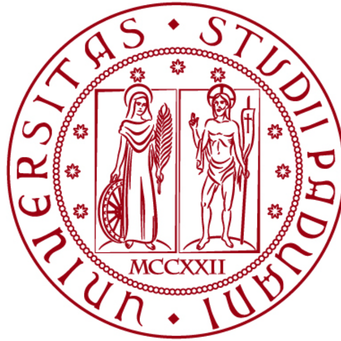
UNIVERSITÀ DEGLI STUDI DI PADOVA

DIPARTIMENTO DI BIOLOGIA

Corso di Laurea magistrale in Marine Biology



TESI DI LAUREA

Effects of a novel Perfluoroalkyl Substance HFPO-DA in a changing environment on *Mytilus galloprovincialis*: an *in vitro* and *in vivo* study

Relatore: Prof. Gianfranco Santovito
Dipartimento di Biologia

Correlatore: Prof.ssa Rosa Freitas
Department of Biology - Universidade de Aveiro

Laureando: Claudio Bortot

ANNO ACCADEMICO 2023/2024

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to Professor Freitas for the invaluable opportunity to be part of this research. Her guidance, generosity in sharing knowledge, and support have been fundamental throughout this journey. I am also thankful to the incredible team at the University of Aveiro laboratory. The collaborative spirit within the lab have enriched my experience, contributing significantly to my professional and personal growth. Special thanks go to Professor Santovito, whose support, enthusiasm, and vast knowledge have inspired and propelled me forward. As both my professor and the internal supervisor of my thesis, he has played a crucial role in shaping my academic path, and for that, I am immensely grateful. To my friends, thank you for being my constant source of joy, support, and meaningful conversation: your company has been my anchor. Lastly, and most importantly, my heartfelt appreciation goes to my family. Your steadfast belief in me, boundless love, and encouragement to always give my best have been the foundation of my strength and perseverance. I could not have achieved this without you.

INDEX

1. Introduction **1**

1.1. Fluorinated substances 1

1.2. Climate changes 4

1.3. Bivalves and test organism 7

1.4. Oxidative stress and biomarkers 10

1.5. Aim of the study 14

2. Materials & Methods **15**

2.1. Study area 15

2.2. *In vitro* assay 16

2.2.1. Experimental setup and sample processing 16

2.3. *In vivo* assay 19

2.3.1. Collection and maintenance condition 19

2.3.2. Experimental setup and sample processing 19

2.4. Biochemical analyses 23

2.4.1. Metabolic capacity 23

2.4.2. Energy reserves 24

2.4.3. Antioxidant capacity 26

2.4.4. Biotransformation capacity 28

2.4.5. Redox Balance 29

2.4.6. Cellular damage 30

2.4.7. Neurotoxicity 30

2.5. Statistical analyses 32

3. Results **33**

3.1. *In vitro* assay 33

3.1.1. Choice of the time of exposure 33

3.1.2. Multivariate analysis 34

3.1.2.1. Principal Coordinate analysis (PCO) 34

3.1.2.2. Effect of factors 35

3.1.3. Biochemical responses 36

3.1.3.1. Antioxidant capacity 36

3.1.3.2. Biotransformation capacity 38

3.1.3.3. Oxidative damage 39

3.1.3.4. Neurotoxicity 39

3.2. *In vivo* assay 41

3.2.1. Multivariate analysis 41

3.2.1.1. Principal Coordinate analysis 41

3.2.1.2. Mortality rate 42

3.2.1.3. Effect of factors 42

3.2.2. Biochemical responses 43

3.2.2.1. Metabolism 43

3.2.2.2. Energy reserves 43

3.2.2.3. Antioxidant capacity 46

3.2.2.4. Biotransformation capacity 49

3.2.2.5. Redox Balance 51

3.2.2.6. Cellular damage 53

3.2.2.7. Neurotoxicity 53

4. Discussion 57

4.1. *In vitro* assay 57

4.1.1. Multivariate analysis general perspective 57

4.1.2. Antioxidant capacity 58

4.1.3. Biotransformation capacity 60

4.1.4. Oxidative damage 60

4.1.5. Neurotoxicity 60

4.2. *In vivo* study 62

4.2.1. Multivariate analysis general perspective 62

4.2.2. Metabolism and energy reserves 63

4.2.3. Antioxidant capacity and redox balance 64

4.2.4. Biotransformation capacity 67

4.2.5. Oxidative damage 68

4.2.6. Neurotoxicity 69

5. Conclusions & Future perspectives 71

6. Bibliography 75

ABSTRACT

Per- and polyfluoroalkyl substances (PFAS) are emerging contaminants with persistent environmental and biological presence, raising concerns about their health impacts. This thesis focuses on the toxicological effects of a novel PFAS, namely hexafluoropropylene oxide dimer acid (HFPO-DA), also known as GenX. This contaminant is known among the scientific community for its ability to induce oxidative stress and disrupt protein function. However, its effects on marine organisms, specifically *Mytilus galloprovincialis*, are still unknown. A new *in vitro* methodological approach was adopted to assess the impact of this PFAS by focusing on specific biomarkers, including lipid peroxidation, antioxidant enzyme activity and acetylcholine esterase. It has been shown how HFPO-DA, combined with salinity alteration, induced toxicity *in vitro* and *in vivo*. Key findings indicate that GenX induced oxidative stress at low and high contamination levels, producing reactive oxygen species (ROS) and, consequently, oxidative damage. The *in vitro* assay reported an inhibition of the antioxidant enzymes, which may be responsible for the observed oxidative damage. While mussels tend to suffer mainly salinity changes, alteration even at hypo and hypersaline conditions may indicate that the contaminant may induce toxicity at different conditions. The *in vitro* approach offers a reliable and reproducible method for assessing PFAS-related oxidative damage, providing a valuable tool for future toxicological research. These findings contribute significantly to the broader understanding of HFPO-DA impacts on ecosystems, potentially informing future research and mitigation strategies.

1 - INTRODUCTION

1.1 – Fluorinated substances

Poly- or per- fluoroalkyl substances (PFAS) are compounds known worldwide as per- or poly-fluorinated carbon chains, and they have been used for stain-resistant coating in firefighting foams or non-stick cookware for the past decade (Bonato et al., 2020; Savoca & Pace, 2021). However, the definition is changing nowadays, considering a plethora of new pharmaceuticals and pesticides with fluorinated carbons, independently on the chain length (Lukić Bilela et al., 2023). In 2018, nearly 5000 different PFAS were commercialised (OECD, 2018a). These chemicals are known to be stable, as the bond between carbon and fluorine is challenging to metabolise biologically and physically, as it resists processes like photooxidation and hydrolysis (EFSA, 2008). Moreover, the danger of these substances lies in their tendency to bind primarily to proteins, leading to harmful effects in both vertebrate and invertebrate species (Bonato et al., 2020; Alesio et al., 2022; Hou & Zhang, 2024). Specifically in humans, these are carcinogenic, altering the functioning of the thyroid, the metabolism and the reproductive system (Melzer et al., 2010; Fisher et al., 2013; Itoh et al., 2016). Most of these deleterious effects have been mainly attributed to increased reactive oxygen species (ROS) production, mainly driven by the interaction with the peroxisome proliferation pathway or mitochondrial dysfunctions (Bonato et al., 2020). This increase in ROS may not only increase the antioxidant responses but also lead to oxidative damage in case the dose of PFAS turns out to be excessive, with toxic compounds like aldehydes produced (Regoli & Giuliani, 2014; Bonato et al., 2020).

Among these substances, perfluorooctanoic acid (PFOA) and Perfluorooctanesulfonic acid (PFOS) are known to show relevant concentrations all around the world, and due to their link to cancer and their persistence in the environment and animals, they have been banned from commerce (EFSA, 2008; Geng et al., 2024). These substances are constantly released into the environment, making them available in different environmental matrixes, like soil, groundwater and rivers, and the increase in production at the beginning of the decade led to a growth of their concentration even in the marine environment (White et al., 2015; Heydebreck et al., 2015; Song et al., 2018; Joerss et al., 2020). For instance, the production of PFOA went on until their proven toxicity: since the year 2000, their production and adoption have decreased in both America and Europe, also thanks to their ban at the Stockholm convention (Bonato et al., 2020; EUR-Lex, n.d.). The concentration in drinking waters of PFAS can range up to 25 ng/L, while in river samples, the variation is wider: from non-detected up to 111 ng/L in the Rhine-Meuse delta (Heydebreck et al., 2015; Gebbink et al., 2017; Gebbink & van Leeuwen, 2020). On the other hand, higher concentrations of PFAS can be detected in various areas of the world. In fact, in the Xiaoquing River in China, concentrations of these contaminants rise to 800 μg/L, and rivers in the Bohai Sea

reported levels up to 13 µg/L (Heydebreck et al., 2015; Zhao et al., 2020). In both the Netherlands and China, a high contribution of fluorinated substances found was given by PFOA, which is the most abundant and the most soluble in freshwater bodies (Joerss et al., 2019; Gebbink & van Leeuwen, 2020; Zhao et al., 2020). The presence of these contaminants in rivers led to their distribution in the estuarine environment (Heydebreck et al., 2015; Zhao et al., 2020; Wu et al., 2023). These chemicals can be detected even in open seas, where the action of currents contributes to the distribution of the compounds: specifically, in the Arctic Sea, the sum of PFAS can be up to 120 pg/L (Kwok et al., 2015; Joerss et al., 2020). The availability of these compounds, even at low environmental concentrations, may pose a threat to the ecosystems by altering the process of carbon sequestration carried out by the phytoplankton (Casal et al., 2017; Niu et al., 2019; Mahmoudnia, 2023). There have been experiments that relate PFAS exposure directly to the bioaccumulation in different organisms. Martín et al. (2019) showed that *Holoturia tubulosa* specimens collected from the environment showed natural PFAS accumulation levels of nearly 400 ng/g. However, there is a need to discriminate between long-chain and short-chain PFAS: the latter are more likely to dissolve in water and not go into the sediment, and because of this, they are claimed to be less toxic than the other long-chained alternatives (Joerss et al., 2019; Fabrello et al., 2021). An experiment was carried out to determine the bioaccumulation factor of various PFAS revealed that long-chained PFAS had the highest bioaccumulation potential (Martín et al., 2019). Again, in Charleston, organisms of *Tursiops truncatus* have been shown to accumulate PFAS even at a concentration of 1 µg/g, leading to developmental problems in the juveniles, in which these chemicals seemed to have a higher concentration than adults (Houde et al., 2005). Wang et al. (2023) analysed the behaviour of zebrafish exposed to PFOA: these authors showed the movement alteration in these organisms, highlighting a possible impairment of the fish exposed to these contaminants. The accumulation of PFAS in marine bivalves can also lead to metabolic, reproductive and developmental disturbances (Islam et al., 2021). In *Mytilus galloprovincialis*, PFOS accumulates in the tissues, mainly in digestive glands, leading to a possible threat to human consumption of this organism (Cunha et al., 2005). Their embryos' antioxidant responses are affected even at a concentration of 0.1 µg/L of PFOA, making larvae of marine organisms more sensitive to those substances (Fabbri et al., 2014). These kinds of responses may lead to an altered effect on community and ecosystem functioning due to chronic exposure or altering the reproductive capacity of different aquatic organisms, with the induction of oxidative stress being the most common pathway (Lee et al., 2020; Mahoney et al., 2022; Lukić Bilela et al., 2023).

Hexafluoropropylene oxide dimer acid (HFPO-DA), also known as GenX, is a PFAS produced by Du-Pont factories and used to produce fluoropolymers (Gebbink & van Leeuwen, 2020). Its use has been adopted worldwide as a PFOA replacement: at the beginning, the compound was claimed to be less toxic than its

long-chained counterpart due to the shorter chain, but there were still concerns about its stability (Kudo et al., 2001; Li et al., 2021; Yang et al., 2022). In recent years, the compound quickly spread in freshwater pools, rivers and groundwater near fluorochemical plants and has been detected in lagoons worldwide, especially in areas with wastewater treatment plants (Heydebreck et al., 2015; Mahoney et al., 2022). Its concentration ranges from 0 ng/L to 100 ng/L in estuaries worldwide, with Europe and China being the major countries affected by the release of these PFAS (Heydebreck et al., 2015; Sun et al., 2016; Gebbink et al., 2017; Song et al., 2018; Mullin et al., 2019; Joerss et al., 2019; Gebbink & van Leeuwen, 2020; Zhao et al., 2020; ARPAV, 2024; Yuan et al., 2024). Moreover, the compound has been detected in the Arctic Sea, even though its presence is limited to pg/L, demonstrating that the oceanic currents may affect the transportation of this contaminant even in remote regions of the world (Joerss et al., 2020). The threat posed by the availability of this chemical in the environment has been studied and proven to cause rat diseases, like problems with liver toxicity, immune system disruption, and potential links to cancer: the main cause of this is the alteration the compound causes to the lipid metabolism of the organism (Conley et al., 2020). Furthermore, the exposure alters lipid metabolism also in zebrafish, generating behavioural and developmental issues while causing down-regulation of the peroxisome proliferation genes as well as the ones related to cell apoptosis (Wang et al., 2023). In the aquatic environment, it is known that the compound may impair the photosynthetic abilities of *Chlorella*, negatively affecting its growth, while also inhibiting part of the antioxidant responses (Niu et al., 2019; Li et al., 2021). As the effects of this PFAS are slightly different from its banned alternative (PFOA), there is an increased need for studies on the impact of the substance on marine animals to provide information about its bioavailability and bioaccumulation (Wang et al., 2024). Exposure of mussels to this category of contaminants generally leads to an increase in antioxidant enzyme activity, and an alteration of this response may also lead to an increase in lipid peroxidation (LPO) (Lee et al., 2020; Xu et al., 2022). Furthermore, some substances may be affected by the stability in different media, leading to a transformation of the chemical, making that more or less prone to alter the organism's response (Ohoro et al., 2024).

1.2 - Climate changes

During the past decades, climate change has affected the environment due to increased greenhouse gasses in the atmosphere, which worsens the greenhouse effect (IPCC, 2023). Their atmospheric accumulation leads to higher temperatures, which triggers various environmental alterations, including melting glaciers in mountains and polar regions, shifting precipitation and drought patterns (Fenoglio et al., 2010; IPCC, 2023). The latter is especially true for transition environments exposed to fluctuation in salinities that depend on both inputs from freshwater and saltwater from the sea. Analyses in estuarine environments predict that, due to increasing temperature, the salinity in estuaries will increase due to the lack of an appropriate availability of freshwater (Vargas et al., 2017; Bal et al., 2021). In fact, according to IPCC, the increase in ocean surface temperature of 0.88 °C compared to the past century is worrying, which affects acidification and salinity, ultimately altering fishing and aquaculture worldwide (IPCC, 2023; FAO, 2024). Future climate change projections indicate an even greater risk of global warming, with rising emissions, which will potentially push temperatures to 1.5°C or higher. This is expected to intensify heatwaves, extend drought periods, and severely affect ecosystems and humans (IPCC, 2023). Nowadays, estuaries, coastal environments of transition zone between rivers and sea, are subjected to an increase in salinity, referred to as salinisation (Herbert et al., 2015; Bal et al., 2021). This process may be due to both natural or anthropogenic sources: the former causes are phenomena like the sea spray, frequency of rain, sea level rise and melting glaciers; the latter instead refers to alteration caused by humans, like changes in hydrology or widespread removal of deep-rooted perennial plants (Herbert et al., 2015). The increasing trend of salinisation of coastal ecosystems may lead to rapid salinity shifts, hindering freshwater tidal species' competitiveness against euryhaline species, ultimately reducing biodiversity, limiting species distribution, and potentially altering ecosystem services in estuaries (Costanza et al., 1998; Herbert et al., 2015; Röthig et al., 2023). These environments are, in fact, one of the most valuable ecosystems in the world, providing nutrient cycling, food production and coastal protection from erosion, creating optimal and protected conditions for various plant and animal species (Costanza et al., 1998). Many euryhaline organisms use these environments. For instance, the crab *Carcinus maenas* thrives in estuaries to live and feed. This adaptation allows the organism to spread around different estuaries worldwide and compete with native species (DeRivera et al., 2005). Humans have long exploited estuaries for their productivity and accessible locations, particularly targeting bivalves. Activities such as fishing and harvesting these animals have been practiced for generations (FAO, 2009). For instance, *Mytilus galloprovincialis* or the crab *Carcinus aestaurii* are species of economic interest, and they became invasive in other regions of the world, as they are well adapted to coastal and estuarine environments (Steffani & Branch, 2003; FAO, 2009; Matozzo et al., 2013a). Organisms living in coastal environments are adapted

to changing environmental conditions, as these environments undergo cyclical fluctuation of parameters like water currents or tidal change, by conforming their internal fluids to the external salinity or regulating the osmotic pressure, maintaining an adequate environment inside their organs (Freire et al., 2011; Bal et al., 2021). For example, organisms like crabs, mussels and clams live in these environments and exhibit different strategies. Sessile organisms, like mussels or clams, are directly exposed to sunlight and air; this changes their ability to breathe, but they react by lowering their metabolism to avoid damage (Velez et al., 2017; Andrade et al., 2019). On the other hand, mobile species can move in the environment, avoiding stressors to find suitable conditions. Creating unsuitable salinity conditions or environmental parameters inside the range of tolerance for a species may alter energy metabolism (Freitas et al., 2017; Leite et al., 2023). For instance, when exposed to higher salinities, the mussel *M. galloprovincialis* tends to decrease the metabolic rate, but membrane damage increases due to coping with oxidative stress (Andrade et al., 2021, 2024a). As previously reported, lagoons are important ecosystems that may provide invaluable benefits to the environment and humans (Costanza et al., 1998). For instance, lagoons in Portugal and Spain were used to produce salt, which has been a factor in both economic income and preservation of the ecosystem, as salt production significantly affects the biodiversity of organisms present in those environments (Rodrigues et al., 2011). Therefore, the loss of these structures, along with the action of increasing water level, poses a significant threat to estuaries and the preservation of some birds, which depend on some invertebrates and microbiota that reach their optimal proliferation in salty environments (Rodrigues et al., 2011).

However, estuaries are not only subject to environmental alterations due to the variation in tides and riverine input but also due to the actions of mankind (Freire et al., 2011; Herbert et al., 2015; Vargas et al., 2017). Not only salinisation but also chemicals coming from rivers are another stressor in lagoons, as these contaminants are going to accumulate in the water column, in sediments and living organisms, potentially influencing the wealth and well-being of organisms in lagoons (Rodrigues et al., 2011). In the past decade, many contaminants in the aquatic environment have been identified, those being mostly metalloids and Persistent Organic Pollutants (POPs), like bisphenol A, microplastics and PAHs (Freitas et al., 2019b; Andrade et al., 2021; OECD, 2023; Andrade et al., 2024a). Nowadays, research focuses more and more on manufactured chemicals that can be released into the environment, mainly known as Contaminants of emerging concern (CEC) (Freitas et al., 2020; Wu et al., 2023). These contaminants belong to the classes of UV filters, Personal Care Products (PCPs), Polycyclic Aromatic Hydrocarbon (PAHs), Pharmaceuticals and antibiotics, pesticides, halogenated flame retardants and PFAS (Aminot et al., 2019; Bordalo et al., 2022; Wu et al., 2023). Different sources of these contaminants are present in the environment: agricultural fields, landfills and emission of ultrafine particles and micro and nano

plastics also contribute to the spreading of these pollutants, leading to further threats in open ocean and arctic regions too (Joerss et al., 2020; Xu et al., 2022). However, the discharge of high quantities of these contaminants in rivers not only led to a high concentration in the riverine environment but also an increase in the presence of the contaminant worldwide, due to first the riverine discharges at the levels of estuaries but also marine currents, that bring these contaminants in the most remote regions of the world (Zhao et al., 2012; Kwok et al., 2015). These compounds are released into the environment as a consequence of improper disposal. They can act as endocrine disruptors (EDs). These substances impair the endocrine system by causing hormonal imbalance: they have been linked to a range of adverse health effects in humans, including disruptions in thyroid, kidney, and metabolic functions, as well as impacts on reproductive health and steroid hormone regulation (OECD, 2023). These compounds present an elevated concentration near wastewater treatment plants (WWTPs), generally placed in riverine areas and discharge chemical compounds in the rivers without proper disposal or degradation (Gebbink et al., 2017). Furthermore, contaminants act with other environmental factors or other chemical components already present. They can be associated with plastics, which are present in ocean environments and their threat to organisms and ecosystems is well known. For instance, many organisms can ingest microplastics and their pollutants, resulting in a possible way of transfer of associated contaminants on the trophic chain (Scott et al., 2021; Lukić Bilela et al., 2023; Wang et al., 2024). Therefore, testing the possible implications of the effect on organisms exposed to PFAS and environmental changes may lead to a deeper understanding of other implications in marine organisms. For these reasons, a deep understanding of the effect of the contaminant in the environment, especially in marine biomonitor organisms like bivalves, is required.

1.3 - Bivalves and test organism

To assess the possible stress caused by pollutants present in the environment, bivalve molluscs have been widely adopted as they are filter-feeding organisms that thrive in coastal waters (Lockwood & Somero, 2011; Fabrello et al., 2021; Curpan et al., 2022). Bivalves may be well adapted to face various stressors that range from differences in salinity to air exposure and temperature variations. For example, in the intertidal habitat, these organisms face conditions where the shell may reach up to 30 °C in temperature (Freire et al., 2011; Denny et al., 2011). Salinity changes may be due to saltwater input in lagoons or increased freshwater input in others, causing mussels to face seasonal environmental changes. In particular, in the case of *Mytilus edulis*, it is known that the increase in transcription of heat shock protein (HSP) genes, which function is to stabilise the proteins in case of unsuitable environmental conditions, occurs when exposed to low salinities (Lockwood & Somero, 2011; Barrett et al., 2022).

Furthermore, bivalves thrive in estuaries, where factors such as fluctuations in salinity and higher concentrations of contaminants are present and can be stressful to those organisms (Funes et al., 2006; Freire et al., 2011). For these reasons, bivalves have developed different responses to the fluctuation in environmental parameters: the adaptation of antioxidant defences and transcription of molecules that may be triggered in hypo or hypersaline conditions, depending on the organism (Freire et al., 2011). Due to their wide availability in the environment and their presence in different estuaries, bivalve molluscs are used as biomonitoring species to assess the health of the environment they live in: their sedentary behaviour and their filter-feeding strategy are common characteristics of those animals, which also allow for the quantification of the contaminants they are exposed to (Regoli & Principato, 1995; Bocchetti et al., 2008; Cravo et al., 2009). Therefore, as the human consumption of mussel and fish products is increasing, there is a need to control and understand the possible health issues related to these organisms (Carella et al., 2018; FAO, 2024).

