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Long-term effects of bisphenol S at environmentally relevant concentrations on the zebrafish life-cycle

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Abstract

Contaminants of emerging concern (CECs) are a group of chemicals whose effects on the environment and public health are still largely unknown. More than 700 CECs are known to be present in aquatic environments originating from a variety of sources: plasticizers, pharmaceuticals, microplastics, etc., and their presence in the aquatic environment is ubiquitous. One of these CECs is Bisphenol A that, due to harmful effects on multiple organisms had to be replaced. Bisphenol S (BPS) is one of the recent alternatives to BPA that is used to produce many everyday products, and so, is present in almost every aquatic environment with potential toxicity to the aquatic life. Recent studies have showed that BPS can cause effects on non-target aquatic organisms including endocrine disruption, neurotoxicity and immunotoxicity. Despite that, there is still gaps of knowledge regarding its long-term effects. Therefore, in order to explore the long-term effects of this chemical on *Danio rerio*, three environmental relevant concentrations were selected (400 ng/L, 2000 ng/L and 10000 ng/L) and the model species was exposed for 4 months (F0) where apical and biochemical endpoints were then analyzed, and the non-exposed offspring (F1) were followed until 8 days post fertilization to assess behavior endpoints. This study showed that BPS increased the female gonadosomatic index, had negative effects on fertility, disrupted acetylcholinesterase activity and several markers of oxidative stress in f1 exposed generation and affected the behavior of non-exposed larvae (F1).

These results help expanding the knowledge of the long-term effects of environmentally relevant concentrations of BPS, demonstrating the significant impact that this chemical can have on aquatic organisms.

Resumo

Os contaminantes de preocupação emergente (CECs) são um grupo de químicos cujos efeitos no meio ambiente e na saúde pública são maioritariamente desconhecidos. São conhecidos mais de 700 CECs encontrados no meio aquático e originam de diversas fontes: plastificantes, fármacos, mioplásticos, etc., e a sua presença no meio aquático é ubíqua. Um destes CECs é o bisfenol A (BPA) que devido aos seus efeitos prejudiciais em vários organismos teve de ser substituído. O bisfenol S (BPS) foi um dos substitutos ao bisfenol A (BPA) que devido à sua ampla utilização acaba por estar presente em quase todos os ambientes aquáticos. Estudos recentes têm vindo a provar que o BPS tem efeitos em organismos não alvo, incluindo disrupção endócrina, neuro e imunotoxicidade. Apesar disto ainda há uma grande falta de conhecimento acerca dos efeitos do BPS a longo prazo. Assim sendo, de forma a explorar os efeitos a longo prazo deste composto no *Danio rerio*, três concentrações ambientalmente relevantes foram selecionadas (400 ng/L, 2000 ng/L e 10000 ng/L), a espécie modelo foi exposta durante 4 meses (F0) e foram recolhidos dados apicais e bioquímicos, e a descendência não diretamente exposta (F1) foi seguida até 8 dias após a fertilização de forma a avaliar possíveis diferenças comportamentais. Este estudo mostrou que o BPS aumentou o indicie gonadossomático das fêmeas, prejudicou a fertilidade, afetou a atividade da acetilcolinesterase e perturbou o comportamento de larvas não diretamente expostas (F1).

Estes resultados ajudam a expandir o conhecimento acerca dos efeitos de contração ambientalmente relevantes de BPS a longo prazo, demonstrando o impacto significativo que este químico pode ter em organismos aquáticos.

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List of Abbreviations and Acronyms

ACh – Acetylcoline

AChE – Acetylcholinesterase

BOGA – Biotério de Organismos Aquáticos

BPA – Bisphenol A

BPAF – Bisphenol AF

BPF – Bisphenol F

BPS – Bisphenol S

CAT – Catalase

CECs – Contaminants of emerging concern

CIIMAR - Centro Interdisciplinar de Investigação Marinha e Ambiental

dpf – Day(s) post fertilization

DTT - Dithiothreitol

EDTA – Ethylenediaminetetraacetic acid

ERs – Estrogenic receptors

erα – Estrogen receptor alpha

F0 – Generation of fish exposed to BPS

F1 – Generation of unexposed fish, resulting from reproduction of F0 fish

GnRH3 - Gonadotropin-releasing hormone 3

GPx - Glutathione peroxidase

GSH – Glutathione

GSI – Gonadosomatic index

GST – Glutathione S-transferase

hpf – Hour(s) post fertilization

HPG – Hypothalamus-pituitary-gonad

HSI – Hepatosomatic index

K – Fulton’s condition factor

kiss1 - KiSS-1 metastasis suppressor

LAM – Locomotor activity motor

LC-MS/MS – Liquid Chromatography-Tandem Mass Spectrometry

LPO – Lipid Peroxidation

MDA – Malondialdehyde

RNA-seq - Ribonucleic acid sequencing

ROS – Reactive oxygen species

SOD - Superoxide dismutase

TBA - Thiobarbituric acid

VTG – Vitellogenin

WHO – World Health Organization

WWTPs – Wastewater treatment plants

CHAPTER I. Introduction

1.1. Contaminants of emerging concern

Numerous recent studies have documented that a broad group of “novel chemicals”, known as contaminants of emerging concern (CECs), are present in many aquatic ecosystems (Tang et al., 2020). These chemicals, that originate from a variety of sources, including plasticizers, pharmaceuticals, personal care products, nanoparticles, microplastics, etc. (Figure 1), are ubiquitous in aquatic ecosystems, deleterious to non-target organisms and their effects on the environment and public health are still largely unknown despite its potential toxicity (Khan et al., 2022). Only in recent years, CECs started to become a topic of great interest within the scientific community due to the development of more sensitive analytical techniques that allowed the detection of different CECs at low concentrations (ng to µg/L range), especially in aquatic ecosystems (Aguilar-Aguilar et al., 2023). Since then, an increased attention has been given to CECs in environmental waters, as well as to improve the assessment of their impacts and to develop more efficient water treatment technologies to reduce the concentration of CECs in wastewater treatment plants (WWTPs). (Puri et al., 2023). However, the actual knowledge about the persistence and risk of CECs in aquatic environments and global policies to control the presence and discharges of these chemicals are still lacking (Puri et al., 2023). CECs investigation mainly focus on three classes: “new” emerging contaminants, new compounds or molecules that were not previously know or haven’t been studied yet; contaminants that were already known but whose risk wasn’t fully understood and “old” compounds whose contamination became relevant again due to environmental and public health risks (Sauvé et al., 2014). In the last decade the presence and contamination of aquatic environments with CECs are mostly due to anthropogenic activities such as domestic, agricultural, and industrial actions (Kasonga et al., 2021). The main contamination pathways are surface run-off from agricultural land, industrial activities, hospital waste, sewage dischargers and outflows from WWTPs. WWTPs do not have the capacity to completely remove all CECs with their conventional water treatment processes (Rizzo et al., 2019) becoming a source of contamination. More than 700 CECs are known to be present in aquatic environments, additionally many of these can end up in drinking water (Matamoros et al., 2016). Once release into the environment, these contaminants can undergo a variety of transformations since they are subjected to bio, chemical and photochemical degradation. These processes can lead to the production of metabolites whose behavior with the surrounding environment and ecotoxicological profile will be different from the original form of the parental chemicals (La Farre et al., 2008).

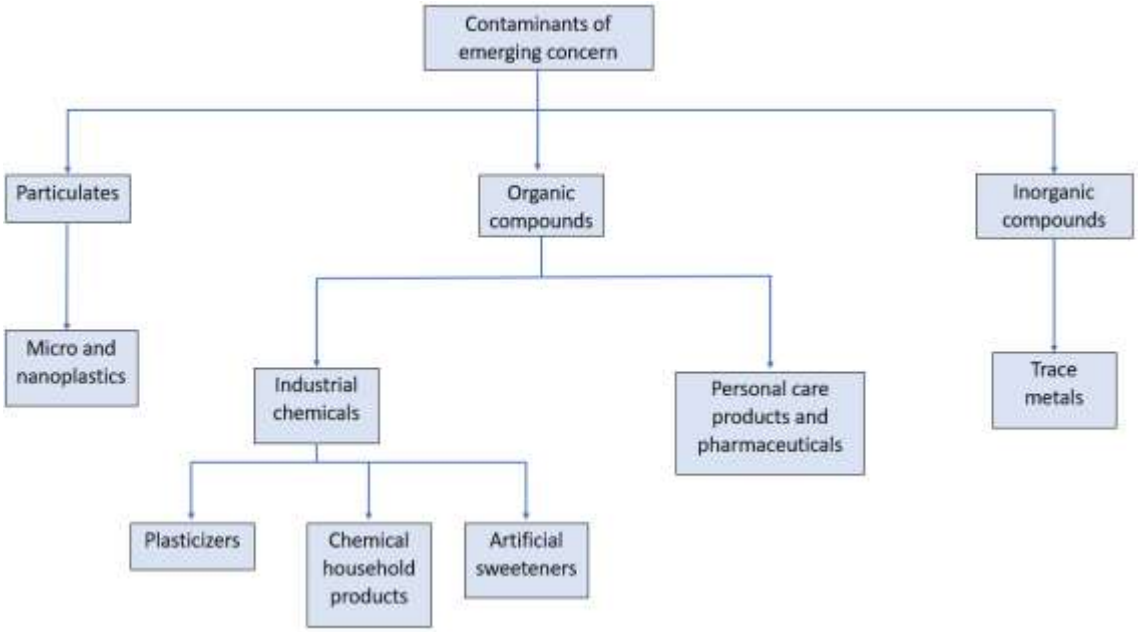

Figure 1- Representative scheme of contaminants of emerging concern. (adapted from Antunes et al., 2021).

