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JOEL MARTINS LOPES

IMPACTS OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS MIXTURES IN MARINE BIVALVES

IMPACTOS DAS MISTURAS DE FARMACEUTICOS E PRODUTOS DE HIGIENE PESSOAL EM BIVALVES MARINHOS

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Eco-toxicologia e Análise de Risco, realizada sob a orientação científica do Professora Doutora Rosa de Fátima Lopes de Freitas, Professor Auxiliar do Departamento de Biologia da Universidade de Aveiro e do Professor Associado Gianluca Polese do Departamento de Biologia da Universidade de Nápoles “Frederico II”

o júri

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palavras-chave

estresse oxidativo, metabolismo, alterações histopatológicas; alterações comportamentais, contaminantes emergentes.

resumo

A mistura de contaminantes frequentemente determina as respostas biológicas das espécies marinhas, dificultando a interpretação dos dados toxicológicos. Entre esses contaminantes estão produtos farmacêuticos e de cuidados pessoais (PCPs), como 17 α-etinilestradiol (EE2) e Lauril Sulfato de Sódio (SLS). Devido à sua grande produção e uso diário pela população humana, eles acabarão entrando no meio marinho, principalmente por meio de águas residuais. Uma vez no ambiente marinho, EE2 e SLS podem interagir com organismos e induzir efeitos tóxicos em bivalves. Junto com a contaminação, os organismos também estão expostos às mudanças climáticas, responsáveis pelo aumento gradual da temperatura dos oceanos, que pode causar danos fisiológicos e bioquímicos aos organismos aquáticos, além de aumentar a sensibilidade dos organismos aos poluentes. Além disso, já foi relatado que o aquecimento pode alterar as propriedades e a toxicidade dos poluentes. O presente estudo avaliou os efeitos de EE2 em condições de aquecimento e EE2 e SLS em *Mytilus galloprovincialis* em temperatura de controlo. Para isso, os mexilhões foram distribuídos em duas salas climáticas para manter os organismos em duas temperaturas diferentes: 17 ± 1 e 21 ± 1 °C. As concentrações testadas usadas se assemelham a locais de baixa a altamente poluídos para a primeira experiência com EE2 a 17 e 21 °C foram 5.0; 25.0; 125.0 e 625.0 ng/L, na segunda experiência 125.0 ng/L para EE2, 4.0 mg/L para SLS e a mistura de ambas as substâncias (EE2 + SLS) na temperatura de controle. A exposição durou 28 dias e no final foram avaliadas, concentrações nos tecidos do mexilhão, alterações histopatológicas, análises bioquímicas, comportamentais e quantitativas de RT-PCR. Os resultados obtidos mostraram claramente quando os organismos são expostos ao EE2 em duas temperaturas diferentes, impactos tóxicos mais elevados são revelados sob condições de aquecimento em comparação com os mexilhões expostos à temperatura de controlo, sugerindo que o aumento da temperatura pode aumentar significativamente a sensibilidade dos bivalves, indicando um efeito interativo induzido pela combinação de EE2 e temperatura. Isso é demonstrado pelas alterações histopatológicas, inibição da superoxide dismutase (SOD), maiores níveis de peroxidação lipídica (LPO), maior capacidade metabólica e diminuição da atividade da acetilcolinesterase (AChE) na presença de EE2 a 21 ° C em comparação com mexilhões expostos à temperatura de controlo. Além disso, demonstra, impactos de EE2 e SLS, agindo isoladamente ou combinados, para o bivalve *M. galloprovincialis*. Os resultados mostraram que ambos os contaminantes representam uma ameaça para os mexilhões. O EE2 é mais prejudicial ao sexo feminino e o SLS ao masculino, causando alterações no comportamento, capacidade metabólica e estado oxidativo. No entanto, não foi evidente um efeito sinérgico quando os dois contaminantes atuaram juntos. Além disso, em relação às alterações histopatológicas nas brânquias de bivalves com ambos, os contaminantes induziram impactos negativos nos mexilhões, corroborando a análise de qPCR que mostrou resposta clara à exposição aos contaminantes. Os resultados obtidos evidenciam a capacidade nociva de ambos os contaminantes, mas são necessários mais estudos nesta matéria, nomeadamente considerando diferentes cenários de alterações climáticas.

keywords oxidative stress, metabolism. histopathological alterations; behavioural alterations, emerging contaminants.

abstract

Mixture of contaminants often determine biological responses of marine species, making difficult the interpretation of toxicological data. Among these contaminants are pharmaceuticals and personal care products (PCPs) such as 17 α-ethinylestradiol (EE2) and Sodium Lauryl Sulfate (SLS). Due to their large production and everyday use by the human population, they will eventually enter the marine environment, manly through wastewater. Once in the marine environment EE2 and SLS may interact with organisms and induce toxic effects, in bivalves. Beside contamination, organisms are also exposed to climate change, responsible for a gradual increase in the ocean temperature, which can cause physiological and biochemical impairments in aquatic organisms as well as increased the sensibility of organisms to pollutants. Furthermore, it is already reported that warming may change the properties and toxicity of pollutants. The present study evaluated the effects of EE2 under warming conditions and EE2 and SLS in *Mytilus galloprovincialis* at control temperature. For this, mussels were distributed into two climatic rooms to maintain organisms at two different temperatures: 17 ± 1 and 21 ± 1 °C. The tested concentrations used resemble low to highly polluted sites for the first experiment with EE2 at 17 and 21 °C were 5.0; 25.0; 125.0 and 625.0 ng/L, at the second experiment 125.0 ng/L for EE2, 4.0 mg/L for SLS and the mixture of both substances (EE2+SLS) at control temperature. The exposure lasted 28 days and at the end, concentrations in mussel's tissues, histopathological alterations, biochemical, behavioural and quantitative RT-PCR analyses were evaluated. Results obtained clearly showed when organisms are exposed to EE2 at two different temperatures, higher toxic impacts are revealed under warming conditions in comparison to mussels exposed control temperature, suggesting that temperature rise may significantly increase the sensitivity of bivalves indicating an interactive effect induced by the combination of EE2 and temperature. This is demonstrated by the histopathological alterations, inhibition of superoxide dismutase (SOD), greater lipid peroxidation (LPO) levels, higher metabolic capacity and decreased acetylcholinesterase (AChE) activity in the presence of EE2 at 21 °C compared to mussels exposed to control temperature. Furthermore demonstrates, impacts of EE2 and SLS, acting alone or combined, to the bivalve *M. galloprovincialis*. Results showed that both contaminants represent a threat to mussels. EE2 is more harmful to females and SLS to males, causing alterations on their behaviour, metabolic capacity, and oxidative status. Nevertheless, it was not evident a synergistic effect when both contaminants were acting together. Moreover, regarding histopathological alterations in bivalve's gills with both contaminants induced negative impacts in mussels, corroborating qPCR analysis that showed clear response to contaminants exposure. The results obtained highlight the harmful capacity of both contaminants but further research on this matter is needed, namely considering different climate change scenarios.

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Chapter 1

Introduction

1. INTRODUCTION

1.1 Marine coastal systems: major stressors

Among the most productive ecosystems in the world are marine coastal systems (Dayton et al., 2005). These dynamic areas are usually defined as the interface between land and sea being constantly subjected to natural and anthropogenic changes (FAO, 1998). Estuaries and lagoons are coastal water bodies that connect terrestrial to aquatic systems and for this reason they are the ultimate sink of contaminants (Islam and Tanaka, 2004; Dauvin and Ruellet, 2009). In fact, these ecosystems have been exposed to a variety of contaminants, such as personal care products (PCPs) (Cizmas et al., 2015; Wang et al., 2019; Wang et al., 2021) or pharmaceuticals (Desbiolles et al., 2018; Fernández-Rubio et al., 2019). Climate change associated phenomena are also considered major threats to coastal waters bodies, and it is expected that these coastal systems and the inhabiting organisms will become increasingly challenged under the eminence of environmental changes (e.g., IPCC, 2019). Some of the most prevalent climate change related factors that can affect aquatic organisms include seawater acidification, salinity changes and temperature rise (Ani and Robson, 2021; Freitas et al., 2021). Studies suggested that the increase of temperature and the mean sea level, with the resulting salinization of transitional environments, may be the factors that most affect these ecosystems (Breda et al., 2018; Naumann et al., 2018; Mackie et al., 2020). Especially, calcareous species (e.g., mussels, oysters, corals) are particularly vulnerable to climate change as CO_2 interferes with ocean pH and the saturation level of aragonite, influencing formation of their calcium carbonate ($CaCO_3$) structures (Fabry et al., 2008; Ries et al., 2009). Though it is less understood, climate change also affects various physiological processes in marine invertebrates, such as acid-base regulation, metabolism and aerobic scope, as well as sensory abilities, reproduction and development (Fabbri and Franzellitti, 2016 ;Moreira et al., 2018; Costa et al., 2020). These effects can lead to changes in population dynamics such as growth, survival, and fecundity, and ultimately affect marine ecosystem biodiversity (Kroeker et al., 2013). Among the

most important climate change related factors that may affect marine species is temperature rise (Ko et al., 2014; Thiyagarajan and Ko, 2012). The increase of anthropogenic greenhouse gas emissions (e.g. CO_2) have proven to lead to unprecedented oscillations in temperature regimes in several marine ecosystems, especially in areas where exchange of water is limited (e.g., coastal lagoons, estuaries) (IPCC, 2019). In particular, projections for temperature rise by the end of the 21st century indicate an increase that may reach 4 ºC (IPCC, 2018). Several studies already demonstrated that warming affects feeding, growth and respiration in bivalves (Mackenzie et al., 2014; Pörtner and Farrell, 2008; Bramwell et al., 2021; Ani and Robson, 2021). Besides the direct biological effects of temperature rise, increased temperature may also change organism's responses when exposed to stressors, namely pollutants, through alterations in biochemical processes (Banni et al., 2014; Nardi et al., 2017; Coppola et al., 2018). Indeed, histological alterations have been recorded in mussel's gills, gonads and digestive tubules (Cuevas et al., 2015; Pinto et al., 2019; Leite et al., 2020), since these organs are in direct contact with contaminants present in water during mussel's filtration and respiration, being considered sensitive organs to changes in water quality (Au, 2004; Rajalakshmi and Mohandas, 2005; Marigómez et al., 2013).

1.1.1. Emerging Contaminants

The exponential population growth from 1 billion in 1800 to 7.9 billion in 2020 and the development of the chemical, agrochemical, cosmetic and pharmaceutical industries has led to the increased manufacture and usage of numerous compounds (Starling et al., 2019; Jensen and Creinin, 2020). Part of these compounds are Emerging contaminants (ECs), which are a heterogeneous group of compounds that include agricultural products, pharmaceuticals, personal care products (PCPs) or household cleaning products, endocrine disrupting chemicals (EDCs), among others (Martín-Pozo et al., 2019).

Globally, thousands of different PCPs are produced per year, and the release in the environment remains an unavoidable by-product of a modernized lifestyle (Caldwell et al. 2012; Wang et al., 2021; Xiang et al., 2021). The precise amount introduced into aquatic systems is not well characterized (Schwarzenbach et al. 2006). Nevertheless, a multitude of studies have identified trace amounts of PCP-related compounds in aquatic ecosystems, waste or drinking water (Cizmas et al., 2015; Parida et al., 2021). These substances have been increasingly more monitored as they are closely related to human daily life and wide use (Wang et al., 2021; Wilkinson et al., 2017; Xiang et al., 2021). Several PCPs cannot be completely absorbed and used by humans and are excreted as metabolites, but a large amount of wastewater containing PCPs is emitted from manufacturing sites and hospitals (Kovalova et al., 2013; Patel et al., 2019). In addition, residues of veterinary facilities are discharged directly into the ecosystems.

Given the unique chemical properties, diversity and low concentrations, various PCPs cannot be completely eradicated in wastewater treatment plants (Ebele et al., 2017; Wang et al., 2021). The flow of PCPs in the environment is presented in Figure 1.

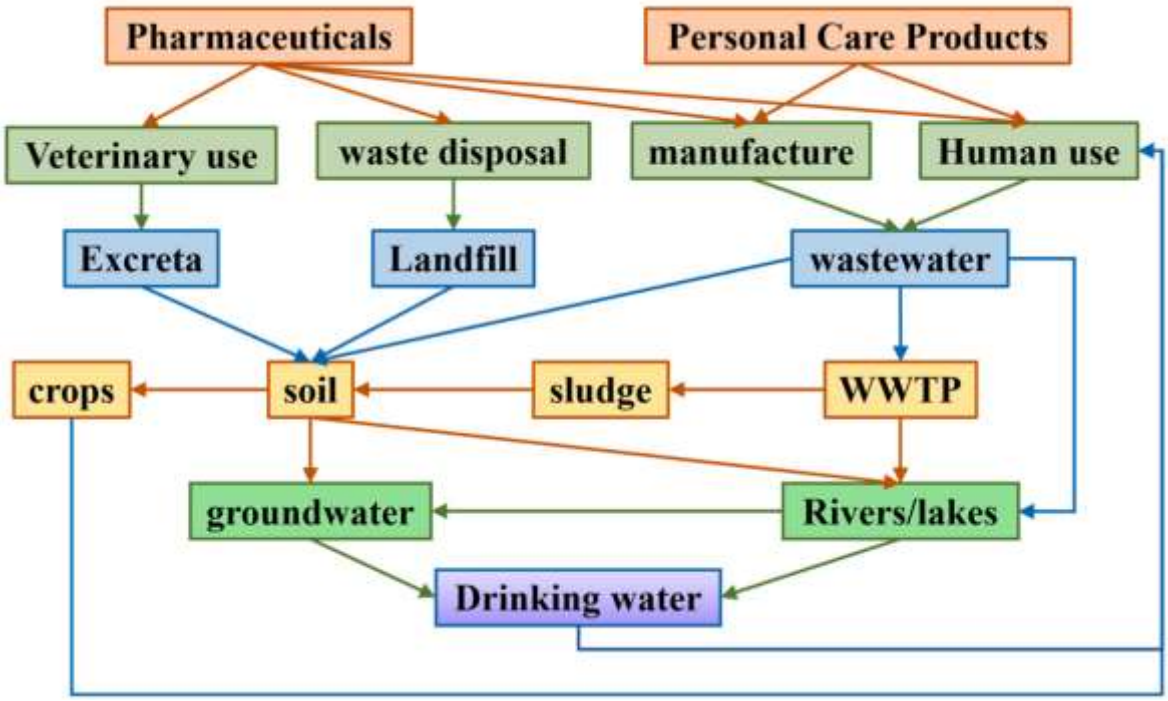

Figure 1. Schematic representation of pharmaceuticals and personal care products (PCPs) flowing in the environment (Xiang et al., 2021).

Although PCPs are traced to a ng/L to µg/L level in the environment, they may cause serious ecotoxicological problems and present extraordinary risks to ecosystems or organisms (Brausch and Rand, 2011; Boxall et al., 2012). Many PCPs can persist in water and may bioaccumulate in organisms, leading to endocrine disorders, drug resistance, primary productivity inhibition, accelerate ecosystem imbalance, and cause irreversible impacts on ecosystems and humans (Chen et al., 2017; Czarny et al., 2019; Wilkinson et al., 2017), due to their specific characteristics (e.g., optical activity, polarity, and semi volatility) (Monteiro and Boxall, 2010; Xiang et al., 2021).

Sodium Lauryl Sulfate (SLS), also known as Sodium Dodecyl Sulfate (SDS) is identified as one of the most used PCPs (Chaturvedi and Kumar, 2010). It is an anionic surfactant used as a cleaning agent in household detergents such as dishwashing soaps; in many personal care products and cosmetics, including toothpaste, shampoos, hand soap, facial cleanser, shower gel, and shaving creams and foams (Chaturvedi and Kumar, 2010). The concentration of SLS found

in these products is very variable, and SLS may be subject to biodegradation in a percentage ranging from 45% to 95% within 24 h (Brunelli et al., 2008). Nevertheless, the massive use of both domestic and industrial products containing SLS and its consequent continuous introduction into the environment keeps the concentration of this xenobiotic high (Brunelli et al., 2008), with values varying from 0.48 to 1.80 mg/L in coastal systems, such as the Bay of Cádiz (Quiroga et al., 1989). Anionic surfactants, like SLS, can bind to the bioactive macromolecules such as proteins, peptides, DNA and lipid component of the cellular membranes, with possible alterations in their structures, even cell lysis (Cserháti et al., 2002; Fatma et al., 2015). Recently, studies conducted by Freitas et al. (2020a) and Messina et al. (2014) observed that SLS (0.1 - 4.0 mg/L) induced increase of antioxidant alterations, osmotic stress, Hsp70 levels and cellular damage in *M. galloprovincialis* exposed to SLS. Also, the SLS inhibited the filtration rate activity as showed by Ostroumov (2003, 2005) in mussels *M. edulis* and in oysters *Crassostrea gigas* especially if associated to a cationic surfactant, the tetradecyltrimethylammonium bromide. Moreover, SLS caused histopathological and histochemical changes in the digestive gland of *Crassostrea angulata* and *M. galloprovincialis* (Rosety et al., 2000).

It is well-established that aquatic wildlife in marine and freshwater ecosystems it is also exposed to natural and synthetic EDCs which can interfere with the hormonal system, thus possibly causing adverse effects on the biology of organisms (Pojana et al., 2007; Schlumpf et al., 2004; Ismail et al., 2017). Studies showed ample evidence that exposure to these chemicals can lead to unusual alterations or disruption of development and reproduction in aquatic species (Smolarz et al., 2017; Aris et al., 2014; Chen et al., 2017). The mechanisms involve agonist or antagonist action for the receptors, interference of metabolic pathways and other physical actions (Annamalai and Namasivayam, 2015). Ultimately turning on, shutting off or modified hormonal signals occur, leading to decreased fertility, increased chance of birth defects, altered sexual expression, and some types of cancers (Caldwell et al., 2012; Costa et al., 2010). Some of the most recorded EDCs are steroidal estrogens, currently the world human discharges approximately 30,000 kg/yr of natural estrogens (estrone (E1), 17β-

estradiol (E2) and estriol (E3)) and an additional 700 kg/yr of synthetic estrogens (17-α Ethynylestradiol (EE2)) exclusively from birth control pills (Adeel et al., 2017; Stanczyk et al., 2013). Nevertheless, the release of estrogens to the environment from livestock is much higher, equal to the global quantity of 18,270 t, in which, humans contributed for 17.0% of global steroid emissions, while animals contributed for the remaining 83.0% (Zhang et al., 2021).

The estrogenic compound 17 α-Ethynylestradiol (also known as EE2), a synthetic hormone (Almeida et al., 2020; Ciocan et al., 2010), it is most commonly found in combined oral contraceptives (Stanczyk et al., 2013) but it is also used as medication for osteoporosis, palliative treatments for prostatic cancer and breast cancer in postmenopausal women, physiological therapy in deficiency states, menopausal and postmenopausal syndrome, among other disorders (Aris et al., 2014; Ying et al., 2002). Annually 700 kg are discharged in aquatic environments in respect to its use in oral contraceptives only, although livestock activities could lead to discharge values close to 80,000 kg/year (Adeel et al., 2017). This organic compound exhibits properties such as non-polarity, hydrophobicity with low volatility and is resistant to biodegradation (Silva et al., 2012). EE2 has relatively low solubility in water (4.8-11.3 mg/L at 27 °C) and has a relatively high octanol-water partitioning coefficient (Kow = 3.67 - 4.2) which makes it persistent and preferentially attached to the organic matter in the aquatic environment (Auriol et al., 2006; Snyder et al., 2008). Laboratory experiments revealed that sediments can behave as a sink for EE2 in marine, estuarine and riverine environments, due to its hydrophobic properties, with concentrations up to a thousand times greater in sediments than in the surrounding water column (Lai et al., 2000). However, in the field, levels of EE2 in sediments may range from 23 ng/g in rivers to 41 ng/g in estuaries and coastal lagoons; while concentrations up to 4 and 98 ng/L were found in water from rivers and estuaries, respectively (Almeida et al., 2020; Aris et al., 2014; Labadie and Hill, 2007; Ribeiro et al., 2009). In aquatic organisms recorded EE2 concentrations ranged between 3 - 38 ng/g in *M. galloprovincialis* at the Venice Lagoon and reached 310 ng/g in *M. trossulus* at the the Gulf of Gdańsk (Almeida et al., 2020; Pojana et al., 2007; Caban et al., 2016). Collected information regarding the predicted no-effect concentration (PNEC) of EE2 for

aquatic wildlife is scarce, however, some authors have pointed values between 0.1 and 0.5 ng/L (Almeida et al., 2020; Laurenson et al., 2014; Caldwell et al., 2012). Furthermore, very limited information is available on the impacts of this hormone in bivalves, with recent studies showing effects on reproductive system in the mussels *Elliptio complanata* and *M. trossulus* and throughout several metabolic pathways, related to energy acquisition (Almeida et al., 2020).

1.1.2. Climate changes: warming

The substantial growth of population and industrialization has been identified as the main driver for climate change, with coastal areas among the most vulnerable marine ecosystems, and it is therefore expected that species found in these habitats are increasingly under threat from these climate changes (Islam and Tanaka, 2004; Pauly et al., 2005; Todd et al., 2019). The ocean plays a crucial role, namely in the supply of food, the hydrological cycle, renewable energy, cultural values, trade and transport, with approximately 40% of the world's population living in a distance of 100 km to the coast (Perry, 2015; D'Odorico et al., 2019; Chakraborty et al., 2020). More than 600 million people currently live-in coastal areas, a number that is expected to exceed 1 billion by 2050 (Boretti and Rosa, 2019; Mackie et al., 2020). It is also in this region that transitional environments such as estuaries are included. Due to their location and characteristics, these areas are especially vulnerable to climate change, particularly regarding changes induced in the structure and function of ecosystem and loss of biodiversity (Breda et al., 2018; Moschino et al., 2012).

Several studies have demonstrated that seawater temperature is rising in oceans and especially in marine coastal systems (Bindoff et al., 2007; Fogarty et al., 2008; Levitus et al., 2009). In particular, a study conducted by Collins et al. (2013) predicted that global ocean warming will increase between 0.5 °C (RCP2.6) and 1.5 °C (RCP8.5) a depth of about 1 km by the end of the century due to the human activity. Nevertheless, lagoons and estuaries are more susceptible to temperature influence, because of their limited heat exchange with open waters

(Newton et al., 2018; Pan and Wang, 2011). Temperature fluctuations are responsible for alterations in geographic distribution of marine invertebrates, affecting larval development and dispersion, with consequences in the establishment and expansion of their life cycle (Marinho et al., 2016; Verdelhos et al., 2015). Higher frequency and duration of extreme warming events may also be responsible for changes in the structure and function of coastal communities, specifically regarding the decline of total abundance and species richness, impacting the food chain (Grilo et al., 2011; Holbrook et al., 2019). At an individual level, studies already showed that the increase of seawater temperature is responsible for metabolic, physiological and biochemical alterations in marine and estuarine invertebrate species with implications namely in growth rates and reproduction (Mackenzie et al., 2014; Velez et al., 2017). Recent studies also show that the increase of the average temperature in transitional systems and the greater exposure to periods of dissection due to prolonged exposure (usually in drought events) are accountable for physiological changes in organism that live in intertidal zones organisms, causing greater accumulation of pollutants (Andrade et al., 2019). The increase in temperature may also be associated with changes in the behaviour and toxicity of pollutants, as well as with a greater sensitivity of organisms to different substances, and there are already studies showing synergistic effects in organisms simultaneously exposed to pollutants and increased temperature (Andrade et al., 2019; Freitas et al., 2019; Pirone et al., 2019). Warming and the quantity of organic matter are correlated with changes in the behaviour and bioavailability of contaminants, changing toxicity patterns of organic and inorganic substances (Maulvault et al., 2017; Nardi et al., 2017; Pirone et al., 2019). Organisms' responses may also be altered when exposed to contaminants, as a result of alterations in biochemical and physiological processes as well as altering bioavailability and toxicity of compounds (Banni et al., 2014; Izagirre et al., 2014; Nardi et al., 2017). Examples of recent studies on the effects of multiple stressors include the assessment of the effects of pharmaceutical (e.g., carbamazepine, cetirizine, diclofenac) under different climate change scenarios (acidification, temperature rise, salinity alterations) (Almeida et al., 2018; Freitas et al., 2016, 2019a, 2019b; Maynou et al., 2021). Since climate change and pollution

act in tandem, it is important to understand how temperature may alter the behaviour and effects induced by pollutants.