M. galloprovincialis (Lamarck, 1819) is a bivalve mollusc belonging to the Mytilidae family, which thrives in estuaries and intertidal environments. Therefore, in their habitat, they constantly face emersion and immersion in the water due to tidal changes, which make this species more likely to be exposed to extreme environmental conditions, one of which salinity changes (Freire et al., 2011). In the case of this animal, *Mytilus* also transcribe for genes functional to synthesise proteins that attach directly to the cytoskeleton, stabilising its structure to prevent rupture in case of an osmotic imbalance (Ji et al., 2014; Barrett et al., 2022). *M. galloprovincialis* has been adopted over the years as a model organism in various ecotoxicological experiments all over the globe to understand the impact of xenobiotic substances on the environment, both using *in vivo* and *in vitro*

approaches (Solé et al., 2021; Andrade et al., 2021; Curpan et al., 2022). For instance, Carella et al. (2018) analysed different biomarkers to assess the quality of life of these mussels in different sites to define the possible stressors in the Campania coast in Italy. Alternatively, the post-mitochondrial fraction allows for the assessment of the disruption of the biochemical response of these organisms (Cruz et al., 2023; Vieira Sanches et al., 2023). This bivalve has a worldwide distribution and is an invasive species living in the intertidal habitat and estuarine environment (Steffani & Branch, 2003; FAO, 2009). The invasiveness of this organism in other regions allows a wide availability and replicability of the experiments and possible comparison of its physiology with other bivalve species. This is the case in the study of Lockwood & Somero (2011), in which the proteome of two species of *Mytilus sp.* was analysed. The aim was to understand the difference in zonation caused by lower salinities, as *M. edulis* is more tolerant than *M. galloprovincialis*. Furthermore, the small dimension of mussels usually allows laboratory experiments to be carried out with little or no difficulties. In those *in vivo* studies, mussels are kept in controlled conditions to understand their physiology in case of one or multiple stressors, allowing them to check how they interact (Freitas et al., 2020; Andrade et al., 2021). There are multiple ways in which factors may interact (additive, synergistic, antagonistic), causing the organism to face different stress levels (Kroeker et al., 2017). For instance, related to the antioxidants in *Mytilus*, in some scenarios, exposure to a particular environmental condition and a contaminant may have different outcomes, leading possibly to inhibition (Orbea et al., 2002; Freitas et al., 2019b; Andrade et al., 2021, 2024a).

This species is also world-renowned for its aquaculture: in 2016, the worldwide production of blue mussels was slightly more than 100 thousand tonnes (FAO, 2024). However, there are also other relevant economic benefits of the presence of this mussel in the environment. They provide ecosystem services in their habitat, like removing phytoplankton from the water column and improving water quality (van der Schatte Olivier et al., 2020). *Mytilus sp.* physiology has been studied over the years, and many different biomarkers have been developed to detect possible contaminants in the environment (Cravo et al., 2009). For instance, while the presence of metal has been associated with an increase in metallothionein, the response, also known as peroxisome proliferation is mainly related to exposure to polycyclic aromatic hydrocarbons (PAHs) and other organic contaminants (Orbea et al., 2002; Cajaraville et al., 2003; Carella et al., 2018). As a consequence of the increase of these organelles, the associated oxidases and the oxidation of fatty acids increase the release of ROS, causing increased oxidative stress (Regoli & Giuliani, 2014; He et al., 2021). Not only histological analyses have been developed, but also, the response of antioxidant enzymes and biotransformation in mussels is crucial to understand their variability with environmental parameters, like pH, temperature and salinity, or the exposure to novel or legacy contaminants (Regoli & Principato, 1995; Freitas et al., 2020; Leite et al., 2023). Another possible

alteration that derives from the exposure to a contaminant is the neurotoxicity. For instance, in *Mytilus sp.*, various substances like pesticides, pharmaceuticals or even POPs have been shown to induce neurotoxicity and impairment in the behaviour of the organism (Escartín & Porte, 1997; Freitas et al., 2019b; Wang et al., 2024). For these reasons, the responses of the organisms may change, possibly leading to behavioural responses like closure of the valves and difficulty breathing, ultimately affecting the welfare of the animals (Grimmelpont et al., 2024). In the case of salinity alteration, *M. galloprovincialis* might either increase or decrease their metabolism and vary the production of antioxidant responses (Andrade et al., 2024a). Alteration of the salinity to high values may alter the osmotic balance of the organism's internal fluid compared to the environment, leading to an overall decrease in the activity of antioxidants, which in turn leads to an increase in oxidative stress (Freitas et al., 2017). While the possible interaction between different factors may not always provide the expected outcome, none of the contaminants are in the environment on their own. While most studies on PFAS focus on the exposure of an organism directly to a particular contaminant, the use of a combined exposure between legacy and novel compounds may provide additional information on the nature of their effects (Xu et al., 2022; Geng et al., 2024). For instance, the impact of exposure to PFOS and PFOA may alter the filtration rate of the green mussel (Liu et al., 2014). The study by Xu et al. (2022) showed that combined exposure to PFOS and nanoplastics may have a more detrimental effect on the amount of ROS produced than exposure to PFOS alone in mussels, leading to increased oxidative stress

1.4 – Oxidative stress and biomarkers

Oxidative stress is the imbalance in reactive oxygen species (ROS) production and their scavenging through different molecules (Freitas et al., 2020; Aranda-Rivera et al., 2022). ROS may be generated in different ways in the cells. For instance, the generation of ROS may happen through triggering the mitochondrial proteins associated with respiration (Regoli et al., 2011a; Andrade et al., 2024b). Those proteins are altered by a possible electron leakage in the mitochondrial membrane, which causes a reaction with oxygen. In the case of mitochondria, the level of ROS production depends on the activity of the electron transport chain and the possible associated electron leakage (Rutter et al., 2010; Regoli & Giuliani, 2014). On the other hand, ROS can be produced by different substances: for instance, substances like metals trigger the Fenton reaction, which implies the reaction of hydrogen peroxide (H_2O_2) with a transition metal, typically a ferrous ion, leading to an increase in hydroxyl radicals and consequent damage to the cell (Viarengo et al., 1999).

On the other hand, the increased release of specific contaminants like PAHs and polychlorinated biphenyls (PCBs) leads to an activation of the pathway of the cytochrome p450, leading to further electron leakage in the cell due to the slow reaction (Regoli & Giuliani, 2014). Most PFAS, instead, are known to disrupt the peroxisome proliferator-activated receptor (PPAR) pathway, leading to an increase in peroxisomes and, as a consequence, enzymes related to lipid metabolism (Conley et al., 2020; Wang et al., 2023). In fact, in the case of increased expression, the amount of H_2O_2 increases in the organism due to increased activity of fatty acid oxygenase, leading to further damage in the cells, as the molecule can cross the membranes (Yeldandi et al., 2000).

Therefore, cells have developed specific molecules that are functional for facing oxidative stress by mitigating ROS generation in different ways through antioxidants. Antioxidant is a general term to refer to all those molecules that bind or reduce the effect of singlet oxygen, H_2O_2 and oxidised organic molecules that attract electrons (Regoli & Giuliani, 2014; Aranda-Rivera et al., 2022). They may have different origins, like carotenoids, that need to be assumed in animal diets through plants; other organic molecules that can be synthesised by the organisms directly, like thioredoxins or glutathione; or enzymes, that carry out reactions to mitigate the reactivity of ROS (Aranda-Rivera et al., 2022). These enzymes are crucial to face oxidative stress as, unlike other molecules that bind directly to ROS and need to be re-synthesized or reused, they can be active or inactive and always present in the cell. Those carry out several reactions, but they can be present in the cell in different isoforms or have different locations in the organelles of the organisms, where their affinity for the substrate plays a crucial role in their activity. For instance, superoxide dismutase (SOD) is present in 3 different isoforms in organisms: SOD1, SOD2 and SOD3. While the second is present mainly in

mitochondria, and it is also known as MnSOD, the other two isoforms refer to the Cu/Zn SOD, which is present in the cytoplasm (SOD1) or in the extracellular medium (SOD3), leading to different functional activities of the enzymes (Manduzio et al., 2003; Letendre et al., 2008).

Furthermore, while the MnSOD is the main one expressed in every organ, the amount of SOD3 may vary: the expression of the enzyme in the extracellular environment is thought to be a way to prevent excessive oxidative damage by carrying out the dismutation of singlet oxygen directly outside biological membranes (Manduzio et al., 2003). In some instances, the reactions in the organisms are different and require different affinities of the enzymes to their substrate, ideal for the organelle or section of the cell they need to protect. Enzymes like catalase (CAT), glutathione peroxidase (GPx) and peroxiredoxins (Prdx) carry out the same reaction: releasing water and oxygen from the dismutation of H_2O_2 (Regoli et al., 2011a; Molavian et al., 2015). However, these enzymes not only carry out the reaction in a different way but also possess different spatial locations: while GPx and Prdx are mainly located in the cytoplasm, CAT is located in peroxisomes (Flohé, 2012; Fransen et al., 2012; Molavian et al., 2015). This spatial distribution of the enzymes influences their activity based on the type of reaction triggered in the cell. For instance, the activation of the PPAR system causes an increased synthesis in the associated enzymes, leading to the increase in oxidases and the consequent increase in the activity of CAT (Regoli & Giuliani, 2014).

On the other hand, the increasing concentration of ROS triggers signalling molecules that lead to the activation of different pathways. For instance, when exposed to metals, mussels tend to use metallothionines to scavenge oxyradicals and activate various transcription factors in the cell DNA to increase the antioxidant enzymes and glutathione (GSH) metabolism (Viarengo et al., 1999). The antioxidant enzymes that are present in the cell require the use of a reducing agent whose purpose is to reduce the ROS. For instance, GPx and Prdx6 couple that reaction with the oxidation of GSH, while other isoforms of Prdx require thioredoxins instead (Molavian et al., 2015). For this reason, a crucial action in recycling these molecules is played by enzymes like the glutathione reductase (GR) or the thioredoxin reductase (TrxR), which make GSH and thioredoxin available again in the cells (Molavian et al., 2015; Aranda-Rivera et al., 2022). The GR catalyse the reaction to convert 1 molecule of GSSG back to 2 molecules of GSH. These enzymes not only cover an important role in the regulation of the substrate availability in the cell without the need to synthesize them again, but also, allow the correct maintenance of the appropriate redox balance condition for the cell when they are exposed to stressful environmental conditions (Freitas et al., 2017). However, the redox balance of the cells does not depend only on the antioxidants, but also on the possible de-novo synthesis of thiols. For instance, GSH is a tripeptide made of cysteine, glutamate and glycine synthesized by the glutamate-

cysteine ligase (GCL) first and the glutathione synthetase then in a 2-step process, which requires ATP (Forman et al., 2009).

Together with the antioxidant enzymes and reducing agents, other enzymes are responsible for protecting the cell from oxidative damage through the detoxification of the cell from xenobiotics. These enzymes are known as biotransformation enzymes. As their name suggests, they are responsible for transforming the xenobiotic to be excreted by the cell more efficiently before they react with other molecules and trigger oxidative damage (Regoli & Giuliani, 2014). One of these enzymes is the glutathione-S-transferase (GST), whose role is to bind the xenobiotic to a molecule of GSH. This reaction may cause either an activation or deactivation of the compound of interest, making it more water-soluble, leading to different effects in the cell (Habig et al., 1974). On the other hand, some compounds do not require an additional reductive agent but carry out the reaction just by transforming molecules containing an ester bond and a carboxylic acid into an alcohol and an acid: the carboxylesterases (CbEs) (Escartín & Porte, 1997; Hosokawa & Satoh, 2001; Solé et al., 2018). Both enzyme's activity varies according to seasonal or environmental fluctuations, leading to different responses depending on possible exposure to pollutants (Escartín & Porte, 1997; Bebianno et al., 2007; Andrade et al., 2021).

However, some enzymes may be destabilised by the presence of environmental contaminants that bind to those molecules in different ways (Andrade et al., 2024b). In general, it has been shown that in zebrafish, algae and bivalves, there could be an alteration of the antioxidant responses when these organisms are exposed to various PFAS, both legacy and novel compounds (Niu et al., 2019; Fabrello et al., 2021; Wang et al., 2023; Geng et al., 2024). An alteration of the functioning of the antioxidant enzymes or the biotransformation and excretion enzymes may ultimately increase the amount of free ROS or contaminant present in the cell. At that point, chain reactions and alteration of a specific cell compartment can be caused. In particular, an increase in ROS may alter cysteine residues in proteins, create a reaction with lipids or damage DNA, altering the genetic information or the transcription of some enzymes, possibly leading to tumorigenesis (Jahngen-Hodge et al., 1997; Zhang et al., 2016; Aranda-Rivera et al., 2022). For instance, one possible consequence of protein damage is the carbonylation of proteins, which occurs when a ROS reacts with an amino acid in the structure of the protein itself. This alteration can be measured by the change in the number of aldehydes and ketones in the cells (Suzuki et al., 2010). A change in protein oxidation response may depend on the environmental change the organism is exposed to. For example, while the changes in salinity do not alter this parameter, they may be modified by a higher temperature or the presence of a contaminant (Andrade et al., 2021; Leite et al., 2023). In addition, ROS may target lipids: the increase in lipid hydroperoxides causes a chain reaction with other lipids, leading to the release of malondialdehyde (MDA) (Regoli & Giuliani, 2014). These

changes can be monitored, as lipids are more sensitive than proteins to peroxidation, making it a widely recognized biomarker for monitoring oxidative damage in bivalves (Freitas et al., 2017; Fabrello et al., 2021; Andrade et al., 2021; Xu et al., 2022).

Regarding PFAS, their toxicity is generally associated with their binding directly to the proteins present in the solution (Sakurai et al., 2013). In this way, they alter their structure and the reaction they carry. One essential protein identified as a neurotoxic biomarker is acetylcholine esterase (AChE), which inhibition in activity is associated with an increased signal and a possible impairment of the organism's response. For instance, the study of (Wang et al., 2023) showed that zebrafish exhibited abnormal behaviour patterns that could potentially affect survival, reproduction, and predator avoidance, highlighting the risk of PFAS at higher ecological levels. This disruption in response mechanisms at the individual level could have cascading effects on populations and ecosystems, making PFAS a significant concern in environmental toxicology.

1.5 - Aim of the study

As PFAS are generally widely distributed worldwide, the study of the possible alteration that these contaminants may cause at reported environmental concentrations in seas and estuaries in the environment may lead to possible alteration to the organisms living there, possibly altering the ecosystem. In particular, there seems to be a lack of knowledge on the effect of HFPO-DA on marine bivalves. Furthermore, previous studies addressing PFAS exposure did not focus on the possible impact of changing environmental conditions, which may lead to different stability of the chemicals or alteration in organism responses to it (Freitas et al., 2017; Yin et al., 2022). To do so, the biomonitoring species *M. galloprovincialis* has been chosen. Being a sessile organism which feeds by water filtering and thanks to its high availability in estuaries around the world, it is considered an ideal biomonitoring organism (Regoli & Principato, 1995; Bocchetti et al., 2008; Cravo et al., 2009). To achieve this, the present study focuses on two approaches: the first is an *in vitro* experiment, which implies the use of a contaminant and directly exposes the s9-subcellular fraction of the organism used. The use of *in vitro* techniques nowadays allows for the detection of possible alteration in the organisms' biochemical performance to certain pollutants, like antibiotics, pharmaceuticals or other substances present in the environment, while limiting the number of organisms sacrificed (OECD, 2018b; Solé et al., 2021; Cruz et al., 2023; Giannessi et al., 2023). In the present case, the homogenate corresponded to the post-mitochondrial fraction, as it is the one related to the enzymes in the cytoplasm and the microsomal component of the organism, including most of the antioxidants, biotransformation and neurotoxicity parameters (Vieira Sanches et al., 2023). If this analysis reported relevant alteration or damage to the cellular structures, an analysis *in vivo*, thus exposing the whole organism to the contaminant, can be considered. Only environmentally relevant concentrations of the contaminant in estuaries were used in the latter experiment. In this way, the possible influence of the contaminant on these organisms was analysed to show whether their response is altered by the presence of HFPO-DA in the environment.

Therefore, this study aims to understand whether the emerging PFAS HFPO-DA may alter the mussel's biochemical responses, and if the salinity changes can influence the impacts caused by the contaminant.

2 - MATERIALS AND METHODS

2.1 - Study area

The Aveiro Lagoon (Ria de Aveiro) is located on the Northwest coast of Portugal: it is 45 km long and spans 10 km in width, covering an area that ranges from 83 km^2 to 66 km^2 at high and low tide respectively (Dias & Lopes, 2006). The bar-built estuary, mainly influenced by tides rather than riverine input, includes different environments like channels, mudflats, and salt marshes, and the only access from the sea to the lagoon occurs thanks to a westside channel. Nevertheless, the estuary presents two fluvial inputs in the eastern part, namely from rivers Vouga and Antuã (Dias & Lopes, 2006; Lopes et al., 2007; Vargas et al., 2017). The estuary is subject to different seasonal environmental changes; the main effect of these variations is on temperature and salinity. The first varies between 12 °C to 24.5 °C across the year, while salinity is influenced not only by the season but also by the tide: for instance, in the mouth of the lagoon during the late spring and summer period, values may range from 25 to 36 (Dias et al., 1999; Lopes et al., 2007). However, during the winter, riverine inputs are higher, and areas located downstream of the channels could reach values as low as 20 (Rodrigues et al., 2012). Additionally, a horizontal variation in salinity can occur within the estuary, ranging from 0 near the river mouth to 36 closer to the ocean (Lopes et al., 2007; Vargas et al., 2017).

The Aveiro lagoon is also known for its tourism as well as the presence of aquaculture facilities, salt extraction, and harbour presence (Vargas et al., 2017; Aminot et al., 2019). In particular, human activities and the consequent release of sewage or drainage from agricultural fields around the lagoon cause phenomena like eutrophication (Lopes et al., 2007). In addition, in the Aveiro lagoon, different chemical compounds have been registered as responsible for stress to the organisms. For instance, in the lagoon, levels of different metalloids, including mercury, have been detected in water, organisms and sediments (Coelho et al., 2005; Stoichev et al., 2020). Furthermore, in recent years, contaminants like halogenated flame retardants, UV filters and PFAS, specifically PFOS, have been detected in this ecosystem (Cunha et al., 2005; Gadelha et al., 2019; Aminot et al., 2019).

2.2 – In vitro assay

2.2.1 – Experimental setup and sample processing

After the first sampling in February, 50 individuals of *M. galloprovincialis* were immediately transported to the laboratory in wet conditions. They were placed in tanks of artificial seawater (Coral Pro Salt Red Sea) at 17±1 °C, pH 8.1±0.1 and salinity 28±1 for 1 week. During this period, mussels were kept on the previously reported conditions and fed every other day using Oceanlife® solution (composed of 1 spoon of Oceanlife Coral Food®, 1 mL of Oceanlife Phyto Marine®, and 4mL of deionized water for 100L), administered at a concentration of 50 µL/L. For this study, 40 mussels were sacrificed: after the acclimation period in the laboratory, individuals were opened and dissected to extract both digestive glands (DG) and gills (G) tissues separately. The collected tissues were joined in 4 pools, corresponding to 4 replicates of 10 organisms per tissue and per treatment, and all the replicates were weighted.

Each pool was then subject to homogenization by using a Potter-Elvehjem homogenizer to process each pool of organs and transfer them to different falcons per different pools. Then, a phosphate buffer (K_2HPO_4, 0.1 M, pH 8) (1:2 p/v ratio) was added to act as a stabiliser for the enzymes of interest (OECD, 2018b). Subsequently, centrifugation at 10000 *g* for 20 min at 4 °C was carried out to obtain the organs' post-mitochondrial supernatant (S9 fraction). After that, the supernatants were collected, and 1 mL was stored in different microtubes: the ones containing the same pool of organisms were marked similarly. These microtubes were then preserved at -80 °C until their use. For each pool, the total volume of supernatant fractions obtained was recorded and used to determine the concentration of protein per gram (g) of tissue, and this was achieved according to the spectrophotometric biuret method adopted by Robinson & Hogden (1940) (the procedure is written below in the "Biochemical analyses" section).

For the *in vitro* assay, a stock solution of HFPO-DA was prepared by diluting 2 mg of the chemical in 10 mL of methanol: this solvent is recommended for storing the contaminant for long periods at room temperature (Liberatore et al., 2020; Zhang et al., 2022). After that, the starting solutions were prepared by serial dilution of the stock solution. Specifically, the process implied first the preparation of an intermediate stock solution by diluting 100 µL of stock solution with 900 µL of distilled water. The subsequent exposure of the subcellular component was made by testing the five concentrations of concentrations of the previously mentioned contaminant with four homogenate replicates each. A volume corresponding to 1 mg of protein per litre (L) of sample was pipetted in 450 µL of phosphate buffer (K_2HPO_4, 0.1 M, pH 8.2) in 5 microtubes. After that, 50 µL of the contaminant were taken from the previously prepared solutions to get the contaminant's final concentration (Figure 1).

Once the contaminant was inserted into the microtubes, all of them were vortexed and put in an orbital incubator at 25 °C ± 2 °C at 1350 *g*. Two exposure times were tested to verify which was the most effective in describing the alteration caused by the contaminant to the biomarkers of mussel S9 fraction: 30 min and 60 min. These *in vitro* tests can be carried out for up to 2 h (OECD, 2018b).

This type of approach reinvolves the study of the alteration of the enzyme activity in response to the exposure of the post-mitochondrial supernatant (S9 fraction) of a pool of organisms. It is helpful to perform an initial screening on the possible alteration of the organism's biochemical responses thanks to its high throughput analysis (Solé et al., 2021; Vieira Sanches et al., 2023). Nevertheless, the choice of concentration for this experiment is critical. The approach relies on acute exposure (the order of minutes) to biological material, so a higher amount of the contaminant should be used than *in vivo* experiments (Solé et al. 2020, Cruz et al. 2023). Therefore, the choice was based on levels of HFPO-DA present in estuaries and freshwater bodies from different surveys. Specifically, the levels chosen for the concentration were 100 ng/L, close to the highest possible found in estuarine environments, 1 µg/L, more common in contaminated freshwaters, 10 µg/L, less common but still possible to be found in these environments, and 100 µg/L, which is a concentration that is still not found in the environment (Heydebreck et al., 2015; Sun et al., 2016; Gebbink et al., 2017; Song et al., 2018; Mullin et al., 2019; Joerss et al., 2019; Gebbink & van Leeuwen, 2020; Zhao et al., 2020; ARPAV, 2024; Yuan et al., 2024).