Some CECs are able to exert biological effects and once released into the environment will cause negative impacts to non-target organisms (Nilsen et al., 2019). Previous studies have shown that CECs can create drug resistance in bacterial communities, inhibit algae growth, cause endocrine disruption in various organism, originate oxidative stress and have carcinogenic effect (Gogoi et al., 2018).

1.2. Plasticizers

Plasticizers are substances that when added to another material make them softer, more flexible and increase their workability, by lowering the glass transition temperature (Cadogan et al., 2000). Plasticizers are frequently used in PVC based products, food packaging, toys and medical tools (Qadeer et al., 2022). Due to the high number of plastic applications, a great number of plasticizers are produced (around 30.000 chemicals) (Daniels et al., 2009), the most used are adipates, azelates, citrates, benzoates, phthalates and phenols, mostly bisphenols (Stuer-Lauridsen et al., 2001). The demand for these products is related to industry growth, which is continuously increasing and so, it is expected that by 2024 the world will consume 9.75 million tons of these substances (Additives Polymers, 2017). Plasticizers, however, are often not covalently bonded to the plastic matrix to which they are applied and can therefore slowly diffuse out of polymers through migration, evaporation or extraction by liquids and cause unwanted changes to plastic products but

mainly will be discharged in aquatic environment affecting all the aquatic organisms (Wei et al., 2019; IJERPH, 2021).

1.3. Bisphenol A (BPA)

BPA was firstly developed in the search for synthetic estrogens but the research on this topic has become redundant due to the discovery of a more effective compound (Oehlmann et al., 2009). BPA was rediscovered in the 1950s, when a chemist used it to create polycarbonate plastic by reacting it with phosgene. Since then, BPA has been used mainly in the plastics sector. (Oehlmann et al., 2009).

The use of BPA is widespread in everyday objects, and it can be found in plastic and infant bottles, children´s toys, food packaging, medical devices, automobile parts and electrical applications (Muhamad et al., 2016). Due to the broad range of applications, it is estimated that more than 3500 million kilograms of BPA were annually produced (Halden et al., 2010) and at least 100 tons are released into the environment every year (Bocharnikova et al., 2020). Therefore, the presence of BPA in the environment is ubiquitous and concentrations in freshwater systems can reach up to 21 µg/L (Crain et al., 2007). This data is very alarming due to the reported effects of BPA in non-target organisms: BPA is part of WHO´s list of endocrine disruptors (Haffner et al., 2014) due to its xenoestrogenic effects (Hafezi et al., 2019). Moreover, BPA has a well-documented list of effects on human health such as causing breast and ovarian cancer, cardiovascular diseases, colon cancer, depression, obesity, reproductive disorders in males, respiratory diseases, and insulin resistance (Trivedi et al., 2021). Due to the mainstream usage, BPA reaches the aquatic environments in increasing concentrations, and it is documented to cause endocrine disruption and development disorders on aquatic organisms, such as zebrafish (Santangeli et al., 2016; Scopel et al., 2020), it also affects zebrafish's normal behavior by interfering with brain functions (oxidative stress and neurotoxic stress) (Pradhan et al., 2023).Table 1 summarizes some of the effects of BPA on various aquatic organisms further cementing its risks to the environment. Due to the evidences of negative effects of BPA in human and environmental health, BPA was regulated for several uses, such as applications in plastics for babies (Flint et al., 2012). Given the restrictions the industry developed BPA derivates to replace BPA. Dozens of chemicals were developed such as bisphenol F (BPF), bisphenol S (BPS), and bisphenol AF (BPAF), among others, that are now being used in everyday products (Harnett et al., 2021). Figure 2 shows a timeline of BPA history and how long it took for its use to be reevaluated.

Table 1- Effects of BPA on various aquatic organisms, adapted from Kang et al., 2007.

Organisms	BPA concentration (µg/L)	Duration	Observed effects
Eunapius fragilis (freshwater sponge)	16,000–160,000	6 days	Abnormal growth inhibition of germination
Mytilus edulis (invertebrate)	50	21 days	Gonad resorption
Chironomus riparius (insect)	1000	2 days	Reduction of weight and delay of moulting
Caiman latirostris **embryo** (reptile) **(33 ° C)**	9000 µg per egg	10 days	100% male to female sex reversal
Rana temporaria **larvae** (amphibian)	1000	10 days	100% developmental malformation
Danio rerio (fish)	1000	21 days	Vitellogenin induction
Oryzias latipes (fish)	200	9 days	Embryonic deformity
Salmo trutta f. fario (fish)	5	2 months	Reduction of sperm quality on males and non-ovulating females

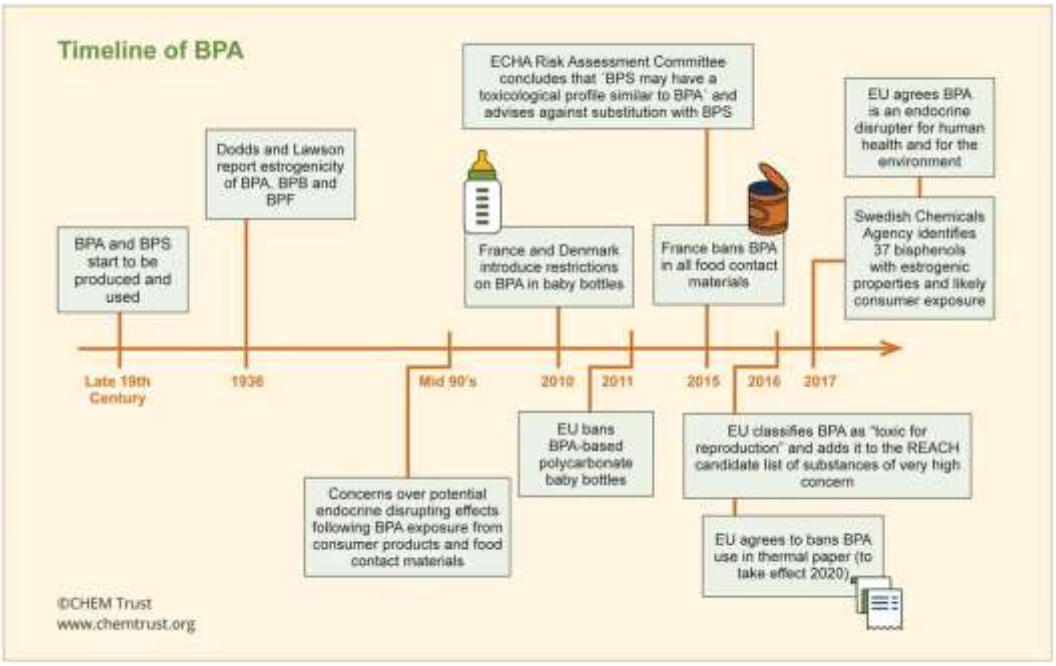

Figure 2- Timeline of BPA (adapted form chemtrust.org, 2023).

1.4. Bisphenol S (BPS)

BPS is the most common substitute for BPA because it is less likely to leach monomers into food and drinks, thanks to its higher photo-resistance and tolerability to heat (Harnett et al., 2021). As its use only began to gain relevance recently, there is still scarce knowledge regarding BPS's toxicity. Considering that non-target aquatic organisms may be continuously exposed to low levels of this compounds for several generations, there is an urging need to assess the chronic effects of environmentally relevant concentrations of BPS.

BPS differs from BPA by the addition of a radical group, as shown in Figure 3. It is commonly used in consumer goods, most predominantly epoxy resins and polycarbonate plastics. Industries that previously used BPA are now mostly replacing it with BPS and so it can be found in food packaging, baby bottles, canned foods and drinks and thermal paper receipts (Thoene et al., 2020). Through its extensive use it contaminates humans, and 81% of urine samples collected from humans contain BPS (Liao et al., 2012), this contamination happens due to the leaching of BPS from the products previously mentioned (Wu et al., 2018).

In humans, previous studies have shown that BPS can act as an endocrine disruptor, since it can trigger estrogenic activity, firstly demonstrated in Kuruto-Niwa et al., 2005, using human cells acutely exposed to BPS (1.75E−06 M). BPS can cause oxidative stress by enhancing the level of reactive oxygen species in human red blood cells after short-term exposure to 250 μg/ml (Maćczak et al., 2017). Furthermore, BPS negatively impacts pig oocyte maturation, when exposed to concentrations found in human blood (0.8–84 nM) for 24h (Žalmanová et al., 2017), and is also linked to obesity by increasing the expression of adipogenic markers in human preadipocytes exposed to 25 μM of BPS (acute exposure) (Boucher et al., 2016). . Since BPS is diffused in all types of environments it ends up discharged in aquatic systems, where it mainly exists due to its high water solubility (Fang et al., 2020), with concentrations reaching 7200 ng/L in aquatic environment (Adyar River, India) (Morales et al., 2020) and the mean concentration in water systems being around 1000 ng/L (Qiu et al. 2019).

