCS (Figure 30 and 44 A, B).

At 32 hpf we observed that TCS induced an increase in pericardial edema on embryos in the 1000 µg/L treatment (Figure 25 B). This abnormality could be an indication of TCS toxicity, since at 80 hpf was lethal to the embryos. On the other hand, M-TCS seemed to affect primarily the cardiovascular system, causing hemorrhages on embryos' pericardial area, but only at 80 hpf (Figure 40 A, B), which resulted on death or severe abnormalities at 144 hpf. Although in different ways TCS and M-TCS seemed to affect primarily the pericardial area. At the end of the assay, M-TCS caused spinal curvature on embryos (Figure 37 A, B) along with yolk-sac abnormalities (Figure 38 A, B) and muscular involuntary contractions (Figure 71 A, B). Similarly, at lower concentrations (400 µg/L), TCS induced yolk-sac abnormalities (Figure 24 B) and muscular involuntary contractions (Figure 27 B) but no tail abnormalities were observed (Figure 23 B). One possible justification for these results could be related with the fact that both compounds can target different signaling pathways. However, the mechanism of action of TCS and its methylation byproduct on aquatic species is not well understood. A non-specific narcosis on tissue is another plausible hypothesis for triclosan toxicity (Lyndall *et al.*, 2010).

For sea urchin bioassay, both endpoints (larval length and percentage of abnormities) were significantly affected by TCS and M-TCS. Although TCS reduced larval length at all concentrations, only at 100 µg/L was significantly different from controls (Figure 31 A). Regarding the percentage of abnormalities, in the two highest treatments (1000 and 400 µg/L), TCS affected larvae development whereas in the highest concentration all larvae were in 2 or 4-cell stage (Figure 32 B). At 400 µg/L, TCS also delayed larvae development, yet, some larvae reached an abnormal *pluteus* stage (Figure 32 B). M-TCS affected significantly the larval length at all concentrations (Figure 46 A), but only the highest concentration (1000 µg/L) increased significantly the percentage of abnormalities (Figure 46 B). Both compounds seemed to be very toxic to the sea urchin larvae. Contrarily to zebrafish assay, M-TCS seemed to affect the organism at lower concentrations than the parental compound. Yet, it was not as toxic as TCS for the two higher concentrations. Thus, for higher concentrations, TCS was more toxic than its metabolite, affecting the larvae in the first hours after fertilization, but for lower concentrations, sea urchin larvae were more sensitive to M-TCS. The concentrations tested were of environmental relevance for triclosan, whereas, M-TCS has been detected in the order of ng/L range (Table 8).

Recently, a study conducted on TCS toxicity with sea urchin reported that TCS affected the larvae development of *Strongylocentrotus nudus* at 113 µg/L (Hwang *et al.*,

2014), which is in agreement with our results. Very few studies have been conducted on M-TCS toxicity to aquatic organisms but a 72 hours bioassay on the algae *Scenedesmus subspicatus* reported an EC_{50} on growth rate at 170 µg/L (Batscher, 2006b) and also, a 48 hours bioassay on *Daphnia magna* immobilization, reported a NOEC of 180 µg/L (Batscher, 2006a). In comparison with our results on sea urchin, the larval length endpoint used appears to be more sensitive.

In general, our results are in agreement with other previous studies. As reviewed by Brausch and Rand, 2011, short-term exposure assays with disinfectants on different species have revealed to be more toxic to invertebrates than fish. Furthermore, among other properties, TCS and M-TCS both show potential to adsorb to the sediment which could be a concerning fact since it can be an indicative of direct exposure to benthic organisms as is the case for the sea urchin (Orvos *et al.,* 2002). Our study contributed to understand how the presence of the metabolite of this disinfectant can adversely affect the organisms, and although it was not as toxic as TCS for the fish, M-TCS was more toxic to the sea urchin than the parental compound, inhibiting the larval length. The impact on larval length could be an indication of an alteration on the normal development of the invertebrate and possibly reducing their mobility and potential for survival. Hence, long-term exposure studies should be conducted on this subject to better understand the possible impact of these chemicals on sea urchin and perhaps other invertebrate phyla.

