DEPARTMENT OF ENVIRONMENTAL SCIENCES
AND ENGINEERING

NOVA SCHOOL OF
SCIENCE & TECHNOLOGY

Marta Sofia Simões Pereira

BSc in Sciences of Environmental Engineering

Ecotoxicological effects of emerging endocrine disruptor pollutants (BDE 99 and Bisphenol A) in juvenile marine fish

MASTER IN ENVIRONMENTAL ENGINEERING
PROFILE OF ENVIRONMETAL SYSTEMS ENGINEERING

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Adviser: Professora Doutora Marta Susana Silvestre Gouveia Martins
Associate Professor
NOVA School of Science and Technology | FCT-NOVA
NOVA University Lisbon

Co-advisers: Doutora Ana Luísa Maulvault
Assistant Researcher
IPMA I.P. – Portuguese Institute for the Sea and Atmosphere, Division of Aquaculture, Upgrading and Bioprospection

Examination Committee:

Chair: Professor Doutor Carlos David da Silva Oliveira dos Santos
Assistant Professor, NOVA School of Science and Technology | FCT-NOVA

Rapporteurs: Professora Doutora Neusa Lindorosa Loureiro Figueiredo Motubane
Invited Assistant Professor, NOVA School of Science and Technology | FCT-NOVA

Adviser: Professora Doutora Marta Susana Silvestre Gouveia Martins
Associate Professor, NOVA School of Science and Technology | FCT-NOVA

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ACKNOWLEDGMENTS

Começo por agradecer à minha orientadora, Professora Doutora Marta Martins, e à minha co-orientadora, Doutora Ana Luísa Maulvault, por todo o apoio, esclarecimentos e acima de tudo compreensão, que demonstraram ao longo deste último ano.

Agradeço ao Centro de Ciências do Mar e do Ambiente (MARE NOVA), ao grupo de investigação Marine Research and Environmental Risk Lab (MARlab, NOVA) e ao Instituto Português do Mar e da Atmosfera (IPMA, I.P.), e também ao projeto EDCs-SEAFOOD - Integrated Assessment of Emerging Endocrine Disruptor Contaminants in Seafood from Portuguese Estuaries (POCI-01-0145-FEDER-028708, PTDC/ASP-PES/28708/2017) por me terem proporcionado todas as condições à realização desta dissertação de mestrado.

Devo também um grande agradecimento à Isa Marmelo, por me ter ajudado tanto e me ter ensinado muito do trabalho em laboratório. Quero também agradecer à Beatriz Matos do MARLab (MARE NOVA) e a toda a equipa do IPMA, que me acolheu e me ensinou muito durante o meu percurso.

Mãe e pai, obrigada por me terem possibilitado chegar até aqui. Por me terem ajudado e apoiado ao longo de todo este percurso, que nem sempre foi fácil, mas durante o qual nunca deixaram de acreditar em mim, mesmo quando eu própria não acreditava. Obrigada pela confiança que têm em mim, mesmo que por vezes isso me irrite. Obrigada por estarem sempre do meu lado, mesmo que às vezes puxe o meu lado insuportável e não o mereça.

Mana, obrigada por seres o meu porto de abrigo, por me ouvires a reclamar, chorar, rir e tudo mais. És a minha pessoa e sem ti, sem o teu apoio, sem a tua amizade, também sem as nossas discussões, tudo teria sido sem graça. Acima de tudo e sem esquecer, obrigada por aturares as minhas crises existenciais e os meus dramatismos (eu sei que no fundo sou a tua fonte de entretenimentos, de nada).

Obrigada madrinha, Cátia, aos meus meninos, por se preocuparem tanto comigo e estarem sempre disponíveis para me ajudar.

Quero também agradecer aos meus amigos, sem eles não teria acabado a tese, a tese teria acabado comigo.

Marga, Cat, obrigada por terem partilhado comigo esta nossa passagem pela FCT. Espero que continuemos a partilhar muitos mais anos e histórias juntas. Não consigo agradecer-vos o suficiente pelo vosso apoio ao longos destes anos. Obrigada por estarem lá em todos os momentos difíceis e obrigada por todos os momentos bonitos que levo da FCT terem sido ao vosso lado. Este curso não teria sido o mesmo sem vocês, alcagoitas.

Marta, obrigada por seres um exemplo para mim. Tenho um orgulho imenso em ti e foste o meu suporte todos estes anos, mesmo quando já não estavas à distância de uma porta. Obrigada por me teres acolhido tão bem desde o primeiro dia. Sem ti tinha caído desamparada no monte, mas felizmente tinha-te a ti para me guiares. Obrigada por todos os conselhos, toda a ajuda, és uma pessoa incrível e não podia ter escolhido melhor madrinha.

Aos meus amigos de casa, aos meus amigos de sempre, "10 sem nome", "Víboras", "Desempregadas", a todos vocês, obrigada! Obrigada por me ouvirem reclamar, por me motivarem, por me distraírem quando já não aguentava mais, obrigada também por me darem na cabeça quando era preciso. Obrigada por estarem comigo, a vossa presença é o suficiente para me sentir uma sortuda.

Por último, mas de todo não menos importante, um obrigada muito, muito especial, a ti Avó, minha Vózinha, foste a minha casa toda a minha vida. O amor mais puro e verdadeiro que alguma vez tive. Obrigada pelo teu apoio incondicional. És a minha luz, sempre. Espero fazer-te orgulhosa. Se um dia tiveres em mim um ínfimo do orgulho que eu tenho em ti, já será maior do que eu alguma vez irei merecer. És a melhor parte de mim. Obrigada!

Obrigada a todos vocês que fizeram parte deste meu percurso pela FCT!

ABSTRACT

In the past years, with the evolution of anthropogenic activities, environmental pollution has increased, affecting several different species and decreasing ecosystems biodiversity. Ecosystems can be altered due to the presence of xenobiotics, such as emergent contaminants. ECs are not legislated and their complexity and low concentrations in the environment makes their detection a current challenge.

Within this context, the aim of this study was to assess the effects of two emerging and non-regulated EDCs, BDE-99 and BPA, alone or in a mixture, on juvenile *Sparus aurata*, for a period of 28 days. The nominal contaminant concentrations used in daily fed in this trial were: [BDE99] = 3 μg g^{-1} dry weight; [BPA] = 15 μg g^{-1} dry weight; [BDE99+BPA] = 3 μg g^{-1} dry weight + 15 μg g^{-1} dry weight.

Several biomarkers related with antioxidant, immune and endocrine responses were analyzed in fish plasma and spleen. The CAT, SOD and GST activities decreased and LPO damage increased when fish were exposed to BDE-99, indicating that the antioxidant defense mechanisms were not activated during exposure leading to lipoperoxidation damage.

VTG content and 17β-estradiol concentration decreased in all tested treatments, while 11-ketotestosterone concentration showed an increase in all tested treatments, especially in BPA.

No effects were detected in the immune responses as a result of the exposure to BDE-99 and BPA, single and combined.

Even though the biomarkers tested showed a reaction to the presence of the tested contaminants, such exposure did not lead to alterations in the animal fitness.

Keywords: Emerging contaminants, BDE-99, BPA, oxidative stress, endocrine disruption, immune responses, *Sparus aurata*

RESUMO

Com a evolução de atividades antropogénicas, tem-se verificado um aumento na poluição ambiental, afetando diferentes espécies e reduzindo a biodiversidade dos ecossistemas. Os ecossistemas podem ser alterados devido à presença de xenobióticos, como os contaminantes emergentes. Os referidos contaminantes não são legislados e a sua complexidade e baixas concentrações no ambiente tornam a sua deteção um desafio.

Neste âmbito, o objetivo do presente trabalho consiste na análise dos efeitos de dois contaminantes emergentes não regulados, BDE-99 e BPA, individualmente ou em mistura, nas respostas antioxidantes, imunológicas e endócrinas da *Sparus aurata*, durante 28 dias. As concentrações de contaminantes introduzidas na ração foram as seguintes: [BDE99] = 3 μg g^{-1} peso seco; [BPA] = 15 μg g^{-1} peso seco; [BDE99+BPA] = 3 μg g^{-1} peso seco + 15 μg g^{-1} peso seco.

Neste estudo, a atividade da CAT, SOD e GST diminuíram e a concentração da LPO aumentou, aquando da exposição ao BDE-99. Posto isto, os mecanismos de defesa antioxidante realizados pela CAT, SOD e GST não foram ativadas pela exposição aos referidos contaminantes, resultando em danos de lipoperoxidação. O conteúdo de vitelogenina e a concentração de 17β-estradiol diminuíram em todos os tratamentos testados, enquanto a concentração de 11-ketotestosterone aumentou em todos os tratamentos em particular no BPA.

A exposição da *S. aurata* ao BDE-99, BPA e à sua mistura não provocou efeitos ao nível das respostas imunológicas. Embora os biomarcadores de stress oxidativo e disrupção endócrina analisados tenham apresentado efeitos devido à exposição dos contaminantes testados, não se verificaram alterações ao nível do *fitness* do animal.

Palavras chave: Contaminantes emergentes, BDE-99, BPA, stress oxidativo, disrupção endócrina, respostas imunológicas, *Sparus aurata*

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ACRONYMS

11-KT	11-ketotestosterone.
AChE	Acetylcholinesterase.
BDE-47	2,2′,4,4′-Tetrabromodiphenyl ether.
BDE-99	2,2′,4,4′,5-pentabromodiphenyl ether.
BDE-209	Decabromodiphenyl ether.
BFRs	Brominated flame retardants.
BPA	Bisphenol A.
BAGDE	Bisphenol A diglycidyl ether.
BPS	Bisphenol S.
CAT	Catalase.
CECs	Contaminants of emerging concern.
DDT	Dichlorodiphenyltrichloroethane.
E_2	17β-estradiol.
ECs	Emerging contaminants.
EDCs	Endocrine Disrupting Compounds.
EQS	Environmental quality standard.
ER	Estradiol receptors.
EROD	Ethoxyresorufin-O-deethylase.
EU	European Union.
GES	Good environmental status.
GST	Glutathione S-transferase.
H_2O_2	Hydrogen peroxidase.
HBCD	Hexabromocyclododecane.
IPMA	Instituto Português do Mar e da Atmosfera.

LPO	Lipid peroxidation.
MSFD	Marine Strategy Framework Directive.
PAEs	Phthalic acid esters.
PBDEs	Polybrominated diphenyl ethers.
PCBs	Polychlorinated biphenyls.
PCDDs	Polychlorinated dibenzo-p-dioxins.
PCDFs	Polychlorinated dibenzofurans.
POPs	Persistent organic pollutants.
RAS	Recirculation aquaculture systems.
ROS	Reactive oxygen species.
SOD	Superoxide dismutase.
TBBPA	Tetrabromobisphenol A.
VTG	Vitellogenin.

1

INTRODUCTION

In the past years, with the evolution of anthropogenic activities, environmental pollution has increased, affecting several different species and decreasing ecosystems biodiversity. Therefore, the preservation of ecosystems biodiversity is one of the biggest challenges for the future (Björklund *et al.*, 2016).

Oceans cover about 71% of the planet surface and account for most of the world's biodiversity (UNEP, 2016), thus it is imperative to study the effects of the release of contaminants into the marine environment, and possible pollution of these ecosystems (Palmer, 2017). Furthermore, oceans and marine ecosystems provide a wide range of services, including supplying oxygen, capturing carbon dioxide and protection against extreme weather events. Additionally, these systems are also a source of food and income, through the fishery and aquaculture sectors (UNEP, 2016; Palmer, 2017).

The release of contaminants into the marine environment can occur as a consequence of atmospheric deposition, agricultural and industrial practices, deforestation, pest control, spills and discarding of dredging materials (Berg *et al.*, 2011; Miglioranza *et al.*, 2004).

The presence and distribution of contaminants depends on diverse factors, such as sources of emission, environmental conditions, hydrogeological characteristics, and the properties of the contaminants (Llamas *et al.*, 2020).

1.1 Environmental pollution

Ecosystems can be altered due to the presence of xenobiotics, that can be introduced by natural or anthropogenic sources. Although xenobiotics are foreign substances in the organism

or ecosystem, they don't always lead to pollution. The presence of xenobiotics implies the concept of contamination, in which substances are present in organisms or ecosystems where they would normally not be detected, or at higher concentration than the natural background. However, pollution only occurs when contaminants cause adverse biological effects to living organisms that have been exposed and pose acute or chronic hazards to human health (Chapman, 2007; Anderson, 2021). With that in consideration, all pollutants are contaminants, but not all contaminants are pollutants, because the effects they may cause depend on their chemical form, bioavailability, environmental factors and the reactions of exposed organisms (Chapman, 2007).

In the context of marine environment, pollution is defined, by The United Nations Convention on the Law of the Sea, as "the introduction by man, directly or indirectly, of substances or energy into the marine environment, including estuaries, which results or is likely to result in such deleterious effects as harm to living resources and marine life, hazards to human health, hindrance to marine activities, including fishing and other legitimate uses of the sea, impairment of quality for use of the sea water and reduction of amenities" (Islam & Tanaka, 2004).

There are many sources of contamination, such as human activities and resource use, namely energy use; military industry; agricultural activities; or construction and infrastructural expansion for industrial, commercial, or urban developments (Anderson *et al.*, 1994; Islam & Tanaka, 2004).

As contaminants have the ability to associate with aerosols, they can be transported from one place to another and, thus, be detected in areas where their use has not been reported (Alharbi, Khattab & Ali, 2018).

Reports state that coastal areas are affected by pollutants, which consequently affects coastal and marine fisheries (Islam & Tanaka, 2004). Although most xenobiotics occur at low concentrations, long-term exposure to low concentrations can lead to severe impacts in marine environment. Additionally, due to the high lipophilicity of these pollutants, they can have great potential to lead to biomagnification and so these xenobiotics also affect predators at higher trophic levels, including human beings, through their diet (Islam & Tanaka, 2004).

1.2 Policies in the field of environmental pollution

Coastal and marine environment face severe pressure from diverse sources of pollution, originated from both land and ocean. In order to protect these ecosystems and minimize the impacts of pollution, the European Union (EU) has adopted several instruments to accomplish these objectives. An example of this is the implementation of The Marine Strategy Framework Directive (MSFD) (Directive 2008/56/EC), adopted in 2008, by the European Union (EC, n.d.).

The MSFD was put in place to protect the marine environment, through "the application of an ecosystem-based approach to the management of human activities, enabling a sustainable use of marine goods and services" (Marine Institute, 2022). This approach aims to help the EU Member States to achieve a good environmental status (GES), following the monitorization of 11 descriptors (EC, n.d.).

In European legislation (Water Framework Directive, Article 2(29)) a contaminant is defined as "substances (i.e. chemical elements and compounds) or groups of substances that are toxic, persistent and liable to bio-accumulate and other substances or groups of substances which give rise to an equivalent level of concern" (EC, n.d.).