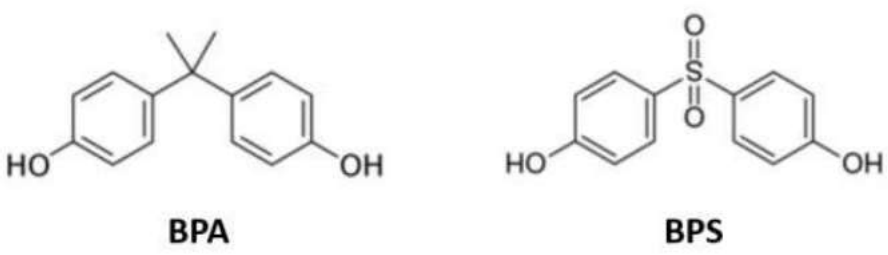

Figure 3- Chemical structures of BPA and BPS adapted from (Harnett et al., 2021).

Therefore, it is necessary to develop studies regarding the toxicity of this chemical, predominantly in the aquatic environment since it is one of the environments most contaminated with BPS. In fact, some studies have already demonstrated that BPS, as BPA, induce effects in non-target aquatic organisms but the ecological risk assessment of BPS is still in its infancy.

Studies on zebrafish have shown that BPS has adverse effects on the endocrine system inducing vitellogenin after 7 days exposure to 0.1 μM of BPS (Le Fol et al., 2017) , reducing testosterone levels in males, egg production and the gonadosomatic index in females. Moreover, the hatching of their offspring was also compromised, and the size of males reduced when exposed to > 0.5 μg/L of BPS for 21 days (Ji et al 2013). Furthermore, BPS exposure can affect hypothalamus development and cause hyperactive behavior on zebrafish larvae acutely exposed to 0.0068 μM (Kinch et al., 2015). BPS is also known to cause acute toxicity in *Daphnia magna* when exposed to 76 mg/L for 24h (Chen et al., 2002). Table 2 shows the studies available on the effects of BPS on different aquatic organisms. As seen, there is still a lack of information regarding the effects of BPS exposure, especially long-term. The majority of the toxicity studies available in the literature involving BPS have been based on acute toxicity tests, with concentrations that are far above environmental relevance. Considering that non-target aquatic organisms may be continuously exposed to low levels of this compounds for several generations, there is an urgent need to assess the chronic effects of environmentally relevant concentrations of BPS.

Despite being mainly introduced into the industry to minimize BPA impacts on non-target organisms, it is already recognized that BPS also is an agent of toxicity with various negative effects, figure 4 shows a comparison of the toxic effects of BPA and its analog BPS.

*Table 2- BPS toxicity data for several groups of organisms. Concentrations expressed as μg/L. * - the organisms were directly exposed via injection.*

Organisms	BPS concentration (μg/L)	Duration	Observed effects	Reference
Clorophyta				
Chlorella vulgaris	2000 – 40000	6 days	Oxidative stress; photosynthesis inhibition	(Li et al., 2018)
Rotifera				

Brachionus koreanus	500-100000	24 hours	Decreased population; Higher levels of ROS and GST activity	(Park et al., 2018)
Crustacean				
Diaphanosoma celebensis	920-23000	48 hours	Alteration of the endocrine system; effects on reproduction	(Fabrello et al., 2022)
Daphnia magna	100	21 days	Decreased body length and heart rate; oxidative stress	**(**Quian et al., 2022)
Fish				
Danio rerio	10-100	75 days	Growth suppression and female-skewed sex ratio; reproductive impairments.	(Naderi et al., 2018)
	300-3000	6 days	Neurotoxicity; decreased locomotor behavior	(Gu et al., 2019)
Salmo truta **(at 10 ºC)**	20 mg/kg *	14 and 56 days	Endocrine disruption; Cytotoxicity.	(Frenzilli et al., 2021)
Cyprinus carpio	10-1000	60 days	Oxidative stress; pro-inflammatory effect	(Qiu et al., 2019)

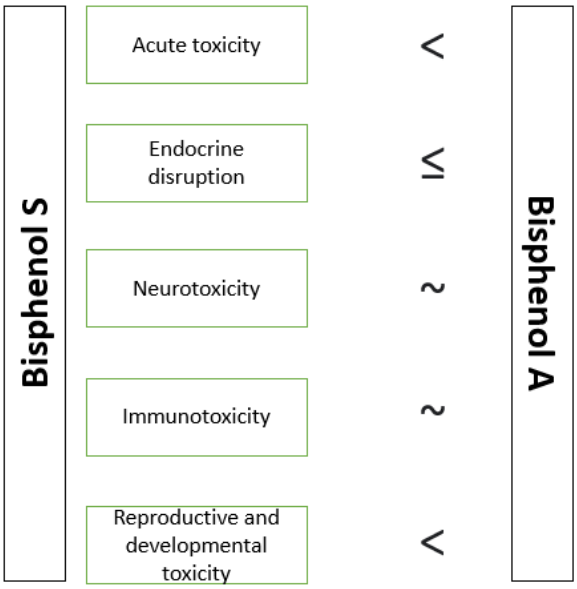

Figure 4: Compared toxicity of BPS and BPA (adapted from Qiu et al., 2019).

1.5. Model species: *Danio rerio* (zebrafish)

In order to understand the potential risk of environmentally relevant concentrations of BPS, *Danio rerio*, commonly known as zebrafish, was chosen as the model organism. Zebrafish a vertebrate from the Cyprinidae family, has already a well described life-cycle and individual development, extensive behavioral comprehension and a fully sequenced genome (Howe et al., 2013. Moreover, functional, biochemical and molecular data are available for this species. (Ruzicka et al., 2019). The short life-cycle of zebrafish, low cost maintenance and handling, small size and high fecundity rates make this species an excellent model organism for ecotoxicological studies to access the adverse effects of different chemicals. The results can later be extrapolated to humans (Zhang et al., 2003), with some caution, since humans and zebrafish share 70% of their protein-coding genes (Howe et al., 2013). In recent years zebrafish has been one of the most popular species to investigate mode of action and evaluate the toxicity of environmental toxicants, like CECs, being utilized as a model in chemical toxicity screening, drug development and developmental neurotoxicity (Horzmann et al., 2018). Zebrafish embryos are also commonly used for toxicological assays because of the low cost and transparency of the chorion that allows to observe the full development of the fish and detect possible modifications at different important endpoints in the embryos (Scholz et al., 2018). Considering all the listed characteristics and previous studies, zebrafish, is an ideal model organism to carry out the research presented here.

1.6. Objectives

The present study aimed to investigate the long-term effects of BPS in the model organism, *Danio rerio* (zebrafish), following a full life-cycle exposure to environmentally relevant concentrations. We used 2 months post fertilization (mpf) zebrafish that were exposed to BPS for 4 months (F0) and the approach combined the study of biochemical endpoints in brain, i.e., (Acetylcholinesterase, Catalase, glutathione S- transferase and Lipid Peroxidation assays) and apical endpoints, i.e., weight, length, hepatosomatic index, gonadosomatic index, condition factor- K and reproductive performance) in adults. A behavior assay was also carried out in the offspring of F0 generation (F1) that were not directly exposed to BPS. Additionally, a fish embryo toxicity test was performed with F0 zebrafish embryos to also investigate the potential acute toxicity of environmental relevant concentrations of BPS. This study provides further insights on BPS effects in aquatic

ecosystems and contribute to improve environmental hazard and risk assessment. This study will hopefully bring attention to the problematic of BPS contamination.

