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 sulfônico (PFOS) e do ácido perfluorooctanoico (PFOA), outros compostos homólogos têm sido produzidos com o intuito de substituir os anteriores, como o ácido perfluorobutano sulfônico (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, respectivamente. 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.
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List of Abbreviations and acronyms

µg Microgram
bpm Beats Per Minute
CECs Contaminants of Emerging Concern
DMSO Dimethylsulfoxide
EC₅₀ Effect Concentration for 50% of the population
ECF Electrochemical fluorination
g Gram
hpf Hours Post Fertilization
LC₅₀ Lethal Concentration for 50% of the population
LOEC Lowest Observed Effect Concentration
log Kₒₒ Soi Organic Carbon-Water Partition Coefficient
log Kₒₒw 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 Perfluoroalkyls 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-triclosan (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, bioaccumulates (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.
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.

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 Perfluoroalkyls carboxyls acids (PFCAs) and more recently the Perfluoroalkyls phosphonates acids (PFPAs) (Figure 3) (Lau et al., 2012).
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).
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 perfluoroalkyl compounds chemical formula.

Regarding their distribution, PFAAs are ubiquitous in the environment, being already found in some remote areas such as the Arctic 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.
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.
Regarding toxicity studies on these two chemicals, several studies have been conducted recently on zebrafish embryos (Shi et al., 2008; Huang et al., 2010; Hage naars et al., 2011; Zheng et al., 2012; Ding et al., 2013; Ulhaq et al., 2013a,b; Hage naars 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). Hage naars 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 (Hage naars 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 *Chydomus 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.

![Chemical Structures](image)

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 Shenyang, 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 Daphnia 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.

![Perfluorodecanoic acid (PFDA) chemical structure.](image)

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.

![Figure 9 – Perfluoroundecanoic acid (PFUnA) chemical structure.](http://www.chemspider.com/Chemical-Structure.69649.html)


**PFUnA**

\[
\text{C}_{11}\text{HF}_{21}\text{O}_2
\]

log\(K_{oc}\): 3.30 ± 0.11 L/kg\(^1\)

VP: -0.98\(^2\)
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.

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<tr>
<th>Abbreviation</th>
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<td>TCS</td>
<td>Triclosan</td>
<td>3380-34-5</td>
</tr>
<tr>
<td>M-TCS</td>
<td>Methyl-triclosan</td>
<td>4640-01-1</td>
</tr>
<tr>
<td>PFOS</td>
<td>Perfluorooctanesulfonic acid</td>
<td>2795-39-3</td>
</tr>
<tr>
<td>PFOA</td>
<td>Perfluorooctanoic acid</td>
<td>335-67-1</td>
</tr>
<tr>
<td>PFBS</td>
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<td>375-73-5</td>
</tr>
<tr>
<td>PFBA</td>
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<td>Perfluorodecanoic acid</td>
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</tr>
<tr>
<td>PFUnA</td>
<td>Perfluoroundecanoic acid</td>
<td>2058-94-8</td>
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</tbody>
</table>

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

<table>
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<th>Molecular formula</th>
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<td>NaCl</td>
<td>Sodium chloride</td>
<td>7647-14-5</td>
</tr>
<tr>
<td>NaHCO₃</td>
<td>Sodium bicarbonate</td>
<td>144-55-8</td>
</tr>
<tr>
<td>MgSO₄</td>
<td>Magnesium sulfate</td>
<td>7487-88-9</td>
</tr>
<tr>
<td>MgCl₂·6H₂O</td>
<td>Magnesium chloride hexahydrate</td>
<td>7791-18-6</td>
</tr>
<tr>
<td>CaCl₂</td>
<td>Calcium chloride</td>
<td>10043-52-4</td>
</tr>
</tbody>
</table>
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 10-15°C in winter to 18-25°C 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*)](https://pixabay.com/pt/ouri%C3%A7o-do-mar-animal-natureza-597313/)

Source: Pixabay Database.
In our study, the organisms were all collected in intertidal areas in the Northern Portugal, Vila Nova de Gaia, Granja (N41° 2’ 26,18”, W -8° 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).](image)

### 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 20°C 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.

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.

![Zebrafish (Danio rerio)](https://commons.wikimedia.org/wiki/File:Zebrafisch.jpg)

**Figure 13** – Zebrafish (*Danio rerio*).
Source: Wikimedia Commons repository.

The zebrafish stock was obtained from local suppliers in Singapore and kept in laboratory in a 160L aquarium with dechlorinated water at 28°C ± 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.
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 26.5°C ± 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.
**Figure 15** – Experimental design for zebrafish bioassay. C – Control; C+S – Solvent Control; T – Treatment.