Decreasing the effects these contaminants have on marine ecosystems is one of the main objectives of the MSFD, in particular, in the measures presented in Descriptor 8 of this Directive. Descriptor 8 aims to regulate the levels of contaminants, such as pesticides, anti-foulants, pharmaceuticals and heavy metals, in marine habitats, preventing an increase in pollution (EC, n.d.).

Due to the importance of seafood, such as fish, crustaceous, mollusks and seaweed, in the human diet, it is important to establish safety levels for its consumption. Therefore Descriptor 9 of the MSFD exists to make sure that "contaminants in marine fish and other seafood for human consumption do not exceed levels established by Community legislation or other relevant standards" (EC, n.d.).

The Environmental Quality Standard Directive (Directive 2008/105/EC) is another European legislation that establishes the maximum concentration of a contaminant without causing harm. This concentration is defined as the Environmental Quality Standard (EQS) and is determined with tests in standard organisms in laboratory, in order to assess the lowest toxic effect observed in the organisms (EC, n.d.).

1.3 Emerging contaminants

Pollution by emerging pollutants is an increasing concern due to their wide presence in the environment and their harmful potential. These types of contaminants can be found in common products used in everyday life and, with the increase of population and the development of industry, their release into the environment will also increase (Rodriguez-Narvaez *et al.*, 2017; Taheran *et al.*, 2018).

Emerging contaminants (ECs) are used in personal care products, plasticizers, pharmaceuticals, pesticides, flame retardants, among others. Since they are present in a various range of products, their presence in the environment is also of great importance (Taheran *et al.*, 2018).

Furthermore, ECs are not legislated and their complexity and low concentrations in the environment makes their detection a current challenge. However, even though ECs can be found in low concentrations, their effects may be chronic and may be inheritable (Taheran *et al.*, 2018; Llamas *et al.*, 2020). These water pollutants can have negative effects on human and wildlife endocrine systems, thus, often being referred as Endocrine Disrupting Compounds (EDCs) (Rodriguez-Narvaez *et al.*, 2017).

Directive 2008/105/EC requires the establishment of a watch list of substances that should be monitored by Member States, including monitoring matrices and analytic methods, in order to support a prioritization of contaminants of emerging concern (CECs) (Sousa *et al.*, 2019). The first watch list was published in the Commission Implementing Decision (EU) 2015/495, and it included ten substances or group of substances, an indication of the monitoring matrix, possible cost-effective analytical methods and maximum acceptable method detection limits. It was also defined that the watch list is to be updated every two years (Commission Implementing Decision (EU) 2020/1161 of 4 August 2020).

A group of pollutants that generates great concern for the health of ecosystems are the persistent organic pollutants (POPs), which are pollutants that have long half-lives in soils, sediments, atmosphere, and biological systems, remaining for decades in soil or sediment and several days in the air (Jones & De Voogt, 1999).

The fact that POPs can exist in both the gas and particle phases, in diverse mediums, makes them more susceptible to long range transportation and, as such, they can be found in environments far from their source of emission (Ashraf, 2017; Alharbi, Khattab & Ali, 2018; Jones, 2021).

Under certain environmental temperatures, POPs volatilize from soils and water bodies into the atmosphere, being deposited in different areas (Jones & De Voogt, 1999). Additionally,

these pollutants have a low metabolism and are highly hydrophobic and lipophilic, which means they partition into organic matter, specially into lipids in the organism, leading them to bioaccumulate and magnify in the trophic chains (Jones & De Voogt, 1999; Fiedler *et al.*, 2019; Jones, 2021).

Considering the characteristics of persistent organic pollutants, they pose a severe threat to the environment, wildlife, marine biota and, ultimately, humans (Ashraf, 2017). Some of the health hazards from POPs pollution consist of endocrine disruption and damage to the immune system, which can alter their reproductive system and increase the organism's susceptibility to diseases (Jones & De Voogt, 1999; Minh *et el.*, 2000). Additionally, some POPs are also known to have carcinogenic effects (Jones & De Voogt, 1999).

Some of these pollutants are chlorinated and brominated aromatics, such as polychlorinated biphenyls (PCBs), polychlorinated dibenzo-p-dioxins (PCDDs), polychlorinated dibenzofurans (PCDFs), polybrominated diphenyl ethers (PBDEs) and organochlorine pesticides, like dichlorodiphenyltrichloroethane (DDT) and its metabolites (Jones & De Voogt, 1999). These pollutants come from the application of pesticides from agricultural practices; industrial chemicals, wastes and by-products of industrial processes. POPs can also be emitted by natural sources such as volcanic activities and wildfires (Alharbi, Khattab & Ali, 2018).

1.3.1 Flame retardants

Flame retardants are a group of chemicals used to revest plastics and textiles, with the aim to slow the process of combustion. There are more than 175 different types of flame retardants, which can be divided into the following groups: halogenated organic, normally brominated, or chlorinated; phosphorus-containing; nitrogen-containing; and inorganic flame retardants. Among these groups, the organobromine compounds (i.e. brominated flame retardants, BFRs) are the most common, accounting for almost 20% of the total commercialized flame retardants, due to their low cost and high-performance efficiency (Bimbaum & Staskal, 2004).

There are 3 major groups of BFRs: tetrabromobisphenol A (TBBPA), hexabromocyclododecane (HBCD) and polybrominated diphenyl ethers (Bimbaum & Staskal, 2004; Darnerud, 2008).

Brominated flame retardants are lipophilic, bioaccumulable and, their chemical structure and the presence of bromine atoms, also makes them persistent contaminants in the environment (Darnerud, 2008; Raldúa *et al.*, 2008; Yang, Zhao & Chan, 2017). Compounds with more bromine atoms have lower volatility and water solubility and a stronger adsorption on sediments, which makes them less mobile in the environment and consequently reduces their

bioaccumulative capacity. On the other hand, compounds with less bromine atoms have a higher volatility and water solubility and are more bioaccumulative (Darnerud, 2008).

For humans, the main sources of exposure to brominated flame retardants are through food consumption and the inhalation or ingestion of dust. Children can also be exposed to them through breast milk (Tagliaferri *et al.*, 2010; Yang, Zhao & Chan, 2017).

Some of the toxic effects of brominated flame retardants are neurotoxic and morphological effects, oxidative stress, and endocrine disrupting activity (Darnerud, 2008; Tagliaferri *et al.*, 2010).

Polybrominated diphenyl ethers are chemical substances used as flame retardants and can be found in textiles, carpets, and electrical objects. The most common congeners of PBDEs found in the environment and in human tissues are 2,2′,4,4′-Tetrabromodiphenyl ether (BDE-47) and 2,2′,4,4′,5-pentabromodiphenyl ether (BDE-99) (Albina *et al.*, 2010; Tagliaferri *et al.*, 2010). Decabromodiphenyl ether (BDE-209) is more commonly found in indoor dust and in atmospheric particles (Yang, Zhao & Chan, 2017).

One of the effects from the exposure to PBDEs is the formation of reactive oxygen species (ROS). In fact, according to Albina *et al.* (2010), the metabolization of the congener BDE-99 that occurs in the liver produce metabolites that function as unstable reactive intermediates that can provoke liver injury from protein oxidation, lipid peroxidation, DNA damage and modifications of the mitochondrial membrane permeability.

1.3.2 Plasticizers

Plasticizers and additives, like bisphenol plasticizers and phthalic acid esters (PAEs), are EDCs found in textiles, food containers, detergents, hygiene products, insecticides, and paint (Mukhopadhyay & Chakraborty, 2021). Among bisphenol plasticizers, the most common are bisphenol A (BPA), bisphenol S (BPS) and bisphenol A diglycidyl ether (BADGE), with BPA being the most well-known (Liu *et al.*, 2019).

BPA is a widely used endocrine-disrupting chemical, being recognized as a synthetic estrogen. This chemical is used to produce polycarbonate plastics that can be incorporated in several different products, such as optical products, electrical devices, household appliances, construction and medical materials, packaging, among other (Vandenberg *et al.*, 2007; Eladak *et al.*, 2015).

BPA can also be used as a component of epoxy resins, that case metallic cans used in food and beverage industry. The leaching of this contaminant from food and beverages cans

is one of the main sources of contamination (Vandenberg *et al.*, 2007; Eladak *et al.*, 2015; Molina *et al.*, 2018).

The presence of these contaminants can mimic or block endogenous hormones, affecting the reproductive system of the exposed species (Molina *et al.*, 2018). Although BPA causes histological alterations, molecular effects can develop earlier, which is why yolk protein vitellogenin (VTG) is used as a toxicological endpoint to study the effects of BPA exposure in fish (Molina *et al.*, 2018).

1.4 Ecotoxicology and its importance to assess environmental quality

With the increase of pollutants in the environment, the uncertainties involving this issue arise. To address the challenges that come with environmental pollution it is necessary to assess the environmental risks and the different scenarios that can come from contaminant exposure (Dong *et al.*, 2015).

Connell *et al.* (2009) defined ecotoxicology as "the study of the pathways of exposure, uptake and effects of chemical agents on organisms, populations, communities and ecosystems". Ecotoxicological tests can help distinguish contamination from pollution, since contamination implies the increase of contaminants above normal parameters, while, for pollution to occur, these contaminants need to cause harm to the ecosystem and populations (Chapman, 1995).

Ecotoxicological studies usually focus on laboratory tests using biological models from high trophic levels (Backhaus & Faust, 2012). These studies are often based on biomarkers, which are biological parameters that measures the individual challenge to compensate or tolerate alterations in the environment, allowing to detect behaviors, physiology, biochemistry, cell integrity and genomic structure and expression changes (Chapman, 1995; Vasseur & Cossu-Leguille, 2003).

Biomarkers detect effects that occur at low levels of biological organization, so the individuals are affected before the community, which allows to measure the state of the ecosystem towards degradation or restoration (Vasseur & Cossu-Leguille, 2003). Biomarkers can detect changes that occur due to environmental stress factors that can lead to toxicity in organisms, causing the extinction of individual, species, and disruption in communities (Vasseur & Cossu-Leguille, 2003).

For these studies to be accurate, it is necessary to know the baseline in which the test organisms are found, as well as the influence of sex, reproductive phase, seasonal and climate conditions. If these parameters are determined, conclusions can take into consideration environmental factors and the possibility of misinterpretation is reduced (Vasseur & Cossu-Leguille, 2003).

Moreover, biomarkers can determine the exposure to a disturbed environment, through the measure of primary responses, such as adaptive and compensatory responses. When the stress continues or detoxifying mechanisms are not enough, toxicity occurs, resulting in cell damage and physiological changes, which can be detected by biomarkers of toxicity. These biomarkers are a tool that allows to correlate changes in individuals to effects observed at population level (Vasseur & Cossu-Leguille, 2003). Figure 1.1 represents the processes explained above:

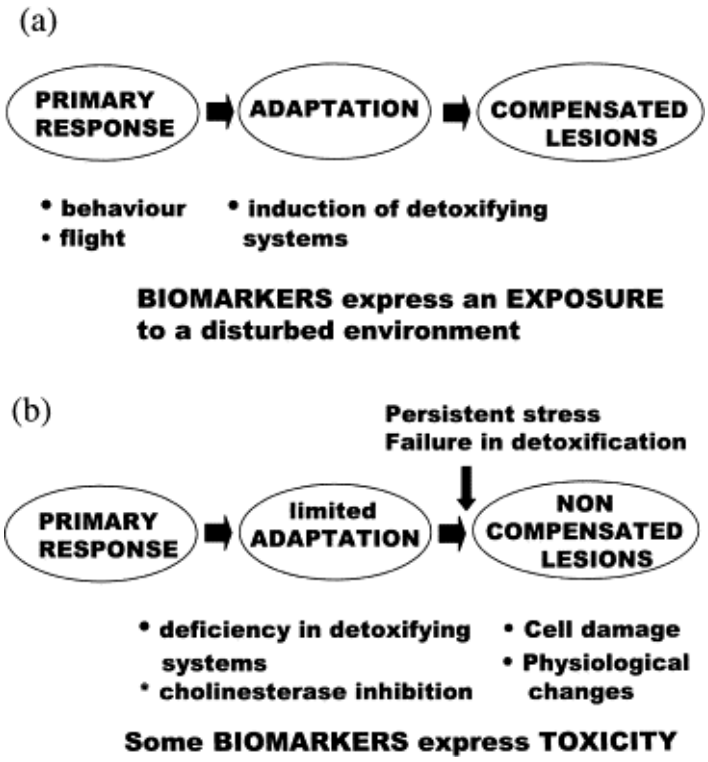

Figure 1.1.1. Patterns of response to environmental stress factors, leading to adaptation (a) or toxicity (b) in the exposed species, and identified by biomarkers (Source: Vasseur & Cossu-Leguille, 2003).

As the exposure to contaminants can interfere with diverse biochemical processes in organisms and cells, ecotoxicological studies commonly involve well established methodologies focused on the activity of enzymes involved in crucial biological pathways (e.g. oxidative stress, metabolism, biotransformation). Depending on the expected effects or target organs,

establishing the toxicological attributes of a given compound may also involve the determination of molecules (e.g. proteins, hormones, neurotransmitters) associated with specific responses (e.g. immunity, endocrine functioning, neurotransmission). Sections 1.4.1, 1.4.2 and 1.4.3 provide an overview and description of some of the most commonly used biomarkers indicative of marine organisms' welfare, antioxidant, immune and endocrine responses upon contaminant exposure.

1.4.1 Oxidative stress biomarkers

Oxidative stress occurs when there is an imbalance between the formation of ROS and antioxidant defenses, which leads to the accumulation of oxidative products (Carillon *et al.*, 2013). Thus, oxidative stress can be monitored by the quantification of antioxidant defenses, such as catalase (CAT) activity, superoxide dismutase (SOD) activity, glutathione S-transferases (GST) activity and lipid peroxidation (LPO). These antioxidant defenses are mechanisms to protect cells and tissues from oxidative stress and neutralize the toxicity of ROS (Souid *et al.*, 2013).