CHAPTER II. Materials and Methods

2.1. Experimental Design

To investigate the effects of different environmentally relevant concentrations of BPS in aquatic ecosystems, zebrafish were chronically exposed to concentrations of 400 ng/L, 2000 ng/L and 10000 ng/L from 60 dpf to 180 dpf. The bioassay was performed at Biotério de Organismos Aquáticos (BOGA) located at CIIMAR, in compliance with the European Directive 2010/63/EU on the protection of animals used for scientific purposes, and the Portuguese 'Decreto Lei' 113/2013.The solutions of BPS were prepared using acetone as the solvent (0,0003%), and for this reason a solvent control treatment of acetone was also conducted. About 800 zebrafish embryos with 4 hours post fertilization (hpf) were grown in 10 L plastic boxes with clean water, with a water temperature of 28 ± 1 °C, a 14:10 h (light:dark) photoperiod and a pH of 7.5 ± 0.2. Feeding started at 5 days post fertilization (dpf), three times a day, with the commercial fish diet zebrafeed (SPAROS) and a supplement with live brine shrimp (*Artemia spp*). Up to 20 dpf, zebrafish larvae were fed ad libitum, and from 20 dpf onward, the amount and size of food supplied were adjusted to fish development, in equal proportion for all aquaria (Barros et al., 2022). At 60dpf, zebrafish were randomly selected and transferred to 27 L aquariums with 35 fish (15 males, 15 females and 5 randomly selected fish; in duplicate for the BPS treatments and triplicate for solvent control) to start the exposition to the different BPS concentrations. BPS exposure was conducted under a flow-through system, calibrated 1 week prior to the beginning of the exposure, where the water flow was maintained at 1.06 L per hour, by means of a peristaltic pump (ISM 444, ISMATEC) supplied with dechlorinated water. In order to maintain the selected concentrations of BPS in the aquaria during the assay, working solutions of BPS were manually dosed directly into the aquaria three times a day, in the morning, in the beginning of the afternoon (4 hours later) and in late afternoon (another 4 hours later), in a volume that was equivalent to the contaminant renewal during those periods. Each aquarium was maintained with a water temperature of 28 ± 1 °C, 14:10 h (light:dark) photoperiod, pH 7.5 ± 0.2 and a mean ammonia concentration of 0.08 ± 0.04 mg/L. The fish were exposed until 180dpf. At the end of the BPS exposure, the fish were sacrificed with an anesthetic overdose of 300 mg/L tricane methanesulfonate (MS-222) buffered with the same amount of sodium hydrogen carbonate. Fish were measured, weighted and their liver and gonads were collected in order to measure the Hepatosomatic index (HSI) ((Liver weight (g) / Fish weight (g)x100) the Gonadosomatic (GSI) index ((Gonad weight (g) / Fish weight (g) x 100) and the Fulton's condition factor ($K = (\text{weight} / \text{length}^3) \times 100$). Brains were also collected and frozen in liquid nitrogen and stored at - 80ºC to perform future biochemical analysis.

Figure 5- Long-term toxicity bioassay's setup.

2.2. Fish Embryo Acute Toxicity (FET) Test 236

Fertilized embryos obtained from an unexposed zebrafish stock maintained at BOGA in CIIMAR, were exposed to the three BPS concentrations tested in the present study (400 ng/L, 2000 ng/L, 10000 ng/L) plus an acetone solvent control. 24 well plates were used, with wells filled with 2 mL of the different BPS concentrations, in which one embryo per well was placed. The first row of each plate was used as a solvent control. The assay was carried out for 96h at the end the larvae were sacrificed using Lugol and 10 larvae per plate were later measured. Every day the embryos were individually checked, using an Inverted Microscope Nikon - EclipseTS100, for development malformations and mortality. At 48hpf the heartbeats of 5 embryos per plate were counted. The solution medium of each well was changed every day (about 1.5 ml). This process was carried out in 2 plates per

concentration (n = 40 for the concentrations; n = 48 for the solvent control). This study was carried out based on the OECD Fish Embryo Acute Toxicity (FET) Test 236 (OECD, 2013).

2.3. Breeding Tests

Two pairs of fish per aquarium and per replica were randomly selected and allocated to breeding chambers for reproduction. In order to reproduce, they were kept in these chambers during the night and in the following morning their offspring was collected. During the day the couples were kept at tanks contaminated with BPS according to the treatment they were originally exposed (Solvent control, 400 ng/L, 2000 ng/L, 10000 ng/L). At 2-3hpf, the eggs produced were then counted and divided in fertilized or unfertilized. This process was done for 7 straight days, with the same couples. The couples were returned to their original tanks once the week was finished.

Figure 6- Breeding tests setup.

2.4. Biochemical Assays

The brains of the F0 sacrificed fish preserved at -80 ºC were used for the determination of biochemical markers: acetylcholinesterase (AChE), catalase (CAT) and glutathione S-transferase (GST) activities and lipid peroxidation (LPO) levels. Brains were firstly separated in groups of three (same concentration and same gender) and homogenized in phosphate buffer [100 mM (K2HPO4/KH2PO4), 150 mM KCl, 1 mM DTT, 0.1 mM PMSF, 1 mM ethylenediaminetetraacetic acid (EDTA); pH 7.4]. The protein content of each sample was determined following Lowry's method (Lowry et al., 1951) and then normalized to 1 mg/ml and divided in 3 aliquots: 2 were normalized using the previous buffer and 1 was normalized using Phosphate buffer 0.1 M, pH = 7.2 [K2HPO4 0.1 M, KH_2PO_4 0.1 M]., the single aliquot normalized with phosphate buffer was made to be used in the AChE activity assay since AChE activity could be compromised by DTT (Rotundo, 2017).
CAT was measured in 96-well UV microplates (Thermo Fisher Scientific). CAT activity was determined by measuring the consumption of H2O2 at 240 nm during 2 min at 15 s intervals and expressed as µmol/min/mg protein. This method was adapted from Ferreira et al., 2007. LPO was determined by the quantification of malondialdehyde (MDA), the result of lipid peroxidation. MDA was measured by the thiobarbituric acid (TBA). The homogenates were incubated at 100 °C, for 30 min with TBA 1%, NaOH 0.05 M and butylated hydroxytoluene 0.025%. LPO levels were measured at 530 nm and expressed in nmol of MDA equivalents per mg of protein. This process followed adapted methodologies from Pinheiro et al. (2019).

GST was measured using a reaction mixture of glutathione (GSH) 10 mM in HB (0.1 M, pH 6.5) and 1-chloro-2,4-dinitrobenzene (CDNB) 60 mM in ethanol read at 340 nm for 5 min in 20 s intervals. GST activity was expressed as nmol/min/mg protein, the protocols followed methodologies described in Pinheiro et al. (2019).

AChE activity was determined in a reaction containing 0.1 M phosphate buffer, 10 mM acid dithiobisnitrobenzoate (DTNB) and acetylthiocholine iodide 0.075 M. The AChE activity was performed at 412 nm during 5 min and expressed in nmoles/min/mg protein, this assay followed the work described in Barros et al. (2017).

2.5. Behavioral Tests

F1 embryos of zebrafish were collected from the different aquariums (solvent control, 400 ng/L, 2000 ng/L and 10000 ng/L) and grown in clean water for 4 days. 30 of the 4dpf larvae per treatment and per replica were then selected, located in individual tubes, and placed in a Locomotor Activity Monitor (LAM) system (figure 7). Their movement and activity were

measured using infrared (IR)-based activity developed by Trikinetics (TriKinetics, Waltham, MA), for 5 days, by counting the times that each larvae crossed an infrared beam of light that was placed in each tube. The data collected was then analyzed using Rtivity software. Rtivity is a R programming language base software that collects data from infrared monitors and expresses them in counts per time format, this allows the program to analyze the behavior of different animal like activity or sleep. Sleep and activity parameters per day and per light phase (light/dark (14:10h) were then analyzed (Silva et al., 2022).

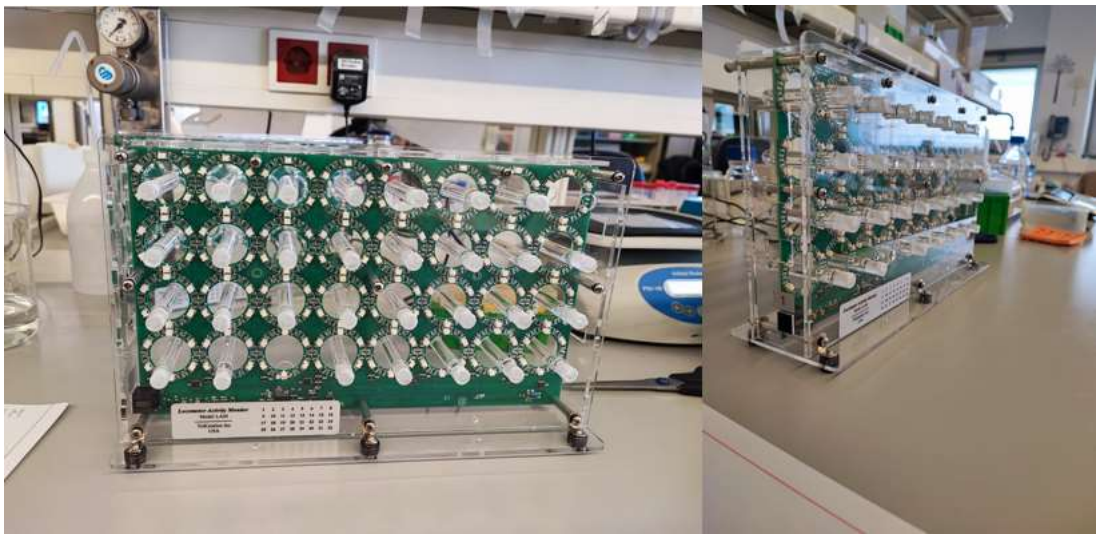

Figure 7- Locomotor Activity Monitor (LAM, Trikinetics).

2.6. BPS analytical chemical quantification

Actual BPS concentrations were determined in water samples by Liquid Chromatography-Tandem Mass Spectrometry (LC-MS/MS). The measured concentrations were slightly different from the nominal ones, instead of 400 ng/L, 2000 ng/L and 10000 ng/L, LC-MS/MS detected mean concentrations of 500 ng/L, 2000 ng/L and 16000 ng/L BPS.