1.2. *Mytilus galloprovincialis* as bioindicator species

Among the organisms inhabiting coastal systems, bivalves are commonly used in ecotoxicological studies as bioindicator species to evaluate and monitor substances contamination levels and impacts due to their bioaccumulation capacity, ecological role, wide distribution and high abundance in several aquatic systems as well as for their huge economic value (Ahmad et al., 2011; Velez et al., 2015). A biological indicator is an organism (or a community of organisms) which represents the impact of environmental pollutants on an ecosystem, community or habitat (Markert et al., 2003). A good bioindicator must demonstrate wide geographical distribution, display a sessile lifestyle or a restricted territory, be easily collected and be well documented in biochemical, physiological and biological terms (Lower and Kendall, 1990). Mussels are commonly used as bioindicators of pollution in coastal environments and, in fact, mussels have wide distribution, dominate coastal and estuarine communities, accumulate and respond to many pollutants, do not show a prolonged handling stress, and many characteristics of their biology and responses to intrinsic and extrinsic factors are well known (Livingstone et al., 1989). *M. galloprovincialis* (Lamarck, 1819) (Figure 2), also known as Mediterranean mussel, is widely distributed from warm temperate to subpolar areas, it is present in intertidal zones to 40 m deep attached to rocks and piers, within sheltered harbours, estuaries and on rocky shores (FAO, 2021). The species is native to Mediterranean coasts, but has successfully colonized several regions around the globe, such as South Africa, Hong Kong, Japan, Australia, Hawaii, California, and the west coast of Canada, and it is established as an invasive species (Branch and Steffani, 2004). This species plays a significant ecologic and economic role in marine ecosystems, being commonly used as bioindicator (Viarengo et al., 2007; Banni et al., 2014). This species is also used in biomonitoring programs to evaluate the distributions and biological

effects of pollutants (Catsiki and Florou, 2006; Benali et al., 2017; Smolarz et al., 2017).

Figure 2. Mytilus galloprovincialis (FAO, 2021).

1.3. Objectives

In most aquatic environments, especially coastal systems, both pollution and climate change related factors, as warming, act in combination, and so it is important to understand how temperature may change the effects induced by EE2 and SLS as well as the sensitivity of *M. galloprovincialis* to these pollutants. Therefore, the present study aimed to evaluate: i) the influence of temperature on the impacts caused by different concentrations EE2 in *M. galloprovincialis*, by evaluating the biochemical and histopathological alterations caused in mussels exposed to control temperature (17 ºC) and predicted temperature rise (21 ºC) ii) the impacts induced by EE2 and SLS acting alone or as a mixture, in *M. galloprovincialis*, by measuring the alterations at the behavioural, histopathological, metabolic, oxidative status and genetic expression levels.

Chapter 2

Material and Methods

2. MATERIAL AND METHODS

2.1. Experimental design

Mytilus galloprovincialis specimens were collected in the Mira channel (Ria de Aveiro lagoon, Portugal), with a mean length of 5.8 ± 0.5 cm and a mean width of 3.6 ± 0.5 cm. Mussels were transported from the field, in plastic containers, to the laboratory where they were kept in artificial seawater (salinity 35 ± 1, representing salinity level at the study area during sampling) prepared with reverse osmosis water and artificial salt (Tropic Marin® SEA SALT from Tropic Marine Center), temperature 17 ± 1 °C, pH 8.0 ± 0.1 and constant aeration for a depuration week. Every 2-3 days seawater was changed, and mussels were fed with Algamac protein plus (150.000 cells/animal/day) after the first three days. Subsequently, mussels were submitted to a week of acclimation during which salinity was gradually decreased to 30 ± 1 (mean salinity at the sampling area), while the remaining parameters were maintained as during the depuration period.

2.1.1. First setup: influence of temperature on the impacts of 17 α-Ethynylestradiol

After acclimation 180 individuals were equally distributed in 30 glass aquaria of 3 L (6 per aquarium), divided into two groups with different temperatures (17 ± 1 °C and 21 ± 1 °C). Temperature was gradually increased from 17 to 21 ºC. A temperature of 17 °C was selected as representative of mean values measured at the sampling site, while temperature of 21 °C was selected as representative of the predicted increase of global mean surface temperature by the end of the 21st century (2.6 °C - 4.8 °C under RCP 8.59) (IPCC, 2018). At each temperature organisms were exposed for 28 days to four concentrations plus control (CTL, 0.0 ng/L): 5.0, 25.0, 125.0 and 625.0 ng/L of EE2. For each treatment 3 aquaria were used (18 mussels per treatment). EE2 used in the experiment was obtained from Sigma-Aldrich, Milan, Italy; chemical purity ≥ 98%; molecular weight 296.41. The concentrations of EE2 used were selected based on

published literature, in the attempt of reflecting environmental concentrations and laboratory exposures (Almeida et al., 2020; Caldwell et al., 2012; Ribeiro et al., 2009).

On a weekly basis, the exposure medium was renewed, after which EE2 concentration was re-established. Water samples were collected from each aquarium, immediately after spiking and used for EE2 quantification analysis to compare nominal and real exposure concentrations. Due to procedure limitations, water samples from each treatment (3 replicate aquaria per treatment) were pooled and measured together. Every 2-3 days, during the exposure period, mussels were fed with AlgaMac Protein Plus (150 000 cells/animal/day).

To evaluate EE2 stability in saltwater medium during experimental period, a set of aquaria without organisms, but under the same conditions (5.0, 25.0, 125.0 and 625.0 ng/L of EE2 concentrations; temperatures 17 ± 1 °C and 21 ± 1 °C; pH 8.0 ± 0.1; salinity 30 ± 1 and 12h light: 12h dark photoperiod) were prepared. For this, 2 aquaria for each condition were prepared, in the first week water samples were collected immediately after spiking and before water renewal to quantify EE2 concentrations. Due to procedure limitations, for each sampling period (after spiking and before water renewal) water samples from both aquaria were pooled and measured together.

After the 28-days exposure period, biochemical analyses and EE2 quantification in mussels' soft tissues were performed. The whole soft tissue of 3 mussels per aquarium (9 per treatment) were homogenized manually using liquid nitrogen. From each individual, the homogenized tissue was divided into aliquots of 0.5 g fresh weight (FW).

2.1.2. Second setup: impacts of 17 α-Ethynylestradiol and Sodium Lauryl Sulfate

For the exposure, each mussel was previously placed in a beaker filled with 500 mL of water and, through the injection of 1 mL KCl 0.5 M into the mussels shell, spawning was induced (Gago and Luís, 2011) to differentiate males from females, which were maintained in two distinct tanks by gender, for one week until the beginning of the experiment. Mussels were then divided in four different treatments, including control (CTL), 17 α-Ethynylestradiol 125 ng/L (EE2), sodium lauryl sulfate 4 mg/L (SLS) and the mixture of both substances (EE2+SLS). Three aquaria with three male and three female organisms each were used per treatment. The concentration of EE2 used in this study was based on EE2 concentration found in marine bivalves, (between 125 and 295 ng/g DW (dry weight)) (Chiu et al., 2018; Almeida et al., 2020) as well as the results of the first experiment. The SLS concentration was selected according to a previous study showing that 4 mg/L SLS induced toxicity to *M. galloprovincialis* (Freitas et al., 2020a) and concentrations found in the environment, namely in contaminated aquatic systems (Gibson et al., 2016).

Mussels were subjected to a chronic exposure of 28 days, during which temperature, salinity and mortality were regularly checked. Organisms were fed with Algamac protein plus (150.000 cells/animal/day) every 2-3 days. During the experimental period, the seawater was weekly renewed, conditions re-established in each aquarium and mussels' behaviour (i.e., valves closure) was recorded. Footage was later analysed, where the amount of time in seconds that mussels' valves were closed was quantified, through the observation of the recording.

Samples of seawater were collected from each aquarium for EE2 and SLS quantification every week: i) in the exposure aquaria after spiking to assess real concentrations; and ii) in blanks (aquaria maintained under the same conditions but without organisms) after spiking and before water renewal to assess contaminants stability. As a result of procedure limitations, each sampling period (after spiking and before water renewal) water samples from both aquaria were pooled and measured together.

At the 28th day of exposure mussels were sacrificed for EE2 and SLS concentration quantification, biochemical, histopathological and quantitative RT-PCR analyses. No mortality was recorded at the end of the experimental period. After exposure, 3 individuals (1 male and 2 female) per aquarium (9 individuals per treatment) were used for biochemical analyses and EE2 and SLS quantification, 1 individual (male) per aquarium (3 individuals per treatment) was used for histopathological analysis and 1 individual (male) per aquarium (3 individuals per treatment) was used for quantitative RT-PCR analysis.

2.2. 17 α-Ethynylestradiol and Sodium Lauryl Sulfate quantification in water and mussel's tissues

In both experimental setups, EE2 and SLS quantification in water samples was performed, samples from each treatment and sampling moment were pooled due to the limited amount of water collected during the experiment. For this reason, no replicates are available for these measurements. Regarding tissues, samples of males and females from each aquarium were polled and analysed together. In this case, replicates for each treatment are available.

Concentrations of EE2 were measured in water and soft tissues by using a high-performance liquid chromatography-fluorometric (HPLC-FL) detection method. Water samples were analysed by using the method of Patrolecco et al. (2013) with slightly modifications. Water samples were filtered and extracted with solid phase extraction (Oasis HLB 6cc 150 mg solid-phase extraction cartridges, Waters), followed by HPLC analysis. Tissue samples of males and females (3 g from each individual) were dehydrated and sonicated at 50 ºC for 1 min using 5 mL of acetonitrile (10 mL) as the extraction solvent. The supernatant was collected after centrifugation and diluted using Milli-Q grade water and then purified with solid phase extraction as reported for water samples (Oasis HLB 6cc 150 mg solid-phase extraction cartridges, Waters). The chromatographic analyses were carried out using an HPLC system with a PerkinElmer Series 200 variable flow pump, coupled to a JASCO FP-1520 fluorescence detector (JASCO, Tokyo,

Japan). The detection was performed using an excitation wavelength of 230 nm and an emission of 302 nm. The system was controlled by a PerkinElmer interface module (NCI 900 Network Chromatography Interface) and chromatograms were processed by a PerkinElmer TotalChrom Navigator software. An Haisil HL C18 5 µm 250x4.6 mm chromatographic column (Higgins Analytical, Inc.) was used. The analyses were performed at room temperature at a flow rate of 1.1 ml/min and an injection volume of 100 µL was used. The mobile phase consisted of acetonitrile/water (45/55%, v/v) acidified at pH 3.6 with glacial acetic acid. The recovery was >73% for water samples and >74% for soft tissues. The detection limit, calculated as a signal-to-noise ratio of 3:1, was 1 ng/L for water samples and 10.0 ng/g for soft tissues.

The determination of SLS as Methylene Blue Active Substances (MBAS) was performed according to Latif and Brimblecombe (2004) and Arand et al. (1992). Water concentrations were determined, using the sample solution (15 mL), an alkaline buffer (2 mL) and 1 mL of neutral methylene blue solution followed by chloroform (6 mL) were added to a 50 mL vial. The vial was tightly closed and vigorously shaken for one minute in a vortex mixer. It was then left to stand until the phases had separated, after which the chloroform layer was transferred to a second vial containing distilled water (22 mL) and 1 mL of acid methylene blue solution. The second vial was shaken, and the separated chloroform was collected. The absorbance of the chloroform phase was measured spectrophotometrically at 650 nm. A calibration curve with a concentration range 0.1 - 10 ppm was established using SLS as the reference compound. The lower limit of detection for anionic surfactant analysis was 0. 05 ppm (0.05 mg/L). Tissue samples of male and female gender (1.5 g) were extracted with 5 mL of water, vigorously shacked for one minute in a vortex mixer and centrifuged for 5 min at 3000 rpm. The supernatant was collected after centrifugation and treated as reported above for water samples. A calibration curve with a concentration range 0.01 - 10 ppb was established using SLS. The lower limit of detection for anionic surfactant analysis was 0.005 ppm (5 µg/g).

2.3. Behaviour measurements

Behaviour was assessed after exposure to EE2 and SLS. During the exposure period, after medium renewal and conditions re-establishment, male mussels from one aquarium per treatment were recorded in a timelapse video for 2 hr using a video camera (GoPro Silver 4). This procedure was done twice, immediately after spiking and one day after spiking on the 3rd week of the experimental period. Afterwards, the videos were observed and the period (in sec) during which mussels' valves remained closed was determined for each treatment.

2.4. Biochemical parameters

For both experimental periods, for each individual, the tissues were homogenized with liquid nitrogen and divided in aliquots of 0.5 g fresh weight (FW).

In the first experimental setup, biochemical analyses in mussels' soft tissues, shells of the frozen organisms were removed and the whole soft tissue of 3 mussels per aquarium (9 per treatment). At the end of the second experimental period 1 male and 2 females from each aquarium (3 males plus 6 females per treatment) were frozen individually in liquid nitrogen and the soft tissue was sampled to perform biochemical assays.

In an effort to estimate biochemical alterations induced in mussels after exposure to EE2 and SLS and the combination of EE2 and warming, biomarkers related to metabolic capacity (electron transport system (ETS) activity), energy reserves (content of glycogen (GLY) and total protein (PROT)), oxidative stress (activity of antioxidant and biotransformation enzymes (superoxide dismutase (SOD); catalase (CAT); glutathione peroxidase (GPx); glutathione reductase (GRed); carboxylesterases (CbEs); glutathione S-transferases (GSTs)); cellular damage and redox balance (levels of lipid peroxidation (LPO); oxidized glutathione (GSSG) content and the ratio between reduced (GSH) and oxidized (GSSG)); and neurotoxicity (acetylcholinesterase (AChE) activity) were assessed.

The ETS activity has been used to obtain an indication of the individual

metabolic status (De Coen and Janssen, 1997). When organisms are exposed to stressful conditions, they may increase metabolism, in the case of ETS, it occurs in the mitochondria, for the preservation of homeostasis, survival and reproduction (Gagné et al., 2006), ETS activity may decrease or maintain by activating behavioural alterations as valves closure (Gosling, 2003). Usually along with the decrease of ETS activity, organisms can preserve energy reserves (GLY and PROT) as a defence mechanism. Organisms may also decrease the content in GLY and PROT resulting from the expenditure of their energy reserves to augment defence mechanisms.

Under stressful conditions organisms, such as mussels, normally increase production of reactive oxygen species (ROS) and in order to avoid cellular damage the activation of their antioxidant defences develop, including the activity of the antioxidant enzymes (SOD, CAT, GPx and GRed) (Regoli and Giuliani, 2014). Along with the activation of antioxidant defences, organisms exposed to stressful conditions, likewise activate biotransformation enzymes, namely glutathione S-transferases (GSTs) and carboxylesterases (CbEs) (Townsend and Tew, 2003; Sturve et al., 2008; Solé et al., 2018).

Lipid peroxidation (LPO) has been generally used as a biomarker for cell damage (Moreira et al., 2016) resulting from insufficient defence mechanisms to avoid damage in the cells, that are attacked by ROS, promoting an autocatalytic oxidation process (Almeida et al., 2007). Also, oxidized glutathione (GSSG) content and the ratio between reduced (GSH) and oxidized (GSSG) glutathione, is an indicator of redox balance; this is because the decrease of GSH/GSSG ratio and high GSSG content, may show that organisms under these conditions spent more GSH to convert to GSSG to neutralize the ROS. The reason is that GSH is the most abundant cytosolic scavenger, which participates in the antioxidant defence system, aiming to directly neutralize ROS through its oxidation to GSSG (Regoli and Giuliani, 2014).

The activity of AChE is used as a biomarker of exposure to neurotoxic compounds in aquatic organisms. This enzyme is important to the functioning of the neuro-muscular system, and it is responsible for the degradation of neural

transmitter acetylcholine to choline in cholinergic synapses and neuromuscular junctions (Matozzo et al., 2005).

To guarantee the validity of the results, the determination of the biochemical parameters was done in duplicate. For each biomarker, the extraction was performed with specific buffers using a proportion of 1:2 (w/v) with the homogenized tissue. For GLY, PROT, SOD, CAT, GPx, GRed, GSTs, CbEs and AChE the supernatants were extracted in potassium phosphate buffer (50 mM potassium dihydrogen phosphate; 50 mM potassium phosphate dibasic; 1 mM ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1% (w/v) polyvinylpyrrolidone (PVP); 1 mM dithiothreitol (DTT); pH 7.0). For GSH and GSSG, the buffer had 0.1% Triton X-100 (v/v), 0.6% sulphosalisilic acid in phosphate buffer (KPE) (0.1 mM potassium phosphate dibasic; 0.1 mM potassium dihydrogen phosphate; 5 mM EDTA with pH = 7.5). For ETS supernatants were extracted in 0.1 M Tris- HCl buffer (pH 8.5, 15% (w/v) PVP, 153 µM magnesium sulfate (MgSO) and 0.2% (v/v) Triton X-100). For LPO quantification supernatants were extracted in 20% (w/v) trichloroacetic acid (TCA). All samples were sonicated using a TissueLyser II (Qiagen) for 90 s, after which they were centrifuged for 20 min at 10,000 g (3,000 g for ETS) and 4 °C. Biochemical measurements were carried out either immediately or after storage at -80 °C in order to determine responses related with bivalves' metabolism and energy reserves content, oxidative stress status and neurotoxicity (Andrade et al., 2018; Coppola et al., 2019; Pirone et al., 2019). All measurements were done using a microplate reader (Biotek).

2.4.1. Metabolism and energy reserves

The metabolic capacity was assessed through electron transport system (ETS). The activity of ETS was measured using the method of King and Packard (1975) and the modifications implemented by De Coen and Janssen (1997). Absorbance was measured at 490 nm during 10 min with intervals of 25 sec. The amount of formazan formed was calculated using the extinction coefficient Ɛ =

15,900 mM/cm. The results were expressed in nmol per min per g FW.

To analyse the levels of energy reserve, glycogen (GLY) and total protein (PROT) contents were quantified. The GLY quantification the sulfuric acid method was used, as described by Dubois et al. (1956). Glucose standards were used (0 - 10 mg/mL) to produce a calibration curve. Absorbance was measured at 492 nm after incubation during 30 min at room temperature. Results were expressed in mg per g FW.

The PROT content was quantified according to the spectrophotometric Biuret method described by Robinson and Hogden (1940). Bovine serum albumin was used as standards in the range 0-40 mg/mL to obtain a calibration curve. Absorbance was measured at 540 nm and results were expressed in mg per g FW.

2.4.2. Antioxidant and biotransformation defences

Antioxidant defences were evaluated by analysing superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx) and glutathione reductase (GRed) enzyme activities.

The activity of SOD was determined based on the method described by Beauchamp and Fridovich (1971) with adaptations implemented by Carregosa et al. (2014). SOD standards (0.25 - 60 U/mL) were used to obtain a calibration curve. Absorbance was read at 560 nm after 20 min of incubation at room temperature. Results were expressed in U per g FW, where one unit (U) represents the amount of the enzyme that catalyses the conversion of 1 µmol of substrate per min.

The activity of CAT was quantified according to the Johansson and Borg (1988) method and the modifications performed by Carregosa et al. (2014). The standard curve was determined using formaldehyde standards (0-150 µM). Absorbance was measured at 540 nm. The enzymatic activity was expressed in U per g of FW, where U represents the amount of enzyme that caused the formation

of 1.0 nmol formaldehyde per min at 25 °C.

The GPx activity was quantified following Paglia and Valentine (1967). The absorbance was measured at 340 nm in 10 sec intervals for 5 min and the enzymatic activity was determined using extinction coefficient Ɛ = 6.22 mM/cm. The results were expressed as U per g FW, where U represents the number of enzymes that caused the formation of 1.0 µmol NADPH oxidized per min.

The GRed activity was determined using the method described in Carlberg and Mannervik (1985). The absorbance was measured at 340 nm and the enzymatic activity was determined using Ɛ = 6.22 mM/cm. The results were expressed as U per g FW, where U represent the enzymes amount that caused the formation of 1.0 µmol NADPH oxidized per min.

Mussels' biotransformation capacity was assessed by measuring the activities of carboxylesterases (CbEs) and glutathione S-transferases (GSTs) enzymes.

The activity of CbEs was quantified following Hosokawa and Satoh (2001) method with adaptations carried out by Solé et al. (2018). The activity was measured using the commercial colorimetric substrate p-nitrophenyl butyrate (pNPB). Absorbance was measured at 405 nm for 5 min with 15 sec intervals between readings and the extinction coefficient Ɛ = 18 mM/cm was used to determine the activity. Results were expressed nmol per min per g of FW.

The activity of GSTs was quantified following Habig et al. (1974) protocol with some adaptations performed by Carregosa et al. (2014). GSTs activity was measured spectrophotometrically at 340 nm (Ɛ = 9.6 $mM^{-1}cm^{-1}$). The enzymatic activity was expressed in U per g of FW where U is defined as the amount of enzyme that catalysis the formation of 1 µmol of dinitrophenyl thioether per min.

2.4.3. Cellular damage and redox balance

To assess mussels' cellular damage and redox status, was highlighted trough lipid peroxidation levels (LPO) and oxidized glutathione (GSSG) content and the ratio between reduced (GSH) and oxidized (GSSG)) was measured.

The levels of LPO were assessed trough the measurement of malondialdehyde (MDA) content, following the method described by Ohkawa et al. (1979). Absorbance was measured at 535 nm and the amount of MDA formed was calculated using the extinction coefficient Ɛ = 156 mM/cm. The results were expressed in nmol per g FW.

The quantification of GSH and GSSG was determined following the Rahman et al. (2007) method and used as standards (0-60 µmol/L) to obtain a calibration curve. Absorbance was measured at 412 nm every 30 sec for 2 min and the ratio GSH/GSSG was determined considering the number of thiol equivalents (GSH/(2*GSSG)). Both results were expressed in µmol per g FW.

2.4.4. Neurotoxicity

Additionally, to assess neurotoxicity, acetylcholinesterase activity (AChE) was evaluated. The AChE activity was determined following the method of Ellman et al. (1961), with Mennillo et al. (2017) modifications. AChE activity was measured at 412 nm for 5 min and calculated using the extinction coefficient Ɛ = 13.6 mM/cm. Results were expressed in nmol per min per g of FW.

2.5. Histopathological analyses

In the first experiment one mussel for each aquarium (3 per treatment) while in the second experiment one male mussel for each aquarium (3 per treatment) was used for histological analysis. After the experimental period, Mussel's gills and digestive tubules were fixed in Davidson solution for 24 h at room temperature. Afterwards, organisms were gradually dehydrated from ethanol

70 % to absolute alcohol in graded alcohols, cleared in xylene and embedded in paraffin (56 – 58 °C). Sections of 5 µm were cut at microtome (Leica, Jung autocut) and stained with Mayer's haematoxylin to observe the presence of histopathological alterations (Coppola et al., 2020). Histopathological indexes were calculated using the following formula (Costa et al., 2013):

$$I_h = \frac{\sum_1^j w_j a_{jh}}{\sum_1^j M_j}$$

Where *Ih* is the histopathological index for the individual *h*; *wj* the weight of the *jth* histopathological alteration; *ajh* the score attributed to the *hth* individual for the *jth* alteration and *Mj* is the maximum attributable value for the *jth* alteration. The *Ih* was determined following the concepts of the differential biological significance of each analysed alteration (weight) and its diffusion (score). The weights vary from 1 (minimum severity) to 3 (maximum severity) while the score varies from 0 (not present) to 6 (diffuse) (Costa et al., 2013).