Catalases are enzymes that can dismutase hydrogen peroxidase (H_2O_2), which aids in ROS detoxification during stress. Considering catalases reduce the levels of H_2O_2, it is expected that CAT activity increases when stress occurs, in order to reduce the damage in the cell (Ahmad *et al.*, 2010). The enzyme SOD is another mechanism to minimize the damage of stress to the cells, since it converts two superoxide anions into one molecule of hydrogen peroxide and one of oxygen (Carillon *et al.*, 2013). Besides CAT and SOD activity, it is also expected to observe an increase of LPO and GST activity when an organism is exposed to stress. LPO is a major cell injury detected in organisms subjected to oxidative stress. When protective mechanism against oxidative stress damage (e.g. catalase) fail, lipid peroxidation occurs leading to the formation of reactive aldehydes, LPO final products, such as 4-Hydroxynonenal (HNE), malondialdehyde (MDA) and acrolein. The production of these reactive aldehydes increases membrane fluidity, cytosol efflux, loss of membrane protein activities and, in severe cases, membrane disintegration and cell death (Gasparovic *et al.*, 2013). Glutathione S-transferases are enzymes that function in Phase II detoxification reactions by catalyzing the conjugation of reduced glutathione through cysteine thiol, and so the activity of GST is used as a biomarker of oxidative stress in a wide range of organisms, including marine invertebrates (Han *et al.*, 2013).

1.4.2 Immune parameters

The immune system plays an important role in the protection of the organisms against diseases, by identifying and eliminating pathogens and suppressing the emergence of tumors; and maintains homeostasis during the development of the organism and upon inflammation or tissue damage (Magnadottir, 2010).

Immune responses can be determined by the increase or decrease of peroxidase and antiprotease activities. Peroxidases are microbicidal agents involved in the elimination of H_2O_2 and maintenance of the redox balance of immune system. Therefore, peroxidase activity is expected to increase when organisms are exposed to xenobiotics, as a defense mechanism (Guardiola *et al.*, 2014). Similarly, protease inhibitors or antiproteases are also mechanisms of defense against bacterial and parasite infections in organisms (Guardiola *et al.*, 2014). Protease enzymes act in the process of protein synthesis, turnover and function, which enables them to regulate physiological processes such as digestion, fertilization, growth, differentiation, cell signaling/ migration, immunological defense, wound healing, apoptosis, and also facilitate disease propagation. So, antiproteases are crucial to inhibit proteases and, subsequentially, pathogens replication (Leung, Abbenante & Fairlie, 2000; Borgia *et al.*, 2018).

1.4.3 Stress and endocrine responses

Endocrine responses can be detected by the analysis of sexual and stress hormones and vitellogenin (VTG) content. Sexual hormones are linked to fish reproduction, growth, digestion, gut transport, shifts in body composition, intermediary metabolism, osmoregulation, and immunity (Cuesta *et al.*, 2007).

According to Cuesta *et al.* (2007), the increase of 17β-estradiol (E_2) and testosterone (T) in females, and T and 11-ketotestosterone [11-KT] in males during sexual maturation was correlated with immunosuppression and increased disease susceptibility, likely due to the decreased antibody secreting cells and circulating levels. In sparids, when both E_2 and T increased, it was reported an enhancement of gilthead seabream serum complement and agglutinating activities, in the post-spawning period. While in goldlined seabream, *Rhabdosargus sarba*, disease status was correlated with high T plasma but low E_2 levels (Cuesta *et al.*, 2007).

Vitellogenin is a large, bulky phospholipoglycoprotein produced as the yolk protein precursor in the liver of oviparous vertebrates, like fish. Moreover, vitellogenin content is a sensitive biomarker for assessing fish exposure to estrogens (Nilsen *et al.*, 2004). VTG has a role in the immune defense of the organism, acting as multivalent pattern recognition receptors

capable of identifying invading microbes; killing bacteria and virus; and have antioxidant activity, being able to defend the host from oxidant stress (Sun & Zhang, 2015).

The sexual steroid hormone 17β-estradiol is synthesized in the gonads of female fish during sexual maturation and the circulating E_2 is subsequently taken up by hepatocytes, where it binds to estradiol receptors (ER), leading to the transcription of the vitellogenin gene. As such, plasma VTG levels usually indicate the maturational status of female fish. Furthermore, high levels of plasma VTG were also detected in male fish in rivers and streams (Nilsen *et al.*, 2004).

Vitellogenin concentrations in fish plasma can vary between 100 million-fold, a few ng/mL in unexposed male fish, or 50–150 mg/mL or above found in estrogenized salmonids (Nilsen *et al.*, 2004).

Cortisol is used as a biomarker of psychological stress and related diseases, since cortisol is produced in the hypothalamus–pituitary–adrenal axis (HPAA) as an adaptation to stress. Therefore, an increase in stress is expected to lead to an increase in cortisol levels in the plasma of the exposed organisms (Hellhammer, Wüst & Kudielka, 2009).

1.5 Biological model

Gilthead sea bream (*Sparus aurata*) is a teleost fish that inhabits the Atlantic European coast, the Mediterranean Sea and, in less scale, the Black Sea (Madeira, 2016; Arabaci *et al.*, 2010). *Sparus aurata* born in the open sea during the months of October and November and then migrate to coastal areas in the spring, returning to open sea in autumn, where adult fish breed (FAO, 2023). Whereas juveniles are found in shallow areas (around 30 m), adults reach up to 50 m in depth (FAO, 2023).

S. aurata is a euryhaline fish, living in both marine and brackish water habits, like coastal lagoons and estuaries; and a eurythermal species, that lives in environments with temperatures from 11 °C, in the winter, and 24 °C, in summer (Arends *et al.*, 1999; Arabaci *et al.*, 2010). This species is a protandrous hermaphrodite, that matures as a male at around 2 years of age, while sexual maturation in females occurs at 2-3 years of age (Arabaci *et al.*, 2010; Madeira, 2016; FAO, 2023).

This species is highly commercialized and has high value in both fisheries and aquaculture practices. The countries with most production are Greece, with 49% of production in 2002; Turkey, with 15%; Spain, detaining 14%; and Italy, with 6% of production (FAO, 2023). Croatia,

Cyprus, Egypt, France, Malta, Morocco, Portugal, and Tunisia also have a large production of this species (FAO, 2023).

S. aurata have a good market price, high survival rate and are relatively low in the food chain, which contributes to their importance in the human diet and commerce. Therefore, their vulnerability to toxicants will have an impact on human life and, as such, they are considered an important biological model (Madeira, 2016; FAO, 2023).

2

Objectives

In the recent years, the production, discharged and subsequent detection of emerging chemical contaminants in marine ecosystems has substantially increased. Hence, studies on their potential toxicological effects in wildlife and humans are utmost necessary as the information they yield is crucial to refine the current policies and protective actions. Within this context, the aim of this study was to assess the effects of two emerging and non-regulated EDCs, BDE-99 and BPA, acting alone or in a mixture context, in juvenile *Sparus aurata* antioxidant, immune and endocrine responses, during an exposure period of 28 days.

The study was conducted on juveniles since fish in this life stage are more prone to exhibit higher sensitivity to environmental stressors.

3

MATERIAL AND METHODS

The methods described in subsections 3.1. and 3.2. were developed previously to this MSc thesis research work, by researchers from the Instituto Português do Mar e da Atmosfera (IPMA I.P.) and REQUIMTE (Rede de Química e Tecnologia), in the framework of the project EDCs-SEAFOOD - Integrated Assessment of Emerging Endocrine Disruptor Contaminants in Seafood from Portuguese Estuaries (POCI-01-0145-FEDER-028708, PTDC/ASP-PES/28708/2017).

3.1 Experimental setup for the final trials

Juvenile gilthead seabream, *Sparus aurata*, with similar morphometry (weight, W: 14.1±1.5 g; total length, TL: 10.3±0.1 cm; n = 180) reared at IPMA's aquaculture pilot station (EPPO-IPMA, Olhão, Portugal) were transported to the aquaculture facilities of Laboratório Marítimo da Guia (MARE-FCUL, Cascais, Portugal), where the exposure trial took place. Here, fish were distributed, in a random and equitable way, in 12 rectangular shaped incubating glass tanks (50 L each, total volume; i.e. 15 fish per tank) within recirculation aquaculture systems (RAS) with independent functioning.

An acclimation period of 15 days was carried out, during which fish were daily fed with a non-contaminated feed (i.e. CTR feed; ~2% average body weight), produced by SPAROS lda. according to the nutritional requirements of juvenile *S. aurata*, and with appropriate granulometry taking into consideration fish size (1 mm pellets). Three experimental contaminated feeds were also prepared by this feed producing company, using the same chemical composition as in CTR feed but, in these cases, different contaminant solutions (i.e. BDE99, BPA or BDE99+BPA) were also added to the oils and incorporated in feeds during coating step.

Contaminant solutions were previously prepared by solubilizing a given amount of contaminants in a small volume (5 mL) of organic solvent (ethanol for BPA and methanol for BDE 99). Even though the low volume of organic solvents was completely evaporated during feed production, to rule out any potential adverse effect elicited by the use of ethanol and methanol, an additional non-contaminated feed (i.e. Control+Solvent feed) was also prepared containing exactly the same amount of solvents added to the three contaminated feeds.

The nominal contaminant concentrations selected for this trial were: [BDE99] = 3 μg g^{-1} dry weight; [BPA] = 15 μg g^{-1} dry weight; [BDE99+BPA] = 3.00 μg g^{-1} dry weight + 15 μg g^{-1} dry weight, corresponding to ~10x the values usually found in seafood species. The selection of such concentrations was based on two criteria:

1) Concentrations that can be somewhat linked to realistic environmental levels (Vandermeersche *et al.*, 2015);
2) Concentrations that are high enough to elicit contaminant bioaccumulation and toxicological responses within the timeframe of the trial.

In Table 3.1 is presented the concentration of contaminants in each type of experimental feed used in the experiments (or bioassays).

Table 3.1. Contaminants concentration in experimental feeds, in μg/g dry weight.

Experimental feed	Composition (μg/g dry weight)	
	BDE-99	BPA
Control (CTR)	<LOD	<LOD
Control + Solvent (CTR + SOL)	<LOD	<LOD
BDE-99	3.00 ± 0.58	<LOD
BPA	<LOD	16.5 ± 2.59
BDE-99 + BPA (MIX)	3.04 ± 0.37	16.43 ± 1.28

After acclimation to laboratory conditions, the exposure trial was carried and five treatments (each one composed by three replicate tanks) were considered:

1. Control (CTR) treatment;
2. CTR+Solvent treatment;
3. BDE99 treatment;
4. BPA treatment;

5. BDE99+BPA treatment.

In each treatment, fish were daily fed with the respective diet (CTR-feed, CTR+Solvent-feed, BDE99-feed, BPA-feed or BDE99+BPA-feeds; feed amount ~2% of their average body weight), for a period of 28 days. Throughout the experiment, fish were kept under the following abiotic conditions:

i. temperature = 19.0 ± 0.5 °C;
ii. pH = 8.00 ± 0.10 units;
iii. salinity = 35 ± 1 ‰;
iv. dissolved oxygen (DO) > 5 mg L-1;
v. photoperiod = 14 hours light and 10 hours dark.

In each incubation tank, fish faeces and feed leftovers were removed on daily basis, and a 25% seawater renewal was performed. Ammonia, nitrite and nitrate levels were daily checked through colorimetric tests (Tropic Marin, USA), and kept below detectable.

In Figure 3.1, it is presented the experimental setup of the tanks in which the experiment was conducted.

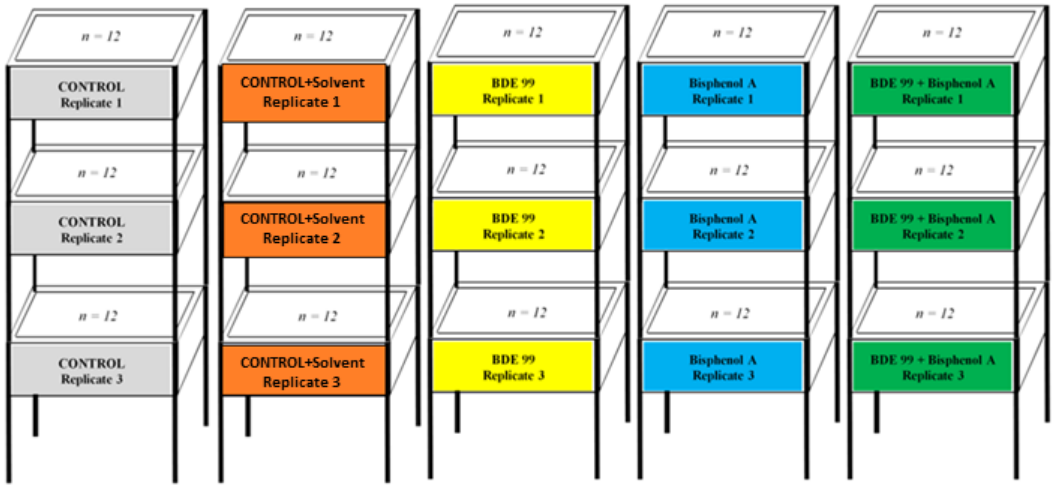

Figure 3.1. Experimental setup

3.2 Samples preparation and haematological parameters

By the end of the exposure period, 5 fish were randomly collected from each treatment in order to assess the ecotoxicological responses. Upon 24h of fasting, fish were euthanized by immersion in an overdosed MS222 solution (2000 mg L^{-1}; Sigma-Aldrich, USA) buffered with

sodium bicarbonate (1 g of $NaHCO_3$ to 1 g of MS222 to 1 L of seawater) for 10 min. Euthanized fish were measured (total length, TL, and weight, W), and peripheral blood was collected by puncture of the caudal vein. A small fraction of blood was used to perform smears on preclean glass microscopy slides, which were allowed to air-dry for 24 h, fixed for in methanol and stained with the ready to use Hemacolor staining reagent (Hemacolor® Rapid staining of blood smear, Sigma-Aldrich) according to the instructions provided in this product, in order to subsequently count blood cells (erythrocytes and leukocytes), as well as to detect the presence of erythrocytes nuclear abnormalities (ENAs) and micronuclei, through optical microscopy. Following staining, the microscope glass slides were mounted with DPX (BDH, Poole, England) and three slides were prepared per individual. A minimum of 300 cells per slide were examined under the microscope (1000 × magnification), following the ENAs and micronuclei classification according to Carrasco *et al.* (1990).

The remaining (and larger) fraction of fish blood was centrifuged (10 min, 10,000 g, 4º C) in order to extract the plasma, which was subsequently frozen and kept at -80 ºC until further analyses. Then, fish spleen was isolated, weighed, individually placed in 1.5 mL Eppendorf tubes, and homogenized in ice-cold conditions with 1.0 mL of phosphate buffered saline (PBS; 140 mM NaCl, 3mM KCl, 10 mM KH_2PO_4, pH = 7.40 ± 0.02; reagents from Sigma-Aldrich, Germany), using an Ultra-Turrax® device (T25 digital, Ika, Germany). Crude homogenates were centrifuged for 15 minutes at 10.000 g and 4 ºC, supernatants were transferred to new microtubes, immediately frozen and kept at -80 ºC until further analyses. All biochemical analyses were performed in duplicate or triplicate and using reagents of pro analysis grade or higher.