2.7. Statistical Analysis

Statistica 12.5 (Statsoft, USA) software was used to calculate all statistical analyses. With the use of the Kolmogorov-Smirnov and Levene's tests, the obtained data was examined for normality and variance homogeneity. Following that, data were examined using a one-way ANOVA and Fisher's least significant difference (LSD) test for posthoc comparisons. The cutoff for significant differences was chosen as $p < 0.05$.

CHAPTER III. Results

3.1. Fish Embryo Acute Toxicity (FET) Test 236

Table 3 show the results obtained from the FET Test 236, it shows the embryos mortality, cardiac beats per minute and length of the larvae of all tested BPS concentrations (solvent control, 400 ng/L, 2000 ng/L, 1000 ng/L). Embryo mortality was checked every day, cardiac beats per minute were counted at 48hpf and the length was measured at 96hpf. No significant differences were found for all of the evaluated endpoints.

Table 3 - Endpoints of acute toxicity of zebrafish embryos.

ENDPOINT	Control	400 ng/L	2000 ng/L	10000 ng/L
Mortality	0%	2.5%	5%	5%
Beats Per Minute	180.8 ± 5.32	182.8 ± 3,10	181.2 ± 4.21	172.8 ± 3.08
Length (µm)	3315,57 ± 38.55	3311.02 ± 49.93	3274.88 ± 37.32	3326.86 ± 28.44

3.2. Biological Endpoints

Table 4 shows the length, weight, Fulton´s condition factor, liver weight, hepato-somatic index, gonad weight and gonado-somatic index of female and male zebrafish after 4 consecutive months of exposure to BPS concentrations (400 ng/L, 2000 ng/L and 10000 ng/L). The gonado-somatic index of the females from the 400 ng/L and 2000 ng/L were significantly increased. All the other parameters did not have any significant differences.

Table 4- Biological endpoints, for both male and female zebrafish after 4 months of exposure to BPS (6 months old). Asterisks (*) indicate significant differences from the control group (- p < 0.05).*

ENDPOINT	Sex	Control	400 ng/L	2000 ng/L	10000 ng/L
Length (cm)	M	3.60 ± 0.02	3.61 ± 0.02	3.56 ± 0.02	3.57 ± 0.02
	F	3.81 ± 0.04	3.80 ± 0.03	3.73 ± 0.03	3.78 ± 0.04
Weight (mg)	M	437 ± 7.19	433 ± 6.88	425 ± 9.01	427 ± 8.77
	F	634 ± 24,6	670 ± 27.0	617 ± 18.9	613 ± 22.7
Fulton's condition factor (K)	M	0.93 ± 0.01	0.92 ± 0.01	0,94 ± 0.02	0.93 ± 0.01
	F	1.13 ± 0.03	1.21 ± 0.04	1.18 ± 0.02	1.12 ± 0.03
Liver weight (mg)	M	5,09 ± 0.25	4.83 ± 0.32	8.75 ± 0.45	4.78 ± 0.23
	F	25.28 ± 2.53	20.11 ± 1.83	22.25 ± 1.41	24.82 ± 3.77
Hepato-somatic index (HSI)	M	1.15 ± 0.05	1.11 ± 0.06	2.23 ± 1.22	1.08 ± 0.05
	F	3.84 ± 0.31	3.05± 0.21	3,65 ± 0.24	3.85 ± 0.58
Gonad weight (mg)	M	7.73 ± 0,42	8.06 ± 0.43	10.34 ± 2.73	8.2 ± 0.36
	F	112.5 ± 13.0	123.8 ± 13.8	166.9 ± 39.9	121.8 ± 10.96
Gonado-somatic Index (GSI)	M	1.77 ± 0.10	1.86 ± 0.09	2.45 ± 0.61	1.86 ± 0.08
	F	16.69 ± 1.38	**20.47* ± 1.38**	**19.94* ± 1.36**	18.44 ± 1.14

3.3. Breeding Trials

Figure 8 shows the mean fecundity (number of eggs per couple) of each of the couples that were exposed to different concentrations of BPS. No BPS exposed group showed significant differences when compared to the control. Figure 9 shows the fertility (percentage of fertilized embryos at 2-3hpf that were collected from the breeding trials between couples of zebrafish exposed to BPS, 400 ng/L – 10000 ng/L). The percentage of fertilized embryos per treatment significantly decreased in zebrafish exposed to 10000 ng/L of BPS.

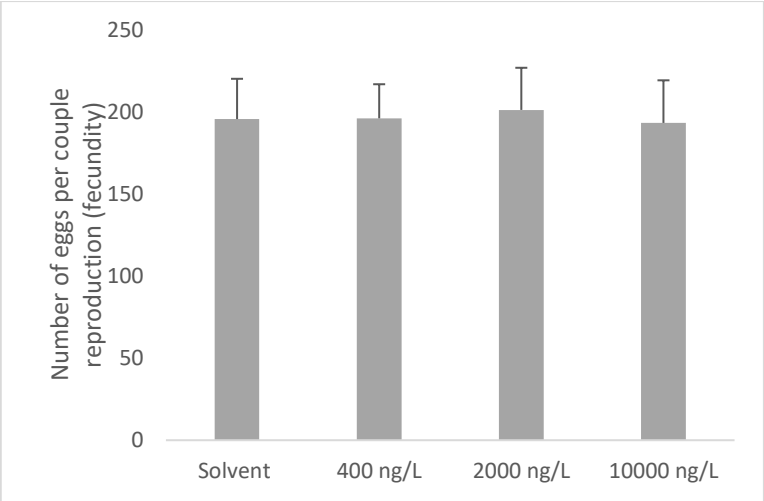

Figure 8- Fecundity of 6 months old zebrafish couples exposed for 4 months to different concentrations of BPS (400ng/L, 2000ng/L, 10000ng/L) as well as a control of acetone (Solvent). Error bars represent de standard error.

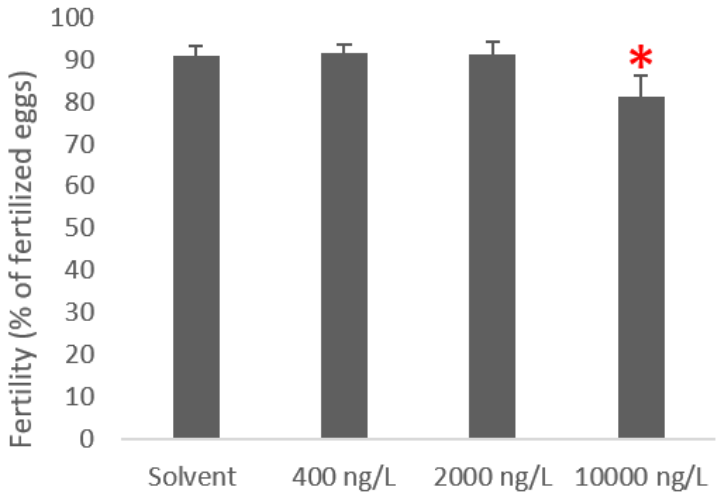

Figure 9- Percentage fertilized eggs (fertility) collected from the breeding trials of 6 months old zebrafish couples exposed for 4 months to different concentrations of BPS (400ng/L, 2000ng/L, 10000ng/L) as well as a control of acetone. Asterisks (*) indicate significant differences from the control group (- $p < 0.05$). Error bars represent de standard error.*

3.4. Biochemical Assays

Figure 10 shows the CAT activity in the zebrafish brains of male and female of the different treatments. CAT activity significantly increased in males exposed to 400 ng/L BPS when compared to the control group.

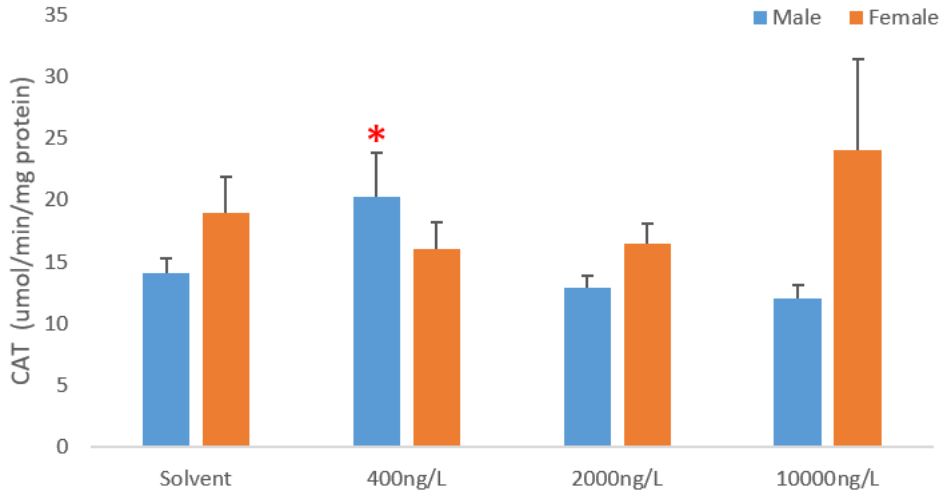

Figure 10- CAT activity in zebrafish brains after being exposed for 4 months to different BPS concentrations (400ng/L, 2000ng/L, 10000ng/L) as well as a control. Asterisks (*) indicate significant differences from the control group (- p < 0.05). Error bars represent de standard error.*

Figure 11 shows LPO levels in the exposed fish's brains, no significant differences were found.