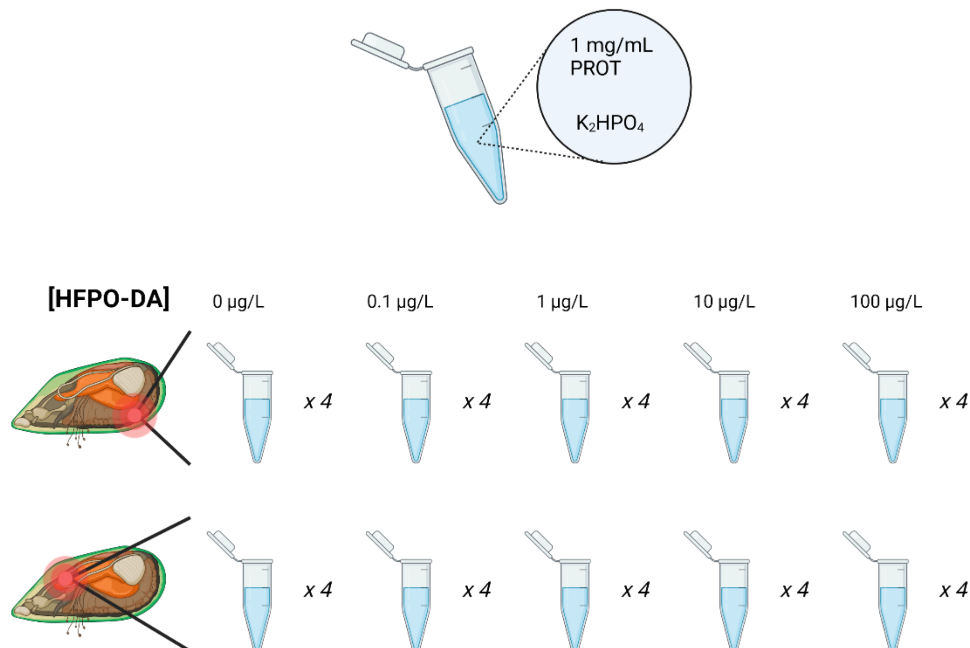


Figure 1: Representation of the experimental design of the in vitro test. Above, samples contain the gills, while on the bottom, the digestive gland. The increasing concentrations of the contaminant with five levels tested horizontally. Each concentration had four replicates.

2.3 - In vivo assay

In vivo testing is the common, most integrative approach for the proper evaluation of the effect of a contaminant on the responses of organisms in the environment (Bernardini et al., 2021; Fabrello et al., 2021; Andrade et al., 2021, 2024a). The choice of this experiment is determined by the need for information related to the effect of contaminants in the environment: for this approach, organisms with well-known biochemical and physiological responses are chosen (Matozzo et al., 2013b; Andrade et al., 2021; Cruz et al., 2023; Vieira Sanches et al., 2023). The concentrations of HFPO-DA selected for this test were based on a typical environmental level of this novel PFAS and the highest concentrations reported in the literature in estuarine environments: respectively 10 ng/L and 100 ng/L (Heydebreck et al., 2015; Gebbink et al., 2017; Joerss et al., 2019; Gebbink & van Leeuwen, 2020; Zhao et al., 2020; ARPAV, 2024).

2.3.1 - Collection and maintenance conditions

After the second sampling (end of February 2024), 300 organisms of *M. galloprovincialis* of similar size (length: 54.101 ± 3.597; width: 30.448 ± 2.176) were transported to the laboratory in wet conditions and were placed in tanks of artificial seawater at 17±1 °C, pH 8.1±0.1 and salinity 30±1 (use of Coral Pro Salt Red Sea). All mussels were cleaned from epibionts the day after the collection and left to rest in tanks for 7 days of acclimation. At the beginning of the second week, mussels were divided into three tanks with an equal number of organisms to allow them to get used to the different salinities. During that week, the changes in the salinity were obtained by 2 ±1 every 2 days until the target levels were reached: 1 tank was kept at 30, 1 tank was brought to salinity 40, while in the third, the salinity was decreased to 20. The synthetic seawater was changed twice in the first week and at each salinity variation in the second week, maintaining consistent pH and temperature conditions during each renewal. During this period after the cleaning, mussels were kept in the previously reported conditions and fed every other day using Oceanlife® solution (composed of 1 spoon of Oceanlife Coral Food®, 1 mL of Oceanlife Phyto Marine®, and 4mL of deionized water for 100L), which was administered at a concentration of 50 µL/L every other day.

2.3.2 – Experimental setup and sample processing

After the acclimation period, different aquaria were prepared, each one with 3L of artificial seawater. Each aquarium held six mussels, with 18 organisms per treatment, as each condition was replicated in three aquaria. The conditions tested involved two orthogonal factors: the concentration of the contaminant (0 ng/L (CTL), 10 ng/L and 100 ng/L of HFPO-DA) and the three different salinities (20,

30 and 40). Therefore, the combination of these factors resulted in nine treatments: 1) uncontaminated mussels at salinity 20; 2) uncontaminated mussels at salinity 30; 3) uncontaminated mussels at salinity 40; 4) 10 ng/L HFPO-DA exposed mussels at salinity 20; 5) 10 ng/L HFPO-DA exposed mussels at salinity 30; 6) 10 ng/L HFPO-DA exposed mussels at salinity 40; 7) 100 ng/L HFPO-DA exposed mussels at salinity 20; 8) 100 ng/L HFPO-DA exposed mussels at salinity 30; 9) 100 ng/L HFPO-DA exposed mussels at salinity 40 (Figure 2). Additionally, two tanks without mussels per salinity level with the highest concentration of HFPO-DA (100 ng/L) were prepared as positive controls. These aquaria's purposes were to assess the stability of the tested PFAS at different salinities and to certify that the changes in concentration in the rest of the aquaria with mussels were related to the organisms' activity and not by external factors. The contaminant concentration in the different treatments was prepared following two different starting solutions with one intermediate solution each to get the same concentration of the solvent (methanol) of 0.0006% in all treatments. This solvent has been chosen for appropriate storage of the contaminant for long periods of time at room temperature, while other less-toxic solvents were not suitable for this aim (Liberatore et al., 2020; Zhang et al., 2022). A methanol control has not been carried out, as the solvent is known to be toxic to marine invertebrates only at high concentrations. Previous studies confirmed that there were no statistically significant differences between control tanks and methanol ones, even when testing for different salinity levels (Helmstetter et al., 1996; Kaviraj et al., 2004; Hutchinson et al., 2006; Bordalo et al., 2022; Cuccaro et al., 2023).

To get the concentration of 10 ng/L, a starting solution of 1.8 mg/L in methanol 100% was prepared; from that, an intermediate solution of 3 µg/L was prepared starting from the first one by pipetting 170 µL of the first stock in distilled water for a total volume of 100 mL; 10 mL of this solution were then pipetted in the nine aquaria. Instead, to get the final concentration of 100 ng/L, a starting stock solution of 18 mg/L has been prepared; the intermediate solution, in this case of 30 µg/L, has been prepared by dissolving 417 µL of the first solution in 250 mL of distilled water. Then, 10 mL of that solution was pipetted into the 15 aquaria (nine conditions with the organisms and in the six blank aquaria).

During the experiment, mussels were fed every other day with a mixture in equal parts of three algae: *Isochrysis galbana*, Provirion Industries NV, BE; *Tetraselmis sp.*, AqualGae; *Phaeodactylum tricornutum*, AqualGae. These organisms were combined in the same quantity (8 mg each) in a solution of 40 mL to get a concentration of 150,000 cells/animal/day, considering that 1g has $3*10^9$ cells. Seawater conditions, including HFPO-DA concentration, salinity and pH, were reestablished each week in aquaria. However, before the water exchange took place, for the first 2 weeks, 50 mL of seawater samples from every pool were collected to assess the stability and the possible changes in concentration of the contaminant. The experiment's mortality has been documented weekly too. For this

experiment, 162 mussels were sacrificed and, at the end of the exposure, divided as follows: at least two individuals for the biochemistry, one for histological analyses, and the remaining organisms for quantifying the tissue.

To analyse the biomarkers and the biochemical analysis, the whole organism tissue of three organisms per aquaria was crushed using a mortar and divided into seven different microtubes of 0.5 ± 0.1 g fresh weight (FW). The remaining tissue was collected in a falcon of 15 mL and weighed. Both microtubes and falcons were stored in a freezer at -80 °C until the moment of the extraction required to analyse the biochemical parameters. The extraction process is achieved by diluting the samples in different extraction buffers specific to groups of biochemical parameters. In fact, for Acetylcholine esterase (AChE), lipid peroxidation (LPO), protein content (PROT), glycogen (GLY), superoxide dismutase (SOD), catalase (CAT), total antioxidant capacity (TAC), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione-S-transferase (GSTs) and carboxyl esterases (CbEs), a standard buffer has been used for the extraction of the supernatant fraction: a 50mM phosphate buffer with 1mM EDTA, 1% Triton X-100, 1mM DTT at pH = 7.0. For the 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide (MTT) extraction, a phosphate buffer (K_2HPO_4, 0.1 M, pH 8) has been used. Instead, for the electron transport system (ETS) supernatant extraction, a 0.1 M TRIS-HCl buffer with 15% PVP, 153 μM Magnesium Sulphate ($MgSO_4$) and 0.2% Triton X-100 at pH 8.5. Reduced glutathione (GSH) and oxidized glutathione (GSSG) were extracted using a KPE extraction buffer with 0.1% Triton X-100 and 0.6% sulfosalicylic acid. In every case, 1 mL of the appropriate buffer was pipetted inside the microtube with 0.5 g of tissue (1:2 p/v ratio).

Afterwards, samples were transferred to a TissueLyser II instrument (Qiagen) set at frequency 20 s^{-1} for 1:30 min to allow tissue breakage. Subsequently, homogenates using the phosphate buffer at pH 7.0 were centrifuged at 4 °C for 20 min at 10,000 *g*, at 4 °C for 20 min at 3,000 *g* for ETS and MTT and the samples for GSH/GSSG at the same temperature for 10 min at 10,000 *g*. The process allows the creation of a supernatant with the cellular material of interest, which is essential for analysing the biochemical parameters. Consequently, 1 mL of the liquid was collected and placed in different aliquots, which were then directly processed for the biomarker analysis or stored at -80 °C until their use.

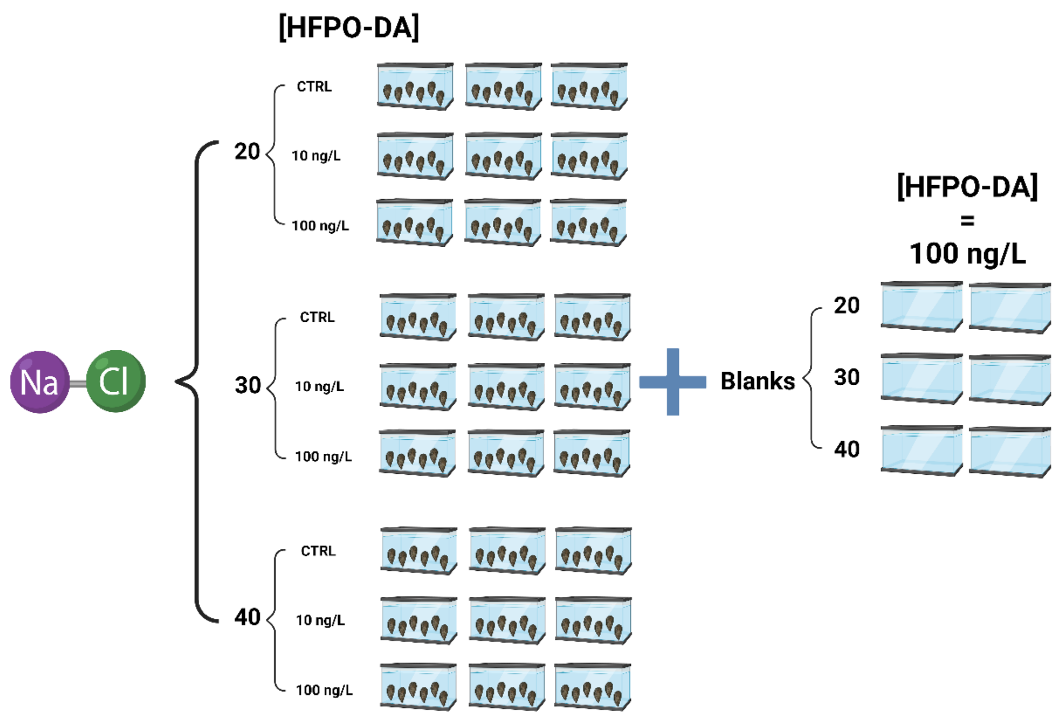


Figure 2: Experimental design for the in vivo experiment. Each treatment presented three different tanks, which were the experimental replicates. Created with BioRender.com.

2.4 – Biochemical analyses

A set of various biochemical parameters was used to assess the biological effect of each tested treatment. In particular, the measurement of metabolic activity occurred using the ETS and MTT assays. The energy reserves have been analysed using GLY and PROT quantification. The measurement of the activity of the TAC and the enzymes SOD, CAT, GPx and GR has evaluated the activity of antioxidants. Biotransformation enzymes that have been assessed are GSTs and CbEs. Redox balance has also been evaluated by analysing reduced glutathione (GSH) and oxidized one (GSSG) level. Oxidative damage was assessed using the LPO assays. Neurotoxicity results from the analysis of the AChE activity.

For the experiment *in vitro*, the biomarkers that have been tested were the antioxidant enzymes SOD, CAT, GPx and TAC; biotransformation enzymes GSTs and CbEs; biomarkers of neurotoxicity AChE and oxidative damage LPO. This set of biomarkers was selected as it covers all the major categories of biochemical responses to oxidative stress: it gives a broad view of the possible oxidative stress caused by these compounds in the subcellular fraction. On the other hand, the *in vivo* experiment included all the previously mentioned biomarkers.

For all the selected biomarkers, *in vitro* and *in vivo* assays, analyses were carried out in technical duplicates (except for GLY and GSSG due to the high reaction speed), and a BioTek Epoch 2 microplate reader did the measurements. Except for PROT, GLY, ETS, MTT, TAC, GSH, GSSG and LPO, which were expressed per fresh weight (FW), the others, as they are enzymes, their activity has been standardized by the PROT of the respective sample, calculated as described in the “Energy reserves” section. For each biomarker, a preliminary test for the dilution with four samples and three dilution levels has been carried out to avoid overflow due to the highest response that the machine could register.

A blank was used in all the biochemical parameters analysed: it was composed of only the extraction buffer for the biomarker of interest and all the reagents present in the assay without the sample. It is used to apply background correction by removing, during the calculation of the activity, the absorbance related only to the reagents. In the case of using the standard, the lowest standard concentration has been used as a blank to apply background correction.

2.4.1 - Metabolic capacity

The ETS in the cells corresponds to the system of dehydrogenase enzymes and cytochrome *c* electron carrier proteins that, through oxygen consumption, produce Adenosine triphosphate (ATP). Its variation from a normal level may be caused by stressors present in the surrounding environment, which may cause either

an increase or a decrease depending on the severity of the alteration (King & Packard, 1975; De Coen & Janssen, 1997; Sokolova et al., 2012)

The ETS activity was measured following the method by King & Packard (1975) and modification by De Coen & Janssen (1997). The method implies the use of the buffer substrate solution (BSS), which is made of 0.13 M Tris-HCl, 0.3% (v/v) Triton X-100 at pH 8.5; a solution which mixes NADH and NADPH, which the ETS uses in concentration 1.7 mM and 250 µM respectively; p-IodoNitroTetrazolium (INT) 8 mM. INT is used as an electron acceptor to register electron transmission rate; in this way, it forms a formazan, which may vary the absorbance measured by a spectrophotometer. After the reaction, the absorbance is read at 490 nm for 10 min in intervals of 25 s. The results are calculated by the extinction coefficient (ε) = 15900 M^{-1} cm^{-1} by the following formula:

$$C = \frac{A}{\varepsilon * b}$$

In this case, the absorbance (A) is divided by the product between the extinction coefficient and the distance travelled by light (b). The results were expressed as nmol per min per g of FW.

Another relevant parameter that gives an idea about the metabolic activity is the MTT assay.

This assay is based on van Meerloo et al. (2011) method with modifications made by Cuccaro et al. (2021). The assay is based on the ability of the succinate dehydrogenase system of the active mitochondria to reduce 3 [4,5-dimethylthiazol-2-y1]-2,5-diphenyltetrazolium bromide to its insoluble form (a formazan): the reaction makes the substance turn from yellow to purple. The absorbance is then read at 560 nm. The amount of formazan in the solution can be calculated using the extinction coefficient (ε) = 51000 M^{-1} cm^{-1} from Bagchi et al. (2001) by the following formula:

$$C = \frac{A}{\varepsilon * b}$$

In this case, the absorbance (A) is divided by the product between the extinction coefficient and the distance travelled by light (b). The results were expressed as nmol per g of FW.

2.4.2 - Energy reserves

Analyzing the quantification of PROT is essential as these molecules are everywhere in the organisms. Their function is crucial in the transportation of compounds, the storage of molecules, and the energy source for the organisms to

cope with defence mechanisms and damages caused, among other things, by the production of ROS (Andrade et al., 2021).

The analysis principle is based on the adaptation of the biuret reaction provided by Robinson & Hogden (1940). The reaction implies that the Copper II exposed in an alkaline environment reacts with the amino acid peptide bonds. In this way, the solution turns to a blue or purple colour. Five standards that report the concentration from 0 to 40 mg/mL using bovine serum albumin (BSA) and distilled water were prepared. The absorbance was read at 540 nm, and the results were calculated through the calibration curve by obtaining the value of the y-intercept and the slope of the standards and using the reverse equation:

$$x = (y - b)/m$$

In which m is the slope, b is the intercept in the y-axis, and y is the absorbance of the sample, the protein concentration in the sample of interest (x) expressed in mg per mL is obtained. The results were expressed in mg per g of FW.

Glycogen is the primary carbohydrate storage form in animals, and its prominent role is to be an energy source. When organisms are under extreme stress, they can decrease their metabolic capacity, avoiding the consumption of their energy reserves. Conversely, in the absence of extreme situations, an increase in ROS and a decrease in reserves can be detected (Sokolova et al., 2012).

The analysis of the amount of GLY is based on the method from DuBois et al. (1956), which relies on the reaction of phenol with sulfuric acid: it can be used for the quantification of sugars, their methyl derivates, oligosaccharides and polysaccharides. Phenol is used at 5% to alter the membrane permeability without killing the cell, while sulfuric acid breaks di- oligo- and polysaccharides to allow the quantification of sugars. The measurements are done through 8 standards of glucose, ranging from 0 mg/mL to 5 mg/mL, which is useful to quantify the amount in the samples. The absorbance read at 492 nm. The results are then calculated through the calibration curve:

$$x = (y - b)/m$$

In which m is the slope, b is the intercept in the y-axis, and y *is* the absorbance of the sample, the protein concentration in the sample of interest (x) expressed in mg per mL is obtained. Results were expressed as mg per g of FW.

2.4.3 – Antioxidant capacity

The antioxidant enzyme SOD is an enzyme that catalyses the dismutation of the superoxide radical ($\bullet O_2^-$), producing H_2O_2 and molecular oxygen (Regoli & Giuliani, 2014).

The activity of SOD was determined following the protocols of (Magnani et al., 2000): it is based on the measurement of the enzyme activity through the inhibition of the pyrogallol autoxidation by the dismutation of the $\bullet O_2^-$. Pyrogallol is subject to autoxidation at 50% in the presence of EDTA at pH 8.2. Calculation of the activity of SOD is obtained in the percentage of inhibition and activity is calculated as follows:

$$\text{\% inhibition of pyrogallol autoxidation} = \frac{\Delta\ \text{Abs sample}}{\Delta\ \text{Abs blank}} \times 100\%$$

$$\text{(Cu-Zn) SOD activity (U/mL)} = \frac{\text{\% inhibition of pyrogallol autoxidation}}{50\%}$$

The results were expressed in U per mg of PROT, which is the activity of the enzyme standardized by the protein content of the sample, where U corresponds to the amount of enzyme that catalyses the conversion of 1 micromole (μmol) of $\bullet O_2^-$ per min.

The enzyme CAT catalyses the decomposition of two H_2O_2 molecules by releasing oxygen and water. Its action is critical to decreasing the presence of H_2O_2 molecules in the cytoplasm, forming oxygen and water (Regoli & Giuliani, 2014).

The activity of CAT was determined following the procedure of Johansson & Borg (1988) with modification from Carregosa et al. (2014). It involves the reaction of the enzyme with methanol based on an adequate concentration of H_2O_2. In this way, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) is used as a chromogen because of the reaction with the formaldehyde so created. The technique requires using the Potassium Periodate to catalyse the reaction between formaldehyde and Purpald, with the consequent formation of Potassium hydroxide. Nine standards from 0 μM to 150 μM of formaldehyde are prepared by dilution in reaction buffer (K_2HPO_4+KH_2PO_4, 50 mM, pH 7.0). The readings are carried out 5 min after the release of the latter reagent at 540 nm, and the results are then calculated through the calibration curve by obtaining the value of the y-intercept and the slope of the standards, the use of the equation:

$$x = (y - b)/m$$

In which *m* is the slope, *b* is the intercept in the y-axis, and *y is* the absorbance of the sample, the concentration of enzyme in the sample of interest (*x*) expressed in

mg per mL is obtained. The final concentration of the parameter is then expressed in the formation of 1nmol formaldehyde per minute per mg of PROT.

The GR is also classified as an antioxidant enzyme that recycles oxidized glutathione (GSSG) to form reduced glutathione (GSH). It maintains the right balance between GSH and GSSG through NADPH consumption, maintaining the redox balance (Regoli & Giuliani, 2014).

The principle of the method, based on an adaptation of the one proposed by Carlberg & Mannervik (1985), is observing the oxidation of NADPH, whose consumption corresponds with the creation of 2 molecules of GSH from one of GSSG. The oxidation of NADPH can be monitored at 340 nm. Hence, the rate of decrease of the measurements carried out during 5 min at intervals of 15 s. The level of dilution used for this biomarker in the *in vivo* experiment is 2x. The quantification occurred by use of the extinction coefficient (Ɛ) 6220 M^{-1} cm^{-1} by the following formula:

$$C = \frac{A}{\varepsilon * b}$$

In this case, the absorbance (A) is divided by the product between the extinction coefficient and the distance travelled by light (b). The results were expressed as nmol per minute per mg of PROT.

The GPx is another antioxidant enzyme whose function is to scavenge the H_2O_2 present in the cell and release water. This is achieved by oxidising the reduced Glutathione (GSH), which is used as an electron donor (Regoli & Giuliani, 2014).