2.6. RNA expression and Gene analysis

To evaluate genetic expression, in the second exposure period with one male mussel for aquarium (3 per treatment) was used for Estrogen receptor 1 (ER1) and Estrogen receptor 2 (ER2) expression analysis. Digestive tubules and gonads were dissected, snap-frozen in liquid nitrogen, ground to a powder by mortar and pestle in TRIzol (Invitrogen, Paisley, UK) for RNA extraction. The samples were further homogenized using a TissueLyser II (Qiagen, Valencia, CA, USA) and steal beads of 5 mm diameter (Qiagen, Valencia, CA, USA). RNA was extracted with Direct-zolTM RNA Miniprep (Zymo Research), following the manufacturer's protocol. The quality and amount of purified RNA were analyzed spectrophotometrically with Nanodrop2000 (Thermo Scientific Inc., Waltham, MA, USA), and Qubit 4.0 (Thermo Scientific Inc., Waltham, MA, USA). Then, 1000 ng of RNA were reverse transcribed with the QuantiTect Reverse Transcription Kit (Qiagen, Valencia, CA, USA), as described by the manufacturer. Afterwards, Real-

Time PCR was performed using the QuantiTect SYBR Green PCR Kit (Qiagen, Valencia, CA, USA) in a final volume of 25 µL containing 100 ng of cDNA, 1 µM of each primer, 12.5 µL of QuantiFast SYBR Green PCR Master Mix (2X). PCR cycling profile consisted of a cycle at 95 °C for 5 min, 40 two-step cycles at 95 °C for 15 sec, at 60 °C for 50 sec. Quantitative RT-PCR analysis was conducted using the $2(-\Delta\Delta C(T))$ method (Livak and Schmittgen, 2001). RT-PCR was performed in a Rotor-Gene Q cycler (Qiagen, Valencia, CA, USA). At the end of each test, a melting curve analysis was done (plate read every 0.5 °C from 55 to 95 °C) to determine the formation of the specific products. Each sample was tested and read in duplicate. No-template controls were included.

2.7. Integrated biomarker response (IBR)

The integrated biomarker response (IBR) was performed to integrate results obtained from biochemical analysis, both for male and female organisms of the second experiment, following Beliaeff and Burgeot (2002) method. Biomarkers were arranged in the following order: SOD, GSH/GSSG, LPO, GLY, GSTs, GPx, ETS, AChE, GRed, PROT, and CbEs-pNPB. Higher and lower values correspond to higher and lower mussels' response, respectively.

2.8. Statistical analysis

Results were divided in two experimental datasets. In the first dataset EE2 and SLS single/mixture exposure were considered at control temperature, and in the second EE2 and temperature at 17 °C and 21 °C treatments were examined.

For the first experimental setup, the biochemical (ETS, GLY, PROT, SOD, CAT, GPx, GSTs, LPO, GSH/GSSG ratio, and AChE) results and *Ih* for gills and digestive tubules data, from all treatments, were subjected to hypothesis testing using non-parametric permutational analysis of variance with one factor design (temperature and EE2 concentration) using PERMANOVA Add-on in Primer v6 (Anderson et al., 2008). The Bray-Curtis similarity matrix was then analysed

following unrestricted permutation of the raw data (9999 permutations) and the calculation of type III sums of squares. Main test with p values lower than 0.05 were considered as significant and followed by pair-wise tests. Pair-wise tests were used to identify statistical differences and represented in figures with lower case letters. The null hypotheses tested were: i) for each biochemical parameter and for each temperature, no significant differences were found among EE2 concentrations (represented with lowercase letters for 17 ºC and uppercase letter for 21 ºC); ii) for each biochemical parameter and for each EE2 concentration, no significant differences were observed between temperatures (differences between temperatures were represented with an asterisk); iii) for EE2 accumulation in mussels tissues, no significant differences were observed between temperatures (differences between temperatures were represented with lowercase letters).

Biochemical and histopathological parameters were also separately submitted to a PERMANOVA analysis with a two factors design: temperature as factor 1 and EE2 concentration as factor 2, to test the interaction between the two factors on each parameter.

The biological descriptors were submitted to ordination analysis, performed by non-parametric multidimensional scaling (MDS) with the software PRIMER. The final diagram, represented in two dimensions (horizontal and vertical axes), includes the respective stress value (Clarke and Warwick, 1994). This value is a measure of the distortion associated with the representation of the multidimensional distance matrix in two dimensions. If it is below 0.10, the representation is considered very good. If it is above 0.30, the final diagram should not be considered a reliable representation of the distance matrix (Clarke and Warwick, 1994).

For the second experimental setup, concentrations of EE2 and SLS in the water and mussels' soft tissues, biomarkers (ETS, GLY, SOD, CAT, GRed, GPx, GSTs, LPO and GSH/GSSG), histopathological indexes, quantitative RT-PCR analysis was separately submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in Primer v7). A one-way hierarchical design was followed in this analysis. The pseudo-F p-values in the PERMANOVA main tests

were evaluated in terms of significance. When significant differences were observed in the main test, pairwise comparisons were performed. Values with $p < 0.05$ were considered as significantly different. The null hypotheses tested were: no significant differences exist in terms of EE2 and SLS concentrations between males and females among treatments (CTL, EE2, SLS and EE2+SLS), both for water and tissue samples; for each biomarker, histopathological index and RT-PCR analysis no significant differences existed among treatments. Differences among each treatment (CTL, EE2, SLS and EE2+SLS) were indicated with different uppercase letters for males and lowercase letters for females, while differences between males and females were represented with asterisks.

Chapter 3

Results

3. RESULTS

3.1. First experiment: influence of temperature on the impacts of 17 α-Ethynylestradiol

3.1.1. 17-α Ethynylestradiol concentrations in water and mussel's tissues

Concentrations of EE2 measured on water samples collected after spiking showed that real concentrations were lower than the nominal concentrations, regardless the EE2 tested concentration and temperature: at 17 ºC the highest loss (30%) was observed at the lowest exposure concentration while the lowest loss (18%) was observed at the highest exposure concentration; at 21 ºC losses of EE2 varied less among exposure concentrations, between 22% at 125 ng/L and 19% at 25 ng/L (Table 1). A similar pattern of EE2 loss was observed in water from blanks collected after spiking: at 17 ºC the highest loss (22%) was recorded at the lowest exposure concentration and the lowest loss (17%) was observed at 125 ng/L; at 21 ºC the highest loss was found at 25 ng/L (27%) while the lowest loss was recorded at 5 ng/L (18%, Table 1).

Table 1. 17-α Ethynylestradiol (EE2) concentrations in water (ng/L) from exposure aquaria (collected immediately after spiking) and blanks aquaria (collected immediately after spiking and before water renewal). LOD for water samples 1 ng/L.

Temperature ºC	EE2 exposure concentrations (ng/L)	Exposure medium	Seawater from blanks	
		After spiking	After spiking	Before water renewal
17	**CTL**	<LOD	<LOD	<LOD
	5.0	3.5	3.9	3.3
	25.0	18.6	24.6	17.5
	125.0	102.4	104.2	99.6
	625.0	512.4	506.3	478.9
21	**CTL**	<LOD	<LOD	<LOD
	5.0	3.9	4.1	3.8
	25.0	20.3	18.3	19.5
	125.0	97.5	94.1	88.2
	625.0	495.3	476.9	467.9

Nevertheless, considering that immediately after spiking concentrations were already lower than the nominal ones, regardless the temperature, concentrations measured immediately before water renewal revealed losses up to 5% at the highest exposure concentration and up to 15% at the lowest exposure concentration. Such results point out the stability of EE2 along one-week exposure and it is hypothesized that lower concentrations measured after spiking could result from an incomplete mixture of EE2 in the water medium when water samples were collected.

Concentrations of EE2 in exposed mussels were only measurable at the highest exposure concentration. Although lower EE2 concentration was found in mussels exposed to 21 ºC, no significant differences were observed between mussels exposed to 17 and 21 ºC. Furthermore, the BCF values were similar between temperatures (Table 2).

Table 2. 17-α Ethynylestradiol (EE2) concentrations in mussel's tissues (ng/g FW) at the end of the experimental period (28 days). BCF: Bioconcentration factor (L/kg). LOD for tissue samples 10 ng/g. Values are the mean ± standard deviation.

Temperature (ºC)	**EE2 exposure concentrations (ng/L)**	**Tissues' concentrations (ng/L)**	**BCF (L/kg)**
17	CTL	<LOQ	-
	5.0	<LOQ	-
	25.0	<LOQ	-
	125.0	<LOQ	-
	625.0	19.9 ± 5.9[a]	39
21	CTL	<LOQ	-
	5.0	<LOQ	-
	25.0	<LOQ	-
	125.0	<LOQ	-
	625.0	14.9 ± 1.6 [a]	30

3.1.2. Biochemical responses

3.1.2.1. Metabolic capacity and energy reserves

At 17 ºC the ETS values were similar among EE2 concentrations, except at 125 ng/L where the activity was significantly lower than values at CTL. At 21 ºC, ETS was significantly higher at 5, 125 and 625 ng/L in comparison to CTL and 25 ng/L treatments. Significant differences between temperatures were found at CTL, 25, 125 and 625 ng/L treatments, with higher values at 17 ºC for CTL and 25 ng/L and higher values at 21 ºC for 125 and 625 ng/L (Figure 3A).

In mussels exposed to 17 ºC significantly higher GLY content was found in mussels exposed to the highest EE2 concentration in comparison to the remaining treatments. At 21 ºC significantly lower GLY content was found in mussels exposed to 25 ng/L in comparison to all the other EE2 exposure concentrations. Significant differences between temperatures were only observed at 125 and 625 ng/L, with higher values in mussels at 21 ºC for 125 ng/L and an opposite response at 625 ng/L (Figure 3B).

Mussels maintained at 17 ºC showed significantly higher PROT content at 5 and 625 ng/L in comparison to CTL and 125 ng/L treatments. At 21 ºC mussels exposed to EE2 showed significantly higher PROT content than the ones at CTL, with the highest values at the two higher EE2 tested concentrations. Differences between temperatures were observed at CTL, 5 and 125 ng/L, with higher values at 17 ºC for CTL and 5 ng/L treatments and an opposite response for 125 ng/L (Figure 3C).

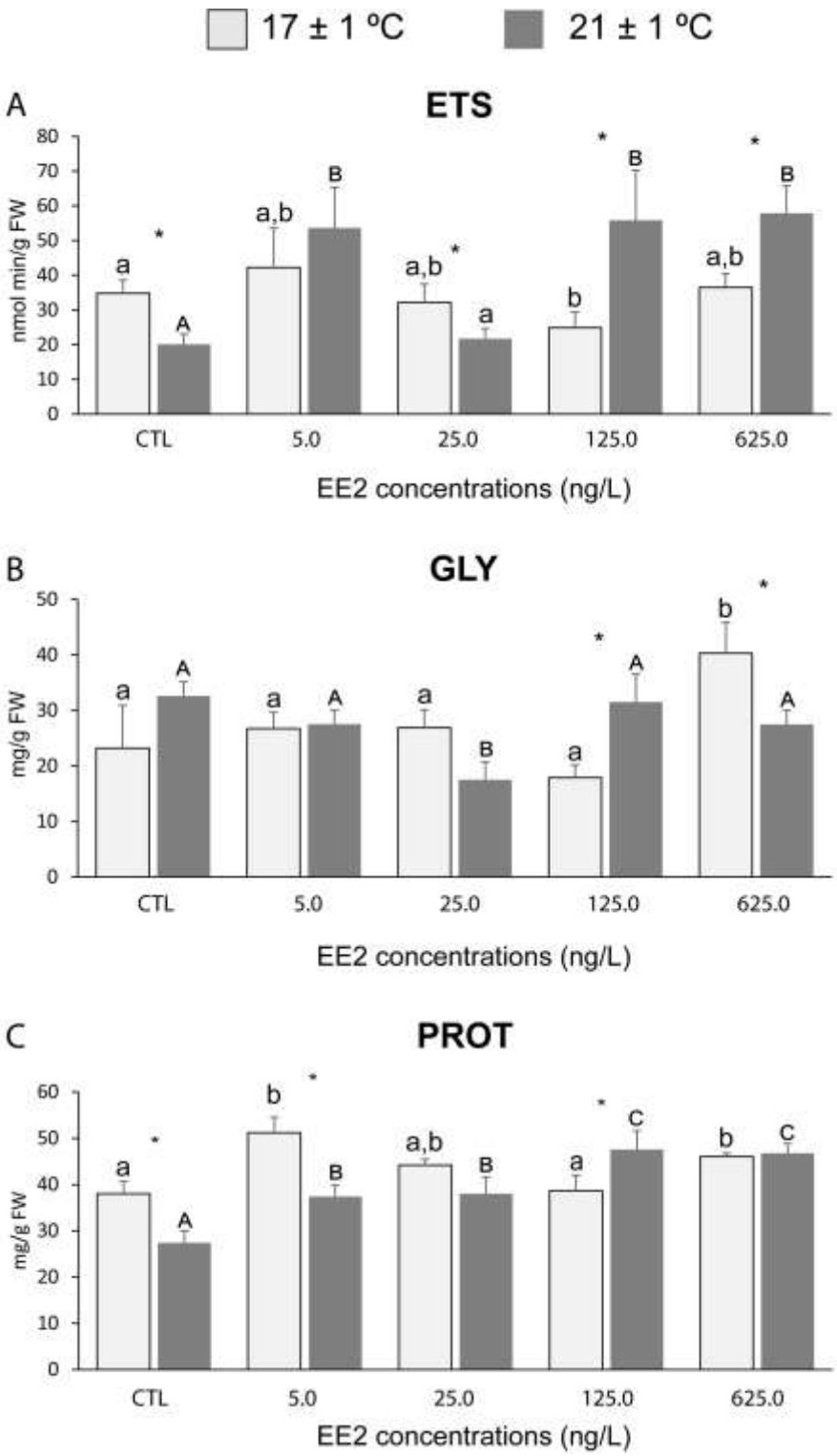

Figure 3. A: Electron transport system activity (ETS); B: Glycogen content (GLY); C: Protein content (PROT) in Mytilus galloprovincialis exposed to each concentration (CTL, 5, 25, 125 and 625 ng/L) and both temperatures (17 and 21 ºC). Values are mean + standard deviation. Significant differences ($p < 0.05$) among concentrations (CTL, 5, 25, 125 and 625 ng/L) for each temperature (17 and 21 ºC) are represented with different letters (lower case letters for 17 ºC, upper case letters for 21 ºC); while significant differences between temperatures for each EE2 concentration are represented with an asterisk.

3.1.2.2. Antioxidant and biotransformation defences

The activity of SOD at 17 ºC was significantly higher in mussels exposed to 125 and 625 ng/L concentrations in comparison to CTL and 5 ng/L treatments. At 21 ºC, significantly lower SOD activity was found in mussels exposed to 25, 125 and 625 ng/L in comparison to CTL and 5 ng/L treatments. Between temperatures significant differences were observed at 25, 125 and 625 ng/L, with higher values in mussels at 17 ºC (Figure 4A).

The activity of CAT showed no significant differences among tested conditions at 17 ºC. At 21 ºC mussels exposed to 5 ng/L showed the lowest CAT activity, with significant differences to CTL, 125 and 625 ng/L treatments. Significant differences between temperatures were found at all treatments, with higher values at 17 ºC (Figure 4B).

At 17 ºC the activity of GPx was significantly higher in mussels exposed to 25 ng/L concentration in comparison to other treatments. At 21 ºC, significantly higher activity was observed at 25, 125 and 625 ng/L concentrations in comparison to CTL and 5 ng/L treatments. Differences between temperatures were only observed at 25 ng/L, with higher values at 17 ºC (Figure 4C).

Mussels maintained at 17 ºC showed significantly higher GSTs activity at 5, 25 and 625 ng/L in comparison to the other treatments. At 21 ºC the GSTs values were similar among EE2 exposed mussels, with differences to the CTL where the activity was significantly higher. Significant differences between temperatures were found at 5, 25, 125 and 625 ng/L treatments, with higher values at 17 ºC (Figure 4D).

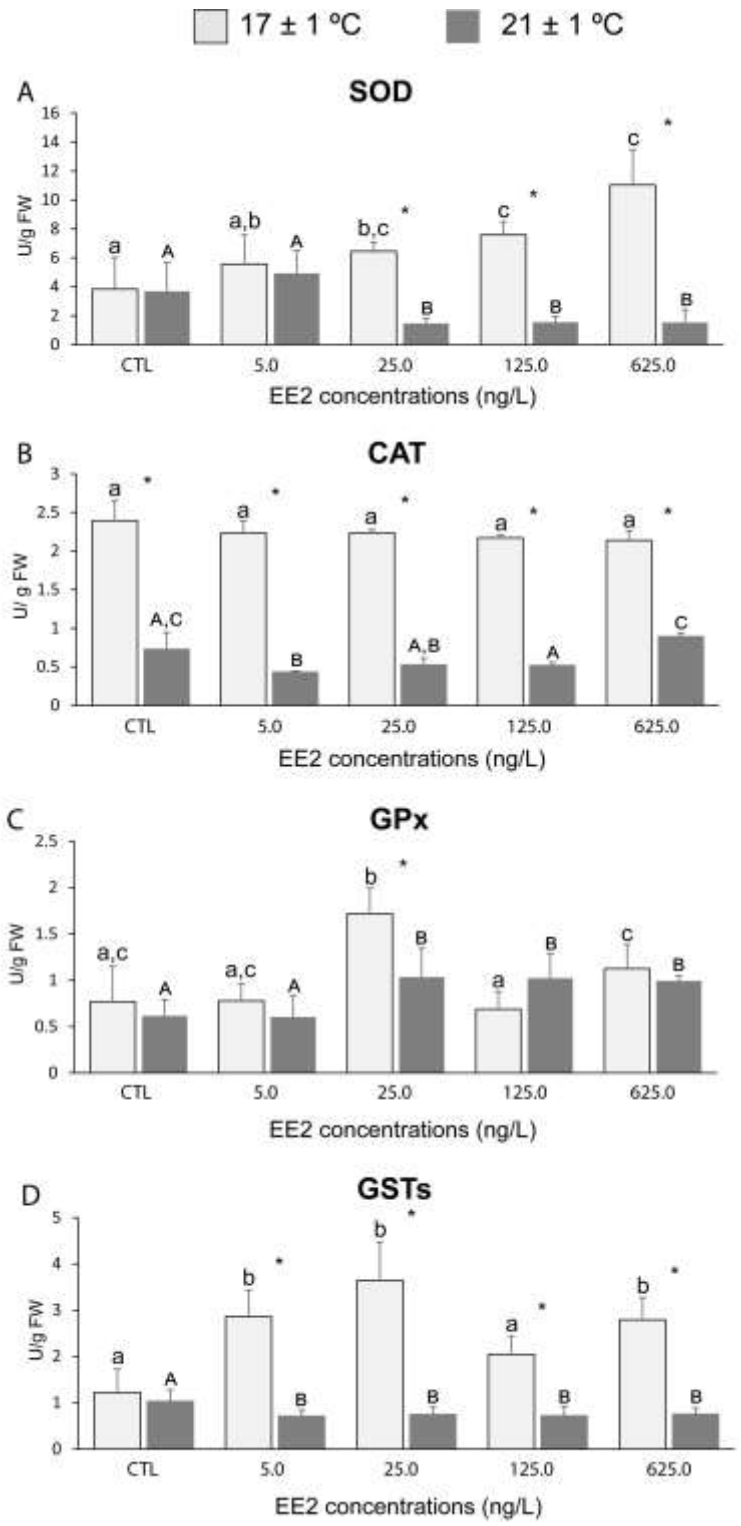

Figure 4. A: Superoxide dismutase activity (SOD); B: Catalase activity (CAT); C: Glutathione peroxidase activity (GPx); D: Glutathione S-transferases activity (GSTs); in Mytilus galloprovincialis exposed to each concentration (CTL, 5, 25, 125 and 625 ng/L) and both temperatures (17 and 21 ºC). Values are mean + standard deviation. Significant differences (p < 0.05) among concentrations (CTL, 5, 25, 125 and 625 ng/L) for each temperature (17 and 21 ºC) are represented with different letters (lower case letters for 17 ºC, upper case letters for 21 ºC); while significant differences between temperatures for each EE2 concentration are represented with an asterisk.

3.1.2.3. Oxidative damage and redox balance

At 17 ºC LPO values showed no significant differences among tested treatments. LPO levels at 21 °C followed the increasing exposure gradient with significantly higher values at 125 and 625 ng/L treatments. Differences between temperatures were observed at 125 and 625 ng/L, with higher values at 21 ºC (Figure 5A).

In mussels exposed to 17 ºC significantly higher GSH/GSSG ratio values were found in mussels exposed to CTL in comparison to the remaining treatments. At 21 ºC, GSH/GSSG ratio was higher at CTL, with significant differences to 5, 25 and 125 ng/L treatments. Significant differences between temperatures were only found at CTL, with higher values at 17 ºC (Figure 5B).

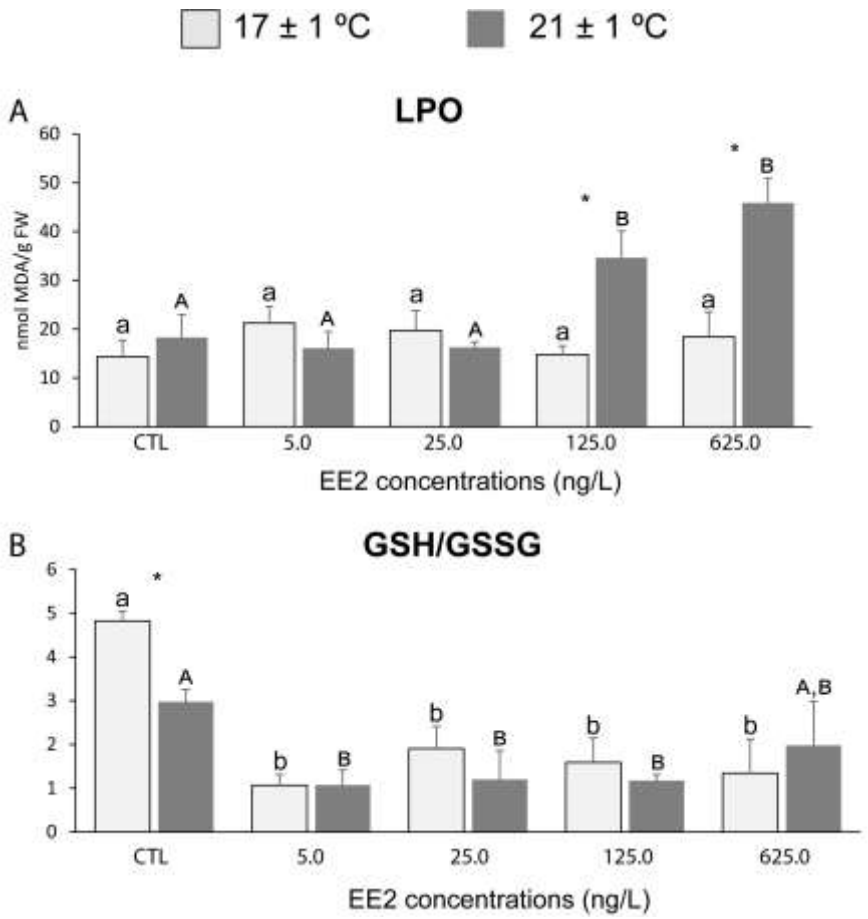

Figure 5. A: Lipid peroxidation levels (LPO); B: Ratio between reduced and oxidized glutathione (GSH/GSSG); in Mytilus galloprovincialis exposed to each concentration (CTL, 5, 25, 125 and 625 ng/L) and both temperatures (17 and 21 ºC). Values are mean + standard deviation. Significant differences (p < 0.05) among concentrations (CTL, 5, 25, 125 and 625 ng/L) for each temperature (17 and 21 ºC) are represented with different letters (lower case letters for 17 ºC, upper case letters for 21 ºC); while significant differences between temperatures for each EE2 concentration are represented with an asterisk.

3.1.2.4. Neurotoxicity

The activity of AChE at 17 ºC tended to decrease in EE2 exposed mussels compared to non-contaminated mussels, but significant differences were only found between CTL and 5.0 ng/L. At 21 ºC a similar pattern was observed, with a decrease in AChE activity in EE2 exposed mussels, but no significant differences were observed among treatments. Comparing temperatures, significantly higher values were observed at 17 ºC for mussels exposed to 25, 125 and 625 ng/L (Figure 6).