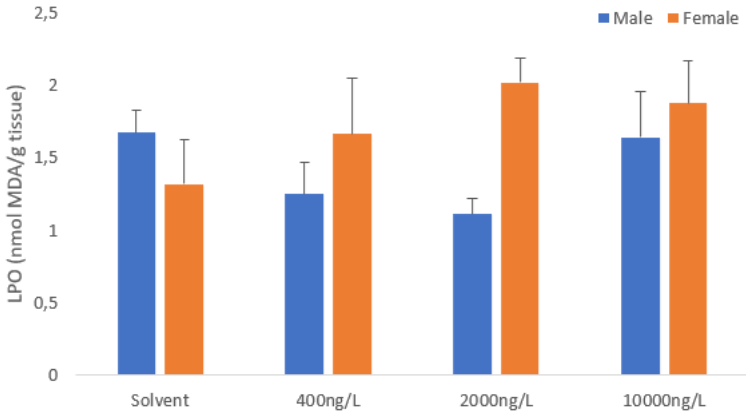

Figure 11- LPO levels in zebrafish brains after being exposed for 4 months to different BPS concentrations (400ng/L, 2000ng/L, 10000ng/L) as well as a control. Error bars represent de standard error.

GST activity in the fish's brain is represented in figure 12. A significant decrease was found between female fish exposed to 10000 ng/L BPS and the control group.

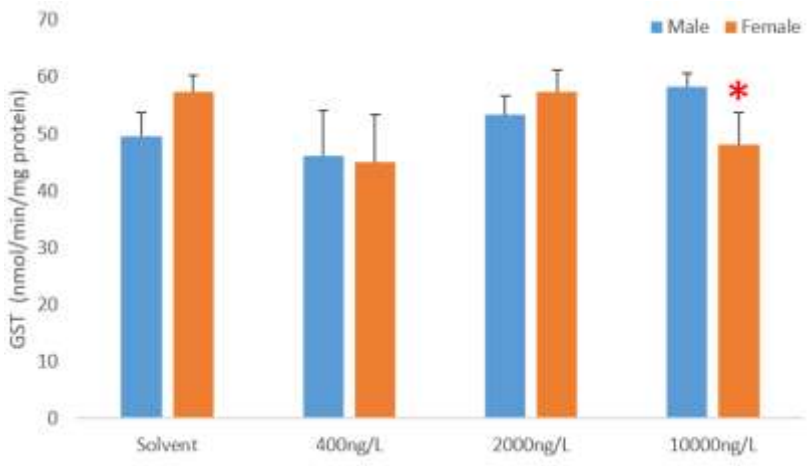

Figure 12- GST activity in zebrafish brains after being exposed for 4 months to different BPS concentrations (400ng/L, 2000ng/L, 10000ng/L) as well as a control. Asterisks (*) indicate significant differences from the control group (- p < 0.05). Error bars represent de standard error.*

Finally, figure 13 shows AChE activity in the used exposed fish's brain. A significant decrease was found between the males exposed to the highest concentration and the control group.

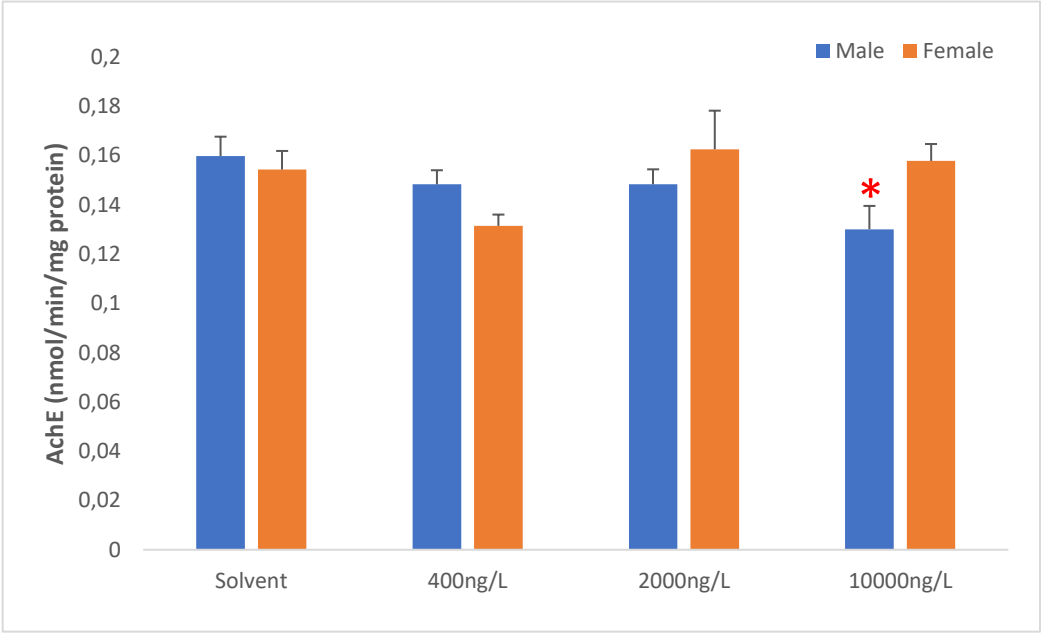

Figure 13- AChE activity in zebrafish brains after being exposed for 4 months to different BPS concentrations (400ng/L, 2000ng/L, 10000ng/L) as well as a control. Asterisks (*) indicate significant differences from the control group (- p < 0.05). Error bars represent de standard error.*

3.5. Behavioral tests

Figures 14, 15, 16 and 17 show several behavior parameters measured in F1 zebrafish larvae (from 4 to 8 dpf) obtained from the different parental treatments. Figure 14 shows the sleeping pattern of the F1 larvae in the different light periods of the day (light and dark). Significant differences were observed between the highest BPS concentration (10000ng/L) and the solvent control group, for both light and dark phases, which means that they spent a higher percentage of the time sleeping. Figure 15 illustrates the sleep ratio per day of the same larvae. significant differences were found between 10000 ng/L and the solvent control group on days 1, 2 and 3, where we found an increased sleep ratio, meaning that the larvae spent more time without moving. Figure 16 exhibits the activity (every time that the larvae crossed the infrared beam of light in their tubes) of the same larvae with and without light (light phase and dark phase, respectively). No significant differences were found for this parameter. Figure 17 displays the activity of the tested larvae in each of the testing days, where no significant differences were found.

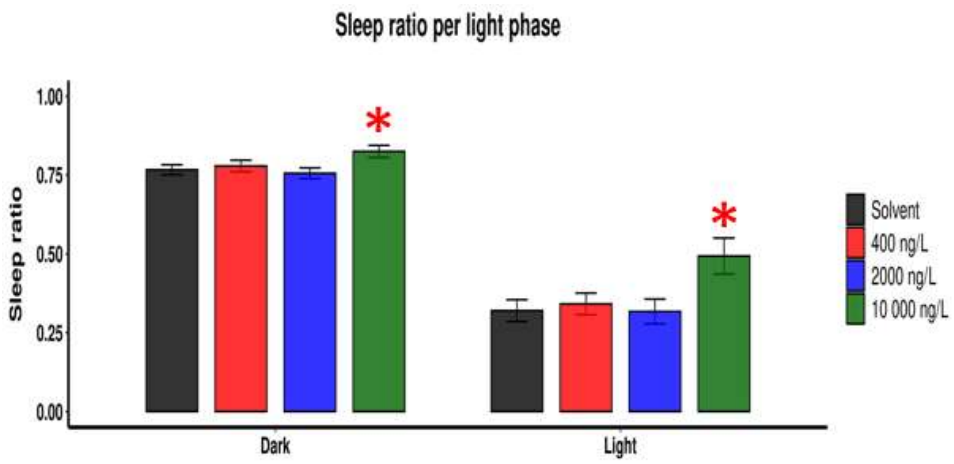

Figure 14- Sleep ratio during dark hours and light hours of 5 days old zebrafish larvae born from parents exposed to BPS for 4 months (Solvent, 400ng/L, 2000ng/L, 10000ng/L). Asterisks (*) indicate significant differences from the control group (- p < 0.05). Error bars represent de standard error.*