The enzyme was analysed using the method described by Paglia & Valentine (1967), which is based on the reduction of cumene hydroperoxide through the oxidation of GSH to form GSSG. GR promptly reduces the product with the reformation of GSH and consequent conversion of NADPH to NADP+, which can be identified spectrophotometrically. The readings are carried out in 5 min with a reading every 15 seconds at 340 nm, and the reaction shows a decrease in absorbance proportional to the GPx concentration in the sample. The quantification occurred by use of the extinction coefficient (Ɛ) 6220 M^{-1} cm^{-1} by the following formula:

$$C = \frac{A}{\varepsilon * b}$$

In this case, the absorbance (A) is divided by the product between the extinction coefficient and the distance travelled by light (b). The results were expressed as nmol per min per mg of PROT.

The TAC measures a biological sample's ability to neutralize free radicals or ROS (Benzie & Strain, 1996). The analysis of this parameter is based on the protocol of Benzie & Strain (1996), modified by Wootton-Beard et al. (2011) and Capó et al. (2020): it consists of the use of the Ferric reducing antioxidant power (FRAP) solution, in which occurs the reduction of a ferric ion (Fe^{3+}) to a ferrous ion (Fe^{2+}) by the antioxidants present in the sample. The result is a formation of the ferrous tripyridyl triazine complex, whose absorbance can be measured with a sequence of readings every 15 s for 4 min at 593 nm. Twelve standards from 0 µM to 1000 µM of Fe (II) are prepared by dilution in distilled water. The results are then calculated through the calibration curve by obtaining the value of the y-intercept and the slope of the standards, the use of the equation

$$x = (y - b)/m$$

in which *m* is the slope, *b* is the intercept in the y-axis, and *y is* the absorbance of the sample, the protein concentration in the sample of interest (x) expressed in mg per mL is obtained. The TAC was expressed in µmol per g of FW.

2.4.4 - Biotransformation capacity

The biotransformation enzyme GSTs is one of the major phase II detoxification enzymes found mainly in the cytosol, and they catalyse the conjugation of the reduced form of GSH with the electrophilic centres of several compounds, for instance, xenobiotics, and the inactivation of lipoperoxidation products formed during oxidative damage (Habig et al., 1974; Regoli & Giuliani, 2014)

It has been analysed using the methodology of Habig et al. (1974). In particular, the enzyme catalyses the conjugation reaction of the 1-Chloro-2,4-dinitrobenzene (CDNB) with the GSH. This forms a thioether in which the extinction coefficient can be monitored spectrophotometrically through a sequence of reads. The results are presented as the increase in absorbance at 340 nm every 15 s for 5 min. The quantification occurred by use of the extinction coefficient (Ɛ) 9600 M^{-1} cm^{-1} by the following formula:

$$C = \frac{A}{\varepsilon * b}$$

In this case, the absorbance (A) is divided by the product between the extinction coefficient and the distance travelled by light (b). The results were expressed as nmol per min per mg of PROT.

The other biotransformation enzymes analysed are the CbEs, which are classified as phase 2 detoxification, and there are different isoforms in the cytoplasm (Hosokawa & Satoh, 2001; Solé et al., 2018).

The method is based on the Hosokawa & Satoh (2001) method, with corrections provided by Solé et al. (2018), and it focuses on using p-nitrophenyl butyrate. A continuous spectrophotometric enzyme assay determines the hydrolysis rate of this compound. The measurements are carried out at 405 nm every 15 s for 5 min. The level of dilution used for this biomarker in the *in vivo* experiment is 2x. The quantification occurred by use of the extinction coefficient (Ɛ) 18000 M^{-1} cm^{-1} by the following formula:

$$C = \frac{A}{\varepsilon * b}$$

In this case, the absorbance (A) is divided by the product between the extinction coefficient and the distance travelled by light (b). The results were expressed as nmol per min per mg of proteins.

2.4.5 - Redox balance

The evaluated biomarker is the ratio between GSH and GSSG (GSH/GSSG) to determine the redox balance. The ratio of the two forms of glutathione is important, as it indicates the oxidative stress in the cells (Regoli & Giuliani, 2014). Both GSH and GSSG are based on the principle of Rahman et al. (2006), and in both cases, the absorbance is read at 412 nm and 750 nm in a sequence of 5 reads in 2 min (one every 30 s). Results are then calculated through the respective calibration curve: by obtaining the value of the y-intercept and the slope of the standards, the use of the equation

$$x = (y - b)/m$$

in which m is the slope, b is the intercept in the y-axis, and y is the absorbance of the sample. The protein concentration in the sample of interest (x) expressed in nmol per g of FW for the single parameters is obtained. At the same time, no unit of measurement is used for the ratio of the two glutathione forms.

The analysis of GSH relies on the principle that the oxidation of GSH occurs by the sulfhydryl reagent 5,5′-dithio-bis (2-nitrobenzoic acid) (DTNB) and forms the 5′-thio-2-nitrobenzoic acid (TNB). The protocol requires the use of 7 standard concentrations that range from 0 to 90 µmol/L of GSH and imply the mixing of the GSH stock solution with KPE buffer.

On the other hand, the quantity of GSSG in the sample can be determined by the activity of the GR; in particular, the protocol is based on the measurement of NADPH consumption by the enzyme. To bind the GSH, 2-vinylpyridine is used, and the excess of this compound is neutralized with triethanolamine. The protocol requires the use of 7 standard concentrations that range from 0 to 90 µmol/L of GSSG and imply the mixing of the GSSG stock solution with KPE buffer.

2.4.6 - Cellular damage

Cellular damage refers to the event in which ROS alter the structure of cellular components. Due to their reactivity, cellular components like lipids can be damaged and form compounds like lipid peroxyl radical (LOO) and lipid hydroperoxides (LOOH), which are highly reactive: these start a chain reaction until compounds which is quenched by radical scavengers or by other enzymes (e.g. GSTs) (Regoli & Giuliani, 2014).

The principle of the method by Ohkawa et al. (1979), with modification by Carregosa et al. (2014), is based on the estimation of LPO through the quantification of the thiobarbituric acid reactive substances (TBARS). These can be formed during the reaction between LPO by-products with 2-thiobarbituric acid (TBA).

The calculation is done considering the extinction coefficient of (ε) = 156000 M^{-1} cm^{-1} of MDA by the following formula

$$C = \frac{A}{\varepsilon * b}$$

in which the absorbance (A) is divided by the product between the extinction coefficient and the distance travelled by the light (b). The results were expressed as nmol of MDA per g of FW.

2.4.7 - Neurotoxicity

The class of enzymes of the cholinesterase (ChEs) that hydrolyse mainly choline-based esters used as neurotransmitters. One of the most studies is the acetylcholine esterase (AChE), which is the primary biomarker of cholinergic impairment: the enzyme is responsible for breaking down the acetylcholine released in the synaptic, but its activity may be inhibited by different compounds (Monserrat et al., 2007, 2011).

The protocol is based on Ellman et al. (1961) and (Mennillo et al., 2017) for the adaptation to microplates. The method uses the Acetylthiocholine iodide (ATChI, 5 mM) as a substrate to split to determine AChE activity. The sequence of

reads is carried out at 412 nm for 5 min every 15 s. The results are calculated using the molar extinction coefficient of (ε) = 13600 nM^{-1} cm^{-1} by the following formula:

$$C = \frac{A}{\varepsilon * b}$$

In this case, the absorbance (A) is divided by the product between the extinction coefficient and the distance travelled by light (b). The results were expressed as nmol per min per g of proteins.

2.6 - Statistical analyses

Biochemical data from *in vivo* or *in vitro* conditions (ETS, MTT, GLY, PROT, SOD, CAT, GR, GPx, TAC, GSTs, CbEs, GSH, GSSG, GSH/GSSG, LPO, AChE) and from all treatments were submitted firstly to a process of outlier removal, secondly to hypothesis testing using a permutational multivariate analysis of variance (PERMANOVA) by using the PERMANOVA add-on in PRIMER v6 (Anderson, 2008). The null hypotheses were different for the two approaches.

For the *in vitro* assay, two null hypotheses were tested: (1) no changes in antioxidant defences, biotransformation enzymes, oxidative damage, or neurotoxicity parameters can be detected under different concentrations of HFPO-DA, and (2) no changes occur in these biochemical responses with different exposure durations. Data analysis was done by importing the dataset into PRIMER, transforming, normalizing, and calculating the Euclidean distance matrix. Statistical differences were assessed using a two-factor PERMANOVA test: contaminant concentration (five levels) and exposure time (three levels), with the tissues (DG and G) set as a random factor (two levels). Univariate permutational ANOVA was performed on each response variable, with a significance level of $\alpha = 0.05$. Significant differences between exposure times at the same concentration were marked with an asterisk, while discrepancies between concentrations at the same exposure time were indicated with different letters.

For the *in vivo* assay, the null hypotheses tested were: (1) no differences across salinities for non-contaminated organisms can be found, (2) no differences across salinities for HFPO-DA contaminated mussels are present, and (3) no differences between controls and HFPO-DA contaminated organisms within each salinity level can be recorded. The data analysis was done using nine organisms per treatment and performing the average per aquarium (three organisms per aquarium were used). After square root transformation and normalization of the dataset, the Euclidean distance matrix was subjected to a two-factor PERMANOVA test (HFPO-DA concentration and salinity were the factors, both with three levels each). Univariate permutational ANOVA tests followed for each parameter without normalization, with $\alpha = 0.05$.

Principal Coordinate Analyses (PCOs) based on the Euclidean distance similarity matrix were calculated across all biochemical parameters per treatment for both assays. In the *in vitro* assay, they were used to visualize the difference at 30 minutes of exposure. For the *in vivo* assay, a single PCO illustrated treatment differences. In both assays, Spearman correlation vectors of biochemical descriptors with correlations above 75% were added as supplementary variables in the graphs.

3 - RESULTS

3.1 – In vitro assay

3.1.1- Choice of the time of exposure

This study focused on the 30-min response over the 60-min response due to the observed variability and potential inaccuracies at longer exposure times. Analyses show that there is either a higher or non-significant difference in response at 30 min for most biomarkers. In contrast, at 60 min, the results become inconsistent, particularly for GPx and TAC. These inconsistencies may be attributed to protease activity, which can degrade enzymes and proteins over time, leading to lower or less reliable measurements at 60 min (OECD, 2018b). Studies by Giannessi et al. (2023) and Vieira Sanches et al. (2023) also support the observation of a more robust biomarker response at 30 min.

Moreover, the extended exposure time may introduce additional variability due to factors like reagent handling and enzyme degradation, further justifying the selection of 30 min for more accurate and consistent results. 30 min is the ideal time chosen for these kinds of experiments: altering the exposure times may lead to problems in the interpretation of the results as an alteration of the adopted standard time (OECD, 2018b; Solé et al., 2021; Cruz et al., 2023; Vieira Sanches et al., 2023). The analysis of longer exposure times of the supernatant may, however, be relevant to observe a possible time-dependent response of the enzymes. For this aim, protease inhibitors may further assist this type of analysis, improving the response by making it more accurate and reliable.

3.1.2 – Multivariate analysis

3.1.2.1 - Principal Coordinate analysis (PCO)

The Principal Coordinate analysis (PCO) is a type of graph that shows the overall multivariate response of the different biochemical parameters that have been analysed. Specifically, in the case of this study, the variability of responses between gills (G) and digestive gland (DG) combined with the concentration of the contaminant allows us to summarise the primary trend of the results (Anderson, 2008; Andrade et al., 2021).

A PCO graph has been created to allow for a more simplified understanding of the trend of the results. It is possible to see in Figure 1 that the highest variability can be shown between the organs, as the horizontal axis explains 90.7% of the overall dataset variability. This isolates G, placed on the left part of the graph, and the DG, which is located on the right instead. Most parameters explain this trend, except the AChE, which tends to have a more vertical influence, creating a concentration gradient from the lowest to the highest part of the graph (Figure 3). The biochemical parameters reported in the graph are related to the biological responses using Pearson correlation coefficients. The parameters that explain the variation in the horizontal axis are CAT, CbEs, GPx, GSTs, LPO, SOD and TAC: while CAT, GSTs, CbEs, LPO and TAC are pointing toward the digestive gland, GPx, and SOD are pointing to the gills, indicating a possible higher response of these biomarkers on that organ.

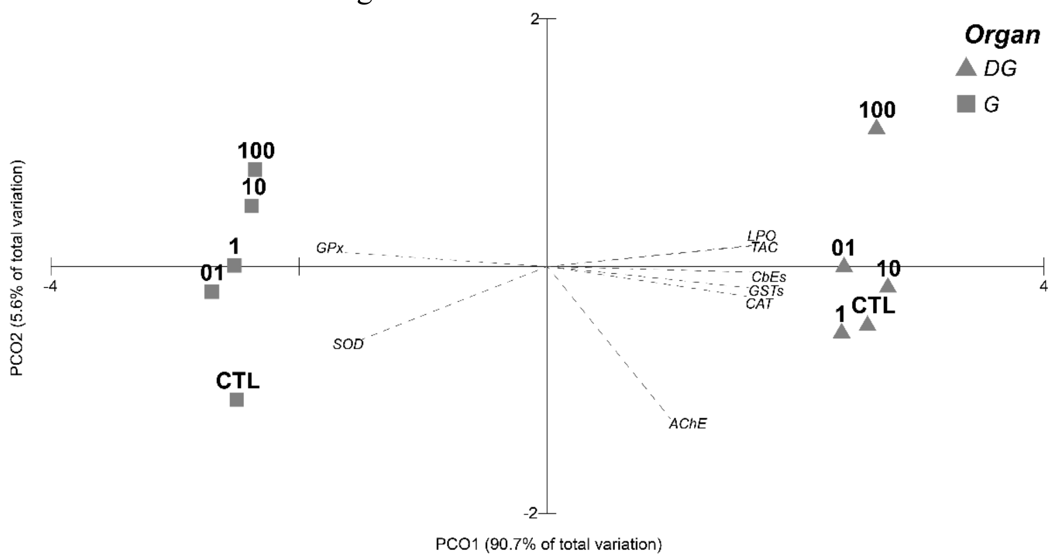


Figure 3: Principal Coordinate analysis (PCO) with data related to 30 min from the in vitro analysis. In the graph, the contaminant concentrations have been reported in µg/L except 0 µg/L (reported as CTL). The legend indicates the two different organs tested, represented in the graph by different symbols. Pearson correlation vectors are superimposed as supplementary variables with r > 0.75: AChE, CAT, CbEs, GPx, GSTs, LPO, SOD and TAC.

3.1.2.2 - Effect of factors

PERMANOVA results show a significant variation of the single random factor organ in the analysis, as the main PCO (Figure 3) also clearly indicates that the two organs are entirely separated. The concentration itself does not significantly influence the results. However, its effect may depend on the organ analysed, as the *p*-value of the interaction is close to the significance level adopted (Table 1).

Table 1: PERMANOVA table of the results. On the left, the factors (Co = Concentration; Or = Organ) with their individual effect (2nd and 3rd rows) and their interaction (4th row)

Source	df	SS	MS	Pseudo-F	P(perm)	Unique perms	P(MC)
Co	4	18.123	4.5308	2.1664	0.2379	7224	0.1827
Or	1	255.41	255.41	254.52	0.0001	9936	0.0001
CoxOr	4	8.3655	2.0914	2.0841	0.0763	9933	0.0773
Res	30	30.104	1.0035				
Total	39	312					

3.1.3 – Biochemical responses

3.1.3.1 - Antioxidant capacity

The results show that the activity of SOD in the DG was significantly higher at 1 µg/L than the control, while at higher concentrations, the trend highlights a significantly lower activity than the control (Figure 4A). The G instead report a decrease in activity for SOD proportional to the concentration (Figure 4E).

Related to the CAT, in the DG, it is possible to see a significant inhibition of the enzyme activity regardless of the contaminant concentration (except for the concentration of 10 µg/L) (Figure 4B). In the G, there is a significant inhibition of the activity of the enzyme at the highest concentration (100 µg/L) (Figure 4F).

In the case of the GPx, in the DG, a significant decrease in enzyme activity was reported only at the intermediate concentration (1 µg/L) (Figure 4C). Conversely, in G, the enzyme activity increased at the highest concentration compared to the control (Figure 4G).

The TAC content in the DG showed a significant increase at 10 µg/L compared to the control (Figure 4D). In G, there is a significant increase in at 0.1 µg/L, 1 µg/L and 100 µg/L compared to the control, while no significant difference at 10 µg/L can be observed neither against the control nor against the other concentrations (Figure 4H).

ANTIOXIDANT ENZYMES

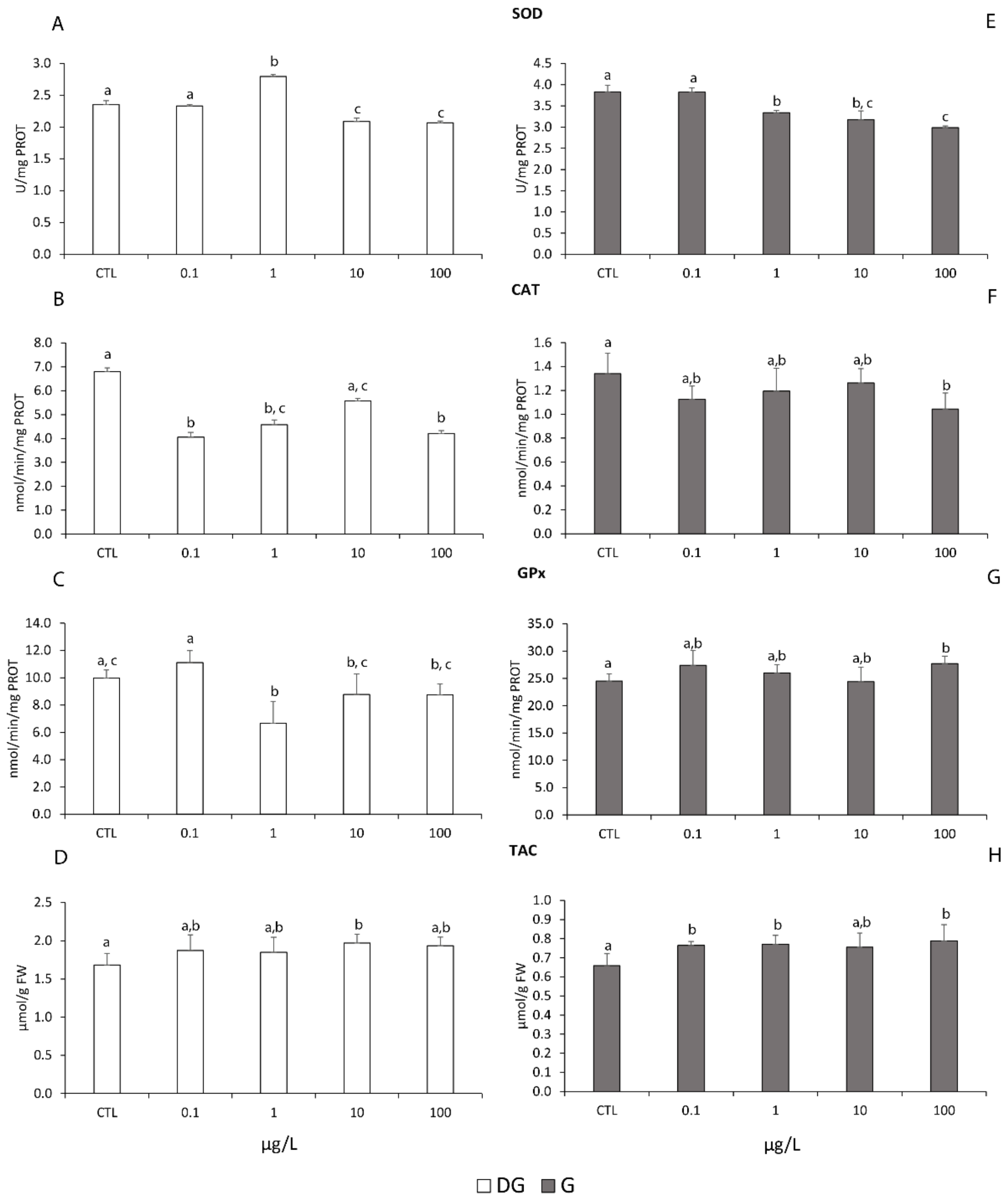


Figure 4: Antioxidant capacity: Superoxide dismutase (SOD), Catalase (CAT), Glutathione peroxidase (GPx), Total antioxidant capacity (TAC). A-D: digestive gland; E-H: gills. "CTL" identifies 0 µg/L. Different letters represent a significant difference among treatments.

3.1.3.2 - Biotransformation capacity

Related to the GSTs in the DG, the results show that a significant decrease in the activity can be reported at 10 µg/L compared to the control. In contrast, there is no significant difference with the other concentrations tested (Figure 5A). In the G, a significant decrease in the enzyme activity was observed at 1 µg/L compared to the control (Figure 5C).

The activity of CbEs in the DG does not show any significant change in all levels of the contaminant compared to the control. Nevertheless, a significant inhibition is presented between 0.1 µg/L and 10 µg/L and a subsequent rise in activity between 10 µg/L and 100 µg/L (Figure 5B). In the G, there are no significant differences among treatments (Figure 5D).

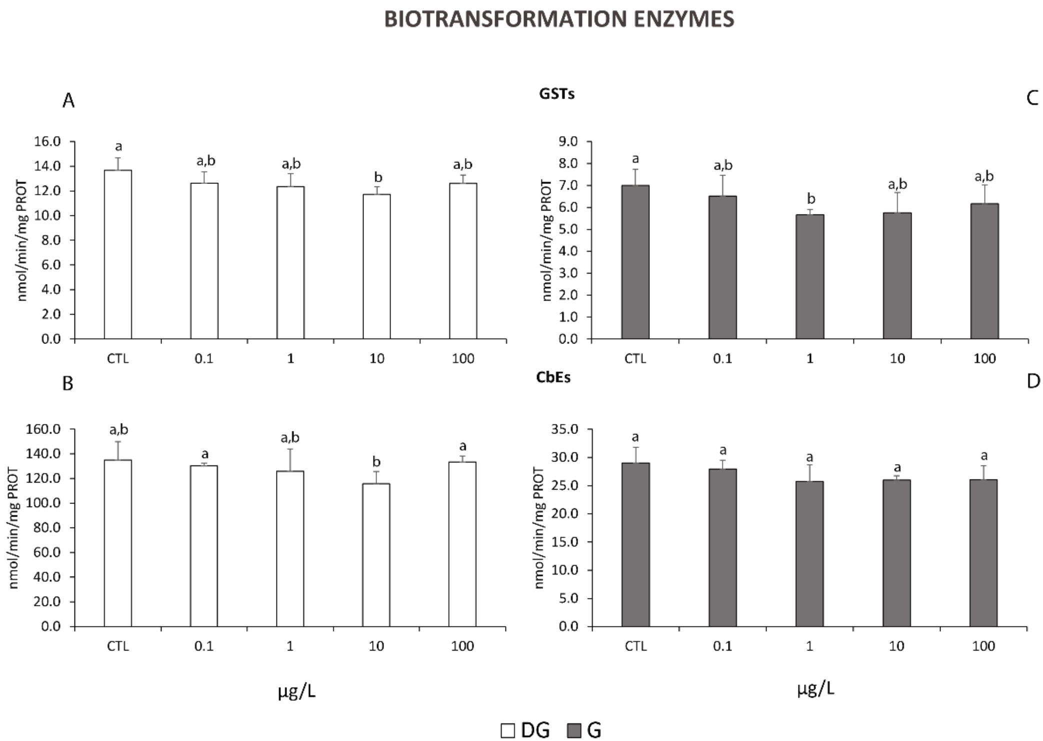


Figure 5: Biotransformation enzymes: Glutathione S-transferases (GSTs) and Carboxilesterases (CbEs) in Mytilus galloprovincialis. A-B: digestive gland; C-D: gills. "CTL" identifies 0 µg/L. Different letters represent significant differences (p<0.05) among treatments.