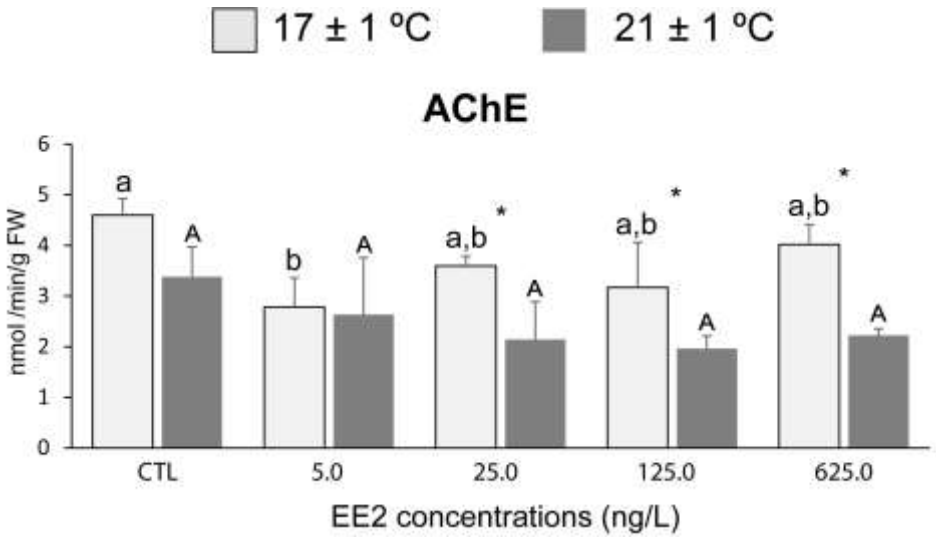

Figure 6. Acetylcholinesterase activity (AChE); in Mytilus galloprovincialis exposed to each concentration (CTL, 5, 25, 125 and 625 ng/L) and both temperatures (17 and 21 ºC). Values are mean + standard deviation. Significant differences ($p < 0.05$) among concentrations (CTL, 5, 25, 125 and 625 ng/L) for each temperature (17 and 21 ºC) are represented with different letters (lower case letters for 17 ºC, upper case letters for 21 ºC); while significant differences between temperatures for each EE2 concentration are represented with an asterisk.

3.1.3. Histopathological parameters

Histopathological alterations were revealed in *M. galloprovincialis* gills and digestive tubules. The exposure to EE2 at different concentrations led to an increase of damage severity in a dose dependent manner in mussels' gills, under both temperatures (Figure 7). At 17 ºC exposed mussels' gills displayed a

progressive increase of haemocytes infiltration, lipofuscin aggregates and loss of cilia. At 21 ºC, there was a progressive increase of haemocytes infiltration and lipofuscin aggregates. Under both temperatures, the *Ih* obtained for mussels' gills (Figure 8A) increased significantly along the exposure gradient, with the highest values in mussels exposed to the highest concentration.

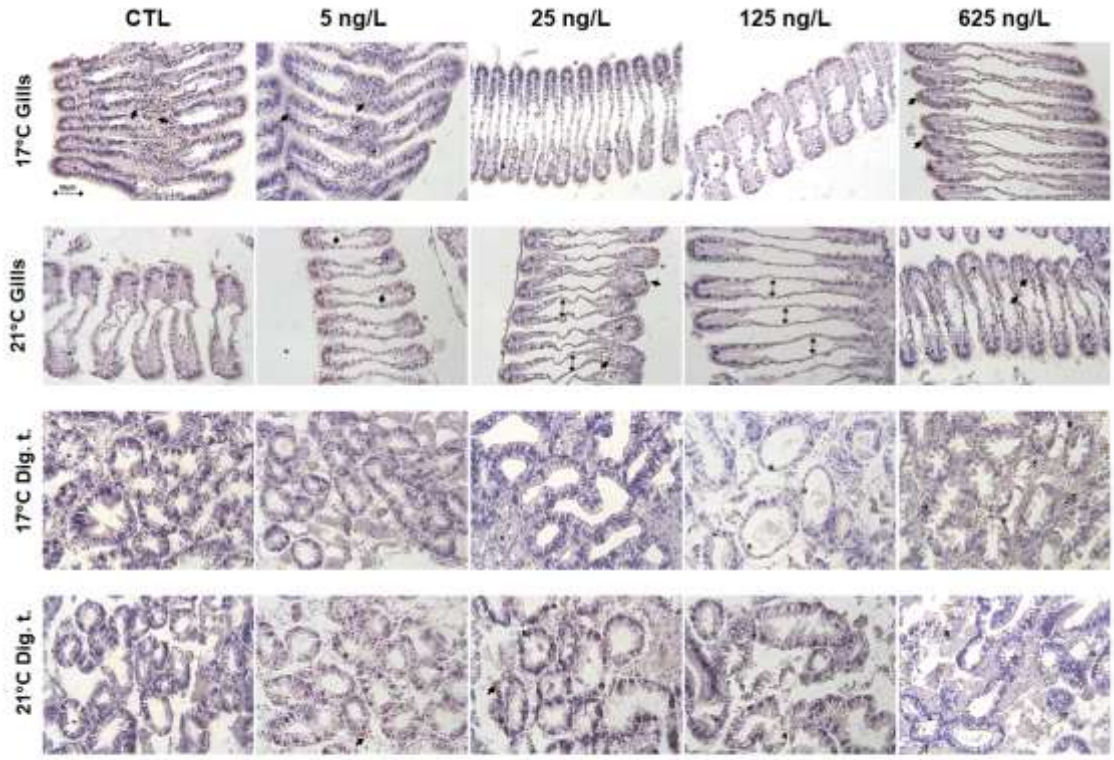

Figure 7. Micrographs of histopathological alterations in the gills; Lower image: Micrographs of histopathological alterations observed in the digestive tubules, of Mytilus galloprovincialis exposed to each concentration (CTL, 5, 25, 125 and 625 ng/L) and both temperatures (17 and 21 ºC), stained with haematoxylin: abundance of lipofuscin aggregates (*); enlargement of the central vessel; and loss of cilia (arrows). Scale bar 50 mm.

Regarding temperature impact, *Ih* was found to be significantly higher at 21 ºC than at 17 ºC in organisms exposed to a concentration of 5.0 and 25 ng/L. The assessment of the digestive tubules (Figure 7) showed that mussels under 17 ºC, presented a progressive increase of haemocytes infiltration, atrophy, and necrosis. At 21 ºC there was also a progressive increase of haemocytes infiltration and necrosis. Under both temperatures the *Ih* obtained for mussels' digestive gland (Figure 8B) was significantly higher in the mussels exposed to 25, 125 and 625

ng/L in comparison to CTL and 5 ng/L. Comparing temperatures, *Ih* was significantly higher at 17 ºC in mussels exposed to 625 ng/L, while significantly higher values were found at 21 ºC in mussels exposed to 25 ng/L.

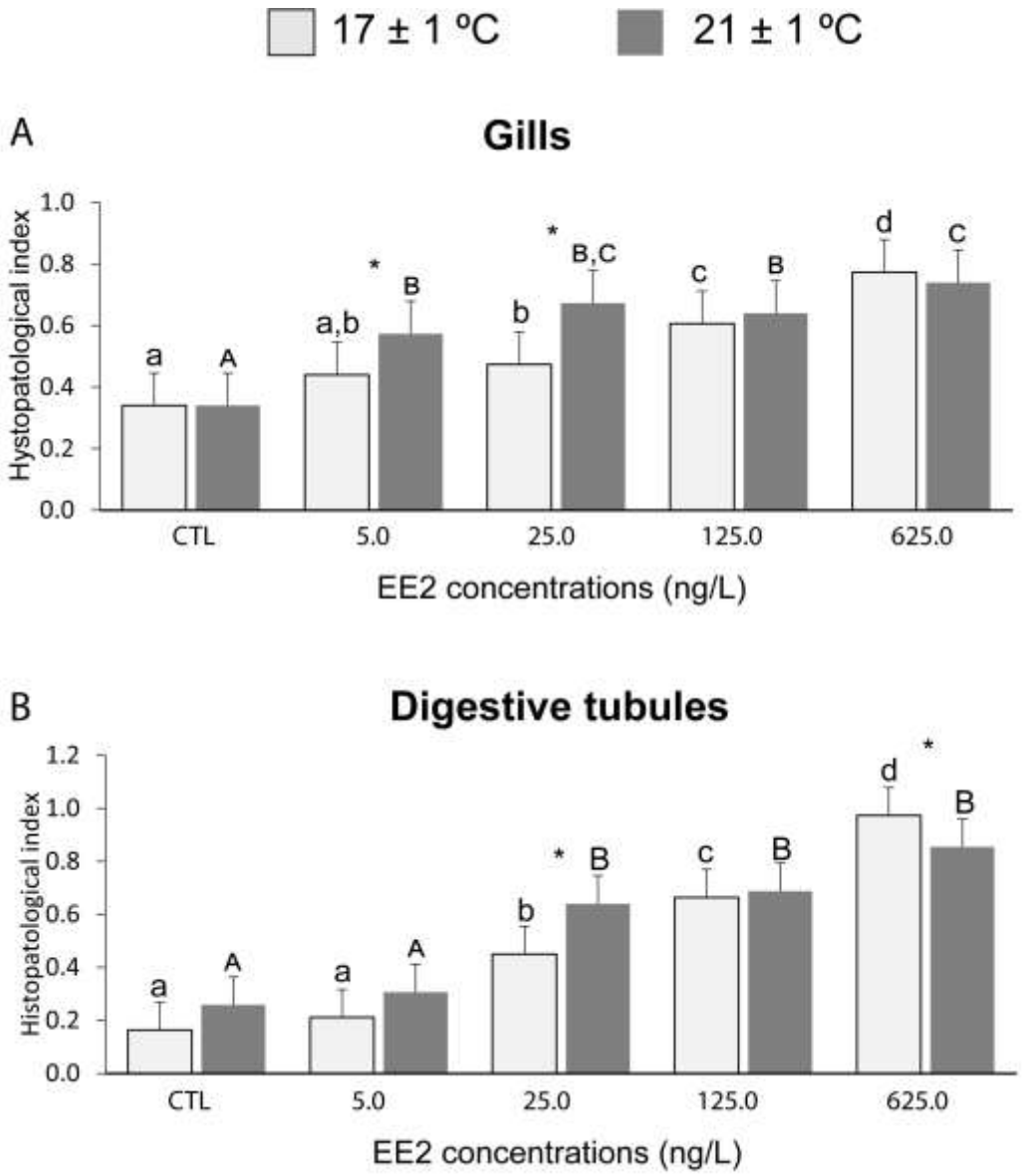

Figure 8. A: Histopathological index in gills (IhG); B: Histopathological index in digestive tubules (IhDG), in Mytilus galloprovincialis exposed to each concentration (CTL, 5, 25, 125 and 625 ng/L) and both temperatures (17 and 21 ºC). Values are mean + standard deviation. Significant differences (p < 0.05) among concentrations (CTL, 5, 25, 125 and 625 ng/L) for each temperature (17 and 21 ºC) are represented with different letters (lower case letters for 17 ºC, upper case letters for 21 ºC); while significant differences between temperatures for each EE2 concentration are represented with an asterisk.

3.1.4. Statistical analysis

The statistical analysis showed a significant interaction between temperature and EE2 concentration for each of the parameters analysed except AChE (Table 3).

Table 3. PERMANOVA results (Pseudo-F and p-value) for each parameter, obtained from an analysis with a two factors design, to test the interaction between temperature (factor 1) and EE2 concentration (factor 2).

Parameter	**Pseudo-F**	***p*-value**
ETS	6.358	0.0003
GLY	5.054	0.0014
PROT	6.644	0.0001
SOD	6.273	0.0003
CAT	3.297	0.014
GPx	3.928	0.005
GSTs	10.328	0.0001
LPO	10.407	0.0001
GSH/GSSG	3.581	0.0103
AChE	0.824	0.507
Ih Gills	4.189	0.0029
Ih Digestive tubules	5.413	0.0011

The ordination analysis, performed by non-parametric multidimensional scaling (MDS) identified two clusters, corresponding to each temperature tested. In both clusters CTL and 625 ng/L treatments are in opposite locations (Figure 9).

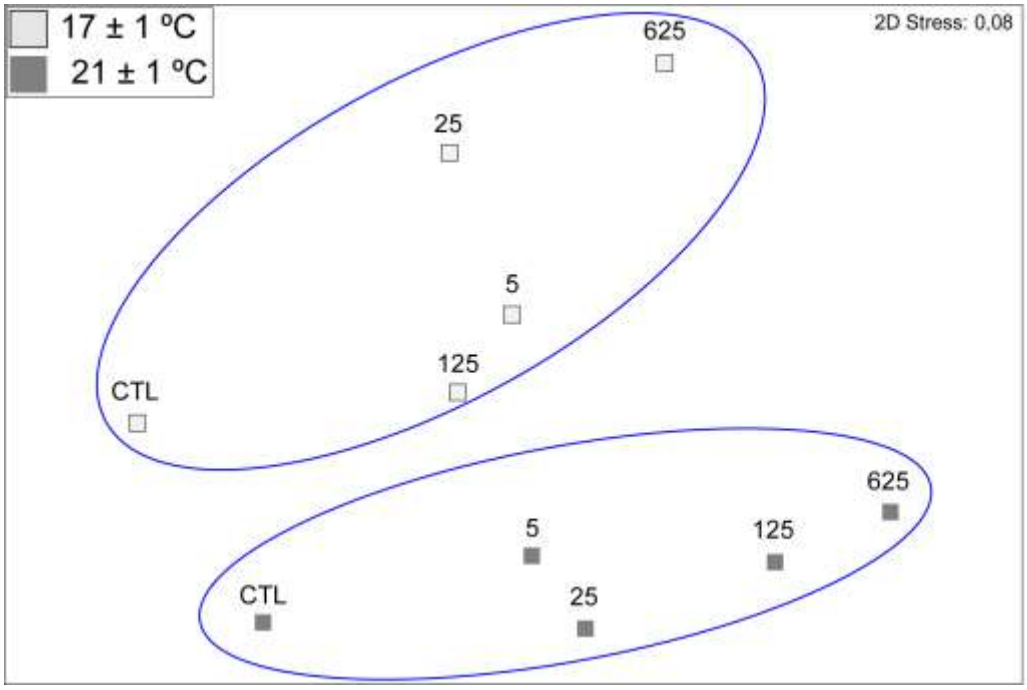

Figure 9. MDS with the average of all biochemical parameters and histopathological index of gills and digestive tubules for each treatment.

3.2. Second experiment: impacts of 17 α-Ethynylestradiol and Sodium Lauryl Sulfate

3.2.1. Concentration of 17 α-Ethynylestradiol and Sodium Lauryl Sulfate in seawater and mussel's tissues

After spiking concentrations of EE2 and SLS were reduced by 8 and 3%, respectively in the exposure medium, while in blanks after spiking concentrations of EE2 and SLS were reduced in 5 and 3% (Table S1 in supplementary material). One week after exposure, concentrations measured in blanks before water renewal revealed a reduction in concentrations up to 12% EE2 while for SLS the concentrations were similar. When both contaminants were acting together similar losses were observed, with greater values found in the exposure medium after spiking, ranging from 12% for EE2 to 6% for SLS (Table S1 in supplementary material). These results demonstrate the stability of both contaminants along one week of exposure, especially in what concerns to SLS (Table S1).

In mussels' tissues, when both contaminants were acting individually, concentrations of EE2 were higher than the ones obtained for SLS (7.9 ± 5.1 and 1.8 ± 0.8, respectively). An opposite result was observed when both contaminants were acting together, with higher SLS concentrations than those of EE2 (3.6 ± 0.6 and <Limit of detection respectively). The results obtained further revealed higher BCF values for EE2 in comparison to SLS (Table S1).

3.2.2. Behaviour alterations

The analysis of the valve's behaviour (opening and closure periods) demonstrates that contamination affected the behaviour of the organisms: immediately after spiking, contaminated mussels remained their valves closed for longer periods in comparison to non-contaminated ones, especially when in the presence of EE2 (EE2 and EE2+SLS treatments) (Figure 10). One day after spiking contaminated mussels were closed for longer periods than those exposed to control, but shorter closure periods were observed when compared to values

found for the spiking day especially noticed when in the presence of EE2 (EE2 and EE2+SLS) while mussels exposed to SLS a similar behaviour was observed comparing to values obtained immediately after spiking (Figure 10; see the QRCode in the supplementary material to watch the videos).

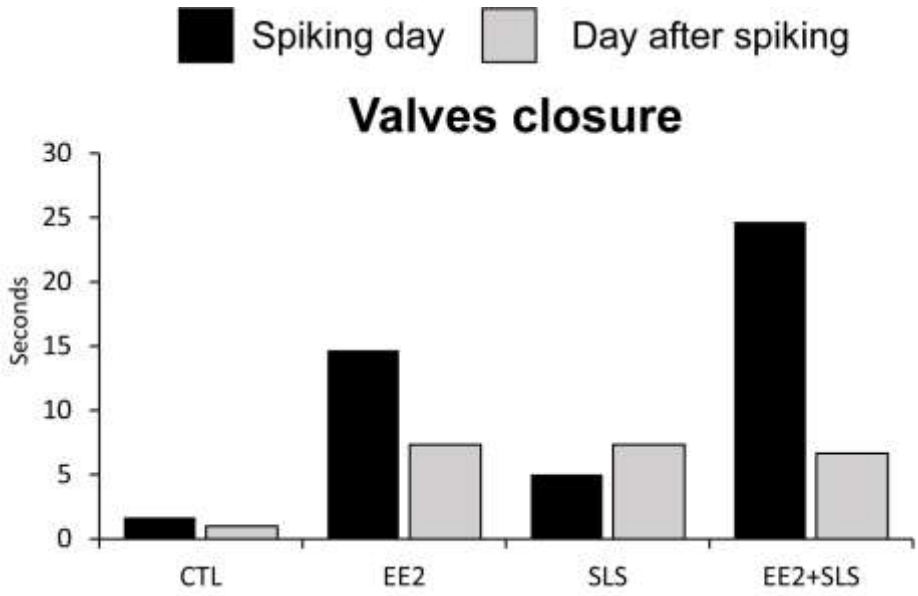

Figure 10. Valves closure time in Mytilus galloprovincialis exposed to different treatments (CTL, EE2, SLS, EE2+SLS) after spiking and 1 day after spiking.

3.2.3. Biochemical responses

3.2.3.1. Metabolic capacity and energy reserves

Regarding males, significantly higher ETS activity was only detected in organisms exposed to EE2+SLS compared to the remaining treatments (CTL, EE2, SLS), while in females significantly higher ETS values were observed in SLS compared to the remaining treatments (Figure 11A). Comparing males and females, significantly higher ETS values were observed in females at CTL, EE2 and EE2+SLS treatments (Figure 11A).

Male mussels exposed to EE2 and SLS showed significantly higher GLY levels compared to CTL and EE2+SLS, with the highest value at SLS treatment (Figure 11B). In females higher GLY content was observed in SLS exposed

organisms, with significant differences to the remaining treatments (Figure 11B). Comparing males and females, significantly higher GLY content was found in females at CTL, while an opposite pattern was observed for EE2 and SLS exposed mussels (Figure 11B).

Regarding PROT, a significant higher PROT level was found in males exposed to EE2 and SLS compared to CTL and EE2+SLS, with the highest value at SLS treatment (Figure 11C). In female mussels, significantly higher PROT level was observed at EE2+SLS treatment compared to the remaining treatments (Figure 11C). Between male and female mussels, significant differences were found at EE2 and EE2+SLS treatments, with the highest value in males for EE2 and in females for EE2+SLS (Figure 11C).

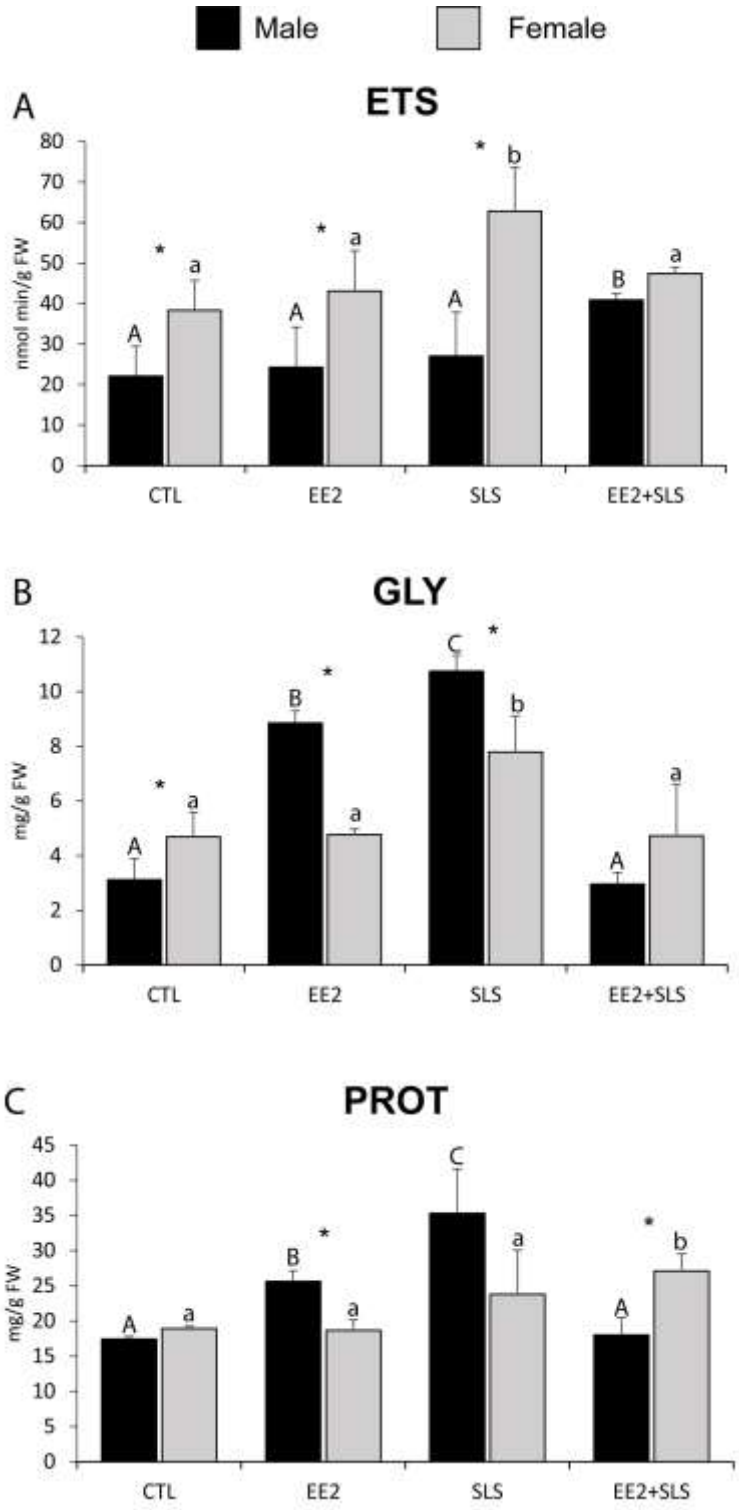

Figure 11. A: Electron transport system activity (ETS), B: Glycogen content (GLY) and C: Total Protein content (PROT), in males and females of Mytilus galloprovincialis exposed to each treatment (CTL, EE2, SLS, EE2+SLS). Values are mean + standard deviation. Significant differences among the conditions are presented with uppercase letters for males, lowercase letters for females and asterisks between males and females.

3.2.3.2. Antioxidant enzymes activity

The activity of SOD was significantly higher in contaminated males compared to control ones, with the highest values at EE2 treatment (Figure 12A). In female mussels, significant differences in SOD activity were found among all treatments, with the lowest activity in mussels exposed to EE2+SLS and the highest in mussels exposed to EE2 (Figure 12A). Between males and females, significant differences were observed at CTL and EE2+SLS treatments, with lower SOD activity in males at CTL and an opposite pattern at EE2+SLS treatment (Figure 12A).