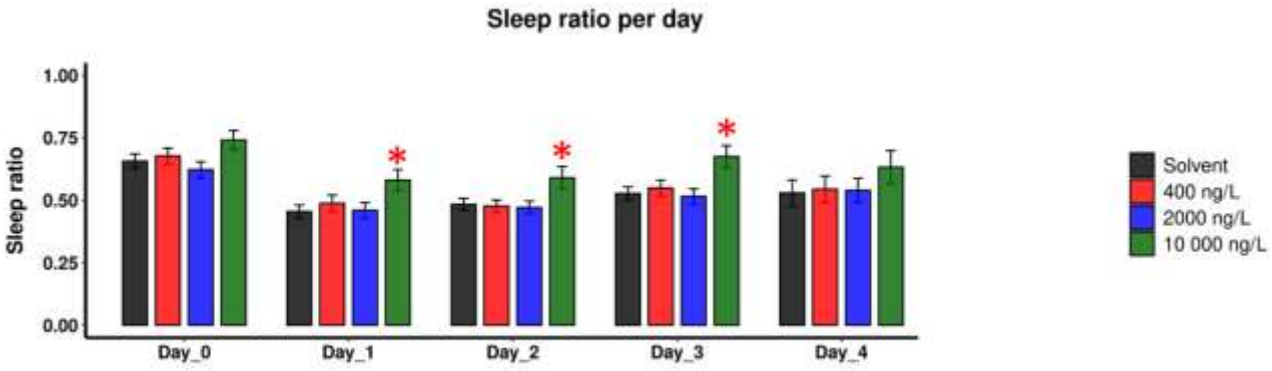

Figure 15- Sleep ratio per day (for 5 days) of 5 days old zebrafish larvae born from parents exposed to BPS for 4 months (Solvent, 400ng/L, 2000ng/L, 10000ng/L). Asterisks (*) indicate significant differences from the control group (- p < 0.05). Error bars represent de standard error.*

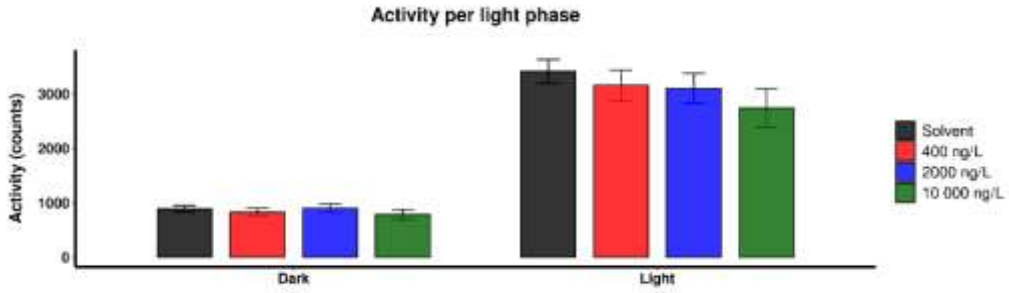

Figure 16- Activity during dark hours and light hours of 5 days old zebrafish larvae born from parents exposed to BPS for 4 months (Solvent, 400ng/L, 2000ng/L, 10000ng/L). Error bars represent de standard error.

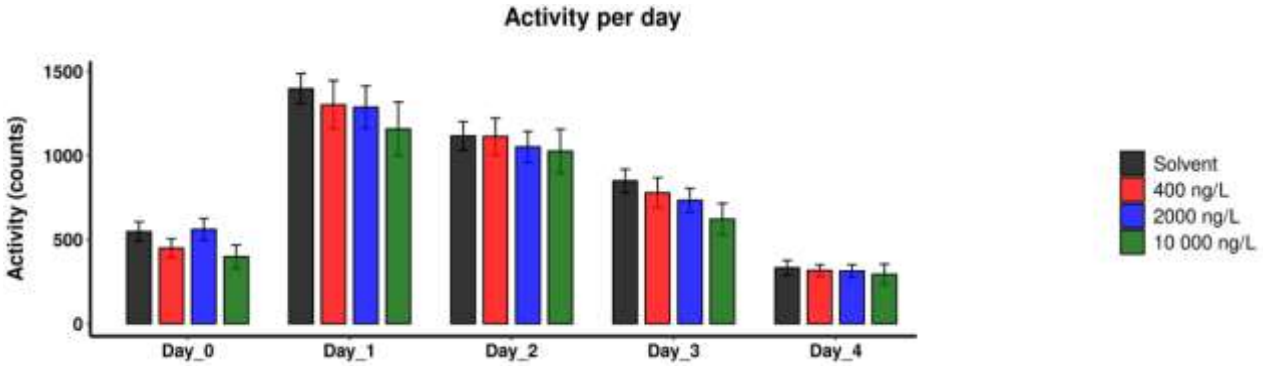

Figure 17- Activity per day (for 5 days) of 5 days old zebrafish larvae born from parents exposed to BPS for 4 months (Solvent, 400ng/L, 2000ng/L, 10000ng/L). Error bars represent de standard error.

CHAPTER IV. Discussion

4. Discussion

BPS has recently received increase attention, due to its potentially adverse health effects on aquatic ecosystems. BPS has emerged as a BPA alternative because it has a higher thermal stability than BPA making its production rise annually. Growing evidences are suggesting that BPS has the potential to disrupt the normal functions of endocrine system producing reproductive toxicity, but also has potential to induce oxidative stress and disturb brain functions in different animals, like mammalians and aquatic organisms (fish and invertebrates). However, most of the studies available in the literature focus on the acute or sublethal effects of BPS using concentrations above ecological relevance. Therefore, there is a gap of knowledge concerning the long-term effects of low doses of this chemical on aquatic organisms (Wu et al., 2018).

In order to address the knowledge gaps, the present study aimed to investigate the chronic effects of BPS on a model organism – zebrafish - after a long-term exposure of environmentally relevant concentrations of BPS (400 ng/L, 2000 ng/L, 10000 ng/L) that lasted 4 months. Several apical endpoints (growth, reproduction, embryonic development), together with potential alterations in behavior, induction of oxidative stress and neurotoxicity were evaluated. This work also intended to study how BPS can affect an indirectly exposed generation, born from exposed parents (F0), where fish were raised in BPS-free water (F1). It is important to gather data from F1, since little is known about generations that are indirectly exposed to BPS, and many chemicals have potential to impair several biological functions of aquatic organisms, not only in the directly exposed generations but also in future non-exposed generations (Neuparth et al., 2022).

In the beginning of this study, an acute assay using zebrafish embryos was conducted in order to evaluate if the concentrations used in the chronic exposure to BPS have acute effects on zebrafish. No significant differences were found for all the studied parameters (mortality, development alterations and heart rate). These results were expected since in a previous study conducted by Mu et al., (2018) using the same protocol, zebrafish's larvae exposed to 50 mg/L of BPS did not show any developmental abnormalities and their heart rate and mortality was also not altered. These results bring attention to the necessity to perform a long-term study with environmentally relevant concentrations of BPS, since they can simulate the real conditions in which aquatic organism live in field conditions. The results of the present research, following 4 months of exposure to BPS (F0), showed a significant increase on the gonadosomatic index (GSI) of females exposed to the two lowest concentrations of BPS (400 ng/L and 2000ng/L). Despite not reaching statistical significance, females exposed to 10000 ng/L also had their GSI increased. GSI is a common

indicator of exposure, since alteration in this index has been described in fish that were exposed to xenoestrogens (Van den Belt et al., 2002). Most of the available data gathered from literature shows that our results regarding female GSI are not in agreement with most of the previous studies. In a study where female zebrafish were exposed to 100 µg/L BPS for 75 days (Naderi et al., 2014), the GSI decreased. Moreover, the study of Ji, (2013), where adult zebrafish pairs were exposed to 0.5, 5, and 50 µg/L of BPS for 21 days, the female GSI also decreased. The GSI findings presented here and compared with the available ones, suggest that the nature of the effects induced by BPS on GSI appears to differ depending on the zebrafish developmental stage, study duration, and tested concentrations. It should be noticed that the present study used lower BPS concentrations and a longer exposure time than most of the available studies. Contrary to the studies previously related (except the present one), Park et al., (2022) found an increased female GSI in zebrafish exposed to 8 µg/mL of BPS for 21 days. According to the Molina et al. (2018), that observed an induction of ovarian development via VTG upregulation caused by BPA and BPS exposure, Park et al., (2022) attributed the observed GSI increase to an augmented level of hepatic vitellogenin levels. This study, despite using higher concentrations and a shorter exposure time, agrees with the findings of our study.

Previous studies indicated that BPS shows estrogenic activities by binding to estrogen receptors (ERs) (Kuruto-Niwa et al., 2005). Low levels of BPs are known from other studies to increase the numbers of hypothalamic Gn*RH3* neurons and increase expression of *kiss1*, *gnrh3*, and *erα* (Qui et al., 2016). These genes have important roles in the reproductive system of fish (function and growth) (Ohga et al., 2018; Paterni et al., 2014; Vom Saal et al., 1998) and since their transcription is known to be affected by BPS, the up-regulated expression of these genes could be another possible explanation for the increased GSI.

In order to clarify the described results regarding female GSI it would be of great interest to explore the histology of the gonads, in order to understand any effects caused by BPS.