3.1.3.3 - Oxidative damage

Considering LPO in the DG, it was possible to see a significant increase with increasing concentration of the contaminant. A further increase was recorded between 10 µg/L and 100 µg/L at the same exposure time (Figure 6A). In contrast, in the G, there is a significant increase in oxidation comparing the control with 0.1 µg/L, 1 µg/L and 100 µg/L (Figure 6B).

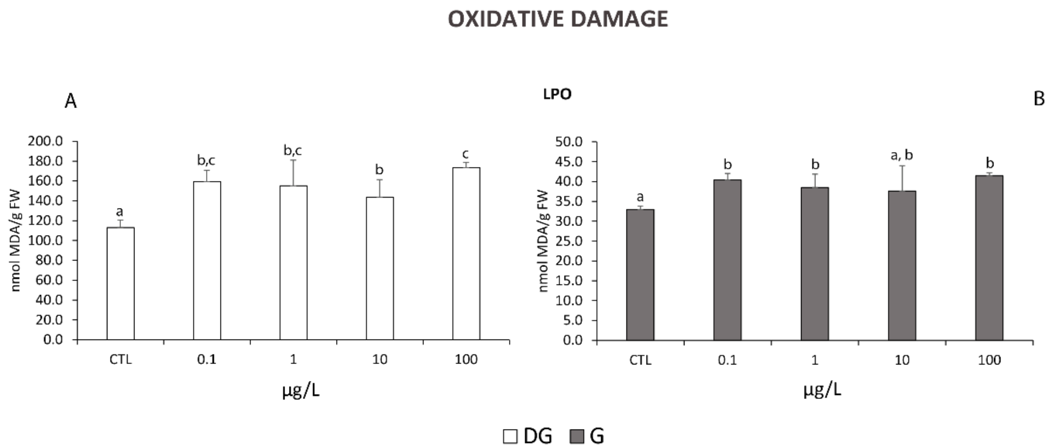


Figure 6: Oxidative damage: Lipid peroxidation (LPO) levels in Mytilus galloprovincialis. A: digestive gland; B: gills. "CTL" identifies 0 µg/L. Different letters mean a significant difference (p<0.05) among treatments.

3.1.3.4 - Neurotoxicity

In the case of the AChE, in the DG, a significantly increased activity can be reported at 10 µg/L than 1 µg/L, both of which do not differ from the control (Figure 7A). On the other hand, in the G, a significant inhibition of the activity was observed between the control and 1 µg/L and also between the control and 10 µg/L (Figure 7B).

NEUROTOXICITY

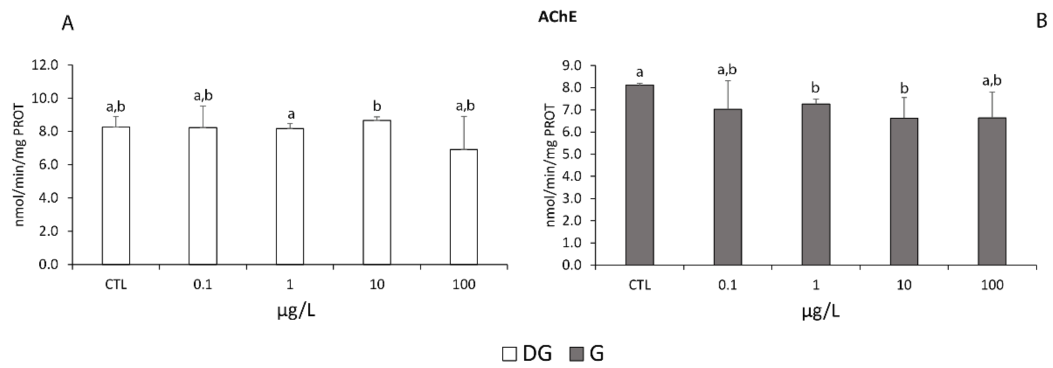


Figure 7: Neurotoxicity: acetylcholinesterase (AchE) activity in Mytilus galloprovincialis. A: digestive gland; B: gills. "CTL" identifies 0 µg/L. Different letters represent significant differences ($p<0.05$) among treatments.

3.2 – In vivo assay

3.2.1 – Multivariate analysis

3.2.1.1 – Principal Coordinate analysis (PCO)

The PCO explains the disparity of responses between the different salinities and concentration of the contaminant *in vivo* (Figure 8). In this case, the main variability is between the 3 salinities, with the PCO1 explaining a horizontal variation of 58.6%. This is explained mostly by AChE, CAT, CbEs, ETS, GPx, GR, GSH/GSSG, GSTs, MTT, SOD and TAC. On the other hand, for the vertical axis, the variability (15.8%) is explained by LPO and PROT. It is possible to see that the treatments are grouped by salinity and isolated one from the other: the salinity 40 is on the left, while 30 is on the bottom and 20 on the right. However, the concentration at 100 ng/L of Gen-X at salinity 30 was located closer to the salinity 20 treatments, and the concentrations of 10 ng/L and 100 ng/L of salinity 40 are separated from the control condition and any other treatment on the vertical axis.

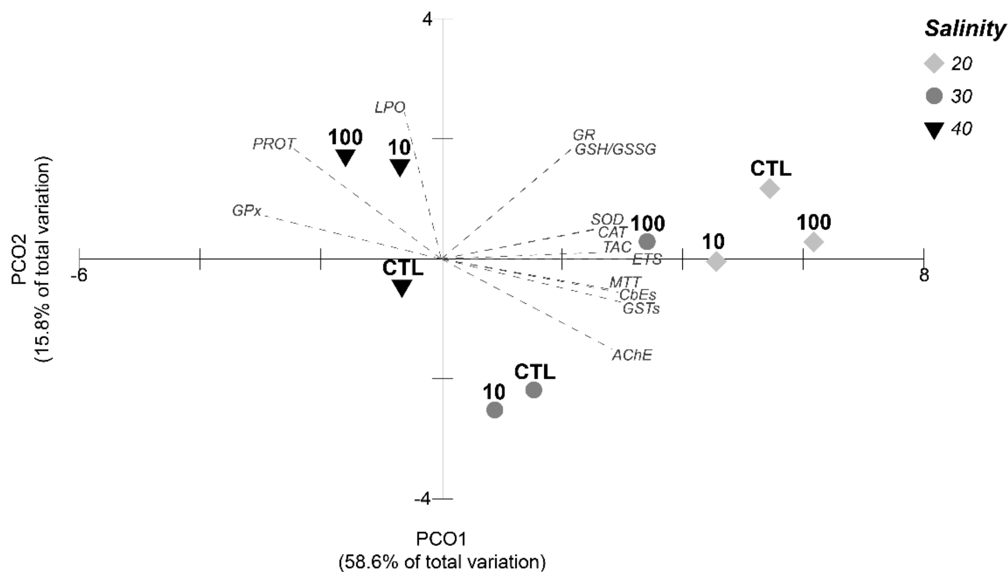


Figure 8: Principal Coordinate analysis (PCO) with all data from the in vivo analysis. The graph reported the contaminant concentrations in ng/L except for 0 ng/L (reported as "CTL"). Different symbols represent three different salinities. Pearson correlation vectors are superimposed as supplementary variables with r > 0.75: AChE, CAT, CbEs, ETS, GPx, GR, GSH/GSSG, GSTs, LPO, MTT, PROT, SOD, TAC.

3.2.1.2 - Mortality rate

The mortality of the organisms is reported in percentages per treatment, which is the ratio between the number of dead mussels and the number of organisms per treatment at the beginning of the experiment (Table 2).

Table 2: Number and percentage of dead organisms per condition. The condition reports the level of the contaminant (CTL = control; 10 = 10 ng/L HFPO-DA; 100 = 100 ng/L HFPO-DA) and the level of salinity later (20, 30 or 40)

CONDITION	PERCENTAGE (%)
CTL-20	0
CTL-30	0
CTL-40	16.7
10-20	0
10-30	0
10-40	5.6
100-20	5.6
100-30	0
100-40	5.6
TOTAL	**3.7**

3.2.1.3 - Effect of factors

PERMANOVA results did not show any significant difference in the effect of concentration, though there is a significant effect of salinity, and the combination of the two factors is significant (Table 3).

Table 3: PERMANOVA table of the results. On the left, the factors (Co = Concentration; Sa = Salinity) with their individual effect (2nd and 3rd row) and their interaction (4th row)

Source	df	SS	MS	Pseudo-F	P (perm)	Unique perms	P(MC)
Co	2	25.714	12.857	1.7464	0.0923	9928	0.1003
Sa	2	199.29	99.643	13.535	0.0001	9926	0.0001
CoxSa	4	58.483	14.621	1.986	0.0279	9894	0.0304
Res	18	132.52	7.362				
Total	26	416					

3.2.2 - Biochemical responses

3.2.2.1 - Metabolism

According to the graph of the metabolism and energy reserves, it is possible to see, related to the controls, that there is a higher activity of the ETS (Figure 9A) at salinity 20 compared to the one at 30 and 40, all 3 showing a significant difference one another. Secondly, at the concentrations of 10 ng/L and 100 ng/L, a significant decrease has been found at the highest salt level compared to the other 2. Furthermore, at control salinity, a significant increase in metabolism can be found at 100 ng/L compared to the other two conditions.

For the MTT assay, there are no significant changes at different salinities for the controls and the lowest concentration of the contaminant. However, at 100 ng/L, there is a significant increase in metabolism at salinity 30 compared to the other two salinities. Moreover, the change at that level is also significantly different from the control condition and the condition of 10 ng/L with the intermediate condition (Figure 9B).

3.2.2.2 - Energy reserves

For GLY content, there are no significant differences between controls at different salinities. However, there is a significant inhibition when considering the condition at 10 ng/L at the lowest salinity compared to the same concentration at the highest level. In addition, for the condition at 100 ng/L, there is a significant increase at salinity 40 compared to the other levels. Furthermore, concentrations of 100 ng/L at salinity 40 are significant also compared to control and 10 ng/L in the same salinity (Figure 10A).

Considering PROT content, there is a significant increase between salinities 30 and 40 at the control condition, while no difference was detected at the concentration of 10 ng/L and 100 ng/L at different salinities. On the other hand, a significant increase was found between control and 100 ng/L at salinity 30 (Figure 10B).

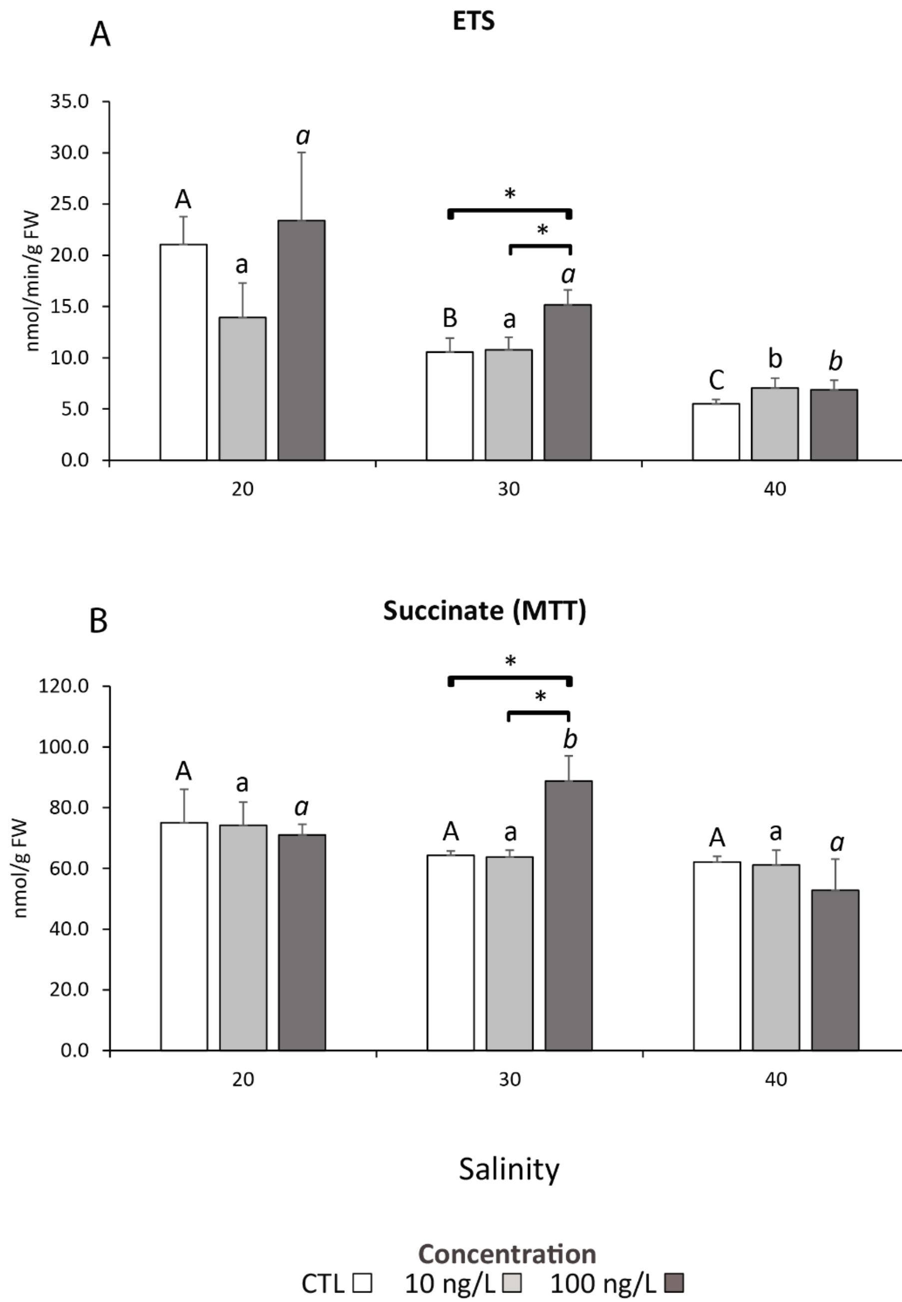


Figure 9: Metabolism parameters in Mytilus galloprovincialis exposed to different salinities (20, 30, 40) and different contaminant concentrations. A: ETS, B: MTT. Different letters identify significant (p<0.05) differences among salinity levels for a given concentration. Asterisks (*) identify significant changes in response between 2 levels of concentration within each salinity.

ENERGY RESERVES

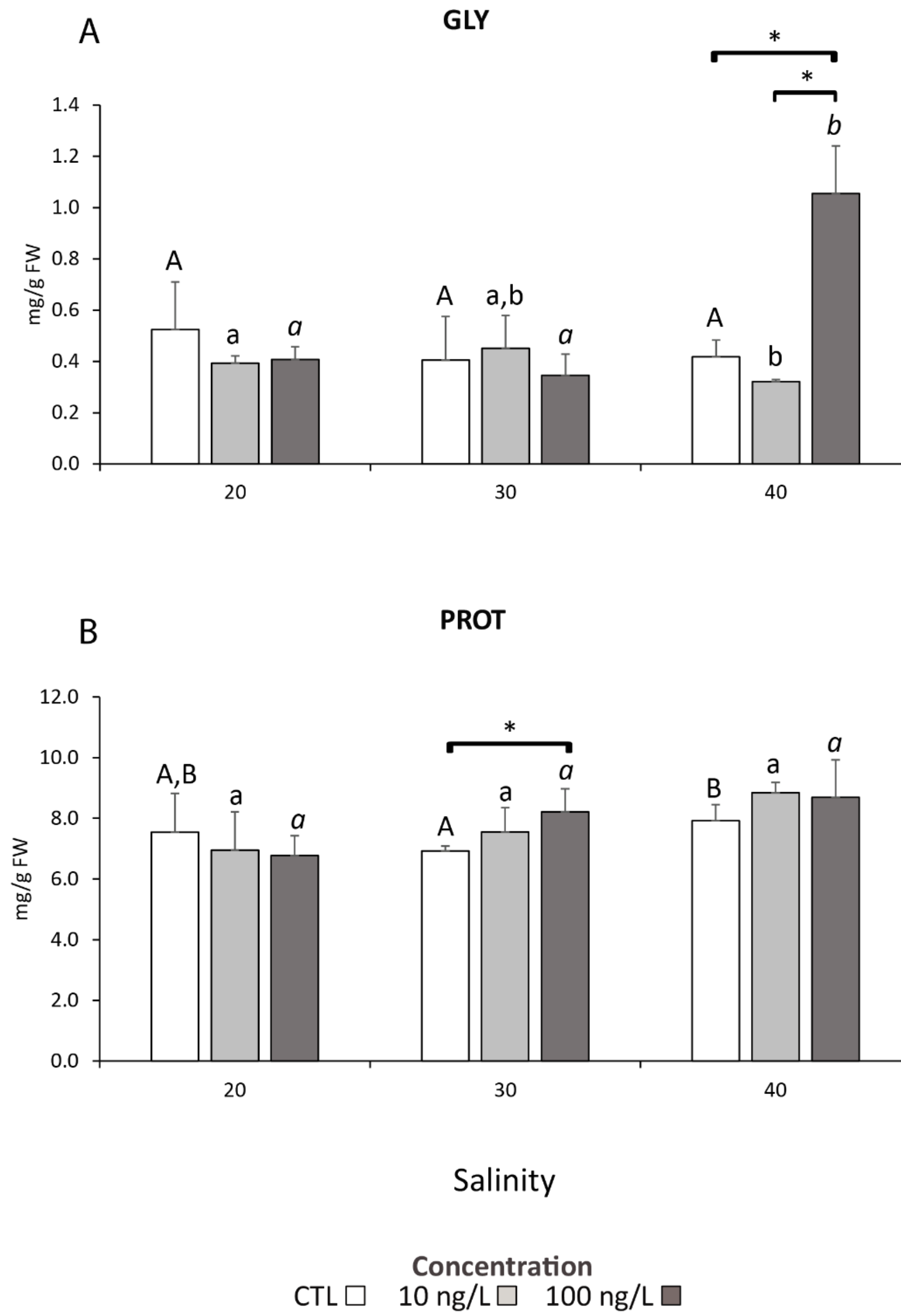


Figure 10: Energy reserves parameters in Mytilus galloprovincialis exposed to different salinities (20, 30, 40) and different contaminant concentrations. A: GLY, B: PROT. Different letters identify significant ($p<0.05$) differences among salinity levels for a given concentration. Asterisks (*) identify significant changes in response between 2 levels of concentration within each salinity.

3.2.2.3 – Antioxidant capacity

For SOD, a significant increase between salinities 30 and 40 at a concentration of 10 ng/L was found. Also, at control salinity, a significant increase in activity between the concentrations 10 ng/L and 100 ng/L has been found (Figure 11A).

Regarding CAT, the controls and concentration of 100 ng/L are significantly higher at salinity 20 compared to the other salinities. In contrast, at 10 ng/L concentrations, the differences are significant only when compared to salinity 30. No significant differences were observed between different concentrations at the same salinity level (Figure 11B).

In GPx instead, a significant increase in the controls and 100 ng/L HFPO-DA exposed mussels at the highest salinity compared to 20 and 30 is shown. In contrast, a significant and directly proportional increase is present among 10 ng/L conditions across the three salinities. Furthermore, there is a significant inhibition at 10 ng/L compared to 100 ng/L at salinity 30 (Figure 12A).

In GR, a significantly higher activity has been found at salinity 20 for both control conditions and 100 ng/L compared to the other two salinities. However, considering 10 ng/L, there is a significant decrease in activity at control salinity compared to the other two conditions. There is a significant decrease between the control and 10 ng/L at salinity 20. Furthermore, at salinity 40, a significant increase is present comparing 10 ng/L to the other two contaminant concentrations (Figure 12B).

Related to TAC, a significantly enhanced content between controls at salinity 20 is found, while a significant decrease is found at 10 ng/L at salinity 40 compared to 30. Significant increase has also been found at salinity 30 comparing 100 ng/L to 10 ng/L and control conditions (Figure 13).