3.3 Biochemical biomarkers

Different biochemical biomarkers indicative of oxidative stress, endocrine disruption and immunological responses were evaluated in fish plasma and spleen samples. All analyses were performed using reagents of pro analysis grade or higher and a Multiskan Go 1510 microplate reader (ThermoFisher Scientific, USA). At least two technical replicates were considered for every sample/analysis.

3.3.1 Total protein content

Bradford assay (Bradford, 1976) was carried out in 96-well microplates (Nunc-Roskilde, Denmark) as to quantify total protein levels in each sample, and so that the subsequent biomarker results could then be normalized (i.e. given in mg of protein). Briefly, 190 µL of

Bradford reagent were added to each well, followed by 10 µL of each sample or standard. Afterwards, absorbance was read at 595 nm in a microplate reader (BioRad, Benchmark, USA). A calibration curve was generated using bovine serum albumin (BSA; Sigma Aldrich, Germany) at different dilutions (at least 7 different concentrations, ranging from 0 to 2 mg mL^{-1}) as standard.

3.3.2 Oxidative stress in fish spleen

3.3.2.1 Catalase (CAT)

CAT activity (EC 1.11.1.6) was determined by following the procedure described by Johansson and Borg (1988) and adapted to 96-well microplates. A calibration curve was built using formaldehyde standards, prepared from a 4,25 mM formaldehyde (Sigma-Aldrich, USA) stock solution. The calibration curve contained concentrations ranging from 0 to 75 µM of formaldehyde.

To each well it was added 100 µL of diluted Assay Buffer - (a solution of 100 mM KH_2PO_4 with pH = 7.0), - 30 µL of Methanol and 20 µL of standards or sample. This step was performed in triplicate.

The reaction was initiated by adding 20 µL of hydrogen peroxide to all wells and then the microplate was incubated for 20 minutes at room temperature. This reaction was then stopped by adding 30 µL of potassium hydroxide (10 M) and 30 µL of Purpald solution and the microplate was incubated on a shaker for 10 minutes, at room temperature.

After 10 minutes it was added 10 µL of potassium periodate solution, the microplate was covered and incubated for 5 minutes on a shaker, at room temperature.

The absorbance was read in a microplate reader Thermo Scientific Multiskan GO at 540 nm.

The formaldehyde concentration of the samples was calculated by using the equation obtained from the linear regression of the standard curve substituting corrected absorbance values for each sample.

Then the following equation (1) was used to calculate the CAT activity:

$$(1)\ CAT\ Activity = \frac{[Formaldehyde]\ \mu M}{tr}$$

Where tr corresponds to reaction time – 20 minutes

Enzyme activity was calculated considering that one unit of CAT is defined as the amount that will cause the formation of 1.0 nmol of formaldehyde per minute at 25 °C. The results were presented as nmol min^{-1} mg $protein^{-1}$.

3.3.2.2 Superoxide dismutase (SOD)

SOD activity was carried out as described by Sun *et al.* (1988), using nitroblue tetrazolium (NBT) and xanthine oxidase (XOD) (both from Sigma-Aldrich, Germany).

This assay was performed in a 96-well microplate and to each well it was added 200 µL of potassium phosphate buffer, 10 µL of EDTA, 10 µL of xanthine, 10 µL of NBT, 10 µL of sample and 10 µL of XOD, in this order. This step was done in duplicate.

The negative control followed the same steps except for adding the sample.

The absorbance was read at 550 nm, every 5 minutes for 20 minutes and results were presented as the percentage (%) of enzyme inhibition, using the following equation (2):

$$(2)\ \%\ inhibition = \frac{Abs\ per\ minute\ (negative\ control) - Abs\ per\ minute\ (sample)}{Abs\ per\ minute\ (negative\ control)} \times 100$$

3.3.2.3 Lipid peroxidation (LPO)

The LPO assay was performed according to the procedure originally developed by Uchiyama and Mihara (1978) and adapted to a microplate by Martins *et al.* (2015).

The calibration curve was prepared by doing successive dilutions in Eppendorf tubes, obtaining a range of concentrations from 0 to 1 µM of malondialdehyde (MDA).

In new Eppendorf tubes it was added 300 µL of sulfosalicylic acid solution (5% m/v) (SSA) and 100 µL of sample or standards. This solution was then mixed by vortex.

The Eppendorf tubes with sample were kept at 2-8ºC for 10 minutes to allow deproteination. Following this step, the samples were put in a centrifuge at 10 000 g for 10 minutes.

To 100 µL of the deproteinated supernatant or the standards mixture it was added 100 µL of thiobarbituric acid solution (1% m/v) (TBA) and that mix was incubated in a boiling water bath for 15 minutes. After that period, the Eppendorf tubes were put on ice to stop the reaction. To a 96-well-microplate it was added 70 µL of each sample or standard. This process was done in duplicate. The absorbance of the pink pigment was read at 535 nm, in a microplate reader (Thermo Scientific Multiskan GO).

3.3.2.4 Glutathione S-transferase (GST)

GST assay was performed according to a procedure adapted from GST Assay Kit from Sigma-Aldrich to a 96-well microplate.

In order to perform the assay, 180 µL of substrate solution was added to 20 µL of sample in each microplate well. This process was done in triplicate. The substrate solution was prepared by adding 16,6 mL of Dulbecco's Phosphate Buffered Saline, 200 µL of 200 mM L-Glutathione reduced and 200 µL 100 mM CDNB.

The absorbance was read at 340 nm in a microplate reader (Synergy HTX, Biotek, USA), every minute for 6 minutes to determine the total enzyme activity. The change in absorbance per minute was determined and used to calculate the GST activity, using the following equation (3):

$$(3)\ GST\ activity\ (\mu mol\ ml^{-1}min^{-1}) = \frac{Abs\ 340\ per\ minute \times V\ (ml) \times dil}{\varepsilon_{mM} \times V_{sample}(ml)}$$

Where:

dil = the dilution factor of the original sample

ε_{mM} ($mM^{-1}cm^{-1}$) – extinction coefficient for CDNB conjugate at 340 nm for test in Sigma 96-well plate = 5.3 mM^{-1} (path length -0.552 cm)

V – the reaction volume in 96-well microplate = 0.2 mL

V_{sample} – the volume of the enzyme sample tested = 20 µL

The reaction rate was determined considering the molar CDNB extinction coefficient of 5.3 mM^{-1}, and results were expressed in relation to the total protein concentration of the sample and presented in µmol min^{-1} mg $protein^{-1}$.

3.3.3 Plasma parameters

3.3.3.1 Plasma parameters

3.3.3.1.1 Vitellogenin (VTG)

Vitellogenin content was determined in fish plasma following a direct ELISA assay based on the protocol of Denslow *et al.* (1999), adapted to 96-wells microplates and described in detail by Diniz *et al.* (2010). The primary and secondary antibodies used in this assay were carp VTG monoclonal antibody (Biosense, Norway; diluted to 1.0 µg mL^{-1} in a 1% BSA solution) and anti-mouse IgG, fab specific, alkaline phosphatase conjugate (Sigma-Aldrich, Germany; also diluted to 1.0 µg mL^{-1} in 1% BSA), respectively.

A calibration curve was performed using serial dilutions of carp VTG standard (Biosense, Norway), for a concentration range of 10 to 1000 ng mL^{-1} of protein. Absorbance was read at 405 nm and results were expressed in µg mg^{-1} protein.

3.3.3.1.2 17β-estradiol (E_2) and 11-ketotestosterone (11-KT)

Plasma concentrations of E_2 and 11-KT were measured using EIA kits (Estradiol EIA kit and 11-KT EIA kit, Cayman Chemicals, USA), in accordance with the kit protocols provided by Cayman Chemicals (2002, 2003).

Cayman's Estradiol ELISA Kit is a competitive assay based on the competition between native estradiol and estradiol acetylcholinesterase (AChE) conjugate - Estradiol AChE Tracer - for a limited amount of Estradiol Antiserum. The Estradiol AChE Tracer binds to the Estradiol Antiserum. The concentration of Estradiol AChE Tracer is constant, while the concentration of native estradiol varies and, the concentration of the Estradiol AChE Tracer that is bid to the Estradiol Antiserum will be inversely proportional to the concentration of native estradiol. The complex formed from the Estradiol AChE Tracer and the Estradiol Antiserum binds to mouse monoclonal anti-rabbit IgG that was previously attached to the well. The wells are washed to remove any unbound reagents and Ellman's Reagent, with the substrate to AChE, is added to the microplate.

This reaction produces a yellow pigment, and the absorbance was read at 414 nm. The intensity of the yellow color is directly proportional to the concentration of Estradiol AChE Tracer bound to the well, which is inversely proportional to the amount of native estradiol present in the well during the incubation.

Similarly, to the Estradiol assay, the Cayman's Testosterone ELISA Kit is based on the competition between testosterone and Testosterone-acetylcholinesterase (AChE) conjugate, the Testosterone Tracer, for a limited amount of Testosterone Antiserum. Following the same principle, the testosterone tracer concentration is constant in the wells, while testosterone concentration varies. So, the concentration of testosterone tracer that binds to the Testosterone Antiserum is inversely proportional to the concentration of testosterone in the well. The wells had previously attached mouse monoclonal anti-rabbit IgG, to which the testosterone tracer - Testosterone Antiserum complex binds to in the wells. Then the microplate is washed to remove any unbound reagents and the Ellman's Reagent, which contains the substrate to AChE, is added to the well.

This reaction also produces a yellow pigment, however in the case of this assay, the absorbance was read at 412 nm.

To calculate the steroid concentration in the plasma, in both assays, it was prepared a calibration curve, using Estradiol ELISA standard or Testosterone standard, depending on the assay, and serial dilutions to a range between 0,61 and 1000 pg/mL according to information furnished by Cayman Chemical (2002, 2003).

3.3.3.2 Immunological parameters

3.3.3.2.1 Peroxidase activity

The peroxidase activity in the plasma was determined by oxidation of 3,3′,5,5′-Tetramethylbenzidine (TMB), according to Quade & Roth (1997).

In this assay, a first dilution was done by diluting 5 µL of plasma with Hanks's buffer (HBSS) without Ca^{+2} or Mg^{+2} to a final volume of 50 µL in a flat-bottomed 96-well plate. Secondly, 5 µL were diluted in HBSS, to a final volume of 150 µL. As substrate, it was added to the microplate 50 µL of 20 mM of 3,3,5,5,-tetramethyl benzidine hydrochloride (TMB) (Sigma) and 50 µL of 5 mM hydrogen peroxide (H_2O_2).

After a period of 2 minutes, the reaction was stopped by adding 50 µL of 2 M sulphuric acid (H_2SO_4), and optical density (OD) was read at 450 nm.

The negative control followed the same steps, but without the addition of the plasma samples, and their OD values were subtracted from each sample value.

The peroxidase concentration was calculated using the following equation:

$$(4) \quad Peroxidase\ activity\ (450\ nm\ OD) = \frac{(abs_{sample} - abs_{negative\ control}) \times total\ volume}{sample\ volume} \times 10$$

The peroxidase activity, units/ml serum, is determined defining as one unit the peroxidase that produces an absorbance change of 1 OD.

All samples were analysed in duplicate, except for the blanks that were done in triplicate.

3.3.3.2.2 Antiprotease activity

Antiprotease activity was determined by the capacity of the samples to inhibit trypsin activity.

To prepare the plasma sample, in a 1.5 mL eppendorf tube it was added 20 µL of plasma and 20 µL of trypsin; for the positive control, it was added 20 µL of phosphate buffer (NaH_2PO_4), at pH = 7 and 20 µL of trypsin, in a 1.5 mL eppendorf tube; and for the negative control, it was only added 40 µL of phosphate buffer to the 1.5 mL eppendorf tube.

After the trypsin was added, the mixtures were left at room temperature for 10 minutes. Then it was added 100 µL of phosphate buffer and 125 µL of azocasein to each tube, that followed vortex and then let stand at room temperature for 60 minutes.

After the incubation period, it was added to each eppendorf tube 250 µL of trichloroacetic acid (TCA), in a fume cupboard. The eppendorf tubes went through vortex and let stand at room temperature for 30 minutes. Then the mixtures were centrifuged at 10,000g for 5 minutes.

On a 96 well non-absorbent microtray, it was added 100 µl of sodium hydroxide (NaOH) – to stop the reaction - to each well and 100 µL of the supernatant in triplicates in the sample wells and in quadruplicate in the positive and negative control wells.

The absorbance was read at 450 nm and the anti-trypsin activity was determined by the following equation:

$$(5)\ \ Anti-trypsin\ activity = \frac{(abs - abs\ (negative\ control) \times 100}{abs\ (posive\ control)}$$

To express the anti-trypsin activity in reverse, in other words, the percentage (%) of inhibition the value calculated by (5) was subtracted from 100.

3.3.3.3 Cortisol

Plasma concentration of cortisol was determined using Cayman's Cortisol ELISA Kit. This assay is based on the competition between free cortisol and a cortisol-acetylcholinesterase (AChE) conjugate, the Cortisol-AChE Tracer, for a limited number of cortisol monoclonal antibody binding sites. The concentration of free cortisol varies, while the concentration of cortisol-AChE is maintained constant in the wells. So, the concentration of Cortisol-AChE that binds to the cortisol monoclonal antibody is inversely proportional to the concentration of free cortisol in the well. The cortisol-AChE that binds to the antibody is fixed to the wells by binding to the polyclonal goat anti-mouse IgG that has been previously attached to the well. To make sure that any unbound reagents are removed, the microplate is washed and the Ellman's Reagent, that contains the substrate to AChE, is added to the wells.

This reaction produces a yellow pigment, and the absorbance was read at 412 nm. The intensity of the yellow color is directly proportional to the concentration of Cortisol-AChE Tracer bound to the well, which is inversely proportional to the amount of free cortisol present in the well during the incubation.

To calculate the cortisol concentration in the plasma, it was prepared a calibration curve, using Cortisol ELISA standard and serial dilutions to a range between (0 - 2,500 pg mL^{-1}) according to information furnished by Cayman Chemical (2002, 2003).