The breeding trials here performed have shown that the couples exposed to the highest concentration of BPS (10000 ng/L) produced a significantly higher percentage of unfertilized embryos when compared to the solvent control. This finding suggests that although the fecundity was not impacted, the quality of the embryos was decreased. Ji, K. (2013) and Naderi, M. (2014) had already shown the same effects and they credited the decrease in fertilized eggs to a reduction in sperm quality. BPA, that has a very similar chemical structure to BPS, is known to reduce viable egg production and sperm quality and number (Brian et al., 2007), BPS might be affecting reproduction in the same way. Reproduction is a process that involves various elements of the hypothalamus–pituitary–gonad (HPG) axis and so if a

chemical acts at any level of this axis, reproduction could be compromised (Le Fol et al., 2017). Since BPS is known to have estrogenic effect (Kim et al., 2020), it could be affecting the HPG axis leading to reproductive dysfunction by causing changes to hormonal regulation, that might ultimately result in a higher number of unfertilized eggs. To better understand these differences, in future work it would be important to conduct RNA-seq in these fish in order to access any possible changes in gene expression related to the reproductive differences found in this study along with a histological evaluation of the gonads.

Reactive oxygen species (ROS) are generated in cells in response to an exposure to xenobiotics and can result in macromolecular damage when there is an excessive amount of ROS in the cell (oxidative stress) (Ray et al., 2012). Previous studies have shown that the exposure to BPS can cause oxidative stress in zebrafish (Han et al., 2022; Salahinejad et al., 2021). However, the animals have mechanisms that help protecting cells against this stress that can be screened, like it was done in this work using biochemical assays. The oxidative stress was measured in the brain because this organ is directly connected to fish behavior (Randlett et al., 2015) and several studies have already reported the potential neurobehavioral effects of BPS on different vertebrate models, mainly by inducing cell apoptosis or promoting the formation of ROS (Wang et al., 2022) and induction of neurotoxicity. Therefore, by investigating the activities of different antioxidant enzymes, lipid peroxidation, and acetylcholinesterase activity in the brain, we can figure out if the BPS concentrations tested in this work are enough to disrupt some of the normal functions of this organ.

By converting hydrogen peroxide (H_2O_2) to non-reactive molecules, CAT plays an essential role in the detoxification of ROS (Rahman et al., 2007). In the present study CAT was significantly increased in the brain of male zebrafish exposed to 400 ng/L of BPS. In a previous study an acute exposure of zebrafish to 0.3 mg/L also showed an increase in CAT activity (Gu et al., 2019). The induction of CAT is involved in protecting cells against lipid peroxidation and ROS formation (Nunes et al., 2017) which was the most likely event happening in this study, since BPS in known to increase the production of ROS (Qiu et al., 2018). Increased ROS production is a common indicator of neurotoxicity for CECs, since they induce the nerve cells in the brain to increase ROS production and promote inflammation (Wang et al., 2022).

GST primarily known as a biotransformation enzyme that catalyzes chemicals in their metabolites, facilitating their elimination from the organism (Veal et al., 2002), is also required for the cellular resistance to oxidative stress, by eliminating secondary ROS

produced by interactions with cellular constituents, (Veal et al., 2002). In this work GST activity decreased in the brains of females exposed to 10000 ng/L of BPS. These results are concordant with the observed decrease of GST activity in a freshwater fish, *Labeo rohita*, exposed to BPS and can be justified by the buildup of metabolites from the first or second detoxification stages (Shehna Mahim et al., 2021) - the buildup of metabolites in the form of glutathione conjugates after GST-catalyzed reactions result in GST inhibition (Krča et al., 2007).

LPO was measured by the amount of Malondialdehyde (MDA) present in the fish's brains being that MDA is a common marker for oxidative stress (Del Rio et al., 2005) due to its easy reaction with TBA and its high toxicity (Ayala et al., 2014). No significant differences were found in this study between the LPO levels in the brain of fish exposed to BPS and the control which was expected since in a previous study no significant differences were originated in the MDA quantities of zebrafish exposed to less than 3 mg/L BPS (Han et al., 2022).

The biochemical results described above are more relevant when studied together. Since CAT is an antioxidant enzyme its induction usually indicates a response to the presence of ROS, and a decrease in GST levels can be due to an increase in ROS production (Rathore et al., 1998) making this enzyme reach its plateau because of the accumulation of metabolites resulting from its activity, leading to its decreased levels. Lipid peroxidation is one of the main effects produced by the presence of ROS (Juan et al., 2021), because it is a process in which free radical species, namely ROS, remove electrons from lipids generating reactive intermediates that can endure additional reactions. This process can act as a cell death signal, inducing cell death (Su et al., 2019). Antioxidant enzymes like CAT and GST have an important role in controlling free radicals and neutralizing oxidants, by decomposing ROS (He et al., 2017) thus preventing lipid peroxidation and subsequent cell death. In summary, combining the results of these methods let us theorize that there is a of ROS production, indicated by the increase of CAT activity and the reduction of GST activity, but it was not enough to cause lipid peroxidation, probably because of the action the antioxidant enzymes CAT (Barros et al., 2017). Since GST activity was diminished in females exposed to 10000 ng/L BPS, it is possible that the effect of ROS was decreased by other antioxidant enzymes like CAT, superoxide dismutase (SOD), glutathione peroxidase (GPx) etc., but further studies are needed in order to confirm the long-term effects of BPS on other antioxidant enzymes.

AChE is essential for a proper brain function in zebrafish, since it is responsible for hydrolyzing acetylcholine (ACh) to generate choline and acetate as a result, when AChE levels fall, we should expect a greater presence of acetylcholine in the neuronal cleft

potentially damaging the synapse between neurons (Tufi et al., 2016). AChE also has a part in the modulation of motor and behavioral functions and important roles in the nervous system (Heredia-García et al., 2023) because it degrades acetylcholine thus ending the neurotransmission (Richbart et al., 2021). If AChE is inhibited, the neurotransmission will be disrupted (Colovic et al., 2013) which will bring impacts to the movement and subsequent behavior of the organism. The data collected showed a significant reduction in the AChE activity in the brains of male zebrafish exposed to 10000 ng/L of BPS. Shan et al., 2023 showed that after 30 days of exposure to environmentally relevant concentrations of BPS, AChE gene expression is downregulated which agrees with the reduction in AChE activity that we found. Acute exposure of zebrafish to BPS's analog, BPA at 1180 ng/L for 96h, also found decreased in AChE activity in the brain (Heredia-García et al., 2023), and in the present study, although the zebrafish were exposed to lower concentrations of BPS for a longer period of time, the same AChE decrease was found.

Regarding the behavioral assays, our results shown that larvae hatched from eggs produced by zebrafish that were exposed to 10000 ng/L BPS spend significantly more time sleeping when compared to the control group. BPA can induce the decrease of larval movements when zebrafish larvae are exposed to 10 μM for 5 days (Kim et al., 2020) and despite the lower BPS levels tested in the present study, a similar behavior was observed. Previous studies revealed that BPA exposure altered spontaneous movement and swimming speed in response to light stimulation in developing zebrafish (Wang et al., 2013) and, since BPS is an analog of BPA, the results we obtained suggest that the same process could be happening. In study performed by Gu, J. (2019) zebrafish larvae (6 dpf) exposed to concentrations of 3 μg/L of BPS showed significant decreases in their movement which is an indication of neurotoxicity (Gu et al., 2019) caused by this compound. The results of the behavioral assays in the present study follow the same pattern.

As mentioned before, AChE has an important role in the motor capacities of the fish, and the results gathered from the behavioral assays described the loss of movement in larvae obtained from the fish exposed to 10000 ng/L, which lead us to theorize that these two results are connected i.e., the reduction of AChE activity due to BPS exposure and decrease of movement in the indirectly exposed larvae (F1). The inhibition of AChE, induced by BPS, may have resulted in the accumulation of ACh in the synaptic junctions that stopped the connections between the nerves and muscles and altered the behavior of the larvae. These results also mean that parental exposure to BPS can persist in indirectly exposed generations which could affect fish populations. Previous studies with BPS had already proven the capacity of this chemical to induce transgenerational toxicity in zebrafish (Wei et al., 2018), and in the present work we observed intergenerational effects regarding

behavior. Although the detail mechanisms that result in the intergenerational effects of BPS are not fully characterized, they indicate the possible long-term effects of BPS.

In the present study, we gathered important chronic results and some of them were different for male and female, which leads us to hypotheses that BPS effects might be sex dependent. This could be related to BPS's mechanism of action, it is a xenoestrogen that interferes with pathways mediated by estrogen receptors, that play a big part in females physiology and endocrine systems, explaining why the majority of the observed differences were in females.

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CHAPTER V. Conclusion

5. Conclusion

This study contributes to better understanding of the risk of chronic low-level BPS exposure in fish, using zebrafish as a model organism. The data collected demonstrated significant impacts of BPS at ecological, behavioral, and biochemical level. These findings are even more concerning given that BPS is ubiquitously present in the aquatic environment in concentrations similar to those tested here (lowest and medium concentration).

Many doubts about the chronic effects of BPS are still to be solved, since some of the results obtained differed from the ones available in previous literature mostly involving acute exposures. In future studies the same ecological and reproductive endpoints should be evaluated in an unexposed generation descendant from parents exposed to BPS, and the transcriptome of these individuals should also be obtained in order to decipher the signaling pathways affected by BPS that explain the apical effects observed here. Histology should also be performed to evaluate possible BPS effects in gonads.

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