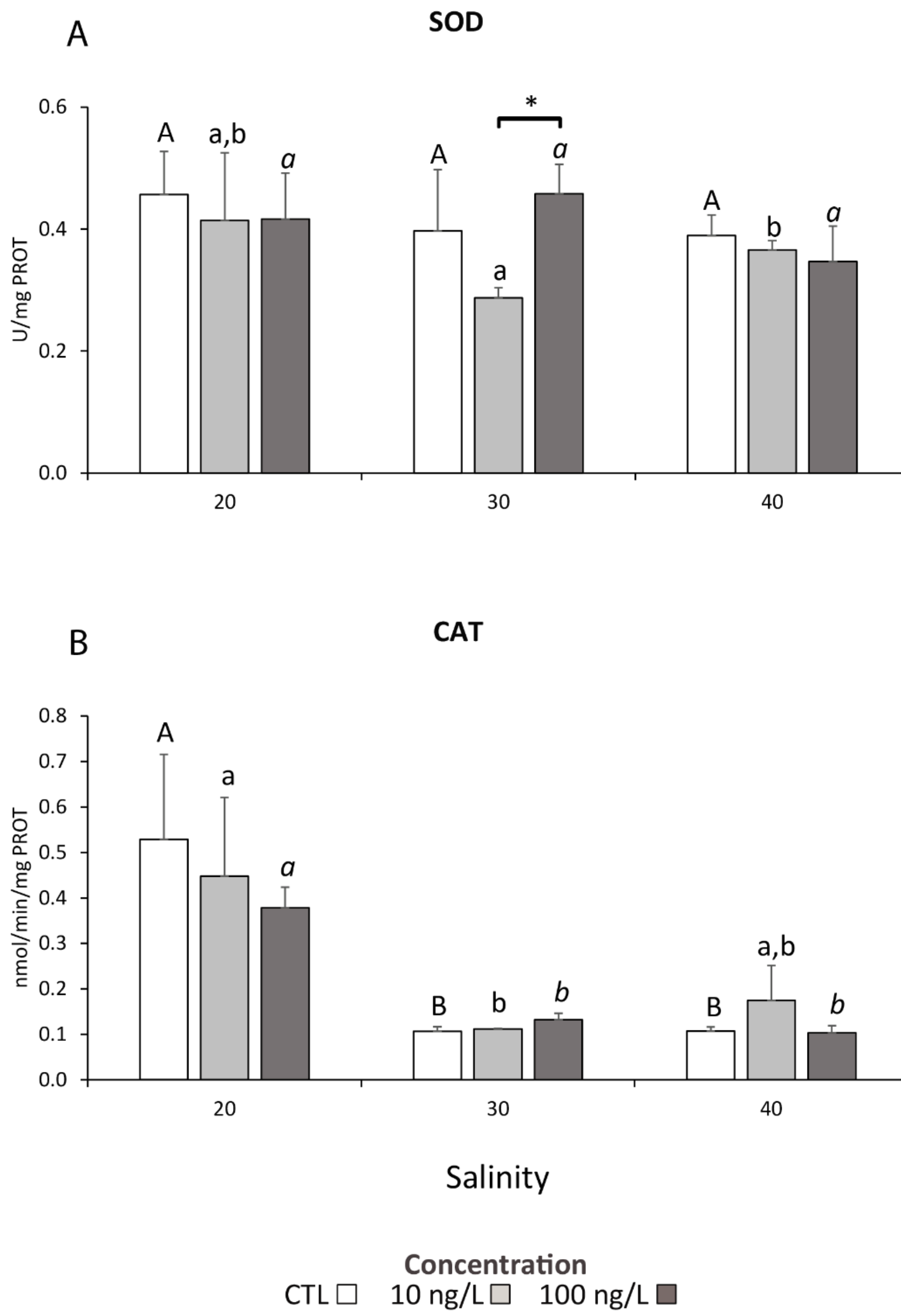


Figure 11: Antioxidant enzymes in Mytilus galloprovincialis exposed to different salinities (20, 30, 40) and different contaminant concentrations. A: SOD, B: CAT. Different letters identify significant (p<0.05) differences among salinity levels for a given concentration. Asterisks (*) identify significant changes in response between 2 levels of concentration within each salinity.

ANTIOXIDANT ENZYMES - 2

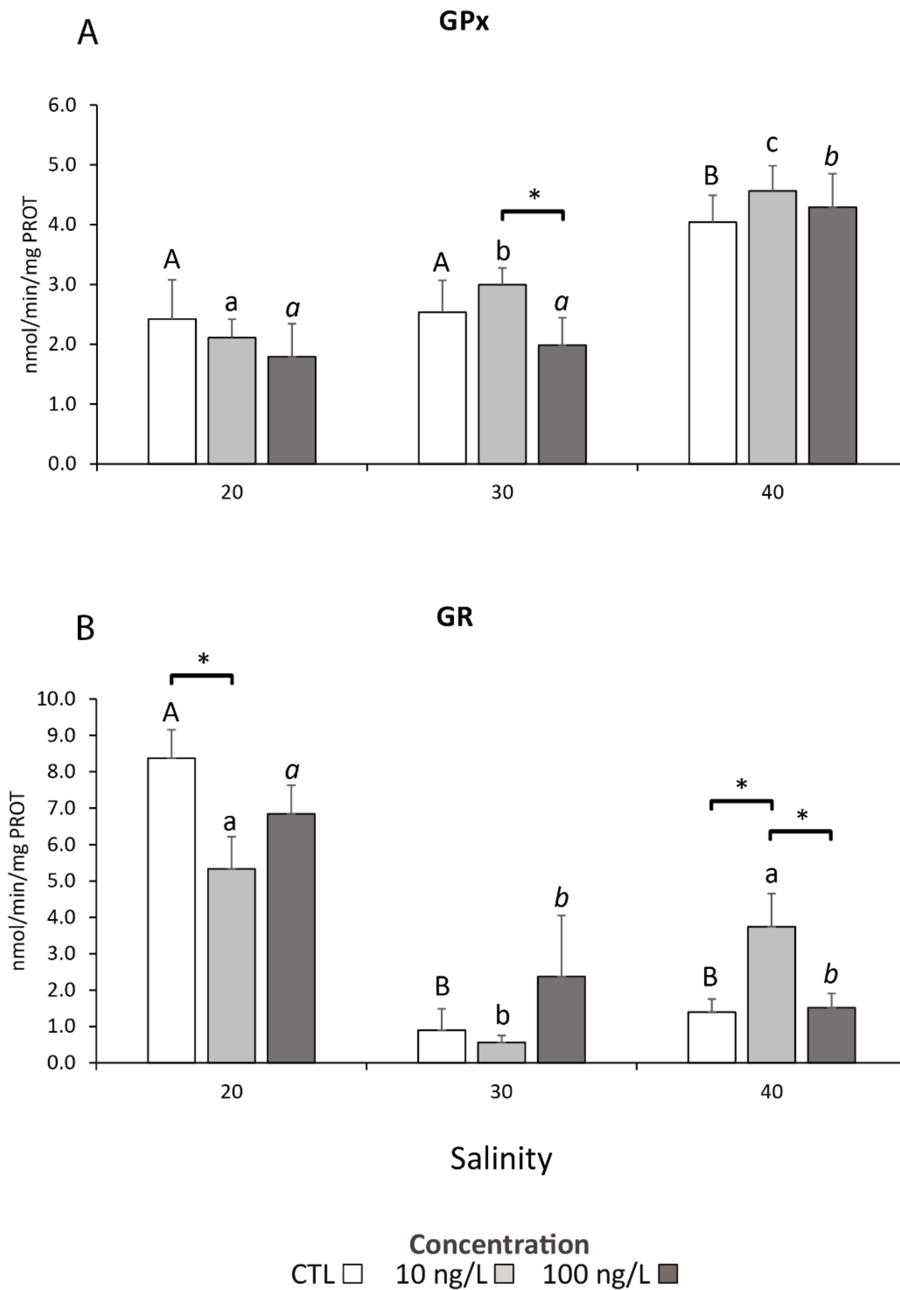


Figure 12: Antioxidant enzymes in Mytilus galloprovincialis exposed to different salinities (20, 30, 40) and different contaminant concentrations. A: GPx, B: GR. Different letters identify significant ($p<0.05$) differences among salinity levels for a given concentration. Asterisks (*) identify significant changes in response between 2 levels of concentration within each salinity.

ANTIOXIDANT CAPACITY

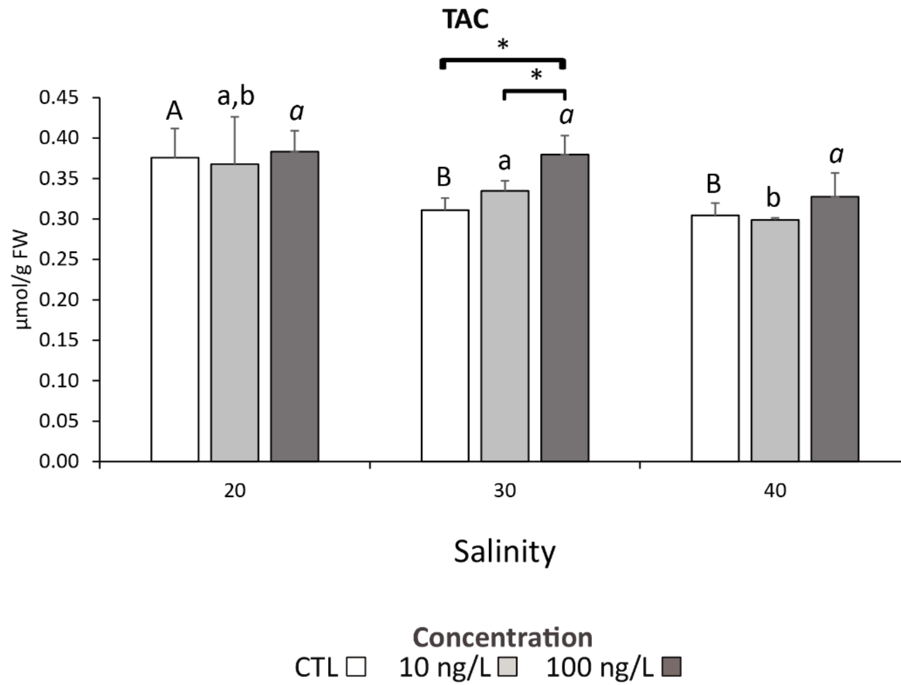


Figure 13: Total antioxidant capacity (TAC) in Mytilus galloprovincialis exposed to different salinities (20, 30, 40) and different contaminant concentrations. Different letters identify significant (p<0.05) differences among salinity levels for a given concentration. Asterisks (*) identify significant changes in response between 2 levels of concentration within each salinity.

3.2.2.4 - Biotransformation capacity

For GSTs activity, a significant inhibition between salinity 20 and 40 at 10 ng/L. Moreover, there is an increased activity at the lowest salinity, considering 100 ng/L, compared to the other two salinity levels. A significant increase exists at salinity 20 between 10 ng/L and 100 ng/L of the contaminant (Figure 14A).

Considering CbEs (Figure 14B), significantly enhanced activity can be shown at salinity 20 to the other two salinities. Moreover, a significant difference between the control and 100 ng/L at the highest salinity level has been found.

BIOTRANSFORMATION ENZYMES

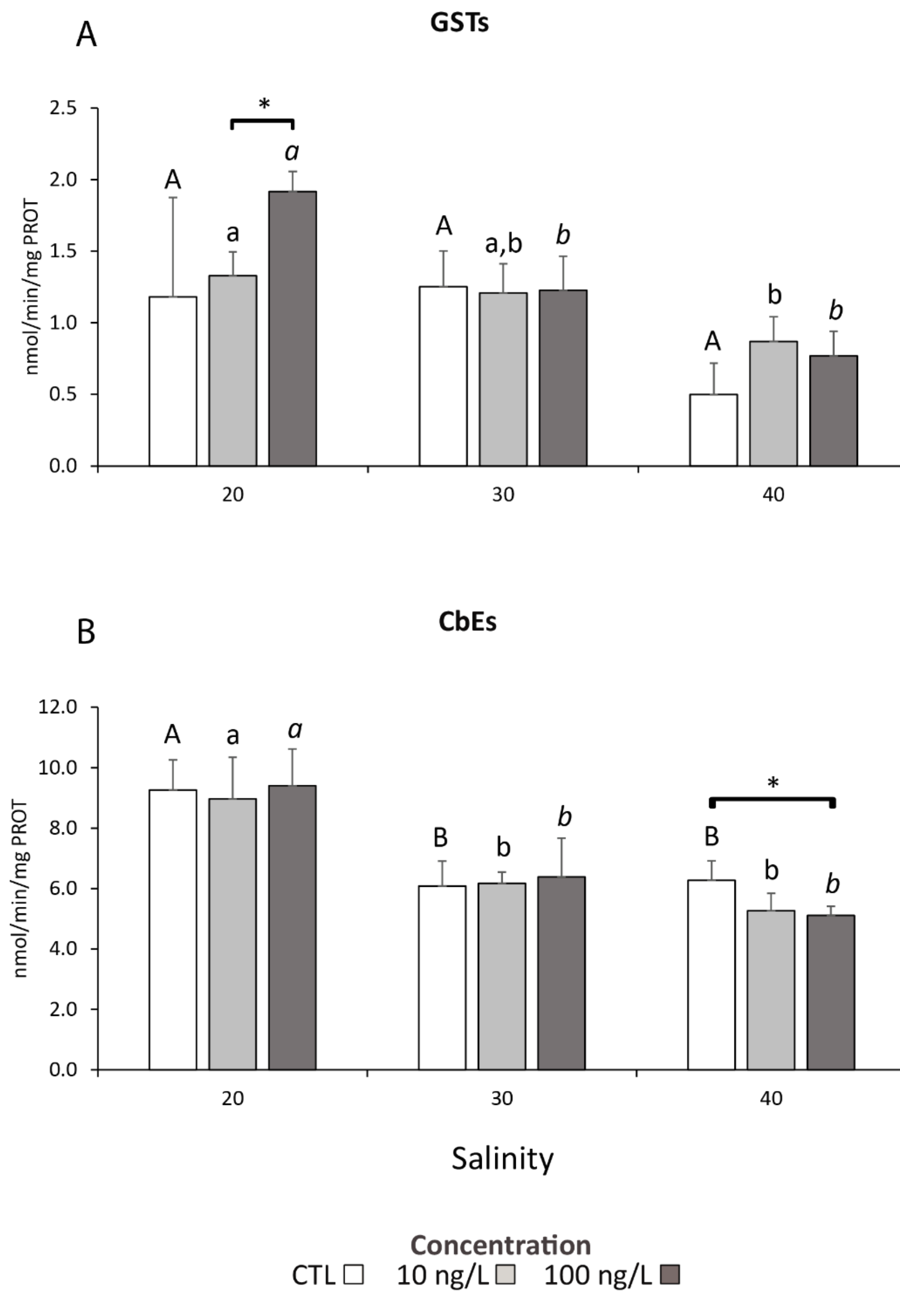


Figure 14: Biotransformation enzymes in Mytilus galloprovincialis exposed to different salinities (20, 30, 40) and different contaminant concentrations. A: GSTs, B: CbEs. Different letters identify significant ($p<0.05$) differences among salinity levels for a given concentration. Asterisks (*) identify significant changes in response between 2 levels of concentration within each salinity.

3.2.2.5 - Redox balance

The GSH content showed a significant decrease at salinity 40 at the concentration 100 ng/L compared to 20. However, there are no significant differences in the contaminant concentration within salinity levels (Figure 15A).

In GSSG analysis, a significant increase is found at salinity 20 compared to the other two salinity tested for the control condition. On the other hand, there are no significant differences in concentration of the contaminant within salinity levels (Figure 15A).

In the ratio of GSH/GSSG, no significant difference has been found between salinities, considering the same concentration, nor within salinities between concentration levels of the contaminant (Figure 16).

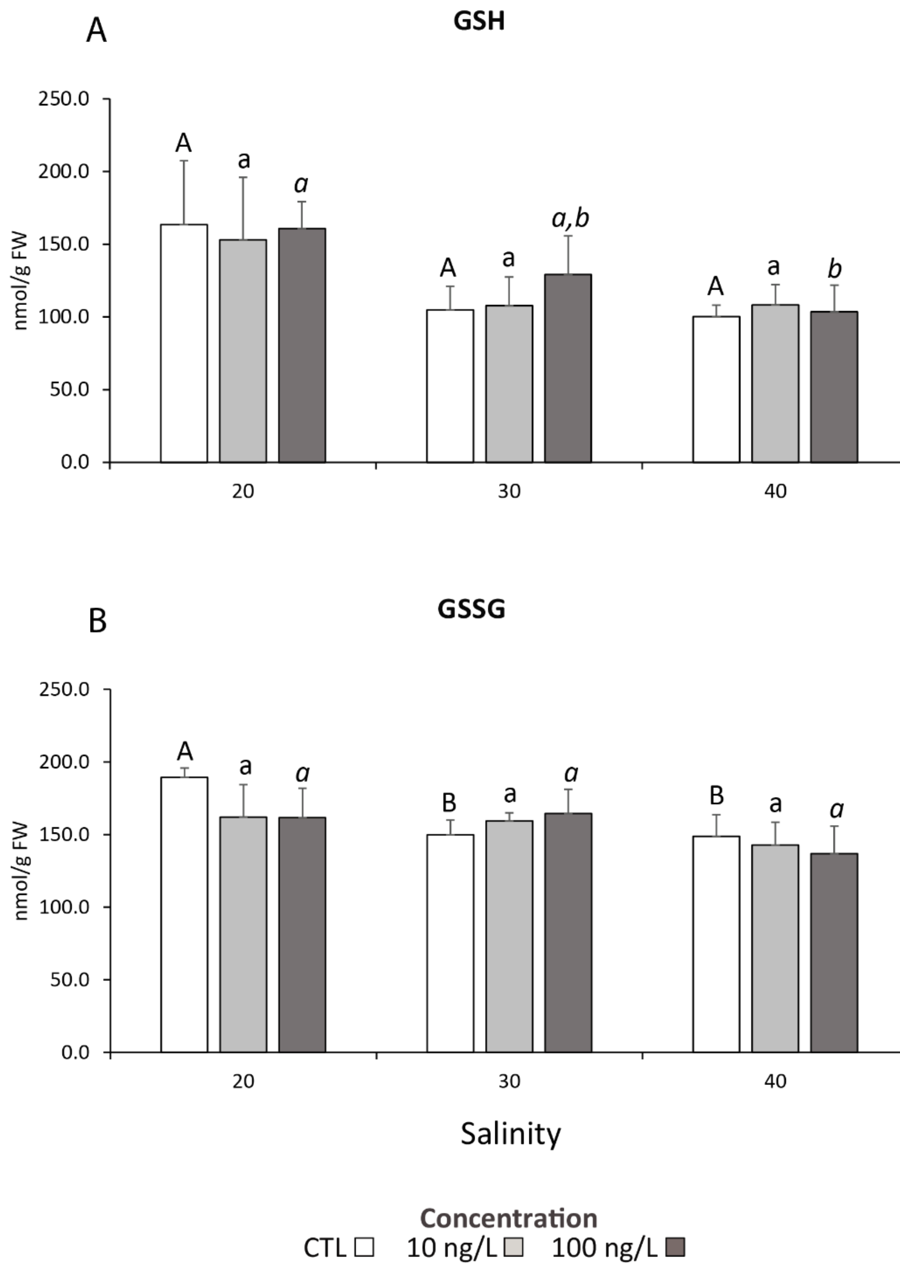


Figure 15: Redox balance of Mytilus galloprovincialis exposed to different salinities (20, 30, 40) and different contaminant concentrations. A: GSH, B: GSSG. Different letters identify significant (p<0.05) differences among salinity levels for a given concentration. Asterisks (*) identify significant changes in response between 2 levels of concentration within each salinity.

REDOX BALANCE - 2

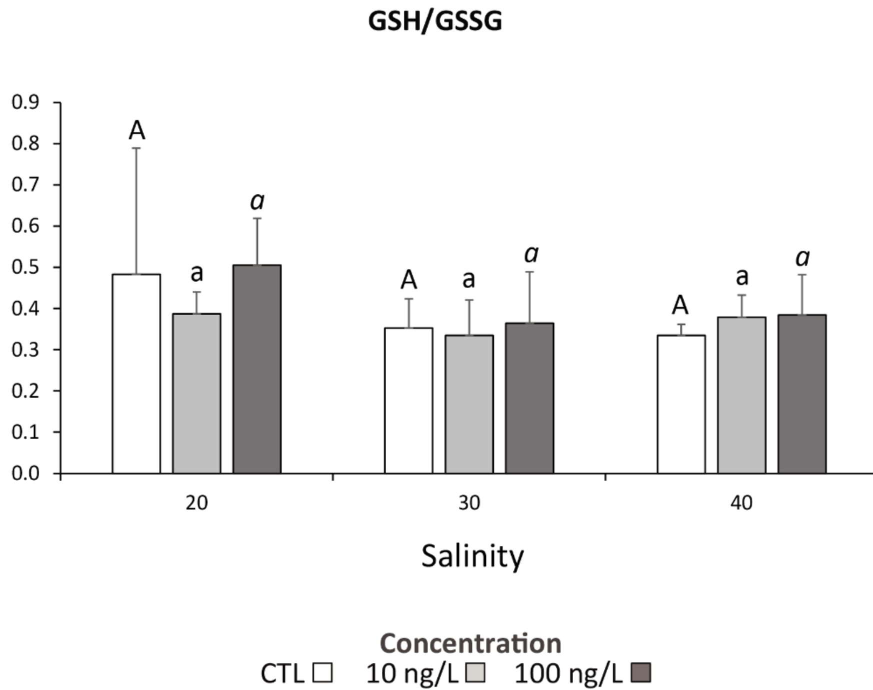


Figure 16: Redox balance (GSH/GSSG ratio) of Mytilus galloprovincialis exposed to different salinities (20, 30, 40) and different contaminant concentrations. Different letters identify significant (p<0.05) differences among salinity levels for a given concentration. Asterisks (*) identify significant changes in response between 2 levels of concentration within each salinity.

3.2.2.6 - Cellular damage

Considering LPO, a significant increase was observed at 10 ng/L concentration comparing salinity 40 and the other two salinities. Furthermore, there was a significant increase in salinity 30 at 100 ng/L compared to the other two concentrations (Figure 17).

3.2.2.7 - Neurotoxicity

Considering AChE, significant decreases were found for concentrations 10 ng/L and 100 ng/L at salinity 40 compared to the same concentrations at the other two salinities. No significant changes were observed within salinities (Figure 18).

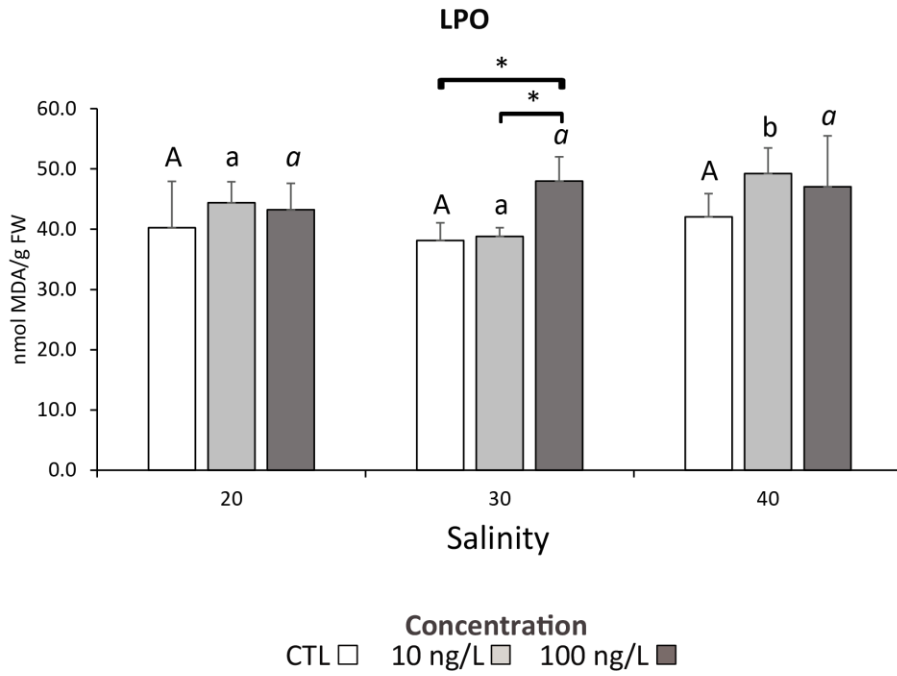


Figure 17: Oxidative damage (LPO) of Mytilus galloprovincialis exposed to different salinities (20, 30, 40) and different contaminant concentrations. Different letters identify significant (p<0.05) differences among salinity levels for a given concentration. Asterisks (*) identify significant changes in response between 2 levels of concentration within each salinity.