Contaminated males showed significantly higher GRed activity than control ones (Figure 12B). In females the activity of GRed was significantly higher at EE2 and SLS treatments (Figure 12B). Comparing males and females, significant differences were observed in contaminated mussels, with higher GRed activity in males exposed to EE2 treatments (EE and EE2+SLS) while females exposed to SLS showed higher activity compared with males (Figure 12B).

Regarding GPx activity, males exposed to both contaminants showed higher values than the ones under control (Figure 12C). A similar response was found in females, with the highest values found in organisms exposed to EE2+SLS treatment (Figure 12C). Between males and females, significant differences were found at SLS and EE2+SLS treatments, with higher values in females (Figure 12C).

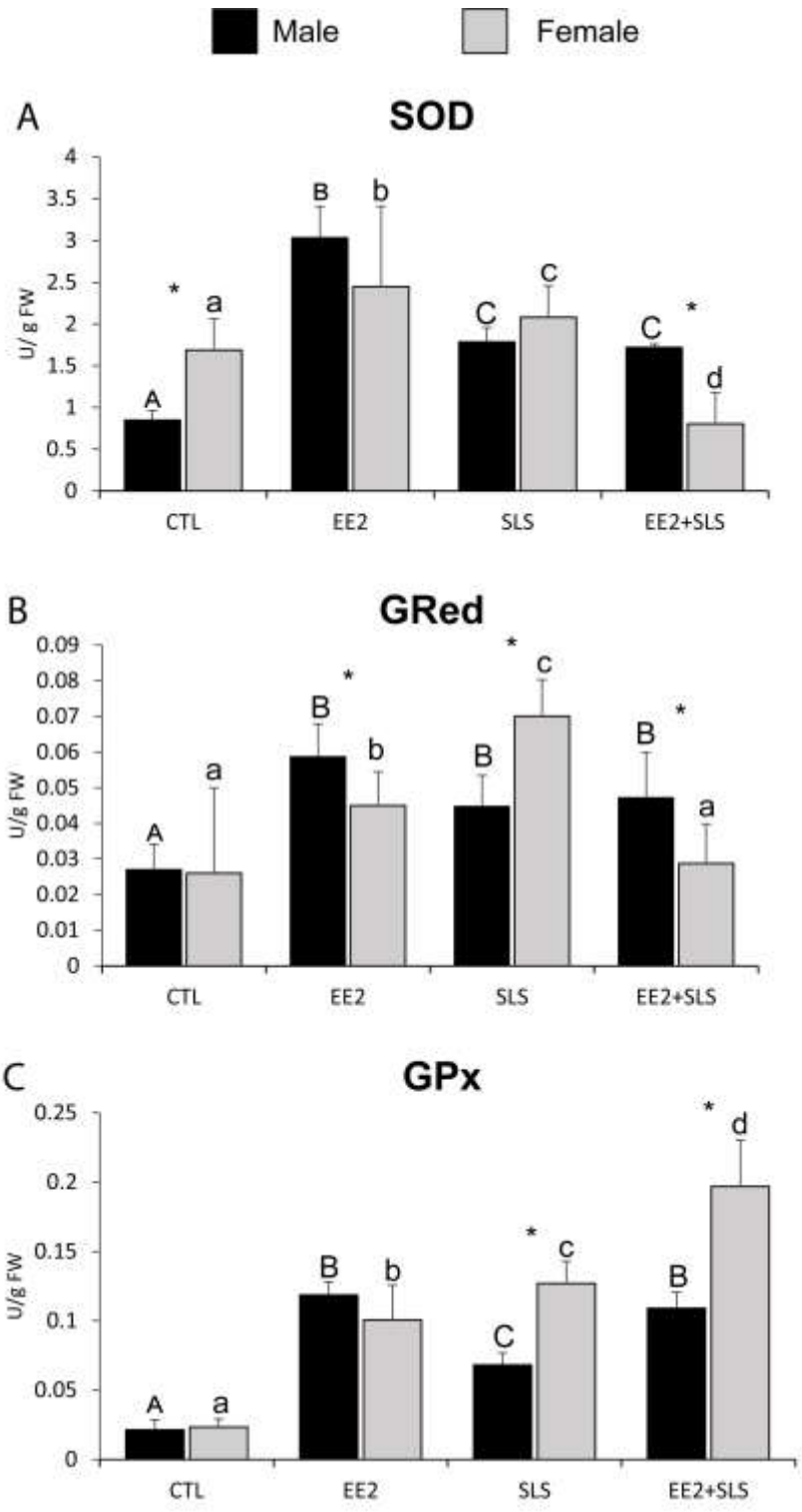

Figure 12. A: Superoxide dismutase activity (SOD), B: Glutathione reductase activity (GRed), C: Glutathione peroxidase activity (GPx), D: Carboxylesterases activity (CbEs) and E: Glutathione S-transferases activity (GSTs), in males and females of Mytilus galloprovincialis exposed to each treatment (CTL, EE2, SLS, EE2+SLS). Values are mean + standard deviation. Significant differences among the conditions are presented with uppercase letters for males, lowercase letters for females and asterisks between males and females.

3.2.3.3. Biotransformation isoenzymes

Regarding CbEs activity, male organisms exposed to EE2 showed significantly higher values compared to males exposed to the remaining treatments (Figure 13A). In females, higher CbEs activity was observed in all EE2 and SLS treatments, with the highest values at SLS exposed females (Figure 13A). Considering the mussels gender, significant differences were found at CTL, EE2 and SLS treatments, with higher values in males maintained at CTL and EE2 while higher values in females were observed at SLS treatment (Figure 13A).

Significantly higher GSTs activity was observed in males exposed to EE2 and SLS in comparison to values obtained for CTL and EE2+SLS treatments (Figure 13B). For females, higher GSTs values were found when in the presence of contaminants, with no differences among EE2, SLS and EE2+SLS treatments (Figure 13B). Between males and females, significant differences were found at EE2, SLS and EE2+SLS treatments, with the highest GSTs activity in males exposed to EE2 and SLS, while females showed higher activity at EE2+SLS treatment (Figure 13B).

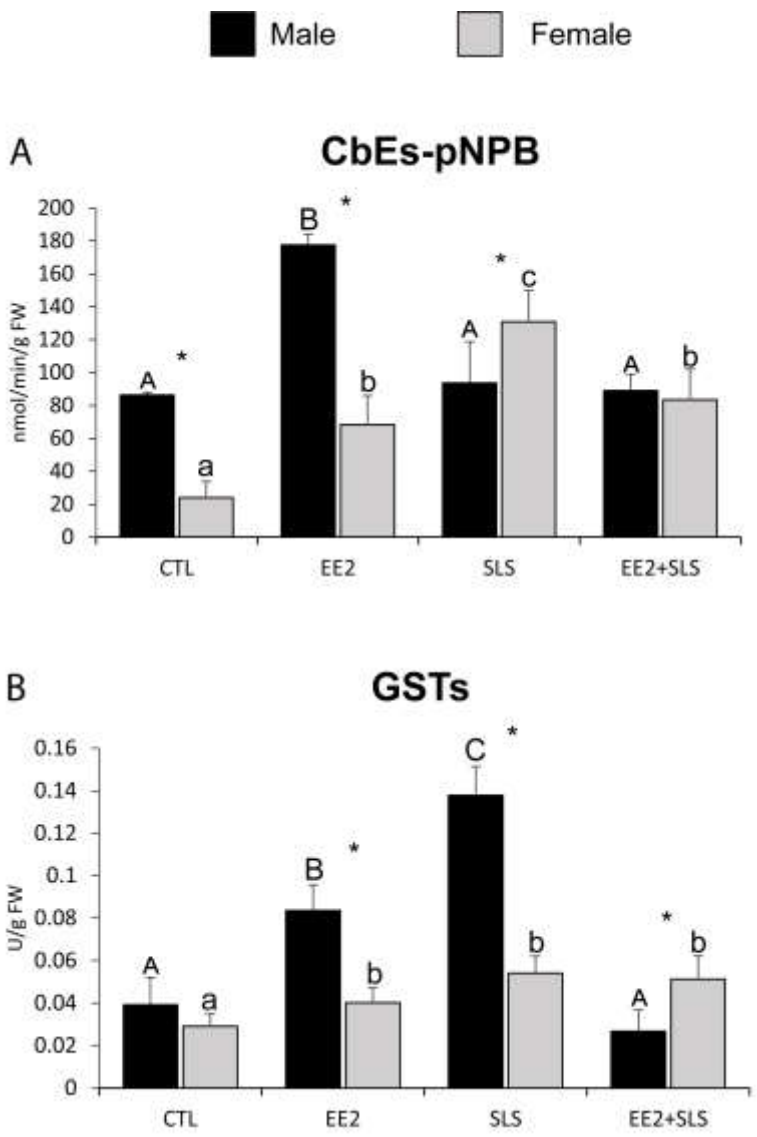

Figure 13. A: Carboxylesterases (CbEs) and B: Glutathione S-transferases (GSTs), in males and females Mytilus galloprovincialis exposed to each treatment (CTL, EE2, SLS, EE2+SLS). Values are mean + standard deviation. Significant differences among the conditions are presented with uppercase letters for males, lowercase letters for females and asterisks between males and females.

3.2.3.4. Cellular damage and redox balance

Significantly higher LPO levels were found in males exposed to EE2 and EE2+SLS compared to CTL and SLS treatments (Figure 14A). Regarding females,

significantly higher LPO was observed at SLS treatment compared to the remaining treatments (Figure 14A). Regardless the treatment, significantly higher LPO levels were found in females than in males (Figure 14A).

In males and females, significantly higher GSSG content was observed in the presence of contaminants, except for males exposed to EE2+SLS (Figure 14B). Males exposed to EE2 showed significantly higher GSSG content than females, while an opposite pattern was observed when both drugs were combined (Figure 14B).

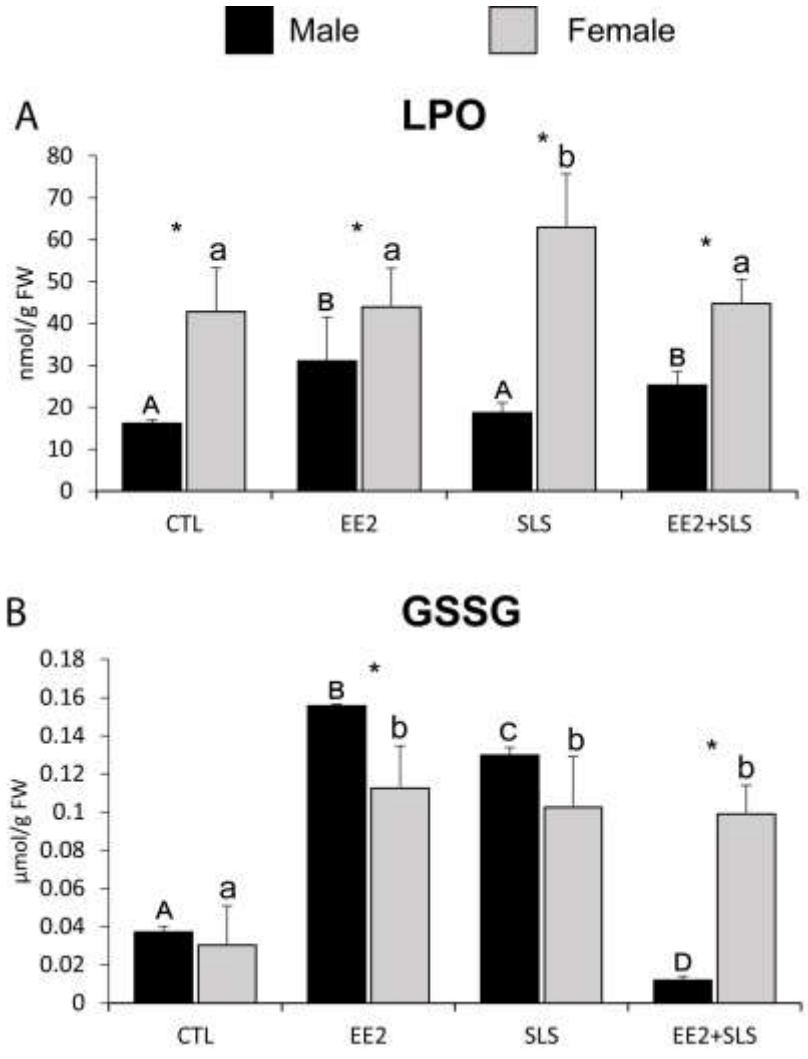

Figure 14. A: Lipid peroxidation levels (LPO) and B: Oxidized glutathione (GSSG), in males and females Mytilus galloprovincialis exposed to each treatment (CTL, EE2, SLS, EE2+SLS). Values are mean + standard deviation. Significant differences among the conditions are presented with uppercase letters for males, lowercase letters for females and asterisks between males and females.

3.2.4. Histopathological measurements

Histopathological alterations in gills are reported in Figure 15. Lipofuscin aggregates (circle) were detected in all treatments, with less incidence in EE2. Also, haemocytes infiltration (arrowhead) was found in all treatments, although less in CTL. Evident enlargement of the central vessel (double-headed arrows) and cilia loss (arrows) were observed in organisms exposed to EE2, SLS and EE2+SLS. Figure 15 reports histopathological alterations present in digestive tubules (D.T.). Lipofuscin aggregates (circle) were found in all treatments. Haemocyte infiltration (arrowhead) was observed prevalently under SLS. Atrophy (At) in digestive tubules was found mainly in SLS and EE2+SLS. No necrosis was detected.

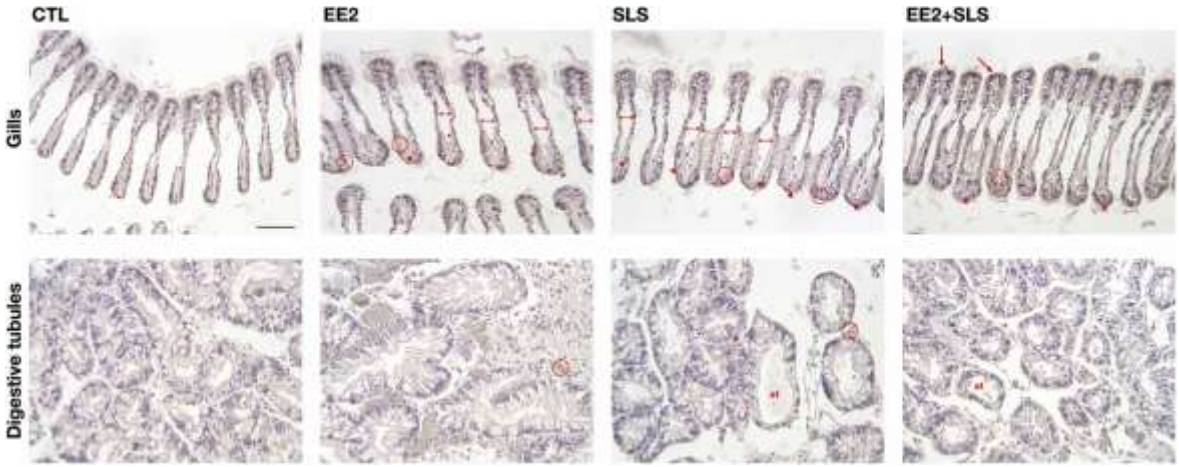

Figure 15. Micrographs of gills and digestive tubules in Mytilus galloprovincialis exposed to different treatments (CTL, EE2, SLS, EE2+SLS) stained with haematoxylin i) Gills: lipofuscin aggregates (circle); cilia lost (arrows), enlargement of the central vessel (double-headed arrows), haemocytes infiltration (arrowhead); ii) Digestive tubules: lipofuscin aggregates (circle), atrophy (At) and haemocytes infiltration (arrowhead). Scale bar = 50 µm.

Regarding histopathological index (Table 4), in gills significantly higher values were observed in contaminated mussels in comparison to control, while in digestive tubules no significant differences were found among treatments.

Table 4. Histopathological indices: Gills (Ih G); digestive tubules (Ih DT). Results are mean + standard deviation.

Treatment	*Ih* G	*Ih* DT
CTL	0.15 ± 0.02^{A}	0.13 ± 0.04^{A}
EE2	0.37 ± 0.09^{B}	0.14 ± 0.10^{A}
SLS	0.3 ± 0.06^{B}	0.25 ± 0.10^{A}
EE2+SLS	$0.\ 37 \pm 0.07^{B}$	0.18 ± 0.05^{A}

3.2.5. Quantitative RT-PCR analysis

Analysis by real-time quantitative PCR showed that the levels of Estrogen receptor 1 (ER1) gene was significantly up regulated in all treatments in digestive tubules compared to CTL (Figure 16A). In particular, EE2+SLS showed a 10 folds higher expression than CTL, and double folds expression than other treatments (Figure 16A). The Estrogen receptor 2 (ER2) gene expression in EE2 mussels was significantly lower than other treatments, furthermore SLS and EE2+SLS treatments showed a significantly higher ER2 expression compared to CTL (Figure 16A).

In gonads ER1 expression was significantly down-regulated in SLS treatment compared to CTL (Figure 16B). On the other hand, ER2 gene showed a significant up-regulation in EE2, SLS and EE2+SLS compared to CTL (Figure 16B).

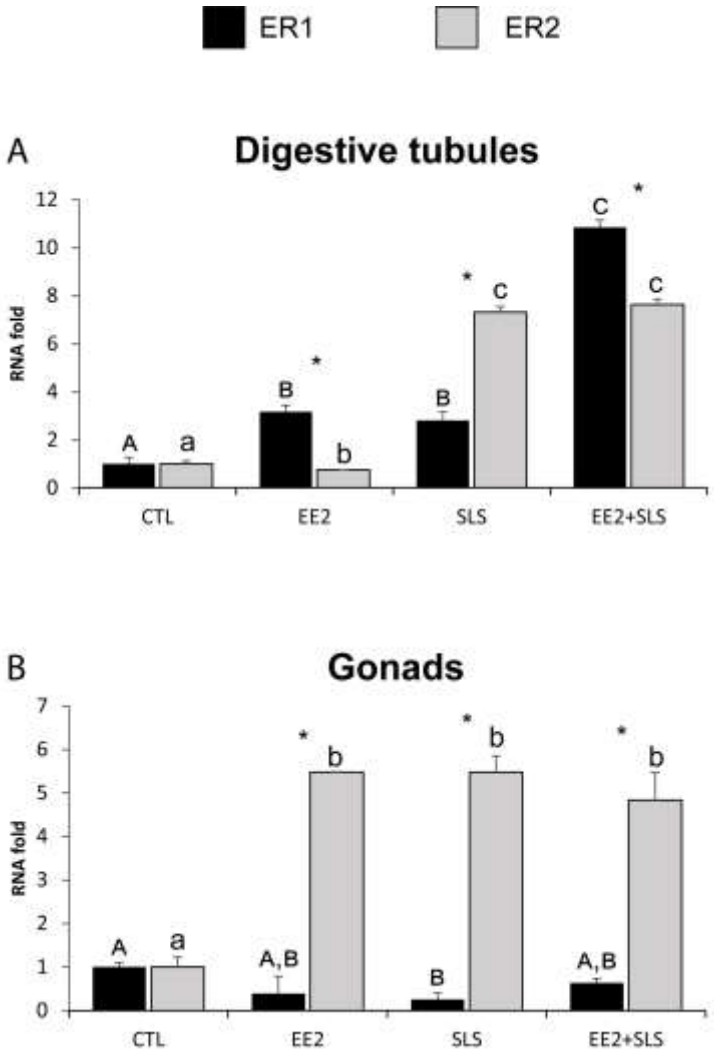

Figure 16. Relative mRNA expression levels of estrogen receptor 1 (ER1) and estrogen receptor 2 (ER2) genes in digestive tubules and gills of male Mytilus galloprovincialis exposed to each treatment (CTL, EE2, SLS, EE2+SLS) at the end of the experiment. Results are mean + standard deviation. Significant differences among the conditions are presented with uppercase letters.

3.2.6. Integrated biomarker response

In males the highest IBR value (4.90) was observed in mussels exposed to EE2, while the lowest was found in mussels exposed to SLS (0.66). By contrast, in females the lowest IBR value was found in organisms exposed to EE2 (0.62) and the highest in mussels exposed to SLS (5.33). IBR values obtained for mussels exposed to EE2+SLS were 1.70 and 1.30 for males and females, respectively.

Chapter 4

Discussion

4. Discussion

The present study evaluated the impacts induced by EE2 and SLS as well as temperature rise in the species *M. galloprovincialis* assessing mussel's EE2 and SLS bioaccumulation, histopathological alterations, biochemical effects and genetic expression, including impacts on organism's metabolism, energy reserves, oxidative and neurotoxic status.

4.1. First experiment: influence of temperature on the impacts of 17 α-Ethynylestradiol

Previous studies already demonstrated that drugs, including EE2, can stimulate intracellular ROS levels in marine bivalves (Mizutani et al., 2016; Tang et al., 2020; Zhou et al., 2021), which will result into oxidative stress. Pharmaceutical compounds as EE2 may also cause alterations on organism's metabolism (among others, Zhou et al., 2019; Annamalai and Namasivayam, 2015; Chen et al., 2017), which may impact their capacity to activate defence mechanisms, enhancing oxidative stress injuries.

The results obtained in the present study evidenced that temperature greatly influenced mussels' biochemical response to EE2, as identified by the MDS analysis, which clearly separated mussels maintained at 17 and 21 ºC in two distinct clusters, indicating a sharp discontinuity between the two groups. Furthermore, the analysis of the data revealed that for all parameters, except AChE, there was a significant interaction between temperature and EE2 concentration with the results showing that the combined effect of both stressors (temperature and EE2) lead to greater toxic impacts than the ones observed at control temperature (17 ºC). Nevertheless, our findings further revealed that EE2 was responsible for biochemical alterations regardless the temperature, with non-contaminated mussels and mussels exposed to the highest tested concentration in opposite sides. This response may be related to greatest EE2 bioaccumulation in mussels exposed to the highest exposure concentration. Furthermore,

temperature influenced the accumulation of EE2 in mussels' tissues, with lower accumulation levels found at 21 ºC. Therefore, because at 21 ºC lower accumulation was observed, greater biochemical responses observed at 21 ºC may mostly result from increased sensitivity of organisms to EE2 due to temperature rise. Lower accumulation at 21 ºC was not explained by increased biotransformation capacity, since higher GSTs activity was observed at 17 ºC, neither by lower metabolism (associated with lower filtration rate and accumulation capacity), as the lowest metabolic capacity was observed at 17 ºC. Thus, differences in accumulation levels, with higher concentrations at 17 ºC might indicate that under control temperature greater accumulation occurred and the detoxification mechanisms although activated were not sufficient to eliminate the drug. Still, more studies are needed to address the accumulation and detoxification of EE2 in marine bivalves, being important to highlight that, in the present study, differences found between temperatures were statistically significant. Previous studies already demonstrated that bivalves increased the accumulation of pollutants along an increasing exposure gradient (Velez et al., 2015, 2016), including the mussel species *M. edulis* exposed for 38 days to ~ 1 µg/L of EE2 (Ricciardi et al., 2016). This can be explained by the fact that EE2 has lipophilic properties, and it can be accumulated in a water body in a solid suspended fraction, rich in organic matter (Caban et al., 2016). Studies assessing the impacts of other pharmaceuticals under warming conditions evidenced contrasting results, with higher accumulation levels of salicylic acid in *M. galloprovincialis* mussels under increased temperatures (Freitas et al., 2020). Thus, according to current findings and results from previously published studies, it is possible to hypothesise that the effects of temperature on pharmaceuticals bioaccumulation might depend on the pollutant.