3.3.4 Animal fitness indexes

The Fulton's K index was directly calculated from the biometric data to determine fish condition, according to the formula:

$$(6)\ K = 100\ x\ \frac{W\ (g)}{TL^3\ (cm)}$$

where W is the fish weight and TL is the total length (Ricker, 1975). The relationship between fish total weight and the respective spleen weight was calculated using the following equation:

$$(7)\ Spleen{:}\ body\ weight\ ratio\ =\ \frac{Spleen\ weight\ (g)}{W\ (g)}\ x\ 100$$

3.3.5 Statistical analysis

As standard procedure, data were first tested for normality and homoscedasticity through Kolmogorov–Smirnov and Levene tests, respectively. To evaluate the presence of significant differences between treatments in fish morphometric data (W and TL), animal fitness indexes (K and Spleen:body weight ratio), hematological parameters (percentage of erythrocytes, leukocytes, ENAs, micronuclei in relation to total blood cells or total erythrocyte counts), and biochemical biomarkers in fish spleen (CAT, SOD, GST and LPO) and plasma (cortisol, E_2, 11-KT, VTG concentration, peroxidase and antiprotease activities), the one-way ANOVA analysis was performed. The post-hoc Tukey HSD test was subsequently conducted to identify significant differences. Whenever the normality and homoscedasticity assumptions of the ANOVA were not verified, even after data transformation (Log or Square-root), the non-parametric Kruskal-Wallis test with multiple comparisons were carried out instead. Finally, potential correlations between biochemical biomarker levels and animal fitness indexes were performed by means of Pearson's correlation analysis. Statistical analyses were performed at a significance level of 0.05, using STATISTICATM software (Version 7.0, StatSoft Inc., USA).

4

RESULTS

The control and control + solvent showed no significant differences in all the parameters and biomarkers analyzed ($p > 0.05$ in annexes A.1). The results observed were expected, since the solvent was completely evaporated and was not expected to have any interference in the bioassays. Considering that, the treatments with BDE-99 and/or BPA will be compared to the control + solvent group for all results obtained.

4.1 Haematological parameters

Juvenile *S. aurata* haematological parameters upon 28 days of exposure to BDE-99 and/or BPA are shown in Figure 4.1, as well as in annexes A.2.

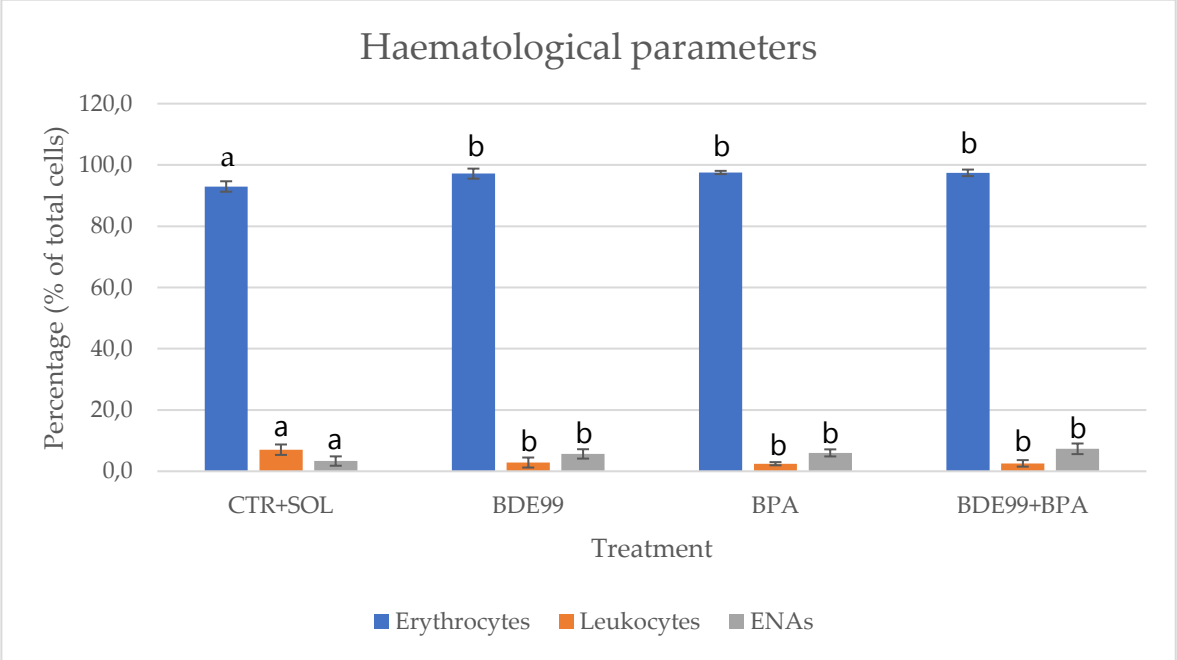

Figure 4.1. Percentage (mean ± SD; n=5) of erythrocytes, leukocytes, and erythrocytes nuclear abnormalities in *S. aurata* upon 28 days of exposure trial. Different letters denote significant differences between treatments within the same haematological parameter ($p < 0.05$).

The results show that the percentage of haematological parameters varied significantly between control+sol and the contaminant treatments ($p < 0.05$). Erythrocytes reached 93% of the total haematological parameters measured, and BDE-99, BPA, and BDE-99 + BPA treatments had a significant increase ($p < 0.001$) of > 4.2% in relation to CTR+SOL. The leucocytes represented 7% in control+sol, and for the BDE-99, BPA, and BDE-99 + BPA treatments these values decreased significantly ($p < 0.001$) down to 4.5% relatively to controls. For both parameters no significant differences ($p > 0.05$) were observed between treatments.

Erythrocytes nuclear abnormalities (ENA) represented 3.3% In the control+sol, and for the BDE-99, BPA, and BDE-99 + BPA treatments these values had a significant increase ($p < 0.001$) of 2.3%, 2.7% and 4.0%, respectively, when compared to controls. The contaminated treatments showed no significant differences among each other.

4.2 Biochemical biomarkers

4.2.1 Oxidative stress in fish spleen

4.2.1.1 Catalase activity

The CAT activity determined in fish spleen are shown in Figure 4.2.

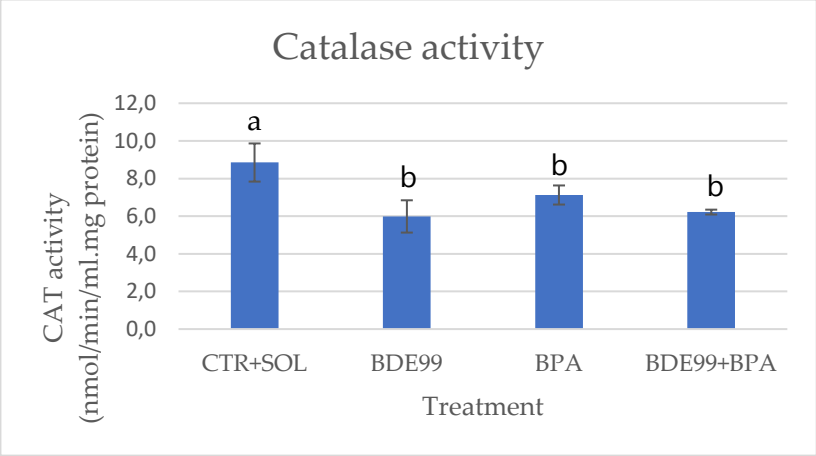

Figure 4.2. CAT activity (mean ± SD) in the spleen of *S. aurata* exposed to BDE-99, BPA and their mixture for 28 days. Different letters denote significant differences between treatments ($p < 0.05$).

CAT activity varied between 6.0 ± 0.5 (BPA) and 9.7 ± 1.0 nmol/min/mg total protein (Control). A significant decrease ($p < 0.05$) was observed for BDE-99, BPA, and BDE-99 + BPA treatments relatively to control+sol group. Yet, no significant differences ($p > 0.05$) were observed among treatments with the test contaminants.

4.2.1.2 Superoxide dismutase activity

The results obtained for SOD dismutase activity are shown in Figure 4.3.

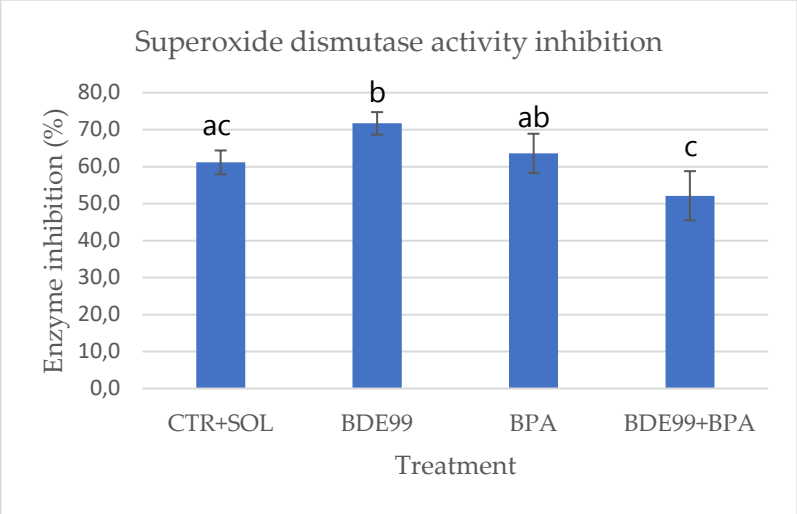

Figure 4.3. SOD activity inhibition (mean ± SD) in the spleen of *S. aurata* exposed to BDE-99, BPA and their mixture for 28 days. Different letters denote significant differences between treatments ($p < 0.05$).

The SOD activity varied between 71.7 ± 5.3 % (BDE-99) and 52.1 ± 5.7 % (BDE-99 + BPA). A significant increase ($p < 0.05$) was observed for BDE-99 treatment relatively to control + solvent (10.5%). Yet, BPA and BDE-99 + BPA treatments showed no significant differences ($p > 0.05$) when in comparison to the control + solvent group.

4.2.1.3 Lipid peroxidation levels (LPO)

LPO determined in fish spleen is presented in Figure 4.4.

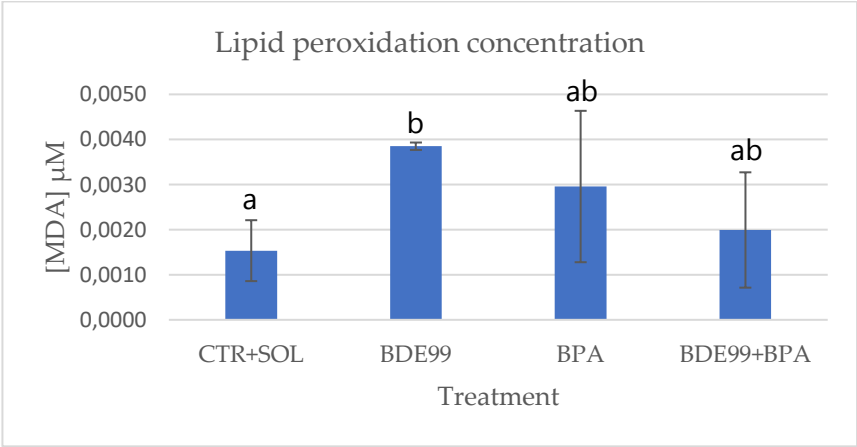

Figure 4.4. LPO concentration (mean ± SD) in the spleen of *S. aurata* exposed to BDE-99, BPA and their mixture for 28 days. Different letters denote significant differences between treatments ($p < 0.05$).

LPO (measured as the concentration of MDA) varied between 1.5 ± 0.1 nM in fish exposed to the control + solvent treatment and 3.8 ± 1.7 nM in fish exposed to BDE-99.

A significant increase of LPO was observed in spleen of fish exposed to BDE-99 when compared to the control + solvent group ($p < 0.05$). No significant differences were observed between control + solvent group, BPA and the mixture of BDE99 and BPA. Also, no significant differences were observed for LPO results among test treatments.

4.2.1.4 Glutathione S-transferase activity

The GST activity measured in fish spleen are presented in Figure 4.5.

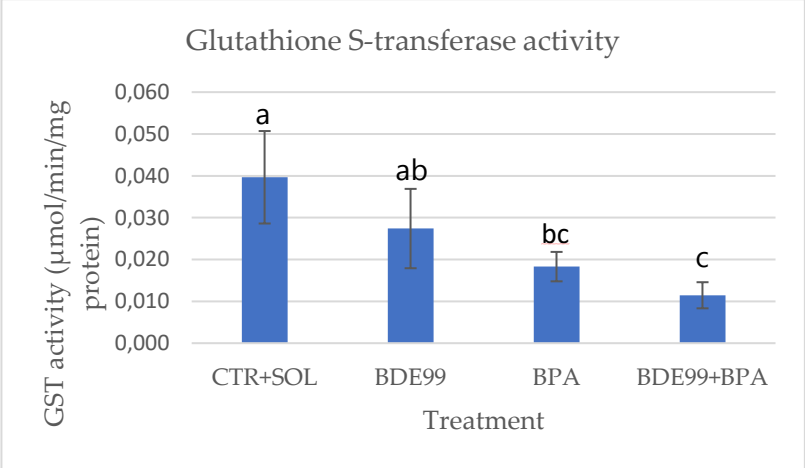

Figure 4.5. GST activity (mean ± SD) in the spleen of *S. aurata* exposed to BDE-99, BPA and their mixture for 28 days. Different letters represent significant differences between treatments ($p < 0.05$).

GST activity varied between 0.043 ± 0.003 µmol/min.mg protein in the control group, and 0.011 ± 0.003 µmol/min.mg protein, in the BDE-99 + BPA treatment.

GST activity showed a significant decrease in fish spleen exposed to BPA and BDE-99 + BPA treatment ($p < 0.001$ in both cases) relatively to control + solvent group. No significant differences were observed between BPA and BDE-99 + BPA treatments and control and BDE99.

4.2.2 Plasma parameters

4.2.2.1 Endocrine disruption parameters

In Figure 4.6 are presented the vitellogenin content determined in fish plasma.

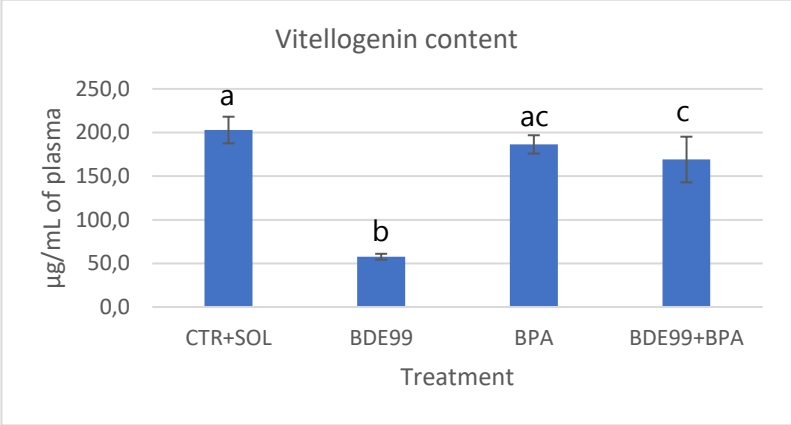

Figure 4.6. Vitellogenin content (mean ± SD) in *S. aurata* plasma, upon 28 days of exposure to BDE-99, BPA and their mixture. Different letters mean there are significant differences between treatments ($p < 0.05$).

Vitellogenin content varies between 213.8 ± 12.4 µg/mL, in the control group, and 57.6 ± 3.4 µg/mL, in the BDE treatment.