PFAAs exhibited a low toxicity to zebrafish embryo assays, being less toxic than PFOS. Our findings are in agreement with the limited studies available in literature where a low toxicity was also reported (Hagenaars *et al.*, 2011; Ding *et al.*, 2013; Ulhaq *et al.*, 2013a). Nevertheless, there is some inconsistency in the true NOEC of PFOS on zebrafish in the literature, e.g. Huang *et al.* (2010) reported an LC_{50} at 120 hpf of 2.20 mg/L, Zheng *et al.* (2012) reported an LC_{50} at 72 hpf of 68 mg/L, and also, Shi *et al.* (2008) reported significant effects on zebrafish only after 84 hpf, detecting differences in the hatching rate at 3 mg/L and abnormalities in all embryos at 132 hpf for the concentration of 1 mg/L. In our assay, no significant differences were detected before the 80 hpf as in Shi *et al.* (2008), but in 144 hpf observation, all embryos were dead in the 10000 µg/L concentration (Figure 48). No significant differences were detected in the hatching rate but apart from the 100 µg/L concentration, it is possible to observe a decrease on embryos hatching, when compared to controls (Figure 50). PFOS development toxicity to aquatic organisms is described to be in the range of 1 – 100 mg/L, which validates our data (Giesy *et* al., 2010). Furthermore, on a subcellular level, Shi *et al.*, 2010 reported an effect of PFOS on hypothalamus-pituitary-thyroid axis' hormones of zebrafish embryos.

PFOA showed no significant effects ($p<0.05$) for any of the endpoints at the concentrations tested (Table 3). Some of the studies conducted in the zebrafish confirm our

data by reporting an EC_{50} at 72 hpf of 200 mg/L (Zhen *et* al., 2012) and Hagenaars *et al.*, reported and EC_{50} of 100 mg/L at 120 hpf. Both studies report effects at concentrations above the levels tested. It is clear that low doses of PFOA do not cause phenotypic alterations on embryos, however, PFOA ability to induce endocrine disruption on zebrafish has been reported. Recently, Du *et al.*, 2013 reported that in a short-term assay, PFOA increased the expression levels of several genes in the signaling pathway of estrogen receptors, early thyroid development and steroid synthesis genes on zebrafish.

Comparing these two PFAAs, it is possible to conclude that zebrafish was more sensitive to PFOS than PFOA. This fact supports the idea of the functional group being important on the PFAAs toxicity (Zheng *et* al., 2012; Ulhaq *et al.*, 2013a,b).

Exposure of zebrafish embryos to PFBS and PFBA did not induce any significant effects ($p>0.05$) for any of the endpoints considered (Table 4 and 5, respectively). Few toxicity studies have been conducted on these two chemicals but limit data indicates that PFBS appears to be more toxic than PFBA, most likely due to the presence of a sulphonic functional group, as is the case for its homologue, PFOS (Hagenaars *et al.*, 2011; Ulhaq *et al.*, 2013a). However, these studies were conducted with a concentration range much higher than the one used in our study. Besides the functional group, the chain length appears to be a possible explanation for PFOS greater toxicity in comparison with its substitute (Hagenaars *et* al., 2011; Ulhaq *et al.*, 2013a,b).

In the present study, PFDA did not affect zebrafish development to the same extent as PFOS, but induced an increased number of abnormalities on embryos, causing spinal curvature (Figure 62 A). Consequently, increased the number of total abnormalities (Figure 65 A) though it was not significant when compared with the solvent control. However, Ulhaq *et al.*, 2013a observed frequently spinal curvature on zebrafish exposed to PFDA, reporting an EC_{50} of 5 mg/L at 144 hpf and so, though our results did not show such high toxicity, could still be an indication of PFDA toxicity on the embryos. As a long-chained perfluorinated compound (C10) it was expected to be more toxic than the shorter-chain length PFAAs (PFBS and PFBA). Our results show significant effects for the highest concentration but only at 144 hpf and only when compared with water control. This could be due to the presence of chorion which may protect the embryos until hatching. Few studies have been conducted on the toxicity of this chemical (Ding *et al.*, 2012; Ulhaq *et al.*, 2013a); one study reported that PFDA had immunomodulatory effects on rats (Nelson *et al.*, 1992) but there is a serious lack on PFDA toxicity assessment to aquatic organisms, especially in marine species.

PFUnA was the second more lethal PFAA to zebrafish but induced toxicity in earlier stages of the embryo development. At 24 hpf, PFUnA induced early chorion softening of the fish on the 10000 µg/L concentration causing almost 35% of embryos hatching (Figure

69 A). Consequently, a decrease on hatching rate at 80 hpf in the same concentration was observed (Figue 69 B). Natural chorion softening in zebrafish is usually due to proteolytic enzymes' digestion of the chorion which are secreted by the embryo during pre-hatching stages (Kim *et al.*, 2006). A possible explanation for this results could be that PFUnA' presence induced the embryo to secrete this enzymes, anticipating the natural process of hatching, or even, the compound itself could have digested the chorion from outside. This effect could adversely affect the embryo on the wild-life since the chorion could no longer offer protection, inducing high embryo mortality.