Once organisms, namely bivalves, are exposed to contaminants the production of reactive oxygen species (ROS) normally increases, leading to the activation of the antioxidant defences, including the enzymes superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) (Regoli and Giuliani, 2014). The present results suggest that at the control temperature (17 ºC), the activity of antioxidant defences was not enhanced along the increasing

exposure concentrations, with the exception of SOD that increased with the increase of EE2 exposure concentration, which may indicate that the concentrations tested were not enough to activate all the antioxidant defences, or, on the other hand, other mechanisms of defence, such as detoxification mechanisms, were enough to prevent impacts especially at higher concentrations. In particular, detoxification of EE2 by GSTs, which main function is to catalyse the conjugation of a diverse array of electrophilic compounds with glutathione (Regoli and Giuliani, 2014), was observed at 17 ºC, with higher GSTs activity in contaminated mussels exposed to 17 ºC compared to 21 ºC. Coppola et al. (2018) demonstrated that at control temperature and in the presence of arsenic, the activity of antioxidant defence such as CAT in *M. galloprovincialis* were not significantly increased which was associated with the capacity of bivalves to activate detoxification mechanisms (GSTs). It is worth of notice that higher GPx and GSTs activities at intermediate concentrations (especially noticed at 25 ng/L of EE2) may indicate an hormesis response, characterized by limited enzyme activation at low stress levels (e.g. GPx at 5 ng/L) and a decline on enzyme response capacity at the highest exposure concentrations (e.g. GPx and GSTs at 125 and 625 ng/L). Under warming conditions (21 ºC), although lower EE2 concentration was found in organisms, the present findings point out for greater impacts on mussels, with the inhibition of SOD and GSTs, while CAT was not activated, indicating the overwhelming stress due to ROS production and the incapacity of cells to activate defence mechanisms when both stressors are combined (temperature rise and EE2). At 21 ºC only GPx was activated in the presence of EE2. Inhibition of antioxidant defences were also observed in combination with temperature rise by Pirone et al. (2019) and Pes et al. (2021). Also, previous studies conducted by Morosetti et al. (2020) revealed the inhibition of GSTs when bivalves were subjected to warming conditions.

Regarding cellular damage, the results here presented showed that no cellular damage was observed in mussels exposed EE2 under control temperature, but lipid peroxidation (LPO) occurred in contaminated mussels at 21 ºC. Such response may result from the mussels' increased capacity to activate their detoxification mechanisms at control temperature, preventing organisms from

cellular damage and oxidative stress. Nevertheless, at increased temperature, cellular damages were observed which were not associated with higher EE2 accumulation but might be related with greater sensitivity of mussels to EE2 when under increased temperature, resulting into a limited biotransformation and antioxidant capacity. Previous studies conducted by Coppola et al. (2017, 2018) already showed that no LPO occurred in contaminated mussels (*M. galloprovincialis*) exposed mercury and arsenic at control temperature, while under higher temperature (21 ºC) LPO levels increased. Additionally, Freitas et al. (2017) showed higher LPO levels in *M. galloprovincialis* exposed to mercury under warm conditions compared to control temperature. Nevertheless, mussel's redox balance was strongly affected by EE2 regardless the temperature tested, which was identified by lower GSH/GSSG levels in contaminated mussels compared to control ones. Such findings highlight that although cellular damage was only observed at 21 °C, loss of redox homeostasis was observed in all EE2 treatments regardless the temperature tested.

The activity of AChE activity was analysed in order to determine if EE2 was able induce a neurotoxic effect in *M. galloprovincialis*. AChE plays a key role in the functioning of the neuromuscular system, preventing continuous muscular contraction (Aguirre-Martínez et al., 2016). The results obtained in the present study showed limited neurotoxic effects in the presence of EE2, although at 21 ºC a slight decrease on AChE activity was observed in contaminated mussels comparing to CTL ones. Again, these results point out for higher threats associated with both stressors acting together, in comparison to organisms exposed to EE2 under control temperature (17 ºC). In accordance, several other studies revealed similar impacts with inhibition of AChE activity when organisms are under the increase of temperature (Coppola et al., 2017, 2018; Freitas et al., 2017; Pirone et al., 2019).

In general, results demonstrated that the effects caused by EE2 on mussels' metabolism were higher under warmer temperature (21 ºC), most probably due to increased sensitivity of mussels under higher temperatures. Mussels exposed to the highest temperature and higher EE2 concentrations

activated their metabolism, most probably to fight against high stress levels. Under control temperature mussels tended to maintain or even reduce their metabolic capacity, which may indicate that mussels under a lower stress conditions tried to avoid accumulation by reducing their filtration rate. At increased temperature this strategy is no longer valid and mussels needed to increase their metabolism to fight against the stress. Previous studies already demonstrated that bivalves have the capacity to maintain their valves closed, to reduce the filtration rate and avoid accumulation of xenobiotics (Gosling, 2003). In fact, under moderate stress conditions and control temperature, metabolic depression was already described in mussels as a response to pollutants exposure, namely in *M. galloprovincialis*, exposed to paracetamol and acetylsalicylic acid (Piedade et al., 2020). Increased metabolic capacity was observed under greater stressful conditions, as it was showed by Piscopo et al. (2021) in *Ruditapes decussatus* exposed to caffeine. In terms of energy reserves, the present study found that mussels were able to increase the PROT content along the increasing EE2 exposure gradient, which may be associated with higher enzymes production (e.g. higher GPx activity at 21 ºC).

In terms of GLY content limited alterations were recorded, except an increase at the highest EE2 concentration and 17 ºC, which reveal the capacity of mussels to preserve this energy reserve in the presence of EE2, regardless the temperature tested. These findings pointed out that the stress induced was not enough to increase the expenditure of mussels' reserves. Previous studies already evidenced the bivalves' capacity to preserve their energy reserves when under stressful conditions, as showed by Costa et al. (2020) in *R. philippinarum and R. decussatus* exposed to triclosan combined with climate change scenario.

The evaluation of histopathological alterations is an important method to evaluate impacts of pollutants in bivalves (Cuevas et al., 2015; Leite et al., 2020). Gills are one of the major target organs for contaminants because they are in direct contact with the surrounding environment, playing an important role in respiration (Au, 2004; Rajalakshmi and Mohandas, 2005). The digestive glands of bivalves are the main organ for xenobiotic biotransformation, a mechanism of

immune defence and homeostatic regulation (Livingstone et al., 2006), that has also been extensively used for toxicity assessments (Marigómez et al., 2013). In the present study, dose-dependent histopathological alterations in gills and digestive glands of mussels exposed to EE2 and temperature were revealed. Concerning mussels' gills, the presence of EE2 mainly caused lipofuscin aggregates which, according to Höhn, and Grune (2013), may suggest oxidative stress in the affected cells, also shown by higher LPO levels observed in mussels exposed to 21 ºC at the most stressful conditions. Additionally, haemocyte infiltration was observed in gills which, according to different authors (Rocha et al., 2016; Cuevas et al., 2015; Costa et al., 2013), is associated with inflammatory responses. Therefore, the present findings are in line with other studies that already demonstrated histopathological alterations in bivalve's gills when exposed to pollutants (Amachree et al., 2014; Coppola et al., 2020; Pinto et al., 2019). Regarding the digestive tubules, for both temperatures, the exposure to EE2 mainly caused haemocytes infiltration and necrosis. At 21 ºC mussels exposed to 25, 125 and 625 ng/L showed signs of necrosis in digestive tubules, which is characterized by cellular rupture (do Amaral et al., 2019). Similarly, studies assessing impacts of contaminants, such as lanthanum (Pinto et al., 2019) cadmium based quantum dots (Rocha et al., 2016) and various metals (Coppola et al., 2020; Cuevas et al., 2015) demonstrated histopathological alterations in digestive glands in mussels.

4.2. Second experiment: impacts of 17 α-Ethynylestradiol and Sodium Lauryl Sulfate

Several studies already demonstrated that bivalves alter their physiological and biochemical performance and even their gene expression when in the presence of contaminants (Burgos-Aceves and Faggio, 2017; Freitas, et al., 2020b; Velez et al., 2016a), with few studies assessing the impacts of mixture of contaminants (Almeida et al., 2021; Freitas et al., 2019; Liu et al., 2010). Considering bivalves, a scarce number of studies evaluated the impacts of contaminants on their behaviour (Costa et al. 2020a; Freitas et al. 2020a;

Giarratano et al., 2014). Overall, the present study clearly demonstrated that the first response of mussels to the presence of contaminants is to close their valves, especially in the presence of EE2. The period of closure was longer in the spiking day than in the day after indicating that valves closure is not a strategy viable for longer periods. Nevertheless, after exposure mussels tend to remain their valves closed to avoid the entrance of contaminants, which was more evident in the presence of EE2. Previous studies developed by Costa et al. (2020a) and Freitas et al. (2020a) in *R. philippinarum* and *R. decussatus* organisms exposed to triclosan and *M. galloprovincialis* exposed to SLS, respectively, demonstrated the decrease of respiration rate in the presence of these contaminants. The capacity to close their valves, was already demonstrated in *Perna viridis* under chlorine exposure, by lowering the filtration rate and preventing bioaccumulation of toxicants, as shown by Venkatnarayanan et al. (2021). Andrade et al. (2019) evaluating the impacts of carbon nanotubes in *M. galloprovincialis* maintained for 28 days to different temperature scenarios showed no significant differences between contaminated and non-contaminated mussels regardless the temperature. Such results may indicate that the capacity to close their valves will greatly depend on the stress level, which seems to be a strategy used under low stressful conditions and/or a short term response.

Corroborating that valves closure is a short term response to the presence of contaminants and it is a strategy used under low stressful conditions, males and females maintained or even increased their metabolic capacity in the presence of contaminants. In particular, the present findings demonstrated increased ETS activity in mussels exposed to SLS (at EE2+SLS for males and at SLS for females) while maintaining their metabolic activity in the presence of EE2 (at EE2+SLS for males). Such findings may indicate that EE2 tested concentration were not stressful enough to alter mussels' metabolism and they were able to limit their filtration to avoid the entrance of this contaminant while the increase of ETS activity may indicate a need to fuel up the activation of defence mechanisms in males exposed to both contaminants (EE2+SLS) and in females exposed to SLS. Similarly, previous studies already demonstrated that when stress is induced by the presence of contaminants bivalves tend to increase their metabolism to fuel up

defence mechanisms and increase for example the activity of antioxidant and biotransformation enzymes to eliminate the excess of reactive oxygen species (ROS) produced (Regoli and Giuliani, 2014; Piscopo et al., 2021). However, previous studies also revealed that when the concentrations of exposure to a given contaminant are not too stressful bivalves can maintain and even reduce their metabolism to avoid the entrance of contaminants and limit the impacts, with no activation of the defence mechanisms (Coppola et al., 2017; Freitas et al., 2019a).

Regarding energy reserves content, the obtained results showed that in general males and females were able to avoid the expenditure of GLY and PROT contents, which may be associated with the maintenance of their metabolism in most of the treatments. Furthermore, even when ETS increased (at EE2+SLS for males and at SLS for females) it seems that there was no need for the use of these energy reserves, corroborating low stress levels induced by tested conditions, or other energy reserves, such as lipids, were used. In fact lipids are the first reserve to be used by organisms when submitted to stressful conditions (Freitas, et al., 2020a; Costa et al., 2020b; Nunes et al., 2017; Velez et al., 2016). Previous studies already evidenced the bivalves' capacity to preserve their energy reserves when under stressful conditions, as showed by Costa et al. (2020a) in *R. philippinarum* and *R. decussatus* exposed to triclosan and Nunes et al. (2017) in *R. philippinarum* exposed to paracetamol. Thus, we may hypothesize that the behaviour observed, in the present study may indicate mussels' defensive behaviour as a response to an increased stress level, reducing the expenditure of energy reserves under an adverse situation.

Biochemical performance of bivalves is normally negatively affected by the presence of xenobiotics, with increased production of ROS and consequent increase of antioxidant defences to avoid cellular damages (Thilagam et al., 2010; Fabrello et al., 2021; Pes et al., 2021). The present study clearly demonstrated that males and females exposed to EE2 and SLS, both alone or in combination, activated their antioxidant defences. While SOD was mostly enhanced both in males and females exposed to EE2, GRed was particularly activated in females

exposed to SLS, while GPx presented the highest values in females exposed to the mixture of both contaminants. Overall, activation of different enzymes in mussels regardless the contamination treatment revealed that although their metabolism was not increased at all treatments (except for SLS in females and EE2+SLS for males) they were still able to activate their mechanisms of defence. Different studies already demonstrated that the presence of pharmaceuticals and surfactants can activate bivalve's antioxidant mechanisms, including in *M. galloprovincialis* exposed to Cyclophosphamide (Queirós et al., 2021), in *M. galloprovincialis* exposed to sodium dodecylbenzene sulfonate and sodium dodecyl sulfate (Liu et al., 2010) and in *M. galloprovincialis* exposed to SLS (Freitas, et al., 2020a).

In terms of biotransformation, both males and females presented higher enzymes activity in the presence of contaminants, with males showing higher capacity than females with higher values for CbEs in males exposed to EE2 and in males exposed to SLS for GSTs. Similarly, the increase of GSTs activity in *R. philippinarum* was observed after exposure to single and mixture of cetirizine and carbamazepine (Almeida et al., 2021), and Queirós et al. (2021) observed increased CbEs activity at the highest concentration in *M. galloprovincialis* exposed to Cyclophosphamide.

Although increased antioxidant and biotransformation capacity was observed in contaminated males and females LPO occurred in males exposed to EE2 (EE2 and EE2+SLS treatments) and in females exposed o SLS treatments. These results indicated that under these treatments the mechanisms of defence activated by mussels were not able to prevent the occurrence of cellular damage and the excess of reactive oxygen species produced caused oxidation of the membrane lipids. Similarly, higher LPO levels were observed in *Lateolabrax japonicus* and *Oryzias javanicus* exposed to 17β estradiol (Thilagam et al., 2010; Woo et al., 2012), as well as in *M. galloprovincialis* exposed to SLS (Freitas et al., 2020a), although defence mechanisms were activated.

Accompanying cellular damages, there was a clear loss of redox balance in males and females regardless the treatment (except for males exposed to

EE2+SLS). Such findings highlight to the toxic capacity of both contaminants which was already demonstrated by Freitas et al. (2020a) and Freitas et al. (2021), under SLS exposure in *M. galloprovencialis* during 28 days, also Brew et al. (2019) exposed EE2 in *C. virginica* in a 10 - days exposure and Cabas et al. (2012), under EE2 in *Sparus aurata* for 29 days.

In terms of histopathological alterations, results obtained demonstrated that EE2 and SLS induced histopathological alterations in gills and digestive glands of mussels. Concerning mussels' gills, EE2 acting alone or in the mix, was the contaminant that affected the most, with histopathological alterations from lipofuscin aggregates, haemocyte infiltration and enlargement of the central vessel. Additionally, in digestive tubules, results showed that SLS caused the highest impact. Other studies, evaluating histopathological effects of pharmaceutical in bivalves also revealed abnormalities such as gill deformations, gonadal atresia, atrophy and vacuolisation in digestive glands exposed to diclofenac in *M. trossulus* (Świacka et al., 2020), also widespread follicle dilatation in gonads, follicle dilatation, gamete degeneration and hemocytic infiltration exposed to paracetamol in *M. edulis* (Koagouw et al., 2021). Therefore, the histopathological alterations in bivalve's gills are more evident probably because they are in direct contact with the surrounding environment, playing a crucial role in respiration (Rajalakshmi and Mohandas, 2005; Giarratano et al., 2014).

In bivalves, the gonadal development as well as gamete maturation and release, seems to be under sex steroids control (Agnese et al., 2019). Moreover, no-reproductive organs, such as digestive gland, express steroids receptors too (Nagasawa et al., 2015). Thus, to elucidate possible changes in gene expression following exposure to EE2, SLS and mix of both, the estrogen receptors genes ER1 and ER2 were selected, and their expression levels in gonad and digestive tubules of male mussels were investigated with RT-PCR analysis. In the present study expression of ER1 and ER2 changed in both tissues. In particular, ER1 expression was induced manly by EE2 in digestive tubules, with the higher expression in the presence of both contaminants, probably due to a synergistic action of EE2 and SLS. In gonads ER1 was weakly down expressed in presence

of SLS, probably due to the fact that gonads were at a spent stage, as in mussels *M. galloprovincialis* ER1 expression changes during the gonadic cycle (Agnese et al., 2019). On the other hand, ER2 was over expressed in digestive tubules exposed to SLS, this could be explained by the estrogenic-like action of SLS (Tasneem et al., 2018). Instead in gonads, ER2 was over expressed under all treatments conditions, in according to Ciocan et al. (2010) who demonstrated ER2 overexpression in gonad tissue of *M. edulis* exposed to 200 ng/L EE2. Since studies revealed a correlation between ERs expression levels and pollution, suggesting their role as biomarkers of reproductive health (Guerriero et al.,2015; Cocci et al., 2017), the present results highlight possible consequences on reproductive outcomes following exposure to common seawater contaminants such as CDCs and PCPs. Furthermore, the changes in ERs expression found in digestive tubules of *M. galloprovincialis* suggest that also a no reproductive organ can be use as marker of estrogenic and/or estrogenic-like compounds contamination.

Chapter 5

Conclusion

5. Conclusion

This study provides information concerning the potential risk of EE2, SLS and warming conditions for the aquatic environment and inhabiting organisms, by assessing biochemical, histopathological, behavioural and genetic effects in *Mytilus galloprovincialis*, a species with high ecological and economical relevance.

When organisms are exposed to EE2 at two different temperatures, higher toxic impacts are revealed under warming conditions in comparison to mussels exposed control temperature, suggesting that temperature rise may significantly increase the sensitivity of bivalves towards pollutants and the existence of an interactive effect induced by the combination of EE2 and temperature. This is demonstrated by the histopathological alterations, inhibition of SOD, greater LPO levels, higher metabolic capacity and decreased AChE activity in the presence of EE2 at 21 ºC compared to mussels exposed to control temperature.

The present study further demonstrates the impacts of effects of a pharmaceutical drug (EE2) and personal care product (SLS), acting alone or combined, to the bivalve *M. galloprovincialis*. Results showed that both contaminants represent a threat to mussels. EE2 is more harmful to females and SLS to males, causing alterations on their behaviour (valves closure), metabolic capacity and oxidative status. Nevertheless, it was not evident a synergistic effect when both contaminants were acting together. Moreover, regarding histopathological alterations in bivalve's gills with both contaminants induced negative impacts in mussels, corroborating qPCR analysis that showed clear response to contaminants exposure.

Overall, the present study represents a step forward to the knowledge on the effect of predicted warming conditions on the impact of pharmaceuticals and personal care products, revealing that the presence of EE2 and SLS in aquatic systems will clearly influence the health status of mussels, which may have consequences to their reproductive and feeding capacity, growth and, consequently, mussels health and survival, which may eventually result in

biodiversity loss and socio-economic impacts in cultures of this species.

In future works it is important to investigate the effects of EE2 and SLS in combination with other climate stressors, such as pH and salinity variations.

Chapter 6

References

6. References

Adeel, M., Song, X., Wang, Y., Francis, D., Yang, Y., 2017. Environmental impact of estrogens on human, animal and plant life: A critical review. Environment International, 99, 107–119.

Agnese, M., Rosati, L., Prisco, M., Borzacchiello, L., Abagnale, L., Andreuccetti, P., 2019. The expression of estrogen receptors during the *Mytilus galloprovincialis* ovarian cycle. Journal of Experimental Zoology Part A: Ecological and Integrative Physiology, 331(7), 367–373.

Aguirre-Martínez, G. V., DelValls, T.A., Martín-Díaz, M. L., 2016. General stress, detoxification pathways, neurotoxicity and genotoxicity evaluated in *Ruditapes philippinarum* exposed to human pharmaceuticals. Ecotoxicology and Environmental Safety, 124, 18–31.

Ahmad, I., Mohmood, I., Mieiro, C. L., Coelho, J. P., Pacheco, M., Santos, M. A., Duarte, A. C. Pereira, E., 2011. Lipid peroxidation vs. antioxidant modulation in the bivalve *Scrobicularia plana* in response to environmental mercury-organ specificities and age effect. Aquatic toxicology, 103(3-4), 150-158.

Almeida, Â., Calisto, V., Esteves, V.I., Schneider, R.J., Figueira, E., Soares, A.M.V.M., Freitas, R., 2021. Can ocean warming alter sub-lethal effects of antiepileptic and antihistaminic pharmaceuticals in marine bivalves? Aquatic Toxicology, 230, 105673.

Almeida, Â., Freitas, R., Calisto, V., Esteves, V.I., Schneider, R.J., Soares, A.M., Figueira, E., Campos, B., Barata, C., 2018. Effects of carbamazepine and cetirizine under an ocean acidification scenario on the biochemical and transcriptome responses of the clam *Ruditapes philippinarum*. Environmental Pollution, 235, 857–868.

Almeida, Â., Silva, M.G., Soares, A.M.V.M., Freitas, R., 2020. Concentrations levels and effects of 17 alpha-Ethinylestradiol in freshwater and marine waters and bivalves: A review. Environmental Research, 185, 109316.

Almeida, E., Bainy, A.C.D., Loureiro, A.P.M., Martinez, G.R., Miyamoto, S., Onuki, J., Barbosa, L.F., Garcia, C.C.M., Prado, F.M., Ronsein, G.E., Sigolo, C.A., Brochini, C.B., Martins, A.M.G., Medeiros, M.H.G.M., Di Mascio, P., 2007.

Oxidative stress in Perna perna and other bivalves as indicators of environmental stress in the Brazilian marine environment: Antioxidants, lipid peroxidation and DNA damage. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 146, 588– 600.

Amachree, D., Moody, A.J., Handy, R.D., 2014. Comparison of intermittent and continuous exposures to inorganic mercury in the mussel, *Mytilus edulis*: accumulation and sub-lethal physiological effects. Ecotoxicology and Environmental Safety. 109, 133e142.

Anderson, M.J., Gorley, R.N., Clarke, K.R., 2008. PERMANOVA+ for PRIMER: Guide to Software and Statistical Methods. PRIMER-E, Plymouth.

Andrade, M., De Marchi, L., Pretti, C., Chiellini, F., Morelli, A., Figueira, E., Rocha, R.J.M., Soares, A.M.V.M., Freitas, R., 2019. The impacts of warming on the toxicity of carbon nanotubes in mussels. Marine Environmental Research, 145, 11-21.

Andrade, M., Soares, A., Figueira, E., Freitas, R., 2018. Biochemical changes in mussels submitted to different time periods of air exposure. Environmental Science and Pollution Research, 25, 8903 - 8913.

Ani, C.J., Robson, B., 2021. Responses of marine ecosystems to climate change impacts and their treatment in biogeochemical ecosystem models. Marine Pollution Bulletin, 166, 112223.

Annamalai, J., Namasivayam, V., 2015. Endocrine disrupting chemicals in the atmosphere: Their effects on humans and wildlife. Environment International, 76, 78–97.

Arand, M., Friedberg, T., Oesch, F., 1992. Colorimetric quantitation of trace amounts of sodium lauryl sulfate in the presence of nucleic acids and proteins. Analytical Chemistry, 207, 73-75.

Aris, A.Z., Shamsuddin, A.S., Praveena, S.M., 2014. Occurrence of 17α-ethynylestradiol (EE2) in the environment and effect on exposed biota: A review. Environment International, 69, 104–119.

Au, D.W.T., Pollino, C.A., Wu, R.S.S., Shin, P.K.S., Lau, S.T.F., Tang, J.Y. M., 2004. Chronic effects of suspended solids on gill structure, osmoregulation,

growth, and triiodothyronine in juvenile green grouper *Epinephelus coioides*. Marine Ecology Progress Series, 266, 255-264.

Auriol, M., Filali-Meknassi, Y., Tyagi, R.D., Adams, C.D., Surampalli, R.Y., 2006. Endocrine Disrupting Compounds Removal from Wastewater, a New Challenge. Process Biochemistry, 41, 525–539.

Banni, M., Hajer, A., Sforzini, S., Oliveri, C., Boussetta, H., Viarengo, A., 2014. Transcriptional expression levels and biochemical markers of oxidative stress in *Mytilus galloprovincialis* exposed to nickel and heat stress. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 160, 23–29.

Beauchamp, C., Fridovich, I., 1971. Superoxide dismutase: Improved assays and an assay applicable to acrylamide gels. Analytical Biochemistry, 44, 276-87.

Beliaeff, B., Burgeot, T., 2002. Integrated biomarker response: a useful tool for ecological risk assessment. Environmental Toxicology and Chemistry, 21, 1316–1322.