Fish exposed to the tested chemicals yielded a decrease in vitellogenin content in fish plasma, being the highest significant decrease observed for BDE99 exposure relatively to control and BPA and BDE-99 + BPA treatments.

Hormone parameters, like 17β-estradiol and 11-ketotestosterone concentrations determined in fish plasma are presented in figures 4.7 and 4.8.

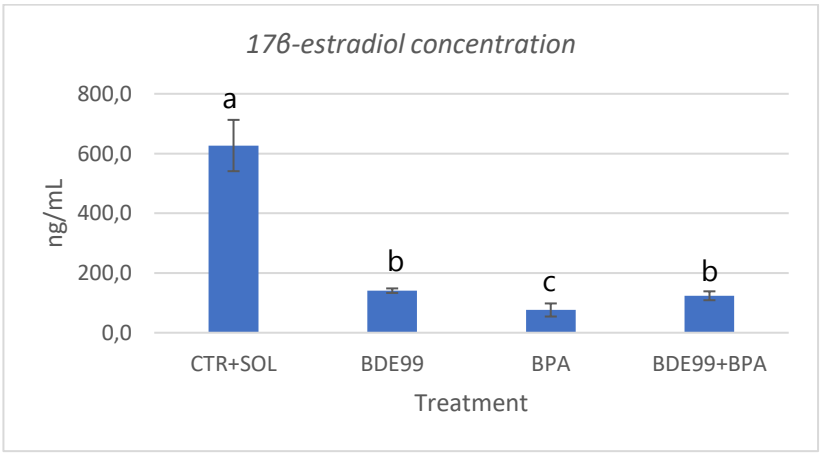

Figure 4.7. 17β-estradiol concentration (mean ± SD) *S. aurata* plasma exposed to BDE-99, BPA and their mixture for 28 days. Different letters represent significant differences between treatments ($p < 0.05$).

17β-estradiol concentration varied between 76.1 ± 14.8 ng/mL, in the BPA treatment and 626.8 ± 7.6 ng/mL, in control + solvent group.

BDE-99, BPA, and BDE-99 + BPA treatments showed a significant decrease ($p < 0.001$), of approximately 20%, relatively to control + solvent treatment. No significant differences were

observed between treatments with the exception of BPA treatment which showed the lowest level of 17β-estradiol.

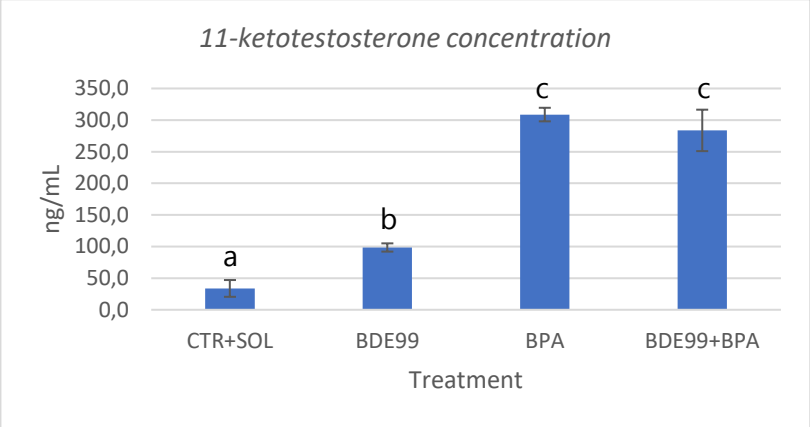

Figure 4.8. 11-ketotestosterone concentration (mean ± SD) in *S. aurata* plasma, upon 28 days of exposure to BDE-99, BPA and their mixture. Different letters mean there are significant differences between treatments ($p < 0.05$).

The concentration of 11-ketotestosterone ranged between 33.8 ± 6.5 ng/mL, which can be observed in the control + solvent group and 308.7 ± 32.8 ng/mL, in BPA treatment.

The highest values were obtained for fish plasma exposed to BPA and BDE-99 + BPA treatments, with values of 308.7 ± 32.8 ng/mL and 283.7 ± 32.3 ng/mL, respectively. Significantly lower values were obtained for BDE-99 group (98.5 ± 10.7 ng/mL).

4.2.2.2 Immunological parameters

4.2.2.2.1 Peroxidase activity

Figure 4.9 shows the graphic representation of peroxidase activity determined in the plasma of fish exposed to BDE-99, BPA, and their mixture.

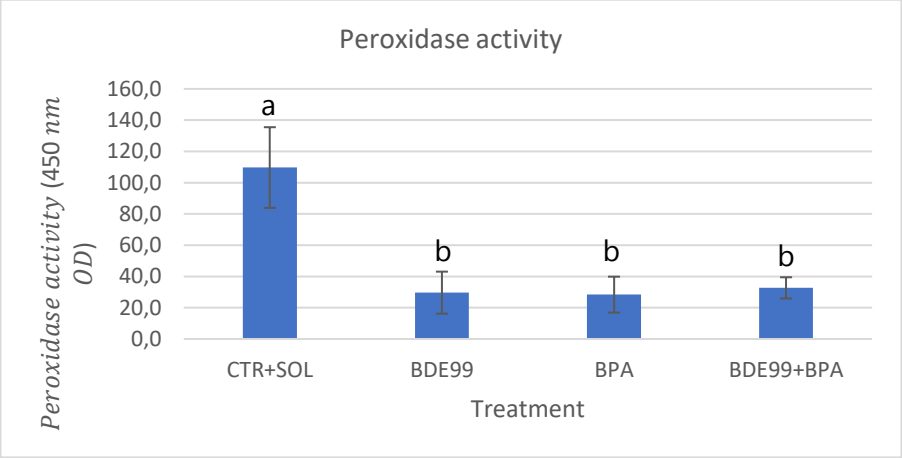

Figure 4.9. Peroxidase activity (mean ± SD) in *S. aurata* plasma, upon 28 days of exposure to BDE-99 and/or BPA. Different letters mean there are significant differences between treatments ($p < 0.05$).

Peroxidase activity varies between 112.0 ± 25.8 450 nm OD (control) and 28.4 ± 6.8 nm OD (BPA treatment).

BDE-99, BPA and BDE-99 + BPA showed a significant decrease ($p < 0.001$) of almost 30% when compared to the control + solvent group. Yet, no significant differences were observed among contaminated treatments.

4.2.2.2.2 Antiprotease activity

The results from antiprotease activity are shown in Figure 4.10.

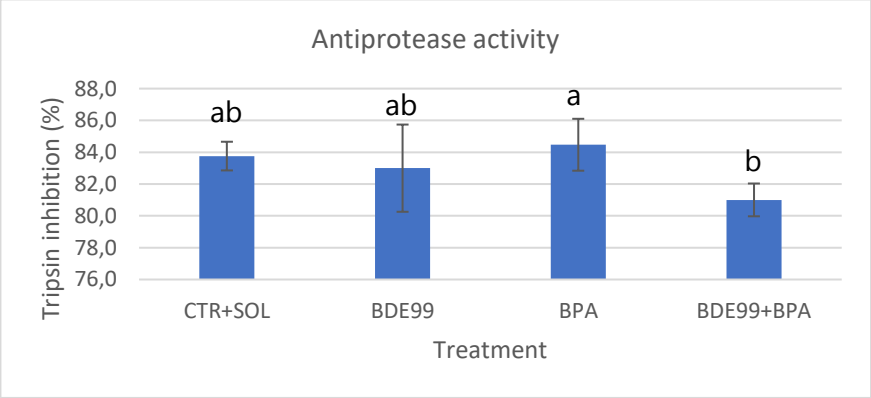

Figure 4.10. Antiprotease activity (mean ± SD) in *S. aurata* plasma exposed to BDE-99 and/or BPA, for 28 days. Different letters mean there are significant differences between treatments ($p < 0.05$).

Antiprotease activity varies between 84.5 ± 1.0 %, in the BPA treatment, and 81.0 ± 1.5 %, in the BDE-99 + BPA treatment.

No significant differences were observed between contaminated treatments and the control + solvent group. However, BDE-99 + BPA treatment showed a significant decrease ($p = 0.03$) of 3.5% when compared to the BPA treatment.

4.2.2.3 Cortisol

Figure 4.11 shows the cortisol concentration determined in the plasma of *S. aurata* exposed to BDE-99 and BPA concentrations, singly and combined.

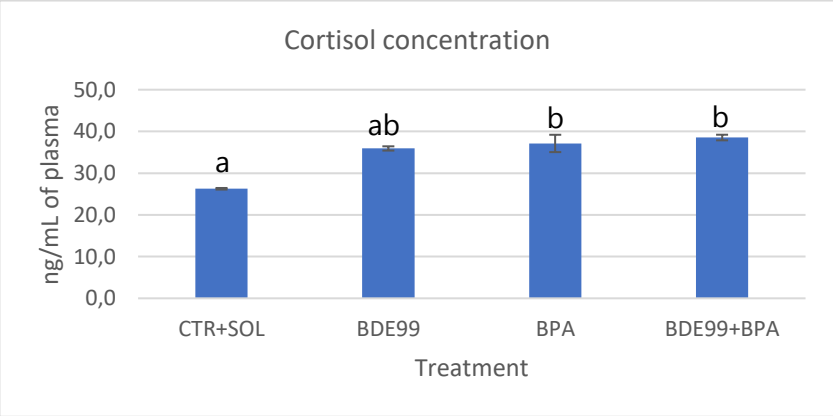

Figure 4.11. Cortisol concentration (mean ± SD; n=5) in S. aurata plasma exposed to BDE-99 and/or BPA, for 28 days. Different letters mean there are significant differences between treatments ($p < 0.05$).

Cortisol concentration determined in the plasma ranged between 38.5 ± 0.2 ng/mL (BDE-99 + BPA treatment) and 26.3 ± 0.5 ng/mL (control + solvent group).

BPA and BDE-99 + BPA treatments showed a significant increase ($p < 0.001$) when compared with the control + solvent group. Yet, BDE-99 showed no significant differences when in comparison to the control + solvent group. Additionally, no significant differences were observed among contaminated treatments.

4.2.3 Animal fitness indexes

The results from the Fulton's K index and the relationship between fish total weight and the respective spleen weight are shown in Table 4.1.

Table 4.1. Fulton's K index and the relationship between fish total weight and the respective spleen weight.

Date	Treatment	Average Weight (g)	STDEV W	Total length (cm)	STDEV TL	Spleen weight (g)	STDEV Spleen weight	K (g/cm)	Spleen: body weight ratio
T28	CTR	20,2	2,6	11,2	0,4	0,020	0,005	1,443 a	0,099 a
	CTR+SOL	21,8	1,2	11,6	0,1	0,031	0,008	1,401 a	0,145 a
	BDE 99	23,2	5,1	11,6	0,8	0,029	0,004	1,495 a	0,123 a
	BPA	23,1	4,4	11,7	0,8	0,039	0,009	1,436 a	0,169 a
	MIX	21,5	1,9	11,4	0,4	0,033	0,012	1,437 a	0,156 a

For both parameters analyzed (Fulton's K index and the spleen:body weight ratio), no significant differences were observed among all treatments.

Fulton's K index values varies between 1.495 g/cm, in the BDE-99 treatment, and 1.401 g/cm, in the control + solvent group. As for the spleen:body weight ratio, it varies between 0.156, in the BPA treatment, and 0.099, in the control group.

5

Discussion

The present research aimed at assessing the effects of BDE-99 and BPA, two endocrine disrupting chemicals, single and combined, in juvenile *Sparus aurata* during 28 days. For this purpose, different biomarkers of oxidative stress, endocrine disruption, and immunological responses were analyzed in fish plasma and spleen. Also, haematological parameters such as erythrocytes, leukocytes and ENAs were also performed in fish blood.

The haematological parameters showed that the presence of BDE-99 and BPA, single and combined, increased the erythrocytes and ENAs in blood cells, relatively to control animals. These results pointed the ability of these two chemicals to affect blood cells of fish at clastogenic levels, as other pollutants known, like Polycyclic Aromatic hydrocarbons (PAH), showing their potential to cause irreversible DNA damage (Martins *et al.*, 2016).

The increase of erythrocytes may indicate a response to oxidative stress, since blood antioxidant capacity is mainly in erythrocytes, as these cells neutralize ROS and protect themselves and the rest of tissues from oxidative damage (Alonso *et al.*, 2010). On contrary, the leukocytes decreased in fish exposed to the referred EDCs.

From a broader perspective, it was possible to observe that animals exposed to BDE-99 showed significant differences with control animals, in almost all biomarkers analyzed. This finding is aligned with the theoretical review that BDE-99 should be more toxic, considering that this pollutant has bromine atoms bonded to aromatic rings, which makes them more persistent in the environment and in organisms (Darnerud, 2008). Lu, Qi & Chen (2013) reported evidence of BDE-99 showing more adverse biological effects than its congener BDE-47, while testing the in vivo effects of BDE-47 and BDE-99 on the following biomarkers: acetylcholinesterase (AChE), ethoxyresorufin-O-deethylase (EROD), GST, SOD and CAT, in goldfish *Carassius auratus*.

To evaluate oxidative stress the following biomarkers were analyzed, in fish spleen: CAT, SOD, LPO and GST. Oxidative biomarkers were analyzed in fish spleen as this organ is mainly composed of blood held in sinuses that are involved in immune reactivity and blood cell formation (Raibeemol & Chitra, 2018).

It was expected to observe a significant increase in these biomarkers, in treatments exposed to BDE-99, BPA, and their mixture. CAT, SOD and GST activities are expected to increase when an organism is exposed to xenobiotics, since these enzymes play an important role on animals' antioxidant defense mechanisms (Ahmad *et al.*, 2010; Carillon *et al.*, 2013; Han *et al.*, 2013). Conversely, when an organism is exposed to severe and/or long-lasting stress condition, the antioxidant mechanisms become depleted or inhibited, failing to prevent lipoperoxidation damage (Gasparovic *et al.*, 2013).

In this study, CAT, SOD and GST showed a decrease of their activity upon exposure to BPA and BDE-99 and such inhibition resulted in increased LPO concentration in the BDE-99 treatment. Such trend clearly evidences that fish were severely affected by the exposure to these contaminants, resulting in oxidative stress and, ultimately, cell damage. In accordance with the present results, Akram et al. (2021) reported a decrease in different antioxidant enzymes, like CAT, SOD, glutathione peroxidase and glutathione reductase and an increase in LPO, in organisms exposed to bisphenol A. Other studies reported that BDE-99 induced GST and SOD activities in goldfish, however, CAT activity presented a dose-dependent decrease (Lu, Qi, & Chen, 2013; 2013; Xie, Lu, & Qi, 2014). To be noted that the referred studies were conducted on the liver and so the results obtained in this study may indicate that the spleen does not have antioxidant mechanisms as active as the liver. Zhao *et al.* (2018) presents evidence that LPO levels in the spleen of zebrafish increase with the increase of ROS production, which goes in line with the results of this study. This allows to conclude that excessive ROS production and the limited antioxidant response by fish induce LPO damage.