NEUROTOXICITY

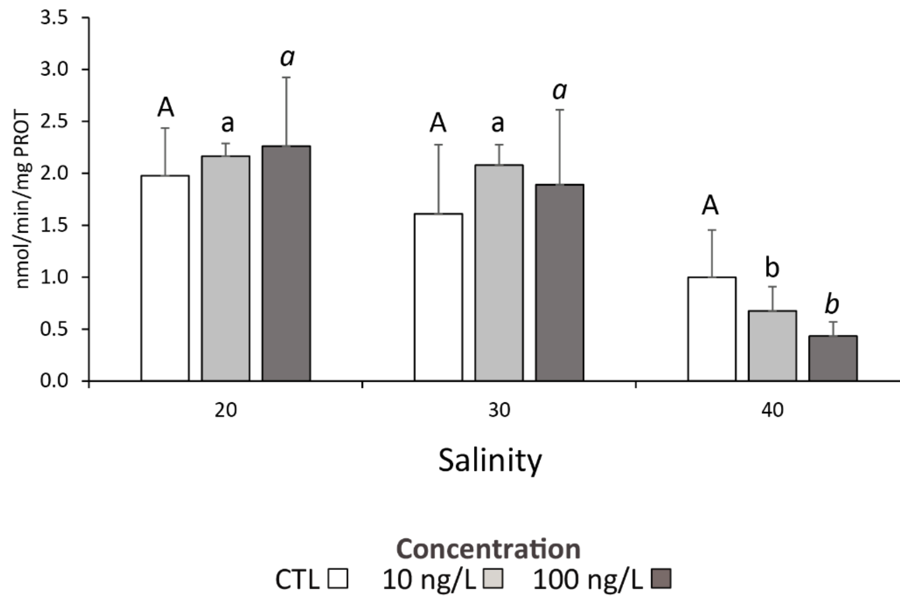


Figure 18: Neurotoxicity (acetylcholinesterase activity, AChE) in Mytilus galloprovincialis exposed to different salinities (20, 30, 40) and different contaminant concentrations. Different letters identify significant (p<0.05) differences among salinity levels for a given concentration. Asterisks (*) identify significant changes in response between 2 levels of concentration within each salinity.

4 - DISCUSSION

Fluoroalkyl substances are widely distributed in the environment, from groundwaters to rivers, reaching estuaries and sea. Due to their environmental persistence, mostly caused by the strong bond between carbon and fluorine molecules, they have been detected both in inorganic and organic matrixes (Heydebreck et al., 2015; Song et al., 2018; Joerss et al., 2020). Their effects range depending on the molecular structure (Niu et al., 2019; Xu et al., 2022; Wang et al., 2023). Nevertheless, most of these compounds are known to interact and bind to proteins directly, altering their functioning, and potentially causing an overall imbalance in the organism's metabolism (Alesio et al., 2022; Wang et al., 2023). One more possible cause of alteration on the welfare of marine mussels is also represented by climate change, which leads also to the alteration of salinity patterns too (IPCC, 2023). With this study, it has been analysed if HFPO-DA alters the protein functioning in *M. galloprovincialis* using the S9 subcellular fraction of the organism, and the possible alteration caused by the contaminant to the mussels directly exposed to it, that has been checked in combination with the reported alteration of salinities in estuaries, to assess whether it can produce different effects.

4.1 - In vitro assay

As previously mentioned, these kinds of studies present a limitation because only the subcellular post-mitochondrial fractions of the organisms are exposed to the chemical, and the short time of exposure plays a role in the regulation of the response too (OECD, 2018b; Vieira Sanches et al., 2023). Therefore, a higher concentration of the contaminant must be used to see whether potential alteration in the oxidative status of the organisms can be seen. In the present study, as carried out in the experiments of Cruz et al. (2023), Giannessi et al. (2023) and Vieira Sanches et al. (2023), the pools of organs were divided by using gills, which are the first organ exposed directly to the water and responsible for the filtration and the creation of currents; and the digestive gland, which instead is the one responsible for the biotransformation and detoxification of the compounds (Livingstone et al., 1992; Regoli & Principato, 1995). In this way, it is possible to define in detail whether the alteration caused by the compound impacts one organ or the other.

4.1.1 – Multivariate analysis general perspective

According to the multivariate analysis, there is an evident distinction between the two organs: gills (G) and digestive gland (DG). This difference has been already reported in other studies, as these organs may present specific isoforms for particular enzymes, or they may show a different expression of antioxidant enzymes compared to biotransformation, maybe also caused by the different

contact with the external medium and functional role of the organs (Livingstone et al., 1992; Regoli & Principato, 1995; Vieira Sanches et al., 2023). In fact, the DG exhibit a higher expression of biotransformation and detoxification enzymes than mussels' gills (Regoli & Principato, 1995). The expression of different enzymes over others is crucial for the system's functioning.

Even though it is not significant according to the analysis, the alteration of the enzyme's activity caused by the contaminant cannot be completely ruled out, as there is a bit of variability between the control and the one with the highest concentration of the contaminant in both organs. This means that there could be a subtle effect of alteration related to the presence of the contaminant that alters the enzymatic response. This might be caused by the tendency of PFAS to alter the activity of antioxidant enzymes, as they bind to proteins (Bonato et al., 2020; Alesio et al., 2022). This has already been shown in other studies involving PFAS, but not *in vitro*, suggesting that the contaminant may alter directly the protein functioning in the system (Niu et al., 2019; Wang et al., 2023). Nevertheless, the inhibition of the activity of the antioxidant enzymes in the present study seems to be correlated with an increase in non-enzymatic responses, as reported by the TAC. This increase in antioxidants may be possible, as these components may be stored in stress granules (SG), smaller cytoplasmatic compartments that present proteins that bind to RNA and regulate their transcription: the rise in activity is not only related to TAC, but also to SOD and GPx, which showed an increase in DG and G respectively with higher concentration of HFPO-DA (Anderson & Kedersha, 2009; Drago et al., 2023). However, this minor increase seems not to be enough to compensate for the rise in LPO in the samples exposed to the contaminant, suggesting that the cellular damage in this preliminary study may be a signal of a more complex regulation of the antioxidant system of in the organism.

4.1.2 – Antioxidant capacity

It needs to be considered that there may be different trends of enzyme expression between the two organs, depending on the isoforms present. In the present study, it has been highlighted that there may be a differential expression of enzyme Cu/Zn-SOD: the expression of the enzyme varies according to the concentration of the contaminant in the DG, and it is possible that the extracellular SOD may be responsible for the creation of different trends (Manduzio et al., 2004; Letendre et al., 2008). As the SOD-3 expression is induced during exposure to contaminants in that organ, it is possible that the presence of the contaminant initially induces the expression of this isoform, but it inhibits the enzyme response at higher concentrations (Manduzio et al., 2004). This increase could be possible in *in vitro* assays in the case there are stress granules (SGs) present in the cytoplasm of the organism that can regulate the expression of the enzyme's transcription (Drago et al., 2023; Piva et al., 2024). These granules have been identified in

organisms like *Botryllus schlosseri* and *Trematomus bernacchii*, but if these enzymes are present in bivalves too, they may influence the response to stressors by activating the transcription of proteins at intermediate concentrations (SOD) or with increasing concentration of the contaminant (GPx and TAC) that may give further assistance to face the threat posed by HFPO-DA exposure.

The enzyme GPx detoxifies the organism from H_2O_2 in the cytosol. The activity of GPx is higher in the gills, in accordance with other studies (Cruz et al., 2023; Giannessi et al., 2023). Even though GPx and CAT have the same function (detoxification from H_2O_2 by creating molecular oxygen and water), their location is different in the cell, and so is the way the reaction occurs: while GPx requires reduced glutathione to carry out the reaction and it is present mainly in the cytoplasm, CAT forms an intermediate enzyme-substrate complex that reacts with a second molecule of H_2O_2, forming oxygen and water in peroxisomes (Johansson & Håkan Borg, 1988; Molavian et al., 2015). This also results in a different affinity for the enzymes to their substrate: while GPx detoxifies one molecule at a time, CAT may be activated only when a high concentration of this ROS is reached (Dobal et al., 2022). Nevertheless, the higher activity in the G seems only slightly affected by the presence of the contaminant, resulting in a statistically significant, even if minimal, increase in activity only at the highest level of exposure. This might indicate that the production of H_2O_2 slightly increases in the cytosol even though there is significant inhibition of SOD in the same organ: this is possible if H_2O_2 is produced by divalent reduction of molecular oxygen, as hypothesised in other studies (Santovito et al., 2005). This may as well explain the high inhibition seen in the SOD: in fact, it is known that most enzymes functioning is inhibited by the product of their reaction to limit the reactivity of the enzyme. Therefore, SOD may be inhibited by the presence of H_2O_2, resulting in a decreased activity of the enzyme, probably also related to an action of the compound itself (Regoli & Giuliani, 2014).

On the other hand, the higher activity of the enzyme CAT in the DG may be linked to the higher density of peroxisomes, cellular organelles that are involved in the degradation of organic substances through the production of H_2O_2 by oxidases (Regoli & Principato, 1995; Cancio et al., 1999; Aranda-Rivera et al., 2022). In other studies, CAT has been found to have higher activity in the DG rather than in the G, and this may imply that the generation of H_2O_2 in digestive glands is not carried out by the SOD but it is generated through other mechanisms (Cruz et al., 2023; Giannessi et al., 2023). Nevertheless, the increase in the concentration of HFPO-DA in the G inhibits this enzyme activity, causing an impairment when facing oxidative stress, especially in this organ. Compared to other enzymes in the DG, like GPx, which is less abundant than in G, the inhibition of CAT caused by the chemical in this organ may be crucial for protecting the cell from oxidative damage generated in peroxisomes.

4.1.3 – Biotransformation capacity

The biotransformation enzymes may be more expressed in the digestive gland, as this organ focuses more on the digestion and excretion of toxic compounds (Livingstone et al., 1992). Thus, a higher activity of enzymes based on the transformation using glutathione or the Carboxylesterases can be possible. Nevertheless, their activity may be influenced by the presence of the chemical, which is why screening these compounds is important to assess the potential biological excretion of a specific contaminant (Cruz et al., 2023). Specifically, while there is no significant variation in the activity of CbEs, there is a trend of inhibition for the GSTs, meaning that the chemical may influence their activity. Other studies *in vitro* with other compounds report increased activity, even though at low concentrations (Giannessi et al., 2023; Vieira Sanches et al., 2023); in the present study, this is not true. As the minor deactivation of GSTs activity is statistically significant, it might indicate a possible alteration in the function of excretion of the compound, with potential effects on the accumulation in the organism.

4.1.4 – Oxidative damage

The observed trend of inhibition of antioxidant enzymes influenced the amount of ROS present in the extract, causing oxidative damage in the cell. This has been assessed by measuring LPO levels, a relevant method to evaluate oxidative damage in ecotoxicological studies (Cruz et al., 2023; Giannessi et al., 2023). However, in this case, the higher amount of ROS in the cells causes a significant increase in LPO even at the lowest level of exposure in the digestive gland. Studies *in vitro* involving other compounds did not show any possible creation of oxidative damage in the cells proportional to an increase in concentration, as in this case (Cruz et al., 2023; Giannessi et al., 2023; Vieira Sanches et al., 2023). This might imply that, due to oxidative damage *in vitro*, even at the lowest tested concentrations, in the present study 100 ng/L, there might be an alteration to organisms directly exposed to the chemical. This is crucial to decide whether to carry out an *in vivo* study, as the oxidative damage may signal increased stress, probably caused by enzyme inhibition, which may damage the organism. Therefore, it may be worth analysing their response *in vivo.*

4.1.5 - Neurotoxicity

One more biochemical parameter to consider is neurotransmission, as HFPO-DA is also known to alter the nervous system, with a possible effect on the behaviour of the organisms too (Wang et al., 2023). This is achieved through the *in*

vitro analysis of AChE, one of the main enzymes that regulate the signalling pathway for activating the action potential in neurons (Escartín & Porte, 1997; Mennillo et al., 2017). In particular, AChE is an enzyme which regulates neurotransmission: in G, the activity of cilia is essential to create the flow of water and allow gaseous exchange and feeding in bivalves (Cruz et al., 2023). Inhibition of the activity of AChE may cause either a prolonged signal or trigger an insensitivity of cells to acetylcholine (Regoli & Principato, 1995; Cruz et al., 2023). In the present study, inhibition of the enzyme's activity has been found, especially in G, meaning that the neurotransmission of the organism may be affected when exposed to HFPO-DA, consequently altering both the feeding and respiration of mussels.

4.2 - In vivo assay

Obtaining significant results on a screening analysis *in vitro* as the one performed in this study, considering the LPO, might imply that there could be a potential alteration in the whole body's response that may adapt to different environmental conditions to face the exposure to the contaminant. As previously mentioned, the effects of climate change are happening worldwide, causing alterations in sea surface temperature, precipitation rates and salinity changes in both open water bodies and estuaries, causing organisms to face different sources of stress (Freitas et al., 2017; IPCC, 2023). Hence, this climate change rate may significantly alter marine organisms' ability to face exposure to certain pollutants, like various PFAS (Niu et al., 2019; Bonato et al., 2020). Most legacy compounds are known to cause oxidative damage mainly through the interaction with the PPAR, causing an alteration in lipid metabolism (Regoli & Giuliani, 2014; Bonato et al., 2020; Shi et al., 2024). Nevertheless, it is believed that not all PFAS directly affect the response by direct interaction with these receptors. In fact, according to Wang et al. (2023) in zebrafish this pathway was affected only by PFOA, while novel chemicals, one of which is Gen-X, did not directly affect it, but it led to the alteration of the lipid metabolism too. The increase in density of peroxisomes increases the fatty acid metabolism and lipid homeostasis through the disruption of a very long fatty acid chain, as well as the production of ROS mainly by oxygenase, like Acyl-CoA oxidase (Cajaraville et al., 2003; Regoli & Giuliani, 2014; He et al., 2021).

4.2.1 – Multivariate analysis general perspective

Starting from the analysis of the PCO, it is possible to see a clear separation of the three salinity treatments: 40 on the left side of the graph, 20 on the right side and 30 in the middle. However, the presence of the treatment of 100 ng/L with 30 of salinity close to 20 may represent a possible threat of the contaminant to normal conditions of salinity, which leads to an increase in all the antioxidant parameters. It is known that *M. galloprovincialis* mostly suffers from hyposaline conditions, in which it deals with increasing antioxidant enzymes activity and regulation of the appropriate redox balance through the increase in glutathione and GR activity, but also in TAC (FAO, 2009; Lockwood & Somero, 2011; Andrade et al., 2024a). However, in the present analysis, it seems that with salinity 30, the concentration of 100 ng/L of HFPO-DA is close to those treatments, meaning that the presence of the contaminant at intermediate salinities may induce higher stress in organisms compared to extreme conditions, which instead do not show clear alteration patterns. It is worth noting that the main effect that the contaminant induces is the increase mainly in non-enzymatic responses, like TAC, GSH and GSSG, leading to alteration of GR, probably to maintain the appropriate redox balance in the cell (David et al., 2007; Regoli & Giuliani, 2014). The increase in non-enzymatic

defences is also suggested by the analysis of metabolism and energy reserves parameters: while the cells do not seem to use GLY, the increase in mitochondrial respiration and protein suggests that there are other mechanisms in action used to cope with the presence of the contaminant. The availability of the chemical in combination with extreme levels of environmental change may lead to further hazards to the ecosystem if bioaccumulation happens (EFSA, 2008; Freitas et al., 2019b; Paciello et al., 2023; Andrade et al., 2024a). None of these effects are detected at other salinity levels, and the results are inconclusive. This implies that salinity changes alone may be the most significant stressor. More research is needed to understand the contaminant's impacts as salinity changes, as some biomarkers, such as GR, GSTs, and CbEs, change with increasing contaminant concentrations at extreme salinities, making it plausible that organisms accumulate HFPO-DA to some extent.

4.2.2 – Metabolism and Energy Reserves

In the present study, the ETS is one parameter that follows a clear pattern with the salinity. This biomarker clearly shows a difference in the three salinity levels that have been tested, with a decreasing trend as the salinity increases. This might imply that lower salinity levels, as expressed before, are less tolerable by *M. galloprovincialis*. Nevertheless, the changes in PROT content at extreme salinities may be mostly related to the increase also of free amino acids and heat shock proteins, which stabilise the organism's osmolarity and proteins (Kube et al., 2006; Podlipaeva & Berger, 2012). Alternatively, the increase in PROT may also be caused by an increased synthesis of antioxidants.

On the other hand, proteins may be altered by the excessive respiration rate in mitochondria, which creates excessive ROS production, damaging proteins and lipids, altering cellular structures, and toxic or reactive compounds need to be excreted from the cell (Regoli & Giuliani, 2014; Aranda-Rivera et al., 2022). This has also been discussed by Andrade et al. (2021), who studied the same salinity levels. In their study, ETS levels decreased with increased salinity but were coupled with increased protein content. Moreover, the increased PROT at the highest salinity level may also partially corroborate the reduced activity of the ETS in mitochondria at different salinities. Other studies report an opposite trend of the protein compared with the one of the ETS, which relates to the fact that organisms may need less energy at the highest salinity (Freitas et al., 2017; Andrade et al., 2021). A higher energetic expenditure may be associated with a specific behaviour of the organisms, like the valve closure, which is helpful to lower the metabolism, even though there may be an increased need for ATP demand (Anestis et al., 2007; Velez et al., 2017; Andrade et al., 2021). Also, a higher energy expenditure at low salinities may be correlated with the need to maintain appropriate cell functioning

by synthesising more protein to maintain the osmotic balance, enhancing their transcription (Lockwood & Somero, 2011).

MTT represents mainly the activity of the succinate dehydrogenase (SDH), which is an enzyme that is not only involved in the electron transport chain but also the Krebs cycle (Rutter et al., 2010; Rai et al., 2018). The evaluation of this biomarker provides information about mitochondrial health and metabolic activity, and it can be used to estimate the cell viability in a medium (van Meerloo et al., 2011; Cuccaro et al., 2021). The double role of SDH allows us to have a broader view of the state of the mitochondria (Bénit et al., 2022). In the present study, the activity is not altered by salinity, even though there seems to be a non-significant decrease in activity with increasing salinity.

As salinity is the main factor influencing the organism's metabolism, the contaminant generates a response visible only at salinity 30, an intermediate environmental condition for mussels. This can be seen not only in the response of the ETS but also in MTT, suggesting that a possible increase in activity on the electron transport system may be caused by the increase in activity of complex II specifically (Bénit et al., 2022). The presence of the contaminant associates an increase in activity with a significant magnification of PROT: this may be possible due to the increased need to synthesise proteins or antioxidants to decrease the possible oxidative stress caused by the exposure to this PFAS (Freitas et al., 2019a; Paciello et al., 2023). This response to exposure has been thought of as a defence mechanism: facing a xenobiotic may be a situation when increasing the production of proteins, specifically antioxidant defences, may prevent or limit cellular damage (Smolders et al., 2003). Other studies have also observed increased mitochondrial activity in mussels exposed to a contaminant (Cunha et al., 2005; Freitas et al., 2017; Paciello et al., 2023). It is known that PFAS may lead to the creation of ROS through an alteration of the activity of peroxisomes, and this might ultimately lead to an impairment of the activity of the ETS via the production of oxygen radicals involving free metal ions in the cell, or an increase in energy demand and ATP synthesis (Regoli et al., 2011b; Fransen et al., 2012; Regoli & Giuliani, 2014). Therefore, even though it has been shown that HFPO-DA does not directly alter that pathway in zebrafish, it may be possible that it indirectly affects the functioning of peroxisomes and lipid metabolism in *M. galloprovincialis*, leading to higher energy expenditure due to increased synthesis of new proteins (Wang et al., 2023).

4.2.3 – Antioxidant capacity and redox balance

On the other hand, an increase in the ETS activity does come with a cost: an increase in the production of ROS due to a higher pace of reaction and possible electron leakage in the chain (Regoli & Giuliani, 2014; Aranda-Rivera et al., 2022). Therefore, the observed increase in protein content may be attributable to a

corresponding rise in various factors, including a potential elevation in antioxidants, which are essential for mitigating oxidative stress and limiting possible structural damages (Smolders et al., 2003; Freitas et al., 2019a). It is known that different antioxidant enzymes are linked to one another by strict regulation mechanisms that are based both on the reaction that they catalyse and their location in the cell (Santovito et al., 2005; Regoli & Giuliani, 2014; Dobal et al., 2022). For this reason, analysing the responses on their own would not give an appropriate view of the overall effects on the biological response. As antioxidants can be identified as an indirect measure of ROS present in the cells of the organisms, an increase in antioxidant response may be linked to a stressful increase in ROS (Freitas et al., 2019a, 2020). The increase in respiration at lower salinities generates more ATP, which is reflected in the activity trend of the other antioxidants. Hence, the increase of more than one biomarker might imply a higher production of oxygen species in that environmental scenario, as it is known to be stressful for the species of mussels analysed here (Lockwood & Somero, 2011). In fact, according to the results obtained, higher metabolism correlates with a higher activity of antioxidant biomarkers, considering enzymes like CAT, GR and TAC. The increase in antioxidant enzymes at lower salinity in *M. galloprovincialis* has already been discussed in Andrade et al. (2021) and Freitas et al. (2017) as a response of the organism to face oxidative stress and increased ROS formation. In some studies, it is possible to see a high correlation between SOD and CAT, as H_2O_2, the product of the former enzyme, is the substrate of the latter (Regoli & Giuliani, 2014; Freitas et al., 2017). However, in the present analysis, there seems not to be a correlation in activity between these two enzymes. While CAT activities vary with salinity, SOD does not display this alteration. This may be due to the different location and generation of ROS during exposure to lower salinities: some studies hypothesise that, in presence of high quantities of $\bullet O_2^-$, the formation of H_2O_2 can happen for divalent reduction of the molecule or by action of the enzymes in peroxisomes, that form the molecule as a consequence of fatty acid catabolism (Santovito et al., 2005; Aranda-Rivera et al., 2022). While it is true that H_2O_2 can be used as a signalling molecule in different pathways, due to its permeability and mobility in the cell, it may cause damage to cellular structures (Zhang et al., 2016). On this matter, CAT is not the only enzyme that dismutases H_2O_2 in water and oxygen: GPx catalyses the same reaction but uses GSH as an electron donor (Molavian et al., 2015). In the present study, compared to the other antioxidants, the activity of the latter enzyme is reversed, being higher at higher salinities. While this result may seem to clash with the activity of GR, which is similar to CAT, the possible reason behind this trend may be associated with an affinity of GPx to work at different salinities. GSH is a reductant in the cell's cytoplasm (Forman et al., 2009). Therefore, a higher activity of the GR, which converts GSSG back to GSH, may be linked to a higher renewal of this reductant, leading to the maintenance of appropriate redox balance without needing an increase in the activity of GPx. Furthermore, a higher rate of conversion of GSH makes it available in the environment, leading to higher use of

the reduced glutathione in the cytosol, where other antioxidants are present too: in particular, the Prdxs. These enzymes are available in the cell, catalysing the reaction to form water from H_2O_2. While most of the isoforms use thioredoxins, the Prdx-6 competes for the substrate with the GPx, as both enzymes rely on glutathione for the reduction of the active site (David et al., 2007; Molavian et al., 2015). In this way, the use of GSH, renewed constantly by GR at salinity 20, means that it is possible to hypothesise a dependency on the salinity between those two enzymes: while GPx activity could be higher at higher salinities, Prdx-6 may be more active at lower environmental values, implying a different detoxification action. One more hypothesis may be instead a biosynthesis of GSH ex-novo in the case of the highest salinity level: in this scenario, the activity of GR may be less preferred with increasing salt content, and instead, the synthesis of GSH and excretion of GSSG is promoted. Glutathione synthesis is carried out by the glutamate-cysteine ligase (GCL), which binds these two amino acids together, catalysing the first step to form GSH. After that, the glutathione synthetase adds a glycine molecule to create the final molecule of GSH (Forman et al., 2009). Therefore, testing the activity of both these enzymes would give even more information related to their dependency on salt content, and there could be a different regulation of both the enzymes' transcription and cell functioning (Campos et al., 2016; Podbielski et al., 2022). According to these possible implications, there may be a different activity of the enzyme to possible environmental variations.