Unlike the PFOS and PFDA, this compound affected the embryos before 80 hpf, since the mortality increase occurred right after the 32 hpf observation, reaching up to 80% of embryos' mortality at the end of the assay (Figure 67). This fact could be associated with the lack of protection from the chorion, being directly exposed to the chemical, inducing toxicity. Furthermore, at 144 hpf PFUnA significantly induced an increase on tail abnormalities on the remaining embryos in the higher concentration (Figure 72 A). Very few studies have been conducted on aquatic organisms for addressing PFUnA toxicity (e.g. Ding *et al.*, 2012), however, several studies were conducted on PFUnA occurrence and distribution on biota (Houde *et al.*, 2011; Lindstrom *et al.*, 2011; Hong *et al.*, 2014). Due to its long-length carbon chain (C11), PFUnA have propensity to accumulate on organisms and though it is not find in great concentrations on surface waters, has been detected in concentrations up to 201 ng/g on birds' egg yolks, being the most dominant compound in the study (Yoo *et al.*, 2008). Furthermore, its ubiquitous presence is concerning. PFUnA was the dominant perfluorocarboxilic acid found in Canadian artic species liver. Concentrations up to 68 ng/g were detected on polar bears (Martin *et al.*, 2004).

Concerning sea urchin bioassay, a similar response in the tested PFAAs was observed. In the larval length endpoint the higher concentration tested (1000 µg/L) was significantly different for all the perfluorinated compounds when compared to the controls apart from (Figure 58 A). Among PFAAs, PFOA inhibited larvae growth the most (Figure 56 A). Regarding the percentage of larvae abnormalities, no significant differences were observed after 48 h for any of the PFAAs.

In PFOS, the 2.56 µg/L treatment also showed a significant effect on larval length, though at higher concentrations no effect was observed, except for the 1000 µg/L concentration (Figure 55 A). Other studies conducted on this subject reported effects only at higher concentrations. A study reported a LOEC at 2 mg/L for growth inhibition of *P. lividus* (Mhadhbi *et al.*, 2012) while another study reported significant differences on larval malformations for organisms exposed to 1 mg/L. In our study, the percentage of larval abnormalities was not significantly different for any of the concentrations tested, however,

for the 1000 µg/L concentration there is a clear increase in comparison with the controls (Figure 55 B) which could confirm PFOS toxicity to larvae.

For PFOA, the larvae length from the 40 and 100 µg/L concentrations were also significantly affected (Figure 56 A). Mhadhbi *et al.* (2012) reported an LOEC of 20 mg/L for growth inhibition of *P. lividus* but in our study, 0.04, 0.1 and 1 mg/L concentrations affected significantly the larval length, which means that the sea urchin larvae were more sensitive to PFOA in our assay. One possible explanation could be the fact that a solvent to deliver the PFOA was not used in Mhadhbi *et* al. (2012) which could impact the chemical solubility in water. In our study, PFOA did not induce significant abnormalities on larvae for any of the concentrations tested (Figure 56 B). In fact, very few studies were conducted on marine invertebrates and information on this subjected is needed.

PFBS inhibited larval growth, but only for the higher concentration was a significant decreased in comparison with the control groups. Also, the 6.4 µg/L treatment induced larval length increase (Figure 57 A). Other studies reported stimulation of larval development when exposed to other PFAAs (Anselmo *et al.*, 2011). Thought it was not detected any significant differences on abnormalities percentage it is possible to observe an increase on abnormalities for higher concentrations, which could be indicative of possible toxicity for long-term exposures. For PFBA, the 100 µg/L concentration was the only concentration significantly different, but only differed from the 2.56, 400 and 1000 µg/L concentrations, showing a stimulation of the larvae development for this concentration (Figure 58 A). In the sea urchin bioassay, the short-chained PFBS seemed to be in the toxicity range of the long-chained compounds PFOS and PFOA. To our knowledge no other study was conducted on PFBS and PFBA on sea urchin and so, more studies on this subject should be considered in order to improve knowledge on possible impact on marine organisms.

PFDA, significantly inhibited larval length at 1000 µg/L in comparison to controls, but no significant differences were reported on larvae abnormalities percentage (Figure 66 A, B). Similarly, PFUnA also did not induce any abnormalities in comparison with controls. As mentioned earlier, the highest concentration inhibited larvae growth but contrarily, the 2.56 µg/L concentration stimulated larval length as in PFBS assay (Figure 75 A, B).