Regarding endocrine disruption parameters, the following biomarkers, were selected and tested in fish plasma: VTG, E_2 and 11-KT. According to theoretical literature, E_2 concentration is expected to increase in organisms exposed to estrogenic chemicals that act as estrogen agonists and mimic the effects of endogenous E_2 (Crain *et al.*, 2012). Recent reports have associated BPA with an estrogenic action (Cuesta *et al.*, 2007). Contrary to what was expected, the present results showed that E_2 concentration decreased in all tested treatments, especially in BPA treatment. These findings indicate that BPA did not lead to a feminizer effect, but rather seemed to have accelerated specimens' sexual differentiation towards masculinity. These results might be justified by the fact that it was selected a juvenile biological model, from a protandrous hermaphrodite species, that first matures as a male at around 2 years of age, and sexual maturation in females only occurs at 2-3 years of age (Arabaci *et al.*, 2010; Madeira, 2016; FAO, 2023). This means that the individuals tested could be male and the presence of pollutants only expedites male maturation.

Although, in general, males don't produce VTG, male hepatocytes do produce to some extent and release VTG into the plasma when exposed to estrogens (Crain *et al.*, 2012). Thus, VTG content in fish plasma is a biomarker for exposure to estrogenic compounds (Crain *et al.*, 2012). So, in the present study, it was expected that the exposure to BPA induced an increase in VTG content. Villeneuve *et al.* (2012) reported that VTG concentration increased in fathead minnows' plasma, exposed to BPA.

However, in the present study, VTG content showed a significant decrease when fish were exposed to BDE-99 and to the mixture containing BDE-99 and BPA, even though BDE-99 showed a more evident decrease. Again, this is consistent with the potential acceleration of fish masculinization process. In the future, confirming this hypothesis would require a comprehensive histological analysis of fish gonads.

Another justification for the results obtained could be the concentration of the contaminants tested, which might have been too low to elicit more evident effects. This research demonstrated negative feedback in response to E_2 concentration, since it was observed a decrease in the steroid concentration as a consequence of an increase of estrogenic levels (Villeneuve *et al.*, 2012).

On the other hand, 11-KT showed an increase in all tested treatments, especially, in BPA, once again corroborating the hypothesis that the sample was mainly constituted by males and that the presence of BPA expedited male maturation. In the case of 11-KT it was not evident a negative control, since exposure to BPA caused an Increase in this steroid concentration (Villeneuve *et al.*, 2012).

Immune responses were tested by analyzing peroxidase activity and antiprotease activity. It was expected to observe an increase in both biomarkers, since peroxidases are defense mechanisms of the immune systems when organisms are exposed to xenobiotics, acting in the elimination of H_2O_2 and maintenance of the redox balance of the immune system (Guardiola *et al.*, 2014). Protease inhibitors or antiproteases are also mechanisms of defense against bacterial and parasite infections in organisms, inhibiting pathogens replication (Guardiola *et al.*, 2014).

In this study, however, peroxidase activity showed a decrease in all treatments tested pointing out to a potential inhibition of fish immune responses upon exposure to BDE99 and BPA. In contrast, antiprotease activity showed no differences to being subjected to the contaminants tested. Hence, the controversial results obtained do not allow to draw conclusions with respect to the action of these two compounds in terms of fish immunity. The lack of literature on this matter does not enable comparisons of the present data. Still, the absence of a

clear effect in these physiological endpoints could be related with the temporal exposition or the tested concentration, which might have not been sufficient to develop effects. Data inconsistency may also be linked with lack of statistical power of the results, which in turn is strongly related with the number of Individuals tested in each treatment. In this study, the number of samples per treatment was 5 fish (n =5). This number could be insufficient to produce more robust and statistically accurate results. However, for ethical reasons, it was chosen not to use a higher number of samples.

The last biomarker tested was cortisol, a biomarker of psychological stress and related diseases (Hellhammer, Wüst & Kudielka, 2009). Therefore, it was expected an increase in cortisol concentration, as a result of the increased stress in the exposed organisms. The results of the study confirm this theoretical hypothesis since cortisol levels showed an increase in the plasma of organisms exposed to BPA and the mixture of BDE-99 and BPA.

Even though the biomarkers tested showed a reaction to the presence of the tested contaminants, such exposure did not lead to alterations in the animal fitness. Both Fulton's K index and the relationship between the fish total weight and the respective spleen weight did not show differences between the various treatments. This leads to conclude that, although there were alterations at a cellular and molecular levels, those changes did not translate in the individual level.

In a broader perspective, the BDE-99 contaminant induced more reaction than other tested contaminants, whether it was an increase or inhibition.

This research brings an innovative view regarding the ecotoxicity of two emerging contaminants for which data is still extremely limited. There are some referenced papers on flame retardants and plasticizers, such as BDE-99 and their congeners and BPA, respectively Lu, Qi & Chen (2013) and Villeneuve *et al.* (2012). However, in the case of PBDEs, these studies mainly approach the effects on the liver of fish species other than *Sparus aurata*.

6

CONCLUSIONS

From this research it is possible to conclude that the exposure to BDE-99, BPA and their mixture contributes to increase erythrocytes and erythrocytes nuclear abnormalities count in *S. aurata* plasma. Additionally, it was possible to conclude that these contaminants induced LPO in fish spleen after 28h of exposure, mainly due to the inefficacy of antioxidant mechanisms or reduced enzyme response by the fish spleen.

The studied contaminants also influenced the endocrine systems, however not the expected effect. Endocrine-disrupting biomarkers, in particular vitellogenin content and E_2, showed a decrease when exposure to these compounds occurred.

In this research no conclusive effects were detected in the immune responses as a result of the exposure to BDE-99 and BPA, single and combined.

In conclusion, it was possible to determine that BDE-99 had more influence in most of the biomarkers analyzed. Therefore, BDE-99 seems to be more reactive than BPA and showed a higher toxicity in this study.

In the present study, and others on the same topic, it is proven that BDE-99 and BPA have effects in fish oxidative stress and endocrine-disruption responses. Additionally, considering BDE-99 and BPA are ECs and their effects are only recently being studied, the present work recommended further research about the toxicity of these chemicals in fish. We suggest, a short-term exposure to understand the acute responses by the fish, and thus elucidate more about the mode of action of these emerging aquatic pollutants.

7

REFERENCES

Ahmad, P., Jaleel, C. A., Salem, M. A., Nabi, G., & Sharma, S. (2010). Roles of enzymatic and nonenzymatic antioxidants in plants during abiotic stress. Critical reviews in biotechnology, 30(3), 161-175.

Akram, R., Iqbal, R., Hussain, R., Jabeen, F., & Ali, M. (2021). Evaluation of oxidative stress, antioxidant enzymes and genotoxic potential of bisphenol A in fresh water bighead carp (Aristichthys nobils) fish at low concentrations. Environmental pollution, 268, 115896.

Albina, M. L., Alonso, V., Linares, V., Bellés, M., Sirvent, J. J., Domingo, J. L., & Sánchez, D. J. (2010). Effects of exposure to BDE-99 on oxidative status of liver and kidney in adult rats. Toxicology, 271(1-2), 51-56.

Alharbi, O. M., Khattab, R. A., & Ali, I. (2018). Health and environmental effects of persistent organic pollutants. Journal of Molecular Liquids, 263, 442-453.

Alonso, V., Linares, V., Bellés, M., Albina, M. L., Pujol, A., Domingo, J. L., & Sánchez, D. J. (2010). Effects of BDE-99 on hormone homeostasis and biochemical parameters in adult male rats. *Food and Chemical Toxicology*, 48(8-9), 2206-2211.

Anderson, S., Sadinski, W., Shugart, L., Brussard, P., Depledge, M., Ford, T., ... & Wogan, G. (1994). Genetic and molecular ecotoxicology: a research framework. Environmental Health Perspectives, 102(suppl 12), 3-8.

Anderson, J. (2021). What is the difference between pollutants and contaminants?. Safeopedia. Assessed in https://www.safeopedia.com/7/4111/physical-agents/what-is-the-difference-between-pollutants-and-contaminants on 25th of February of 2023.

Arabacı, M., Yılmaz, Y., Ceyhun, S. B., Erdoğan, O., Dorlay, H. G., Diler, I., ... & Konçagül, S. (2010). A review on population characteristics of Gilthead Seabream (Sparus auratd).

Arends, R. J., Mancera, J. M., Munoz, J. L., Bonga, S. W., & Flik, G. (1999). The stress response of the gilthead sea bream (Sparus aurata L.) to air exposure and confinement. Journal of endocrinology, 163(1), 149.

Ashraf, M. A. (2017). Persistent organic pollutants (POPs): a global issue, a global challenge. Environmental Science and Pollution Research, 24, 4223-4227.

Backhaus, T., & Faust, M. (2012). Predictive environmental risk assessment of chemical mixtures: a conceptual framework. Environmental science & technology, 46(5), 2564-2573.

Berg, V., Lyche, J. L., Karlsson, C., Stavik, B., Nourizadeh-Lillabadi, R., Hårdnes, N., ... & Ropstad, E. (2011). Accumulation and effects of natural mixtures of persistent organic pollutants (POP) in zebrafish after two generations of exposure. Journal of Toxicology and Environmental Health, Part A, 74(7-9), 407-423.

Birnbaum, L. S., & Staskal, D. F. (2004). Brominated flame retardants: cause for concern?. Environmental health perspectives, 112(1), 9-17.

Björklund, E., Svahn, O., Bak, S., Bekoe, S. O., & Hansen, M. (2016). Pharmaceutical residues affecting the UNESCO biosphere reserve Kristianstads Vattenrike wetlands: Sources and sinks. Archives of Environmental Contamination and Toxicology, 71(3), 423-436.

Borgia, V. F., Thatheyus, A. J., Murugesan, A. G., Alexander, S. C. P., & Geetha, I. (2018). Effects of effluent from electoplating industry on the immune response in the freshwater fish, Cyprinus carpio. Fish & shellfish immunology, 79, 86-92.

Carillon, J., Rouanet, J. M., Cristol, J. P., & Brion, R. (2013). Superoxide dismutase administration, a potential therapy against oxidative stress related diseases: several routes of supplementation and proposal of an original mechanism of action. Pharmaceutical research, 30, 2718-2728.

Chapman, P. M. (1995). Ecotoxicology and pollution—key issues. Marine Pollution Bulletin, 31(4-12), 167-177.

Chapman, P. M. (2007). Determining when contamination is pollution—weight of evidence determinations for sediments and effluents. Environment International, 33(4), 492-501.

Commission Implementing Decision (EU) 2020/1161 of 4 August 2020. Official Journal of European Union. Decisions. Assessed in https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32020D1161&from=EN on 5th of February of 2023.

Connell, D. W., Lam, P., Richardson, B., & Wu, R. (2009). Introduction to ecotoxicology. John Wiley & Sons.

Crain, D. A., Eriksen, M., Iguchi, T., Jobling, S., Laufer, H., LeBlanc, G. A., & Guillette Jr, L. J. (2007). An ecological assessment of bisphenol-A: evidence from comparative biology. *Reproductive toxicology, 24*(2), 225-239.

Cuesta, A., Vargas-Chacoff, L., García-López, A., Arjona, F. J., Martínez-Rodríguez, G., Meseguer, J., ... & Esteban, M. A. (2007). Effect of sex-steroid hormones, testosterone and estradiol, on humoral immune parameters of gilthead seabream. Fish & Shellfish Immunology, 23(3), 693-700.

Darnerud, P. O. (2008). Brominated flame retardants as possible endocrine disrupters. International journal of andrology, 31(2), 152-160.

Denslow, N. D., Chow, M. C., Kroll, K. J., & Green, L. (1999). Vitellogenin as a biomarker of exposure for estrogen or estrogen mimics. Ecotoxicology, 8(5), 385-398.

Diniz, M. S., Maurício, R., Petrovic, M., De Alda, M. J. L., Amaral, L., Peres, I., ... & Santana, F. (2010). Assessing the estrogenic potency in a Portuguese wastewater treatment plant using an integrated approach. Journal of Environmental Sciences, 22(10), 1613-1622.

Directive 2008/56/EC of the European Parliament and of the Council of 17 June 2008. Official Journal of European Union. Directives. Assessed in https://eur-lex.europa.eu/legal-content/EN/TXT/PDF/?uri=CELEX:32008L0056&from=EN on 5th of February of 2023.

Dong, Z., Liu, Y., Duan, L., Bekele, D., & Naidu, R. (2015). Uncertainties in human health risk assessment of environmental contaminants: a review and perspective. Environment international, 85, 120-132.

European Commission (n.d.). EU Marine Strategy Framework Directive. What it is, why it's needed, research contribution to achieving its goals. Assessed in https://research-and-innovation.ec.europa.eu/research-area/environment/oceans-and-seas/eu-marine-strategy-framework-directive_en on 15th of March of 2023.

European Commission (n.d.). Our Oceans, Seas and Coasts. Descriptor 8: Contaminants. Assessed in https://ec.europa.eu/environment/marine/good-environmental-status/descriptor-8/index_en.htm on 15th of March of 2023.

European Commission (n.d.). Our Oceans, Seas and Coasts. Descriptor 9: Contaminants in Seafood. Assessed in https://ec.europa.eu/environment/marine/good-environmental-status/descriptor-9/index_en.htm on 15th of March of 2023.

European Commission (n.d.). Our Oceans, Seas and Coasts. EU Coastal and Marine Policy. Assessed in https://ec.europa.eu/environment/marine/eu-coast-and-marine-policy/index_en.htm on 15th of March of 2023.

Eladak, S., Grisin, T., Moison, D., Guerquin, M. J., N'Tumba-Byn, T., Pozzi-Gaudin, S., ... & Habert, R. (2015). A new chapter in the bisphenol A story: bisphenol S and bisphenol F are not safe alternatives to this compound. Fertility and sterility, 103(1), 11-21.

© FAO 2023. Sparus aurata. Cultured Aquatic Species Information Programme. Text by Colloca, F.; Cerasi, S.. Fisheries and Aquaculture Division [online]. Rome. Updated 2005-05-17. Assessed in https://www.fao.org/fishery/en/culturedspecies/sparus_aurata/en on 13th March of 2023.

Fiedler, H., Kallenborn, R., De Boer, J., & Sydnes, L. K. (2019). The Stockholm convention: a tool for the global regulation of persistent organic pollutants. Chemistry International, 41(2), 4-11.