Benali, I., Boutiba, Z., Grandjean, D., de Alencastro, L.F., Rouane-Hacene, O., Chèvre, N., 2017. Spatial distribution and biological effects of trace metals (Cu, Zn, Pb, Cd) and organic micropollutants (PCBs, PAHs) in mussels *Mytilus galloprovincialis* along the Algerian west coast. Marine Pollution Bulletin, 115, 539–550.

Bindoff, N.L., Willebrand, J., Artale, V., Cazenave, A., Gregory, J., Gulev, S., Hanawa, K., LeQuere, C., Levitus, S., Nojiri, Y., Shum, C.K., Talley, L.D., Unnikrishnan, A.S., 2007. Observations: oceanic climate change and sea level. In: Solomon, S., Qin, D., Manning, M., Chen, Z., Marquis, M. (Eds.), Climate Change 2007: the physical science basis: contribution of working group I to the fourth assessment report of the intergovernmental panel on climate change. Cambridge Univ. Press, Cambridge, 385-432.

Boretti, A., Rosa, L., 2019. Reassessing the projections of the World Water Development Report. Npj Clean Water, 2.

Boxall, A. B. A., Rudd, M. A., Brooks, B. W., Caldwell, D. J., Choi, K., Hickmann, S., Innes, E., Ostapyk, K., Staveley, J. P., Verslycke, T., Ankley, G. T., Beazley, K. F., Belanger, S. E., Berninger, J. P., Carriquiriborde, P., Coors, A., DeLeo, P. C., Dyer, S. D., Ericson, J. F., Gagné F., Giesy, J.P., Gouin, T.,

Hallstrom, L., Karlsson, M.V., Joakim Larsson, D. G., Lazorchak, J.M., Mastrocco, F., McLaughlin, A., McMaster, M.E., Meyerhoff, R.D., Moore, R., Parrott, J.L., Snape, J.R., Murray-Smith, R., Servos, M.R., Sibley, P.K., Straub, J.O., Szabo, N.D., Topp, E., Tetreault, G.R., Trudeau, V.L., Van Der Kraak, G., 2012. Pharmaceuticals and personal care products in the environment: what are the big questions? Environmental Health Perspectives. 120, 1221-1229.

Bramwell, G., Schultz, A. G., Sherman, C. D. H., Giraudeau, M., Thomas, F., Ujvari, B., Dujon, A.M., 2021. A review of the potential effects of climate change on disseminated neoplasia with an emphasis on efficient detection in marine bivalve populations. Science of the Total Environment, 775, 145134.

Brausch, J.M., Rand, G.M., 2011. A review of personal care products in the aquatic environment: environmental concentrations and toxicity. Chemosphere 82, 1518–1532.

Breda, S., Chiesa, S., Freitas, R., Figueira, E., Becherini, F., Gobbo, L., Soares, A. M. V. M., Argese, E., 2018. Biogeochemical dynamics and bioaccumulation processes in Manila clam: Implications for biodiversity and ecosystem services in the Ria de Aveiro Lagoon. Estuarine, Coastal and Shelf Science, 209, 136–148.

Brew, D.W., Henderson, W.M., Santos, M., Rodgers, J., Black, M.C., 2019. Metabolomic investigations of the temporal effects of exposure to pharmaceuticals and personal care products and their mixture in the eastern oyster (*Crassostrea virginica*). Environmental Toxicology and Chemistry, 39, 419–436.

Brunelli, E., Talarico, E., Corapi, B., Perrotta, I., Tripepi, S., 2008. Effects of a sublethal concentration of sodium lauryl sulphate on the morphology and Na+/K+ ATPase activity in the gill of the ornate wrasse (*Thalassoma pavo*). Ecotoxicology and Environmental Safety, 71, 436–445.

Burgos-Aceves, M.A., Faggio, C., 2017. An approach to the study of the immunity functions of bivalve haemocytes: Physiology and molecular aspects. Fish Shellfish Immunology, 67, 513–517.

Caban, M., Szaniawska, A., Stepnowski, P., 2016. Screening of 17α-ethynylestradiol and non-steroidal anti-inflammatory pharmaceuticals

accumulation in *Mytilus edulis trossulus* (Gould, 1890) collected from the Gulf of Gdánsk. Oceanological and Hydrobiological Studies, 45, 605–614.

Cabas, I., Liarte, S., García-Alcázar, A., Meseguer, J., Mulero, V., 2012. 17 α-Ehynylestradiol alters the immune response of the teleost gilthead seabream (*Sparus aurata L.*) both *in vivo* and *in vitro*. Developmental & Comparative Immunology, 36, 547–556.

Caldwell, D.J., Mastrocco, F., Anderson, P.D., Länge, R., Sumpter, J.P., 2012. Predicted-no-effect concentrations for the steroid estrogens estrone, 17β-estradiol, estriol, and 17α-ethinylestradiol. Environmental Toxicology and Chemistry, 31, 1396–1406.

Carlberg, I., Mannervik, B., 1985. Glutathione reductase. Methods Enzymology, 113, 484–490.

Carregosa, V., Figueira, E., Gil, A.M., Pereira, S., Pinto, J., Soares, A.M.V.M., Freitas, R., 2014. Tolerance of *Venerupis philippinarum* to salinity: osmotic and metabolic aspects. Comparative Biochemistry and Physiology - Part A: Molecular & Integrative Physiology, 171, 36–43.

Catsiki, V.A., Florou, H., 2006. Study on the behavior of the heavy metals Cu, Cr, Ni, Zn, Fe, Mn and 137Cs in an estuarine ecosystem using *Mytilus galloprovincialis* as a bioindicator species: the case of Thermaikos gulf, Greece. Journal of Environmental Radioactivity, 86, 31–44.

Chakraborty, S., Gasparatos, A., Blasiak, R., 2020. Multiple values for the management and sustainable use of coastal and marine ecosystem services. Ecosystem Services, 41, 101047.

Chaturvedi, V., Kumar, A., 2010. Toxicity of Sodium Dodecyl Sulfate in fishes and animals. International Journal of Applied Biology, 630–633.

Chen, Y., Li, M., Yuan, L., Xie, Y., Li, B., Xu, W., Wang, R., 2017. Growth, blood health, antioxidant status and immune response in juvenile yellow catfish *Pelteobagrus fulvidraco* exposed to α-ethinylestradiol (EE2). Fish and Shellfish Immunology, 69, 1–5.

Ciocan, C.M., Cubero-Leon, E., Puinean, A.M., Hill, E.M., Minier, C., Osada, M., Fenlon, K., Rotchell, J.M., 2010. Effects of estrogen exposure in mussels,

Mytilus edulis, at different stages of gametogenesis. Environmental Pollution, 158, 2977–2984.

Cizmas, L., Sharma, V.K., Gray, C.M., McDonald, T.J., 2015. Pharmaceuticals and personal care products in waters: occurrence, toxicity, and risk. Environmental Chemistry Letters, 13, 381-394.

Clarke, K. R., Warwick, R. M., 1994. Similarity-based testing for community pattern: the two-way layout with no replication. Marine Biology, 118, 167-176.

Cocci, P., Capriotti, M., Mosconi, G., Palermo, F.A., 2017. Transcriptional variations in biomarkers of *Mytilus galloprovincialis* sampled from Central Adriatic coastal waters (Marche region, Italy). Biomarkers, 22, 537–547.

Collins, M., Knutti, R., Arblaster, J., Dufresne, J. L., Fichefet, T., Friedlingstein, P., Booth, B. B., 2013. Long-term climate change: projections, commitments and irreversibility. In Climate Change 2013-The Physical Science Basis: Contribution of Working Group I to the Fifth Assessment Report of the Intergovernmental Panel on Climate Change (1029-1136). Cambridge University Press.

Coppola, F., Almeida, Â., Henriques, B., Soares, A.M., Figueira, E., Pereira, E., Freitas, R., 2017. Biochemical impacts of Hg in *Mytilus galloprovincialis* under present and predicted warming scenarios. Science of the Total Environment, 601, 1129–1138.

Coppola, F., Almeida, Â., Henriques, B., Soares, A.M.V.M., Figueira, E., Pereira, E., Freitas, R., 2018. Biochemical responses and accumulation patterns of *Mytilus galloprovincialis* exposed to thermal stress and Arsenic contamination. Ecotoxicology and Environmental Safety, 147, 954–962.

Coppola, F., Bessa, A., Henriques, B., Russo, T., Soares, A.M.V.M., Figueira, E., Marques, P., Polese, G., Di Cosmo, A., Pereira, E., Freitas, R., 2020. Oxidative stress, metabolic and histopathological alterations in mussels exposed to remediated seawater by GO-PEI after contamination with mercury. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 243, 110674.

Coppola, F., Tavares, D.S., Henriques, B., Monteiro, R., Trindade, T., Soares, A.M.V.M., Figueira, E., Polese, G., Pereira, E., Freitas, R., 2019.

Remediation of arsenic from contaminated seawater using manganese spinel ferrite nanoparticles: ecotoxicological evaluation in *Mytilus galloprovincialis*. Environmental Research, 175, 200-212.

Costa, D.D.M., Neto, F.F., Costa, M.D.M., Morais, R.N., Garcia, J.R.E., Esquivel, B.M., Ribeiro, C.A.O., 2010. Vitellogenesis and other physiological responses induced by 17-β-estradiol in males of freshwater fish *Rhamdia quelen*. Comparative Biochemistry and Physiology Part C: Toxicology, 151, 248–257.

Costa, P.M., Carreira, S., Costa, M.H., Caeiro, S., 2013. Development of histopathological indices in a commercial marine bivalve (*Ruditapes decussatus*) to determine environmental quality. Aquatic Toxicology, 126, 442–454.

Costa, S., Coppola, F., Pretti, C., Intorre, L., Meucci, V., Soares, A.M.V.M., Solé, M., Freitas, R., 2020a. Biochemical and physiological responses of two clam species to Triclosan combined with climate change scenario. Science of the Total Environment, 724, 138143.

Costa, S., Coppola, F., Pretti, C., Intorre, L., Meucci, V., Soares, A. M. V. M., Freitas, R., Solé, M., 2020b. The influence of climate change related factors on the response of two clam species to diclofenac. Ecotoxicology and Environmental Safety, 189, 109899.

Cserháti, T., Forgács, E., Oros, G., 2002. Biological activity and environmental impact of anionic surfactants. Environment International, 28, 337–348.

Cuevas, N., Zorita, I., Costa, P.M., Franco, J., Larreta, J., 2015. Development of histopathological indices in the digestive gland and gonad of mussels: integration with contamination levels and effects of confounding factors. Aquatic Toxicology, 162, 152–164.

Czarny, K., Szczukocki, D., Krawczyk, B., Skrzypek, S., Gadzała-Kopciuch, R., 2019. Toxic effects of single animal hormones and their mixtures on the growth of *Chlorella vulgaris* and *Scenedesmus armatus*. Chemosphere 224, 93–102.

D'Odorico, P., Carr, J., Dalin, C., Dell'Angelo, J., Konar, M., Laio, F., Ridolfi, L., Rosa, L., Suweis, S., Tamea, S., Tuninetti, M., 2019. Global virtual water trade and the hydrological cycle: Patterns, drivers, and socio-environmental impacts. Environmental Research Letters, 14, 053001.

Dayton, P., Curran, S., Kitchingman, A., Wilson, M., Catenazzi, A., Birkeland, C., Blaber, S., Saifullah, S., Branch, G., Boersma, D., Nixon, S., Dugan, P., Davidson, N., Vörösmarty, C., 2005. Coastal systems. In: Ecosystems and Human Well-being: Current State and Trends, 19, 513–549.

De Coen, W., Janssen, C., 1997. The use of biomarkers in Daphnia magna toxicity testing. IV. Cellular Energy Allocation: a new methodology to assess the energy budget of toxicant-stressed Daphnia populations. Journal of Aquatic Ecosystem Stress and Recovery, 6, 43–55.

Desbiolles, F., Malleret, L., Tiliacos, C., Wong-Wah-Chung, P., Laffont-Schwob, I., 2018. Occurrence and ecotoxicological assessment of pharmaceuticals: Is there a risk for the Mediterranean aquatic environment? Science of The Total Environment, 639, 1334– 1348.

do Amaral, Q.D.F., Da Rosa, E., Wronski, J.G., Zuravski, L., Querol, M.V.M., dos Anjos, B., de Andrade, C.F.F., Machado, M.M., de Oliveira, L.F.S., 2019. Golden mussel (*Limnoperna fortunei*) as a bioindicator in aquatic environments contaminated with mercury: cytotoxic and genotoxic aspects. Science of the Total Environment, 675, 343-353.

Dubois, M., Gilles, K.A., Hamilton, J.K.J., Rebers, P.A., Smith, F., 1956. Colorimetric method for determination of sugars and related substances. Analytical Chemistry, 28, 350–356.

Ebele, A.J., Abou-Elwafa Abdallah, M., Harrad, S., 2017. Pharmaceuticals and personal care products (PPCPs) in the freshwater aquatic environment. Emerging Contaminants, 3, 1–16.

Ellman, G.L., Courtney, K.D., Andres, V., Featherstone, R.M., 1961. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochemical Pharmacology, 7, 88–95.

Fabbri, E., Franzellitti, S., 2016. Human pharmaceuticals in the marine environment: focus on exposure and biological effects in animal species. Environmental Toxicology and Chemistry, 35, 799–812.

Fabrello, J., Masiero, L., Finos, L., Gabriella, M., Matozzo, V., 2021. Effects of a mixture of glyphosate, 17 α-ethynylestradiol and amyl salicylate on cellular

and biochemical parameters of the mussel *Mytilus galloprovincialis*. Marine Environmental Research, 165, 105247.

FAO, Food and Agriculture Organization of the United Nations (2021). Species Facts Sheets: *Mytilus galloprovincialis* (Lamarck, 1819). In: FAO Fisheries and Aquaculture Department [online]. Rome.

FAO, Food and Agriculture Organization of the United Nations. (1998). Integrated coastal area management and agriculture, forestry and fisheries. FAO guidelines. Rome.

Fatma, N., Panda, M., Ansari, W.H., Kabir-Ud-Din., 2015. Environment-friendly ester bonded gemini surfactant: Mixed micellization of 14-E2-14 with ionic and nonionic conventional surfactants. Journal of Molecular Liquids, 211, 247–255.

Fernández-Rubio, J., Rodríguez-Gil, J.L., Postigo, C., Mastroianni, N., López de Alda, M., Barceló, D., Valcárcel, Y., 2019. Psychoactive pharmaceuticals and illicit drugs in coastal waters of North-Western Spain: Environmental exposure and risk assessment. Chemosphere, 224, 379–389.

Fogarty, M., Incze, L., Hayhoe, K., Mountain, D., Manning, J., 2008. Potential climate change impacts on Atlantic cod (*Gadus morhua*) off the north eastern USA. Mitigation and Adaptation Strategies for Global Change, 13, 453-466.

Freitas, R., Almeida, A., Calisto, V., Velez, C., Moreira, A., Schneider, R.J., Esteves, V.I., Wrona, F.J., Figueira, E., Soares, A.M.V.M., 2016. The impacts of pharmaceutical drugs under ocean acidification: new data on single and combined long-term effects of carbamazepine on *Scrobicularia plana*. Science of the Total Environment, 541, 977–985.

Freitas, R., Coppola, F., Costa, S., Manzini, C., Intorre, L., Meucci, V., Soares, A.M.V.M., Pretti, C., Solé, M., 2019b. Does salinity modulates the response of *Mytilus galloprovincialis* exposed to Triclosan and Diclofenac? Environmental Pollution, 251, 756–765.

Freitas, R., Coppola, F., Costa, S., Pretti, C., Intorre, L., Meucci, V., Soares, A.M.V.M., Solé, M., 2019a. The influence of temperature on the effects induced by Triclosan and Diclofenac in mussels. Science of the Total Environment, 663, 992–999.

Freitas, R., Coppola, F., Henriques, B., Wrona, F., Figueira, E., Pereira, E., Soares, A.M.V.M., 2017. Does pre-exposure to warming conditions increase *Mytilus galloprovincialis* tolerance to Hg contamination? Comparative Biochemistry and Physiology Part C: Toxicology, 203, 1–11.

Freitas, R., Coppola, F., Meucci, V., Battaglia, F., Soares, A. M. V. M., Pretti, C., Faggio, C., 2021. The influence of salinity on sodium lauryl sulfate toxicity in *Mytilus galloprovincialis*. Environmental Toxicology and Pharmacology, 87, 103715.

Freitas, R., Marques, F., De Marchi, L., Vale, C., Botelho, M.J., 2020b. Biochemical performance of mussels, cockles and razor shells contaminated by paralytic shellfish toxins. Environmental Research, 188, 109846.

Freitas, R., Silvestro, S., Coppola, F., Costa, S., Meucci, V., Battaglia, F., Intorre, L., Soares, A.M.V.M., Pretti, C., Faggio, C., 2020a. Toxic impacts induced by Sodium lauryl sulfate in *Mytilus galloprovincialis*. Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology, 242, 110656.

Freitas, R., Silvestro, S., Pagano, M., Coppola, F., Meucci, V., Battaglia, F., Faggio, C., 2020c. Impacts of salicylic acid in *Mytilus galloprovincialis* exposed to warming conditions. Environmental Toxicology and Pharmacology, 80, 103448.

Gagné, F., Blaise, C., Fournier, M., Hansen, P.D., 2006. Effects of selected pharmaceutical products on phagocytic activity in *Elliptio complanata* mussels. Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology, 143, 179-86.

Gago, J., Luís, O.J., 2011. Comparison of spawning induction techniques on *Paracentrotus lividus* (Echinodermata: Echinoidea) broodstock. Aquaculture International, 19, 181–191.

Giarratano, E., Gil, M.N., Malanga, G., 2014. Biomarkers of environmental stress in gills of ribbed mussel *Aulacomya atra atra* (Nuevo Gulf, Northern Patagonia). Ecotoxicology and Environmental Safety, 107, 111-119.

Gibson, K.J., Miller, J.M., Johnson, P.D., Stewart, P.M., 2016. Toxicity of Sodium Dodecyl Sulfate to federally threatened and petitioned freshwater mollusk species. Freshwater Mollusk Biology and Conservation, 19, 29.

Gosling, E., 2003. Circulation, respiration, excretion and osmoregulation. In: Bivalve Molluscs: Biology, Ecology and Culture. Blackwell Publishing, 201-225.

Grilo, T.F., Cardoso, P.G., Dolbeth, M., Bordalo, M.D., Pardal, M.A., 2011. Effects of extreme climate events on the macrobenthic communities' structure and functioning of a temperate estuary. Marine Pollution Bulletin, 62, 303–311.

Guerriero, A., Gallo, C.E., Roselli, R., D'Angelo, G., Ciarcia, 2015. Reproductive health assessment and quantitative realtime PCR analysis of estrogen receptor mRNA expression in *Mytilus galloprovincialis* gametes. Experimental and Clinical Endocrinology & Diabetes, 123, LB_02.

Habig, W.H., Pabst, M.J., Jakoby, W.B., 1974. Glutathione S-Transferases. Journal of Biological Chemistry, 249, 7130–7139.

Höhn, A., Grune, T., 2013. Lipofuscin: formation, effects and role ofmacroautophagy. Redox Biology, 1, 140-144.

Holbrook, N.J., Scannell, H.A., Gupta, A.S., Benthuysen, J.A., Feng, M., Oliver, E.C.,Wernberg, T., 2019. A global assessment of marine heatwaves and their drivers. Nature Communications, 10(1), 1-13.

Hosokawa, M., Satoh, T., 2001. Measurement of Carboxylesterase (CES) Activities. Current Protocols in Toxicology, 10(1).

IPCC, 2018: Global Warming of 1.5°C. An IPCC Special Report on the impacts of global warming of 1.5 °C above pre-industrial levels and related global greenhouse gas emission pathways, in the context of strengthening the global response to the threat of climate change, sustainable development, and efforts to eradicate poverty [Masson-Delmotte, V., P. Zhai, H.-O. Pörtner, D. Roberts, J. Skea, P.R. Shukla, A. Pirani, W. Moufouma-Okia, C. Péan, R. Pidcock, S. Connors, J.B.R. Matthews, Y. Chen, X. Zhou, M.I. Gomis, E. Lonnoy, T. Maycock, M. Tignor, and T. Waterfield (eds.)]. In Press.

IPCC, 2019: Climate Change and Land: an IPCC special report on climate change, desertification, land degradation, sustainable land management, food security, and greenhouse gas fluxes in terrestrial ecosystems. P.R. Shukla, J. Skea, E. Calvo Buendia, V. Masson-Delmotte, H.O. Pörtner, D. C. Roberts, P. Zhai, R. Slade, S. Connors, R. van Diemen, M. Ferrat, E. Haughey, S. Luz, S. Neogi, M. Pathak, J. Petzold, J. Portugal Pereira, P. Vyas, E. Huntley, K. Kissick,

M. Belkacemi, J. Malley, (eds.)]. In press.

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.

Ismail, N.A.H., Wee, S.Y., Aris, A.Z., 2017. Multi-class of endocrine disrupting compounds in aquaculture ecosystems and health impacts in exposed biota. Chemosphere, 188, 375–388.

Izagirre, U., Errasti, A., Bilbao, E., Múgica, M., Marigómez, I., 2014. Combined effects of thermal stress and Cd on lysosomal biomarkers and transcription of genes encoding lysosomal enzymes and HSP70 in mussels, *Mytilus galloprovincialis*. Aquatic Toxicology, 149, 145–156.

Jensen, J. T., Creinin, M. D., 2020. Family planning, population growth, and the environment. Contraception, 101(3), 145–147.

Johansson, L.H., Borg, H., L.A., 1988. A spectrophotometric method for determination of catalase activity in small tissue samples. Analytical Biochemistry, 174 (1), 331–336.

Ko, G. W., Dineshram, R., Campanati, C., Chan, V. B., Havenhand, J., Thiyagarajan, V., 2014. Interactive effects of ocean acidification, elevated temperature, and reduced salinity on early-life stages of the pacific oyster. Environmental science & technology, 48(17), 10079-10088.

Koagouw, W., Stewart, N.A., Ciocan, C., 2021. Long-term exposure of marine mussels to paracetamol: is time a healer or a killer? Environmental Science and Pollution Research, 48823–48836.

Kovalova, L., Siegrist, H., von Gunten, U., Eugster, J., Hagenbuch, M., Wittmer, A., Moser, R., McArdell, C.S., 2013. Elimination of micropollutants during post- treatment of hospital wastewater with powdered activated carbon, ozone, and UV. Environmental Science & Technology, 47 (14), 7899–7908.

Kroeker, K.J., Kordas, R.L., Crim, R., Hendriks, I.E., Ramajo, L., Singh, G.S., Duarte, C.M., Gattuso, J.P., 2013. Impacts of ocean acidification on marine organisms: quantifying sensitivities and interaction with warmingGlobal Change Biology, 19, 1884–1896.

Labadie, P., Hill, E. M., 2007. Analysis of estrogens in river sediments by liquid chromatography-electrospray ionisation mass spectrometry. Comparison of tandem mass spectrometry and time-of-flight mass spectrometry. Journal of Chromatography A, 1141(2), 174–181.

Lai, K.M., Johnson, K.L., Scrimshaw, M.D., Lester, J.N., 2000. Binding of waterborne steroid estrogens to solid phases in river and estuarine systems. Environmental Science & Technology, 34, 3890–4.

Latif, M.B. Brimblecombe, P., 2004. Surfactants in Atmospheric Aerosols. Environmental Science & Technology, 2004, 38, 6501-6506.

Laurenson, J.P., Bloom, R.A., Page, S., Sadrieh, N., 2014. Ethinyl estradiol and other human pharmaceutical estrogens in the aquatic environment: A review of recent risk assessment data. American Association of Pharmaceutical Scientists Journal, 16(2), 299–310.

Leite, C., Coppola, F., Monteiro, R., Russo, T., Polese, G., Lourenço, M.A.O., Silva, M.R.F., Ferreira, P., Soares, A.M.V.M., Freitas, R., Pereira, E., 2020. Biochemical and histopathological impacts of rutile and anatase (TiO_2 forms) in *Mytilus galloprovincialis*. Science of the Total Environment, 719, 134886.