Overall, our study encompassed toxicological information on two different classes of chemicals with environmental relevance. Our results show that the disinfectant and its byproduct were more toxic to both organisms than PFAAs, whereas PFOS and PFUnA were the most lethal to zebrafish. This could be explained by the fact that triclosan is conceived to act on microorganisms, contrarily to the perfluorinated compounds. Moreover, in zebrafish bioassay, mortality rate was the endpoint with more significant differences for the tested compounds. As for the sea urchin assay, the larval length was significantly affected for all the tested compounds in this study. For most of PFAAs as for M-TCS, this was a first

approach and our data reveals a higher sensitivity of this endpoint for these chemicals as also reported in Ribeiro *et al.* (2015).

Most of the concentrations tested were above the ones that are reported in the environment (Table 8). However, PFBS stimulated larvae development at 6.4 µg/L, which is within the concentration range detected in the environment (Table 8). Furthermore, PFOS and PFOA exposure to sea urchin larvae showed a significant impact on larvae length at environmental relevant concentrations (2.56 µg/L and 40 µg/L, respectively). Although some of the concentrations above did not affect the larvae development significantly, it is certainly warning and further investigation should be conducted. Furthermore, all PFAAs (except PFBA) induce larvae development delay at 1000 µg/L and previous studies reported accidental PFAAs releases reaching up to 17000 µg/L concentrations of total PFAAs (Moody *et al.*, 2002). Moreover, it is important to highlight that M-TCS inhibited larvae length at very low concentrations, and even though it does not reach such values on surface waters, its parental compound is detected at concentrations much higher than 1 µg/L (Table 8).

Table 8 – Comparison of the LOEC values reported on this study and maximal concentrations on surface waters reported on literature.

	LOEC (*D. rerio*)	**LOEC (*P. lividus*)**	**Maximal concentrations**	**Literature**
TCS	400 µg/L	100 µg/L	22 µg/L	Agüera *et al.*, 2003
M-TCS	400 µg/L	1.024 µg/L	0.19 µg/L	Pintado-Herrera *et al.*, 2014
PFOS	10000 µg/L	2.56 µg/L	21.3 µg/L	Zhou *et al.*, 2013
PFOA	>10000 µg/L	40 µg/L	67 µg/L	Nakayama *et al.* 2004
PFBS	>10000 µg/L	6.4 µg/L	15.3 µg/L	Zhou *et al.*, 2013
PFBA	>10000 µg/L	>1000 µg/L	47.8 µg/L	Zhou *et al.*, 2013
PFDA	>10000 µg/L	1000 µg/L	0.16 µg/L	Knowick *et al.*, 2008
PFUnA	10000 µg/L	2.56 µg/L	0.00352 µg/L	Naile *et al.*, 2010

It would be interesting to understand PFAAs toxicity at a biochemical/molecular level in order to provide a more accurate prediction on the biochemical effects on organisms. Some studies were conducted on that subject (e.g. Shi and Zhou, 2010). Furthermore, it will be important to complement the data obtained here with chronic long-term assays as we cannot discard the possibility of chronic effect due to life-cycle exposure.

CONCLUSION AND FUTURE PERSPECTIVES
CHAPTER V

5. Conclusion and Future perspectives

Our work contributed for a better understanding on the ecotoxicological risk of several emerging contaminants. The compounds exhibiting higher toxicity were TCS in the zebrafish embryos bioassay and M-TCS in the sea urchin larvae bioassay. The PFAAs tested exhibited low toxicity for zebrafish, however, in the sea urchin bioassay, some PFAAs affected the embryo development at concentrations of environmental relevance. The sea urchin larval length has proved to be a very sensitive endpoint for ecotoxicological studies, as reported in the literature, being recognized by the responsible entities as the OECD and EPA.

The toxicological information of some of these chemicals on both organisms is new and so, it is important to carry on the studies on this subject and probably involve long-term exposure to understand the impact of these compounds in full life-cycle tests. For a more thorough risk assessment it would also be interesting to expose the embryos to PFAAs in mixture since it is known that in the environment, combined exposure are usually common. Furthermore it would also be important to expose sea urchin larvae to lower concentrations of M-TCS to refine NOEC values.

As a follow up of this study it will be relevant to carry biochemical and molecular studies to understand potential effects at the subcellular level and the impacted pathways.

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