Gasparovic, A. C., Jaganjac, M., Mihaljevic, B., Sunjic, S. B., & Zarkovic, N. (2013). Assays for the measurement of lipid peroxidation. Cell Senescence: Methods and Protocols, 283-296.

Guardiola, F. A., Cuesta, A., Abellán, E., Meseguer, J., & Esteban, M. A. (2014). Comparative analysis of the humoral immunity of skin mucus from several marine teleost fish. Fish & shellfish immunology, 40(1), 24-31.

Guardiola, F. A., Cuesta, A., Arizcun, M., Meseguer, J., & Esteban, M. A. (2014). Comparative skin mucus and serum humoral defence mechanisms in the teleost gilthead seabream (Sparus aurata). Fish & shellfish immunology, 36(2), 545-551.

Han, J., Won, E. J., Hwang, D. S., Rhee, J. S., Kim, I. C., & Lee, J. S. (2013). Effect of copper exposure on GST activity and on the expression of four GSTs under oxidative stress condition in the monogonont rotifer, Brachionus koreanus. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 158(2), 91-100.

Hellhammer, D. H., Wüst, S., & Kudielka, B. M. (2009). Salivary cortisol as a biomarker in stress research. Psychoneuroendocrinology, 34(2), 163-171.

Islam, M. S., & Tanaka, M. (2004). Impacts of pollution on coastal and marine ecosystems including coastal and marine fisheries and approach for management: a review and synthesis. Marine pollution bulletin, 48(7-8), 624-649.

Johansson, L. H. & Håkan Borg, L. A. A spectrophotometric method for determination of catalase activity in small tissue samples. Anal. Biochem. 174, 331–336 (1988).

Jones, K. C. (2021). Persistent organic pollutants (POPs) and related chemicals in the global environment: some personal reflections. Environmental Science & Technology, 55(14), 9400-9412.

Jones, K. C., & De Voogt, P. (1999). Persistent organic pollutants (POPs): state of the science. Environmental pollution, 100(1-3), 209-221.

Leung, D., Abbenante, G., & Fairlie, D. P. (2000). Protease inhibitors: current status and future prospects. Journal of medicinal chemistry, 43(3), 305-341.

Liu, M., Jia, S., Dong, T., Han, Y., Xue, J., Wanjaya, E. R., & Fang, M. (2019). The occurrence of bisphenol plasticizers in paired dust and urine samples and its association with oxidative stress. Chemosphere, 216, 472-478.

Llamas, M., Vadillo-Pérez, I., Candela, L., Jiménez-Gavilán, P., Corada-Fernández, C., & Castro-Gámez, A. F. (2020). Screening and distribution of contaminants of emerging concern

and regulated organic pollutants in the heavily modified guadalhorce river basin, southern spain. Water, 12(11), 3012.

Lu, G. H., Qi, P. D., & Chen, W. (2013). Integrated biomarker responses of Carassius auratus exposed to BDE-47, BDE-99 and their mixtures.

Magnadottir, B. (2010). Immunological control of fish diseases. Marine biotechnology, 12, 361-379.

Marine Institute (2022). Marine Strategy Framework Directive. Assessed in https://www.marine.ie/site-area/areas-activity/marine-environment/marine-strategy-framework-directive on 15th of March of 2023.

Martins, M., Ferreira, A. M., Costa, M. H., & Costa, P. M. (2016). Comparing the genotoxicity of a potentially carcinogenic and a noncarcinogenic PAH, singly, and in binary combination, on peripheral blood cells of the E uropean sea bass. Environmental toxicology, 31(11), 1307-1318.

Miglioranza, K. S., Aizpún de Moreno, J. E., & Moreno, V. J. (2004). Land-based sources of marine pollution: Organochlorine pesticides in stream systems. Environmental Science and Pollution Research, 11(4), 227-232.

Mihara, M. & Uchiyama, M. Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Anal. Biochem. 86, 271–8 (1978).

Minh, N. H., Minh, T. B., Kajiwara, N., Kunisue, T., Subramanian, A., Iwata, H., ... & Tanabe, S. (2006). Contamination by persistent organic pollutants in dumping sites of Asian developing countries: implication of emerging pollution sources. Archives of Environmental Contamination and Toxicology, 50, 474-481.

Molina, A. M., Abril, N., Morales-Prieto, N., Monterde, J. G., Lora, A. J., Ayala, N., & Moyano, R. (2018). Evaluation of toxicological endpoints in female zebrafish after bisphenol A exposure. Food and Chemical Toxicology, 112, 19-25.

Mukhopadhyay, M., & Chakraborty, P. (2021). Plasticizers and bisphenol A: Emerging organic pollutants along the lower stretch of River Ganga, north-east coast of the Bay of Bengal. Environmental Pollution, 276, 116697.

Nilsen, B. M., Berg, K., Eidem, J. K., Kristiansen, S. I., Brion, F., Porcher, J. M., & Goksøyr, A. (2004). Development of quantitative vitellogenin-ELISAs for fish test species used in endocrine disruptor screening. Analytical and bioanalytical chemistry, 378, 621-633.

Palmer, C. P. (2017). Marine Biodiversity and Ecosystems Underpin a Healthy Planet and Social Well-Being. Nos. 1 & 2 Volume LIV, Our Ocean, Our World. Assessed in

https://www.un.org/en/chronicle/article/marine-biodiversity-and-ecosystems-underpin-healthy-planet-and-social-well-being on 26th of February of 2023.

Quade, M. J., & Roth, J. A. (1997). A rapid, direct assay to measure degranulation of bovine neutrophil primary granules. Veterinary immunology and immunopathology, 58(3-4), 239-248.

Raibeemol, K. P., & Chitra, K. C. (2018). Effects of chlorpyrifos as inducer for oxidative stress in liver, kidney and spleen of freshwater fish, Pseudetroplus maculatus (Bloch, 1795). *Res Rev: J Toxicol*, 8(1), 20-29.

Raldúa, D., Padrós, F., Solé, M., Eljarrat, E., Barceló, D., Riva, M. C., & Barata, C. (2008). First evidence of polybrominated diphenyl ether (flame retardants) effects in feral barbel from the Ebro River basin (NE, Spain). Chemosphere, 73(1), 56-64.

Rodriguez-Narvaez, O. M., Peralta-Hernandez, J. M., Goonetilleke, A., & Bandala, E. R. (2017). Treatment technologies for emerging contaminants in water: A review. Chemical Engineering Journal, 323, 361-380.

Souid, G., Souayed, N., Yaktiti, F., & Maaroufi, K. (2013). Effect of acute cadmium exposure on metal accumulation and oxidative stress biomarkers of Sparus aurata. Ecotoxicology and environmental safety, 89, 1-7.

Sousa, J. C., Ribeiro, A. R., Barbosa, M. O., Ribeiro, C., Tiritan, M. E., Pereira, M. F. R., & Silva, A. M. (2019). Monitoring of the 17 EU Watch List contaminants of emerging concern in the Ave and the Sousa Rivers. Science of the Total Environment, 649, 1083-1095.

Strunjak-Perovic, I., Topic Popovic, N., Coz-Rakovac, R., & Jadan, M. (2009). Nuclear abnormalities of marine fish erythrocytes. *Journal of fish biology*, 74(10), 2239-2249.

Sun, Y. I., Oberley, L. W., & Li, Y. (1988). A simple method for clinical assay of superoxide dismutase. Clinical chemistry, 34(3), 497-500.

Sun, C., & Zhang, S. (2015). Immune-relevant and antioxidant activities of vitellogenin and yolk proteins in fish. Nutrients, 7(10), 8818-8829.

Tagliaferri, S., Caglieri, A., Goldoni, M., Pinelli, S., Alinovi, R., Poli, D., ... & Costa, L. G. (2010). Low concentrations of the brominated flame retardants BDE-47 and BDE-99 induce synergistic oxidative stress-mediated neurotoxicity in human neuroblastoma cells. Toxicology in Vitro, 24(1), 116-122.

Taheran, M., Naghdi, M., Brar, S. K., Verma, M., & Surampalli, R. Y. (2018). Emerging contaminants: Here today, there tomorrow!. Environmental Nanotechnology, Monitoring & Management, 10, 122-126.

Uchiyama, M., & Mihara, M. (1978). Determination of malonaldehyde precursor in tissues by thiobarbituric acid test. Analytical biochemistry, 86(1), 271-278.

UN environment programme. (2016). The marine environment is an essential component of the global life-support system. Assessed in https://www.unep.org/news-and-stories/story/marine-environment-essential-component-global-life-support-system on 26th February of 2023.

Vandenberg, L. N., Hauser, R., Marcus, M., Olea, N., & Welshons, W. V. (2007). Human exposure to bisphenol A (BPA). Reproductive toxicology, 24(2), 139-177.

Vandermeersch, G., Lourenço, H. M., Alvarez-Muñoz, D., Cunha, S., Diogène, J., Cano-Sancho, G., ... & Robbens, J. (2015). Environmental contaminants of emerging concern in seafood–European database on contaminant levels. Environmental Research, 143, 29-45.

Vasseur, P., & Cossu-Leguille, C. (2003). Biomarkers and community indices as complementary tools for environmental safety. Environment International, 28(8), 711-717.

Villeneuve, D. L., Garcia-Reyero, N., Escalon, B. L., Jensen, K. M., Cavallin, J. E., Makynen, E. A., ... & Ankley, G. T. (2012). Ecotoxicogenomics to support ecological risk assessment: a case study with bisphenol A in fish. *Environmental science & technology, 46*(1), 51-59.

Xie, Z., Lu, G., & Qi, P. (2014). Effects of BDE-209 and its mixtures with BDE-47 and BDE-99 on multiple biomarkers in Carassius auratus. *Environmental Toxicology and Pharmacology, 38*(2), 554-561.

Yang, J., Zhao, H., & Chan, K. M. (2017). Toxic effects of polybrominated diphenyl ethers (BDE 47 and 99) and localization of BDE-99–induced cyp1a mRNA in zebrafish larvae. Toxicology reports, 4, 614-624.

Zhao, S. J., Guo, S. N., Zhu, Q. L., Yuan, S. S., & Zheng, J. L. (2018). Heat-induced oxidative stress and inflammation involve in cadmium pollution history in the spleen of zebrafish. *Fish & Shellfish Immunology*, 72, 1-8.

A

ANNEXES

A.1 P-value between the control and control + solvent of parameters and biomarkers analyzed.

Parameter or Biomarker	p-value
Erythrocytes	0.16
Leukocytes	0.16
ENAS	0.5
CAT	0.4
SOD	0.357677
LPO	0.26
GST	0.9
Vitellogenin	0.796
17β-estradiol	0.49
11-ketotestosterone	0.99
Peroxidase activity	1
Antiprotease activity	0.98858
Cortisol	1

A.2 Haematological parameters.

Sample		Total number of cells	Average	Total number of erythrocytes	Average	Total number of leukocytes	Average	Identification of leukocytes	Total number of thrombocytes	Number of ENAS	Average	% erythrocytes	% leukocytes	% ENAS
CTR	1	325	325	311	311	0	0	-	0	14	14	96%	0%	4%
	1	325		311		0		-	0	14				
	2	392	392,5	366	366,5	2	2	2 lymphocytes	0	24	24	93%	1%	6%
	2	393		367		2		2 lymphocytes	0	24				
	3	359	358,5	340	340,5	5	5	5 lymphocytes	0	14	13	95%	1%	4%
	3	358		341		5		5 lymphocytes	0	12				
	4	342	342	336	336	4	4	4 lymphocytes	0	2	2	98%	1%	1%
	4	342		336		4		4 lymphocytes	0	2				
	5	349	350	320	320,5	10	10	10 lymphocytes	0	19	19,5	92%	3%	6%
	5	351		321		10		10 lymphocytes	0	20				
CTR+SOL	1	395	393,5	319	321,5	2	2	2 lymphocytes	0	74	70	82%	1%	18%
	1	392		324		2		2 lymphocytes	0	66				
	2	174	173	143	141,5	0	0	-	0	31	31,5	82%	0%	18%
	2	172		140		0		-	0	32				
	3	212	211	191	189,5	11	11	11 lymphocytes	0	10	10,5	90%	5%	5%
	3	210		188		11		11 lymphocytes	0	11				
	4	523	520	377	375,5	5	5	5 lymphocytes	0	141	139,5	72%	1%	27%
	4	517		374		5		5 lymphocytes	0	138				
	5	282	282	271	271	2	2	2 lymphocytes	0	9	9	96%	1%	3%
	5	282		271		2		2 lymphocytes	0	9				
BDE99	1	445	441	430	426	7	7	7 lymphocytes	0	8	8	97%	2%	2%
	1	437		422		7		7 lymphocytes	0	8				
	2	331	330	315	315	5	5	5 lymphocytes	0	11	10	95%	2%	3%
	2	329		315		5		5 lymphocytes	0	9				
	3	309	308,5	300	299,5	2	2	2 lymphocytes	0	7	7	97%	1%	2%
	3	308		299		2		2 lymphocytes	0	7				
	4	369	367	353	351,5	5	5	5 lymphocytes	0	11	10,5	96%	1%	3%
	4	365		350		5		5 lymphocytes	0	10				
	5	359	357	351	349	5	5	5 lymphocytes	0	3	3	98%	1%	1%
	5	355		347		5		5 lymphocytes	0	3				
BPA	1	391	388,5	373	371	13	12,5	13 lymphocytes	0	5	5	95%	3%	1%
	1	386		369		12		12 lymphocytes	0	5				
	2	237	237	234	234	1	1	1 lymphocyte	0	2	2	98,7%	0,4%	0,8%
	2	237		234		1		1 lymphocyte	0	2				
	3	362	361,5	349	348,5	8	8	8 lymphocytes	0	5	5	96,4%	2,2%	1,4%
	3	361		348		8		8 lymphocytes	0	5				
	4	365	363	348	347	2	2	2 lymphocytes	0	15	14	95,6%	0,6%	3,9%
	4	361		346		2		2 lymphocytes	0	13				
	5	317	315,5	308	307,5	0	0	-	0	9	8	97%	0%	3%
	5	314		307		0		-	0	7				
BDE99+BPA	1	267	268,5	260	261,5	0	0	-	0	7	7	97%	0%	3%
	1	270		263		0		-	0	7				
	2	343	342,5	331	331	0	0	-	0	12	11,5	97%	0%	3%
	2	342		331		0		-	0	11				
	3	340	339	327	326	9	9	9 lymphocytes	0	4	4	96%	3%	1%
	3	338		325		9		9 lymphocytes	0	4				
	4	334	334,5	326	326,5	2	2	2 lymphocytes	0	6	6	98%	1%	2%
	4	335		327		2		2 lymphocytes	0	6				
	5	309	308	300	299,5	0	0	-	0	9	8,5	97%	0%	3%
	5	307		299		0		-	0	8				