<table>
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**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.](image)

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).
3.1.1.3. Embryo development delay

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

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
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.

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.
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).
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).
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 (%)](image)

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 (%)](image)

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 each other.](image)

![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.](image)
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).

![Graph](image)

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.](image)

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.](image)
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).

![Hatching rate](image1)

**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).

![Head Abnormalities](image2)

**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. Bars 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 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.](image1)

![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.](image2)
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).

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).
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 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.

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 10000µg/L in A n=1; for 1000 µg/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).

![Mortality rate graph](image)

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).

![Abnormal cell growth and development delay graphs](image)

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).

![Graph showing hatching rate at 80 hpf (%).](image)

**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).

![Graph showing head abnormalities (%).](image)

**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 (%)](image)

**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 (%)](image)

**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](image)

**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](image)

**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.](image_url)
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<td>Eyes abnormalities</td>
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<td>Yolk-sac abnormalities</td>
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\( ^{a} \)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.](image-url)
Table 4 - Effects of PFBS exposure in zebrafish embryos at 144 hpf. Data are expressed as mean ± SE (n=8).

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<td>Yolk-sac abnormalities</td>
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<td>Hemorrhages</td>
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*(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.](image-url)
Table 5 - Effects of PFBA exposure in zebrafish embryos at 144 hpf. Data are expressed as mean ± SE (n=8).

<table>
<thead>
<tr>
<th>ENDPOINT (%)</th>
<th>TREATMENT</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Water control</td>
</tr>
<tr>
<td>Mortality</td>
<td>11.07 ± 4.5</td>
</tr>
<tr>
<td>Abnormal cell growth&lt;sup&gt;(a)&lt;/sup&gt;</td>
<td>9.82 ± 4.71</td>
</tr>
<tr>
<td>Development delay&lt;sup&gt;(a)&lt;/sup&gt;</td>
<td>6.25 ± 4.38</td>
</tr>
<tr>
<td>Hatching&lt;sup&gt;(b)&lt;/sup&gt;</td>
<td>81.7 ± 4.9</td>
</tr>
<tr>
<td>Head abnormalities</td>
<td>0</td>
</tr>
<tr>
<td>Eyes abnormalities</td>
<td>0</td>
</tr>
<tr>
<td>Tail abnormalities</td>
<td>2.08 ± 2.08</td>
</tr>
<tr>
<td>Yolk-sac abnormalities</td>
<td>4.17 ± 2.73</td>
</tr>
<tr>
<td>Pericardial edema</td>
<td>0</td>
</tr>
<tr>
<td>Hemorrhages</td>
<td>0</td>
</tr>
<tr>
<td>Muscular involuntary contractions</td>
<td>0</td>
</tr>
<tr>
<td>Total abnormalities</td>
<td>4.17 ± 2.73</td>
</tr>
<tr>
<td>Heart rate</td>
<td>122 ± 5.76</td>
</tr>
</tbody>
</table>

<sup>(a)</sup>8 hpf  <sup>(b)</sup>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).

![Mortality rate graph](image)

**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).

![Abnormal cell growth and development delay graphs](image)

**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).

![Hatching rate](image)

**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).
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 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.
3.7.1.7. 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).
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).

![Larval length (µm) and abnormalities rate (%)](image)

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 $8.75 \pm 2.27$ in the water control and $8.75 \pm 3.50$ in the 1000 µg/L concentration to $81.3 \pm 3.5$ 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).

![Mortality rate chart](image)

**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).

![Abnormal cell growth and Development Delay charts](image)

**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](image1.png)  
**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](image2.png)  
**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.](image)

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.](image)
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.](image)

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.](image)
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.

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<th>ENDPOINT</th>
<th>TCS</th>
<th>M-TCS</th>
<th>PFOS</th>
<th>PFOA</th>
<th>PFBS</th>
<th>PFBA</th>
<th>PFDA</th>
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at 80 hpf.

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

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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 completely 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 allows 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 abnormalities) 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.,
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 EC50 at 72 hpf of 200 mg/L (Zhen et al., 2012) and Hagenaars et al., reported and EC50 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 EC50 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 (Figure 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). Though 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 larva 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 larva 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 larva 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.

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

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.
6. References


in fish and suspended particulate matter: Results from the German Environmental Specimen Bank. *Chemosphere*, 91(11), 1517-1524.