Levitus, S., Antonov, J.I., Boyer, T.P., Locarnini, R.A., Garcia, H.E., Mishonov, A.V., 2009. Global ocean heat content 1955-2008 in light of recently revealed instrumentation problems. Geophysical Research Letters. 36, L07608.

Liu, B., Yu, Z., Song, X., Yang, F., 2010. Effects of sodium dodecylbenzene sulfonate and sodium dodecyl sulfate on the *Mytilus galloprovincialis* biomarker system. Ecotoxicology and Environmental Safety, 73(5), 835–841.

Livak, K.J. Schmittgen, T.D., 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2-ΔΔCT Method. Methods, 25, 402–408.

Livingstone, D.R., Dixon, D.R., Donkin, P., Lowe, D.M., Moore, M.N., Widdows, J., 1989. Molecular, cellular and physiological responses of the common mussel *Mytilus edulis* to pollution: uses in environmental monitoring and management. Ecotoxicology, The Institute of Biology, London, 26–30.

Livingstone, D.R., Martinez, P.G., Michel, X., Narbonne, J.F., O'Hara, S., Ribera, D., Winston, G.W., 2006. Oxyradical production as a pollution-mediated

mechanism of toxicity in the common mussel, *Mytilus edulis L.*, and other molluscs. Functional Ecology, 4 (3), 415.

Lower, W.R., Kendall, R., 1990. Sentinel Species and Sentinel Bioassay. In: Biomarkers of Environmental Contamination. Lewis Publishers, 309–331.

Mackenzie, C. L., Ormondroyd, G. A., Curling, S. F., Ball, R. J., Whiteley, N. M., Malham, S. K., 2014. Ocean warming, more than acidification, reduces shell strength in a commercial shellfish species during food limitation. PLoS ONE, 9(1), 1–9.

Mackie, E., Shuckburgh, E., Jones, D., Vaughan, D., 2020. How climate change is affecting sea levels. Weather, 75(9), 280.

Marigomez, I., Garmendia, L., Soto, M., Orbea, A., Izagirre, U., Cajaraville, M.P., 2013. Marine ecosystem health status assessment through integrative biomarker indices: a comparative study after the Prestige oil spill "mussel Watch. Ecotoxicology, 22 (3), 486e505.

Marinho, R.A., Beserra, E.B., Bezerra-Gusmão, M.A., Porto, V. de S., Olinda, R.A., dos Santos, C.A.C., 2016. Effects of temperature on the life cycle, expansion, and dispersion of *Aedes aegypti* (Diptera: Culicidae) in three cities in Paraiba, Brazil. Journal of Vector Ecology, 41(1), 1–10.

Markert, B.A., Breure, A.M., Zechmeister, H.G., 2003. Definitions, strategies and principles for bioindication/biomonitoring of the environment. Trace Metals and other Contaminants in the Environment, 6, 3–39.

Martín-Pozo, L., de Alarcón-Gómez, B., Rodríguez-Gómez, R., García-Córcoles, M.T., Çipa, M., Zafra-Gómez, A., 2019. Analytical methods for the determination of emerging contaminants in sewage sludge samples. A review. Talanta, 192: 508–533.

Matozzo, V., Tomei, A., Marin, M.G., 2005. Acetylcholinesterase as a biomarker of exposure to neurotoxic compounds in the clam *Tapes philippinarum* from the Lagoon of Venice. Marine Pollution Bulletin, 50(12), 1686–1693.

Maynou, F., Costa, S., Freitas, R., Solé, M., 2021. Effects of triclosan exposure on the energy budget of *Ruditapes philippinarum* and *R. decussatus* under climate change scenarios. Science of The Total Environment, 777, 146068.

Mennillo, E., Casu, V., Tardelli, F., De Marchi, L., Freitas, R., Pretti, C., 2017. Suitability of cholinesterase of polychaete *Diopatra neapolitana* as biomarker of exposure to pesticides: In vitro characterization. Comparative Biochemistry and Physiology Part C: Toxicology, 191, 152–159.

Messina, C.M., Faggio, C., Laudicella, V.A., Sanfilippo, M., Trischitta, F., Santulli, A., 2014. Effect of sodium dodecyl sulfate (SDS) on stress response in the Mediterranean mussel (*Mytilus galloprovincialis*): Regulatory volume decrease (Rvd) and modulation of biochemical markers related to oxidative stress. Aquatic Toxicology, 157, 94–100.

Mizutani, T., Mori, R., Hirayama, M., Sagawa, Y., Shimizu, K., Okano, Y., Masaki, H., 2016. Sodium lauryl sulfate stimulates the generation of reactive oxygen species through interactions with cell membranes. Journal of Oleo Science, 65(12), 993–1001.

Monteiro, S.C. Boxall, A.B., 2010. Occurrence and fate of human pharmaceuticals in the environment. Reviews of Environmental Contamination and Toxicology, 202, 53-154.

Moreira, A., Figueira, E., Soares, A.M.V.M., Freitas, R., 2016. The effects of arsenic and seawater acidification on antioxidant and biomineralization responses in two closely related *Crassostrea species*. Science of Total Environment, 545-546, 569-581.

Moreira, A., Freitas, R., Figueira, E., Volpi Ghirardini, A., Soares, A.M.V.M., Radaelli, M., Guida, M., Libralato, G. 2018. Combined effects of arsenic, salinity and temperature on *Crassostrea gigas* embryotoxicity. Ecotoxicology and Environmental Safety, 147, 251–259.

Morosetti, B., Freitas, R., Pereira, P., Hamza, H., Andrade, M., Coppola, F., Maggioni, D., Della Torre, C., 2020. Will temperature rise change the biochemical alterations induced in *Mytilus galloprovincialis* by cerium oxide nanoparticles and mercury? Environmental Research, 188, 109778.

Moschino, V., Delaney, E., Da Ros, L., 2012. Assessing the significance of *Ruditapes philippinarum* as a sentinel for sediment pollution: Bioaccumulation and biomarker responses. Environmental Pollution, 171, 52–60.

Nagasawa, K., Treen, N., Kondo, R., Otoki, Y., Itoh, N., Rotchell, J. M., Osada, M., 2015. Molecular characterization of an estrogen receptor and estrogen-related receptor and their autoregulatory capabilities in two Mytilus species. Gene, 564(2), 153–159.

Nardi, A., Mincarelli, L.F., Benedetti, M., Fattorini, D., D'Errico, G., Regoli, F., 2017. Indirect effects of climate changes on cadmium bioavailability and biological effects in the Mediterranean mussel *Mytilus galloprovincialis*. Chemosphere, 169, 493–502.

Naumann, G., Alfieri, L., Wyser, K., Mentaschi, L., Betts, R. A., Carrao, H., Spinoni, J., Vogt, J., & Feyen, L., 2018. Global Changes in Drought Conditions Under Different Levels of Warming. Geophysical Research Letters, 45(7), 3285–3296.

Newton, A., Brito, A.C., Icely, J.D., Derolez, V., Clara, I., Angus, S., Schernewski, G., Inácio, M., Lillebø, A.I., Sousa, A.I., Béjaoui, B., Solidoro, C., Tosic, M., Caõnedo- Argüelles, M., Yamamuro, M., Reizopoulou, S., Tseng, H.C., Canu, D., Roselli, L., Maanan, M., Cristina, S., Ruiz-Fernández, A.C., Lima, R.F.d., Kjerfve, B., Rubio- Cisneros, N., Pérez-Ruzafa, A., Marcos, C., Pastres, R., Pranovi, F., Snoussi, M., Turpie, J., Tuchkovenko, Y., Dyack, B., Brookes, J., Povilanskas, R., Khokhlov, V., 2018. Assessing, quantifying and valuing the ecosystem services of coastal lagoons. Journal for Nature Conservation, 44, 60-65.

Nunes, B., Nunes, J., Soares, A.M.V.M., Figueira, E., Freitas, R., 2017. Toxicological effects of paracetamol on the clam *Ruditapes philippinarum*: exposure vs recovery. Aquatic Toxicology, 192, 198–206.

Ohkawa, H., Ohishi, N., Yagi, K., 1979. Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. Analytical Biochemistry, 95 (2), 351–358.

Orr, J.C., Fabry, V.J., Aumont, O., Bopp, L., Doney, S.C., Feely, R.A., Gnanadesikan, A., Gruber, N., Ishida, A., Joos, F., Key, R.M., Lindsay, K., Maier-Reimer, E., Matear, R., Monfray, P., Mouchet, A., Najjar, R.G., Plattner, G.-K., Rodgers, K.B., Sabine, C.L., Sarmiento, J.L., Schlitzer, R., Slater, R.D., Totterdell, I.J., Weirig, M.-F., Yamanaka, Y., Yool, A., 2005. Anthropogenic ocean

acidification over the twenty-first century and its impact on calcifying organisms. Nature, 437, 681–686.

Ostroumov S.A., 2003. Studying effects of some surfactants and detergents on filter-feeding bivalves. In: Martens K. (eds) Aquatic Biodiversity. Developments in Hydrobiology, 171. Springer, Dordrecht.

Ostroumov, S.A., 2005. Some aspects of water filtering activity of filter-feeders. Hydrobiologia, 542(1), 275–286.

Paglia, D.E. Valentine W.N., 1967. Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase. Journal of Laboratory and Clinical Medicine, 70(1), 158-169.

Pan, K., Wang, W.X., 2011. Mercury accumulation in marine bivalves: influences of biodynamics and feeding niche. Environmental Pollution, 159, 2500-2506.

Parida, V. K., Saidulu, D., Majumder, A., Srivastava, A., Gupta, B., Gupta, A. K., 2021. Emerging contaminants in wastewater: A critical review on occurrence, existing legislations, risk assessment, and sustainable treatment alternatives. Journal of Environmental Chemical Engineering, 9(5), 105966.

Patel, M., Kumar, R., Kishor, K., Mlsna, T., Pittman Jr., C.U., Mohan, D., 2019. Pharmaceuticals of emerging concern in aquatic systems: chemistry, occurrence, effects, and removal methods. Chemical Reviews, 119 (6), 3510–3673.

Patrolecco, L., Ademollo, N., Grenni, P., Tolomei, A., Barra Caracciolo, A., & Capri, S., 2013. Simultaneous determination of human pharmaceuticals in water samples by solid phase extraction and HPLC with UV-fluorescence detection. Microchemical, 107, 165-171.

Pauly, D., Watson, R., Alder, J., 2005. Global trends in world fisheries: Impacts on marine ecosystems and food security. Philosophical Transactions of the Royal Society B: Biological Sciences, 360(1453), 5–12.

Perry, L. K., 2015. Water Resources Research Institute. Wyoming University Water Resources Research Institute, 44, 4823–4839.

Pes, K., Friese, A., Cox, C. J., Laizé, V., Fernández, I., 2021. Biochemical and molecular responses of the Mediterranean mussel (*Mytilus galloprovincialis*)

to short-term exposure to three commonly prescribed drugs. Marine Environmental Research, 168, 105309.

Piedade, F., Bio, S., Nunes, B., 2020. Effects of common pharmaceutical drugs (paracetamol and acetylsalicylic acid) short term exposure on biomarkers of the mussel Mytilus spp. Environmental Toxicology and Pharmacology, 73, 103276.

Pinto, J., Costa, M., Leite, C., Borges, C., Coppola, F., Henriques, B., Pereira, E., Freitas, R., 2019. Ecotoxicological effects of lanthanum *in Mytilus galloprovincialis*: Biochemical and histopathological impacts. Aquatic Toxicology, 211, 181–192.

Pirone, G., Coppola, F., Pretti, C., Soares, A.M.V.M., Sole, M., Freitas, R., 2019. The effect of temperature on Triclosan and Lead exposed mussels. Comparative Biochemistry and Physiology - Part B, 232, 42-50.

Piscopo, R., Almeida, Â., Coppola, F., De Marchi, L., Esteves, V.I., Soares, A.M.V.M., Pretti, C., Morelli, A., Chiellini, F., Polese, G., Freitas, R., 2021. How temperature can alter the combined effects of carbon nanotubes and caffeine in the clam *Ruditapes decussatus*? Environmental Research, 195, 110755.

Pojana, G., Gomiero, A., Jonkers, N. Marcomini, A., 2007. Natural and synthetic endocrine disrupting compounds (EDCs) in water, sediment and biota of a coastal lagoon. Environment International, 33, 929-936.

Pörtner, H. O., Farrell, A. P., 2008. Physiology and climate change. Science, 322, 690-692.

Queirós, V., Azeiteiro, U.M., Barata, C., Santos, J.L., Alonso, E., Soares, A.M.V. M., Freitas, R., 2021. Effects of the antineoplastic drug cyclophosphamide on the biochemical responses of the mussel *Mytilus galloprovincialis* under different temperatures. Environmental Pollution, 288, 117735.

Quiroga, J. M., Sales, D., Gómez-Parra, A., 1989. Experimental evaluation of pollution potential of anionic surfactants in the marine environment. Water Research, 23(7), 801–807.

Rahman, I., Kode, A., Biswas, S.K., 2007. Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. Nature Protocols, 1, 3159–3165.

Rajalakshmi, S., Mohandas, A., 2005. Copper-induced changes in tissue enzyme activity in a freshwater mussel. Ecotoxicology and Environmental Safety, 62(1), 140-143.

Regoli, F. Giuliani, M.E., 2014. Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. Marine Environmental Research, 93, 106–117.

Ribeiro, C., Tiritan, M.E., Rocha, E., Rocha, M.J., 2009. Seasonal and spatial distribution of several endocrine-disrupting compounds in the Douro River estuary, Portugal. Archives of Environmental Contamination and Toxicology, 56, 1–11.

Ricciardi, K.L., Poynton, H.C., Duphily, B.J., Blalock, B.J., Robinson, W.E., 2016. Bioconcentration and depuration of 14C-labeled 17α-ethynylestradiol and 4-non- ylphenol in individual organs of the marine bivalve *Mytilus edulis. L.* Environmental Toxicology and Chemistry. 35, 863–873.

Ries, J.B., Cohen, A.L., McCorkle, D.C. 2009. Marine calcifiers exhibit mixed responses to CO2-induced ocean acidification. Geology, 37(12), 1131-1134.

Robinson, H., Hodgen, C., 1940. The biuret reaction in the determination of serum proteins: a study of the conditions necessary for the production of a stable color which bears a quantitative relationship to the protein concentration. Journal of Biological Chemistry, 135 (9), 707.

Rocha, T.L., Saboia-Morais, S.M.T., Bebianno, M.J., 2016. Histopathological assessment and inflammatory response in the digestive gland of marine mussel *Mytilus galloprovincialis* exposed to cadmium-based quantum dots. Aquatic Toxicology, 177, 306-315.

Rosety, M., Ribelles, A., Rosety-Rodriguez, M., Carrasco, C., Ordonez, F.J., Rosety, J.M., Rosety Plaza, M., 2000. Morpho-histochemical study of the biological effects of sodium dodecyl sulphate on the digestive gland of the Portuguese oyster. Histology and Histopathology, 15(4), 1137–1143.

Schlumpf, M., Schmid. P., Durrer, S., Conscience, M., Maerkel, K., Henseler, M., 2004. Endocrine activity and developmental toxicity of cosmetic UV filters—an update. Toxicology, 205, 113–22.

Schwarzenbach, R.P., Escher, B.I., Fenner, K., Hofstetter, T.B., Johnson, C.A., Von Gunten, U., Wehrli, B., 2006. The challenge of micropollutants in aquatic systems. Science, 313(5790), 1072-1077.

Silva, C. P., Otero, M., Esteves, V., 2012. Processes for the elimination of estrogenic steroid hormones from water: A review. Environmental Pollution, 165, 38–58.

Smolarz, K., Hallmann, A., Zabrza, S., Pietrasik, A., 2017. Elevated gonadal atresia as biomarker of endocrine disruptors: field and experimental studies using *Mytilus trossulus* (L.) and 17-alpha ethinylestradiol (EE2). Marine Pollution Bulletin, 120, 58–67.

Snyder, S.A., H., Lei, Wert, E.C., 2008. Removal of endocrine disruptors and pharmaceuticals during water treatment. In: D.S. Aga (ed.) Fate of Pharmaceuticals in the Environment and in Water Treatment Systems. CRC Press, New York. 229-259.

Solé, M., Rivera-Ingraham, G., Freitas, R., 2018. The use of carboxylesterases as biomarkers of pesticide exposure in bivalves: A methodological approach. Comparative Biochemistry and Physiology Part C: Toxicology, 212, 18–24.

Stanczyk, F.Z., Archer, D.F., Bhavnani, B.R., 2013. Ethinyl estradiol and 17β-estradiol in combined oral contraceptives: Pharmacokinetics, pharmacodynamics and risk assessment. Contraception, 87(6), 706–727.

Starling, M.C.V.M., Amorim, C.C., Leão, M.M.D., 2019. Occurrence, control and fate of contaminants of emerging concern in environmental compartments in Brazil. Journal of Hazardous Materials, 372: 17–36.

Sturve, J., Almroth, B.C., Förlin, L., 2008. Oxidative stress in rainbow trout (*Oncorhynchus mykiss*) exposed to sewage treatment plant effluent. Ecotoxicology and Environmental Safety, 70(3), 446–452.

Świacka, K., Smolarz, K., Maculewicz, J., Caban, M., 2020. Effects of environmentally relevant concentrations of diclofenac in *Mytilus trossulus*. Science of the Total Environment, 737, 139797.

Taketani, M., Shii, M., Ohura, K., Ninomiya, S., Imai, T., 2007. Carboxylesterase in the liver and small intestine of experimental animals and human. Life Sciences, 81(11), 924–932.

Tang, Y., Rong, J., Guan, X., Zha, S., Shi, W., Han, Y., Du, X., Wu, F., Huang, W., Liu, G., 2020. Immunotoxicity of microplastics and two persistent organic pollutants alone or in combination to a bivalve species. Environmental Pollution, 258, 113845.

Tasneem, A., Laway, G.N., Deva, A.S., Deva, B., Bathia, N., Khan, R.A., 2018. Hazardous effects of Sodium Lauryl Sulfate and Sodium Laureth Sulfate, an overview. World Journal of Pharmaceutical Sciences, 7(5), 283–291.

Thilagam, H., Gopalakrishnan, S., Qu, H.D., Bo, J., Wang, K.J., 2010. 17β estradiol induced ROS generation, DNA damage and enzymatic responses in the hepatic tissue of Japanese sea bass. Ecotoxicology, 19(7), 1258–1267.

Thiyagarajan, V., Ko, G. W. K., 2012. Larval growth response of the Portuguese oyster (*Crassostrea angulata*) to multiple climate change stressors. Aquaculture, 370, 90-95.

Todd, P. A., Heery, E. C., Loke, L. H. L., Thurstan, R. H., Kotze, D. J., Swan, C., 2019. Towards an urban marine ecology: characterizing the drivers, patterns and processes of marine ecosystems in coastal cities. Oikos, 128(9), 1215–1242.

Townsend, D.M., Tew, K.D., 2003. The role of glutathione-S-transferase in anti-cancer drug resistance. Oncogene, 22, 7369–7375.

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R., 2015. Spatial distribution and bioaccumulation patterns in three clam populations from a low contaminated ecosystem. Estuarine, Coastal and Shelf Science, 155, 114–125.

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R., 2016. Combined effects of seawater acidification and salinity changes in *Ruditapes philippinarum*. Aquatic Toxicology, 176, 141–150.

Velez, C., Figueira, E., Soares, A.M.V.M., Freitas, R., 2017. Effects of seawater temperature increase on economically relevant native and introduced clam species. Marine Environmental Research, 123, 62–70.

Velez, C., Teixeira, M., Wrona, F.J., Soares, A.M.V.M., Figueira, E., Freitas, R., 2016. Clam *Ruditapes philippinarum* recovery from short-term exposure to the

combined effect of salinity shifts and Arsenic contamination. Aquatic Toxicology, 173, 154–164.

Venkatnarayanan, S., Murthy, P.S., Kirubagaran, R., Veeramani, P., Venugopalan, V.P., 2021. Response of green mussels (*Perna viridis*) subjected to chlorination: investigations by valve movement monitoring. Environmental Monitoring and Assessment, 193(4), 1–12.

Verdelhos, T., Marques, J. C., Anastácio, P., 2015. Behavioral and mortality responses of the bivalves *Scrobicularia plana* and *Cerastoderma edule* to temperature, as indicator of climate change's potential impacts. Ecological Indicators, 58, 95–103.

Viarengo, A., Dondero, F., Pampanin, D.M., Fabbri, R., Poggi, E., Malizia, M., Bolognesi, C., Perrone, E., Gollo, E., Cossa, G.P., 2007. A biomonitoring study assessing the residual biological effects of pollution caused by the HAVEN wreck on marine organisms in the Ligurian sea (Italy). Archives of Environmental Contamination and Toxicology, 53(4), 607–616.

Wang, H., Xi, H., Xu, L., Jin, M., Zhao, W., Liu, H., 2021. Ecotoxicological effects, environmental fate and risks of pharmaceutical and personal care products in the water environment : A review. Science of the Total Environment, 788, 147819.

Wang, Y., Yin, T., Kelly, B.C., Gin, K.Y.H., 2019. Bioaccumulation behaviour of pharmaceuticals and personal care products in a constructed wetland. Chemosphere, 222, 275–285.

Wilkinson, J., Hooda, P. S., Barker, J., Barton, S., Swinden, J., 2017. Occurrence, fate and transformation of emerging contaminants in water: An overarching review of the field. Environmental Pollution, 231, 954–970.

Woo, S., Won, H., Lee, A., Yum, S., 2012. Oxidative stress and gene expression in diverse tissues of Oryzias javanicus exposed to 17β-estradiol. Mollecular & Cellular Toxicology, 8, 263-269.

Xiang, Y., Wu, H., Li, L., Ren, M., Qie, H., Lin, A., 2021. A review of distribution and risk of pharmaceuticals and personal care products in the aquatic environment in China. Ecotoxicology and Environmental Safety, 213, 112044.

Ying, G.G., Kookana, R.S., Ru, Y.J., 2002. Occurrence and fate of hormone steroids in the environment. Environment International, 28(6), 545–551.

Zhang, Q.Q., Xing, C., Cai, Y.Y., Yan, XT., Ying, G.G., 2021. How much do human and livestock actually contribute to steroids emission and surface water pollution from past to the future: A global research. Science of the Total Environment, 772, 145558

Zhou, W., Tang, Y., Du, X., Han, Y., Shi, W., Sun, S., Zhang, W., Zheng, H., Liu, G., 2021. Fine polystyrene microplastics render immune responses more vulnerable to two veterinary antibiotics in a bivalve species. Marine Pollution Bulletin, 164.

Zhou, X., Li, Y., Li, H., Yang, Z., Zuo, C., 2019. Responses in the crucian carp (*Carassius auratus*) exposed to environmentally relevant concentration of 17α-Ethinylestradiol based on metabolomics. Ecotoxicology and Environmental Safety,183,109501.

Supplementary material

Table S1. 17-α Ethynylestradiol (EE2, ng/L) and Sodium lauryl sulfate (SLS, mg/L) concentrations in water and in mussel's tissues (fresh weight, FW). Water was collected from exposure aquaria (collected immediately after spiking) and blanks aquaria (collected immediately after spiking and before water renewal). Tissues were obtained from animals at the end of the experimental period (28 days). LOD for water samples was 1 ng/L and 0.05 mg/L for EE2 and SLS, respectively. LOD for tissue samples was 10 and 0.005ng/g for EE2 and SLS, respectively. BCF: Bioconcentration factor.

	WATER						TISSUES		
	AFTER SPIKING				BEFORE WATER EXCHANGE				
	Exposure medium		Blanks		Blanks				
Treatment	**EE2**	**SLS**	**EE2**	**SLS**	**EE2**	**SLS**	**EE2**	**SLS**	**BCF**
CTL							<LOD	<LOD	-
EE2	115.1	-	119.2		105.4		7.9±5.1	-	0.06
SLS	-	3.87	-	3.89	-	3.91	-	1.9±0.8	0.00
EE2+SLS	110.7	3.75	118.4	3.93	110.7	3.78	<LOD	3.6±0.6	-