However, when considering the alterations provided to the different antioxidants by the contaminant, it is possible to see that other effects depend on the chemical concentration. First, from a broader view, there are effects mainly at intermediate salinity, except for GR, which seems to be slightly altered at extreme levels. The alteration at extreme levels of the enzymes might indicate a possible accumulation of the contaminant even at higher or lower salinities. This may lead to possible alteration of the responses, making the reductase more influenced by this trend. However, in non-optimal conditions, the chemical structure of the pollutant may be altered, leading to the formation of alternative chemical forms (Zhang et al., 2022). Even though information on the stability of the contaminant at different salinities is not available yet, it is known that the contaminant can be registered even in the open ocean, but the amount may vary due to the dissolution of the contaminant in that environment (Joerss et al., 2020). As GR shows much variability between the control and 10 ng/L at salinities 20 and 40, with the former decreasing and the latter increasing in activity, it might be that for this enzyme specifically, there is a significant variation in the response derived by a combined action of the intermediate concentration and the salinity. In this case, it is possible to hypothesise that there could be different stability of the enzyme at the tested salinities, which may destabilise the molecular structure according to the salt content, altering the possible response of the organisms (Ohoro et al., 2024). However, this should be tested to check for contaminant accumulation in mussels.

While at salinity 30, there seems to be an increase in the activity of CAT in general, even though it is not significant, it has been reported in zebrafish (Wang et al., 2023). There is also a decrease in the activity of GPx: this might imply that, at the same salinity level, the contaminant favours the response of one enzyme over the other, which might be correlated with the different locations of the two enzymes in the cellular compartments. While GPx is mainly located in the cytoplasm, the alteration of the activity of peroxisomes generated by HFPO-DA might favour, at higher concentrations of ROS, an increase in the activity of CAT, which is mostly in those organelles (Regoli & Principato, 1995; Regoli & Giuliani, 2014). This may lead ultimately to the depletion of the generated H_2O_2 directly in peroxisomes, leading to lower activity of GPx. Furthermore, the increase in the activity of SOD at the highest concentration of the contaminant compared to the 10 ng/L conditions, which can also be seen in other organisms like *Chlorella sp.* at the same concentration (Niu et al., 2019). The increase in SOD may lead to an increase in the generation of H_2O_2, enhancing then CAT activity.

This overall enhanced activity of the antioxidants is further associated with an increase in TAC at the highest concentration at salinity 30, suggesting that this alteration of the contaminant may be related to an increase in synthesis or activation of other compounds able to reduce the reactivity of oxygen species (Benzie & Strain, 1996; Franco et al., 2016). An imbalance in the GSH/GSSG ratio, indicating a potential disruption of the cell's redox status, may arise when the capacity of the antioxidant system is overwhelmed by an excess of oxidised glutathione (GSSG) (Regoli & Giuliani, 2014). The fact that no imbalance in the ratio of these compounds is presented, makes it possible to assume that there is not a drastic effect of the contaminant, letting cells maintain their redox balance unaltered, which is essential to prevent oxidative damage in case of inhibition of some enzymes. Nevertheless, the increase in TAC may be related to a non-significant increase in GSH, GSSG and GR activity, corroborating the possibility that non-enzymatic antioxidant responses may be preferred over enzymatic ones. Furthermore, TAC reflects also the general trend of responses of other antioxidants at low salinities possibly due to exposure to osmotic stress.

4.2.4 – Biotransformation capacity

The presence of xenobiotics in the organisms usually correlates with the possible excretion of the contaminant from the organism itself through metabolism from the cells through different mechanisms. In particular, the main enzymes involved in this process are the phase II or Biotransformation enzymes, which are GSTs and CbEs (Regoli & Giuliani, 2014; Solé et al., 2018). These enzymes bind GSH to the molecule or modify the substrate with an ester bound to an alcohol and an acid compound, respectively (Habig et al., 1974; Hosokawa & Satoh, 2001). In this way, the compound may be transformed into an inactive or active compound

and released by the cell through different transport systems, one of which is the Multidrug resistance protein, responsible for both the excretion of excess GSSG and compounds generated by GSTs (Regoli & Giuliani, 2014). According to the present analysis, both enzymes are slightly affected by salinity alteration. Still, the salinity response also alters the reaction caused by the contaminant, primarily visible at extreme levels. As far as CbEs and GSTs respond to salinity, it is possible to see that there is a trend of decrease in activity at the highest level of salinity compared to the lower value, a trend that has already been confirmed by other studies for both enzymes (Bebianno et al., 2007; Andrade et al., 2021). This is due to the effect of salinity shifts, which has been shown to increase the activity of GSTs and CbEs at lower values due to their correlation with antioxidant responses and detoxification, which is enhanced in stressful conditions (Andrade et al., 2021, 2024a). However, it is worth noticing that the presence of the contaminant worsens the alteration caused by the salinity. While for GSTs, the alteration results in increased enzyme activity at salinity 20 at 100 ng/L, CbEs seem to be inhibited at the same concentration at salinity 40. This variability in responses might imply that the activity of the GSTs operates with a higher pace for the detoxification of the compound at lower salinity, which has been found to be possible also in other studies (Andrade et al., 2021). The same kind of increase in activity, even though it is not significant, can be seen at the highest level of salinity and may imply that HFPO-DA has indeed effects on the alteration of GSTs at extreme salinities, and the contaminant does not pose a threat to the functioning of GSTs in intermediate conditions. For CbEs, instead, there is a slight inhibition of the enzyme when the organisms are exposed to the highest level of contamination combined with high salt content: this might be caused by a possible alteration of the enzyme when exposed to HFPO-DA at high salinity, causing a decrease in activity and a possible alteration that is not visible at the other conditions, probably caused by the PFAS altering the structure of the enzyme. GSTs in marine bivalves seem to be altered only at high concentrations of PFAS (Fabrello et al., 2021; Xu et al., 2022). However, this finding at altered salinity conditions may indicate that there could be a significant alteration with altered environmental conditions.

4.2.5 – Oxidative damage

Variations in salinity or other environmental parameters may affect the expression and activity of the antioxidants and the possible oxidative damage that derives from those (Freitas et al., 2017; Andrade et al., 2021). In particular, damage may be caused, depending on the organism, by exposure to high salinity or hyposaline waters (Freire et al., 2011). Andrade et al. (2024) showed that long-term exposure to non-ideal salinity does not influence lipid peroxidation per se, as the blue mussel cells can cope with the stress and act as osmo-conformer accordingly, also by regulating the number of osmolytes present in their tissues (Kube et al.,

2006). The presence of a contaminant may alter the response of organisms, leading to damage to their structures and their cells, which may be or not more pronounced in the case of environmental changes (Freitas et al., 2020; Leite et al., 2023). In the present study, an alteration caused by the novel PFAS is present at both salinity 30 and salinity 40, while at salinity 20 does not display significant changes, with the level of LPO that is stable probably due to the enhanced antioxidant enzyme activity at that level (Freitas et al., 2020).

On the other hand, in hypersaline conditions, the organisms show an increase in lipid peroxidation at 10 ng/L compared to the same concentration at the other two salinities: this may imply that, due to the inhibition of antioxidants at the highest salinity, the effect of the contaminant is worsened by the lower metabolism of the animal at those conditions. However, the main impact of the contaminant can be seen at salinity 30, in which the PFAS, at their highest concentration, induced a peak in LPO. This response may imply that the chemical may cause lipid peroxidation in the organisms. In the study of Fabrello et al. (2021), *Ruditapes philippinarum* is subject to oxidative damage due to exposure to novel perfluorinated compounds, especially when the organism is exposed for a short time. Furthermore, specimens of *Perna viridis* are subject to lipid peroxidation when exposed to high levels of PFOS: even though the study examines a higher concentration of legacy PFAS, it highlights that both legacy and novel PFAS may lead to oxidative damage in marine organisms (Xu et al., 2022). Furthermore, it has been shown that sperms of *M. galloprovincialis* respond to PFOA and PFOS at concentrations as low as 0.1 μg/L (Fabbri et al., 2014).

4.2.6 - Neurotoxicity

As far as neurotoxicity is concerned, AChE is a biomarker that has been widely investigated to assess whether a contaminant may alter the neurotransmission of a signal, especially since it has been discovered to be a relevant marker in the case of organophosphorus pesticides (Ellman et al., 1961; Escartín & Porte, 1997; Mennillo et al., 2017; Freitas et al., 2019a; Fabrello et al., 2021). In particular, previous studies on mussels highlighted a possible influence and variability of the parameter with both salinity and exposure to a contaminant, suggesting that a combination of the factors may influence the response of the organism exposed (Andrade et al., 2024a). In the present study, in accordance with Andrade et al. (2021), there is no variation in the response in the AChE activity between controls at different salinity.

On the other hand, a non-significant decrease due to the contaminant is present at salinity 40. HFPO-DA has been shown to alter the activity of AChE by increasing its activity in zebrafish embryos (Wang et al., 2023). The presence of HFPO-DA combined with the highest possible salinity exposed changes the effect

of the chemical, as it inhibits the enzyme's activity compared to the same concentration at the other two salinities. This trend suggests that the presence of the contaminant may alter the movement of the cilia placed on the gills: this would cause a de-sensibilisation of the organs, leading to possible alteration of the movement of water and, therefore, impaired feeding and accumulation of the contaminant (Escartín & Porte, 1997).

5 - CONCLUSIONS & FUTURE PERSPECTIVES

In recent years, numerous fluorinated compounds have been reported in both estuarine and open sea environments, and their presence in estuarine and marine environments may cause an impact on bivalves (Heydebreck et al., 2015; Fabrello et al., 2021; Geng et al., 2024). With this study, it has been assessed that novel chemicals of the same class may cause disturbances in the antioxidant system of the blue mussel *M. galloprovincialis*, possibly giving insights into the threat that these compounds may pose not only in freshwater environments and humans but also to the marine environment (Bonato et al., 2020; Ohoro et al., 2024).

In the present study, it has been shown, through an *in vitro* first assessment analysis of the gills and the digestive glands of mussels, that there may be an alteration of the enzyme activity; it has been highlighted a general trend of inhibition of the responses of antioxidants associated with an increase in the amount of LPO in the organisms even at the lowest concentration of the chemical. Even though the experiment is based on the procedure adopted by Cruz et al. (2023), the study may have been improved by including a methanol concentration control to compare the study results better. However, methanol is not known to significantly alter marine organisms' response unless the concentration exceeds 2%, which is not the case in this experiment (Helmstetter et al., 1996; Cruz et al., 2023). Due to the limitation of time, stability of proteins and length of the experiment, it has been decided that the lowest concentration of HFPO-DA tested was the highest concentration ever recorded found in estuarine environments (Heydebreck et al., 2015; OECD, 2018b; Vieira Sanches et al., 2023).

PFAS are compounds that alter, directly or indirectly, the activity of peroxisomes in the organisms, leading to a change in the production of ROS directly in these organelles (Bonato et al., 2020; Wang et al., 2023). In peroxisome proliferation, several ROS are produced from the disruption of lipid homeostasis, which mainly leads to the production of H_2O_2, and PFAS may be responsible for the alterations of the associated nuclear genes responsible for the phenomenon (Orbea et al., 2002; Regoli & Giuliani, 2014). Consequently, there may be an increase in different oxygenase enzymes, which are helpful for the catabolism of lipids, and would justify the slight increase in activity of CAT. One way to assess whether the transcription of peroxisome proliferators is happening is to quantify the Acyl-CoA oxidases (AOX), enzymes which transcription is directly correlate to the PPAR pathway (Cancio et al., 1999; Cajaraville et al., 2003). In fact, contrary to the CAT, those enzymes have been widely adopted to analyse the peroxisomes proliferation in different organisms, including *M. galloprovincialis* (Cancio et al., 1999). Therefore, in future studies, it may be worth analysing the activity of the AOX enzymes, to understand if HFPO-DA inhibits its activity or enhances it.

The present study also highlighted the need to analyse more antioxidant enzymes to understand the trend of the response of the combination of a contaminant with salinity, which seems to have a relevant effect in the experiment *in vivo*. In particular, the higher rate of conversion of glutathione made by the GR with a low activity of GPx at the lowest salinity tested suggests that other enzymes may be involved in the redox balance of cells under those environmental conditions. It has been hypothesised that the action of peroxiredoxins may be relevant in this case, especially the 6th isoform, which is known to use glutathione instead of thioredoxins (David et al., 2007; Molavian et al., 2015). Therefore, the need to understand the use of glutathione by *Mytilus* in other studies may give further insights into their physiology. Furthermore, GR presented a low activity, while GPx may present a high activity at high salinities: in this case, given the increased activity of the former enzyme at an intermediate concentration of the contaminant, to assess whether there could be an alteration to the activity or transcription of the glutamate-cysteine ligase in mussels in that scenario (Forman et al., 2009).

Other alterations of the enzymes, caused by the contaminant, are also visible in the biotransformation enzymes, even though it is mainly at extreme levels: an increase in the activity of GSTs at the lowest salinity, an inhibition of CbEs at the highest and different patterns in GR at both altered salinities. For the CbEs, even though the alteration is significant, it is only minor compared to the former. It is conceivable that the accumulation of the contaminant or its derivates, in this instance, may create a condition in which the organism cells, already facing osmotic stress and activating GSTs, face a condition in which the presence of the chemical leads to an increased biotransformation activity, suggesting that the organism, already stressed at extreme salinities, increase significantly the biotransformation due to the accumulation of this xenobiotic. Nevertheless, altering these parameters at extreme salinity levels in mussels might imply that an accumulation of the contaminant may be possible: it may be that its stability in saltwater is be affected only partially by the salt content. In the experiment by Avellán-Llaguno et al. (2020), the increased salinity favoured the bioaccumulation of PFAS in an estuarine fish. Therefore, although HFPO-DA is a short-chain PFAS, its bioaccumulation at altered salinities may still be possible.

The *in vitro* does not predict with high fidelity the trend of antioxidant enzymes in the *in vivo* assay. The former experiment generally reports inhibition of the antioxidant enzymes, especially CAT and SOD, which have been shown to occur also in short-term exposure of other marine algae (Niu et al., 2019). The *in vivo* analysis reports instead an increase in the activity of SOD and no significant increase in CAT. This can be explained mainly by the way the experiment *in vitro* works: the supernatant used is the S9-subcellular fraction, which is deprived of DNA, mitochondria and major organelles, leaving only cytoplasm, microsomes and possibly stress granules in the cell (Richardson et al., 2016; OECD, 2018b; Drago et al., 2023). Therefore, all the alteration caused by the contaminant at the genetic

level, which is common in PFAS, is lost. As most PFAS alter the peroxisome metabolism by stimulating the PPAR pathway, the lack of genetic information or the possibility of activating that pathway does not favour the analysis of those responses, leading instead to a possible activation of alternative mechanisms for the regulation of the transcription (Anderson & Kedersha, 2009; Piva et al., 2024). Therefore, while the study aims to understand whether the contaminant causes an alteration to the organism tissue, which is reported through the generation of oxidative damage, considering an experiment *ex vivo* using cells extracted from *M. galloprovincialis* may give more information on the altered genetic pathway related to enzyme activity and possible transcriptomic responses of the mussel cell. However, that approach has limitations, too, especially in the durability, conservation of the samples, and loss of information on the metabolism of the organism as a whole (OECD, 2018b). Overall, *in vitro* and *ex vivo*, even though they do not consider the whole metabolic variability of the organism, can be preferred as tools for a first screening of the activity or alteration in the cells of interest, to justify further experiments on the whole organism, as happened in this study (OECD, 2018b).

In addition, the organ's division is essential to understanding how a specific tissue might respond to the presence of a contaminant. While analysing a specific biochemical marker gives insights into its function in the organ, the presence of different isoforms of the same enzyme in the same area may provide uncertain results. As previously discussed, the SOD is an enzyme which activity varies according to the organ: however, as the measurement of the activity of an enzyme is an indirect measurement of its quantity, maybe targeting a specific enzyme through the use of a western blot to understand whether a particular isoform is more present than the other in a specific tissue would give better insights on the variability between the organs and further knowledge in the physiology of the organism (Manduzio et al., 2004; Letendre et al., 2008). This will help to get more precise results and possible implications of inhibition directly on specific isoforms in a targeted way.

Overall, both *in vitro* and *in vivo* experiments show that not all the antioxidants are susceptible to the changes on the exposure directly to the chemical: cells have been shown to enhance the use of non-enzymatic systems to compensate for oxidative stress. In particular, the significant increase in TAC in both assays and non-significant increase in both GSSG and GR at salinity 30 *in vivo* might imply that these molecules are preferred to face the oxidative stress present in the cell, maybe because of the alteration of function of the enzymes due to the interaction with HFPO-DA (Bonato et al., 2020; Avellán-Llaguno et al., 2020; Alesio et al., 2022). The alteration of the GR activity may be a physiological response to maintain the accurate redox balance in the cell (David et al., 2007; Regoli & Giuliani, 2014). Therefore, assessing the possible synthesis of other reducing

peptides like other thioredoxins may give more insights into the regulation of the response of the cells (Wang et al., 2013).

To conclude, more studies that analyse the combination of more PFAS, both novel and legacy, should be applied to understand their possible interaction in a changing environment. The combination of the presence of HFPO-DA and the climate changes in this study may suggest the need to regulate the release of this contaminant as well as PFOA and PFOS, which have been banned by the European Union for several years (EFSA, 2008). However, analysis of the oxidative stress status is only one of the many needed to assess the danger that this contaminant represents at the ecosystem level: as sentinel organisms are affected by this contaminant, more studies are required to possibly evaluate the genotoxicity, reproduction or, at the ecosystem level, bioaccumulation potential of this compound.

6 - BIBLIOGRAPHY

Alesio, J. L., Slitt, A., & Bothun, G. D. (2022). Critical new insights into the binding of poly- and perfluoroalkyl substances (PFAS) to albumin protein. *Chemosphere*, *287*, 131979. https://doi.org/10.1016/j.chemosphere.2021.131979

Aminot, Y., Sayfritz, S. J., Thomas, K. V., Godinho, L., Botteon, E., Ferrari, F., Boti, V., Albanis, T., Köck-Schulmeyer, M., Diaz-Cruz, M. S., Farré, M., Barceló, D., Marques, A., & Readman, J. W. (2019). Environmental risks associated with contaminants of legacy and emerging concern at European aquaculture areas. *Environmental Pollution*, *252*, 1301–1310. https://doi.org/10.1016/j.envpol.2019.05.133

Anderson, M. (2008). PERMANOVA+ for PRIMER: guide to software and statistical methods. *Primer-E Limited.*

Anderson, P., & Kedersha, N. (2009). Stress granules. *Current Biology*, *19*(10), R397–R398. https://doi.org/10.1016/j.cub.2009.03.013

Andrade, M., De Marchi, L., Soares, A. M. V. M., Rocha, R. J. M., Figueira, E., & Freitas, R. (2019). Are the effects induced by increased temperature enhanced in *Mytilus galloprovincialis* submitted to air exposure? *Science of The Total Environment*, *647*, 431–440. https://doi.org/10.1016/j.scitotenv.2018.07.293

Andrade, M., Pinto, J., Soares, A. M. V. M., Solé, M., Pereira, E., & Freitas, R. (2024a). How predicted temperature and salinity changes will modulate the impacts induced by terbium in bivalves? *Chemosphere*, *351*, 141168. https://doi.org/10.1016/j.chemosphere.2024.141168

Andrade, M., Pinto, J., Soares, A. M. V. M., Solé, M., Pereira, E., & Freitas, R. (2024b). Yttrium effects on the Mediterranean mussel under a scenario of salinity shifts and increased temperature. *Marine Environmental Research*, *195*, 106365. https://doi.org/10.1016/j.marenvres.2024.106365

Andrade, M., Soares, A. M. V. M., Solé, M., Pereira, E., & Freitas, R. (2021). Salinity influences on the response of *Mytilus galloprovincialis* to the rare-earth element lanthanum. *Science of The Total Environment*, *794*, 148512. https://doi.org/10.1016/j.scitotenv.2021.148512

Anestis, A., Lazou, A., Pörtner, H. O., & Michaelidis, B. (2007). Behavioral, metabolic, and molecular stress responses of marine bivalve Mytilus galloprovincialis during long-term acclimation at increasing ambient temperature. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *293*(2), R911–R921. https://doi.org/10.1152/ajpregu.00124.2007

Aranda-Rivera, A. K., Cruz-Gregorio, A., Arancibia-Hernández, Y. L., Hernández-Cruz, E. Y., & Pedraza-Chaverri, J. (2022). RONS and Oxidative Stress: An Overview of Basic Concepts. *Oxygen*, *2*(4), Article 4. https://doi.org/10.3390/oxygen2040030

ARPAV. (2024). *Concentrazione di sostanze perfluoroalchiliche (PFAS) nelle acque prelevate da ARPAV - Agenzia Regionale per la Prevenzione e Protezione Ambientale del Veneto*. https://www.arpa.veneto.it/dati-ambientali/open-data/idrosfera/concentrazione-di-sostanze-perfluoroalchiliche-pfas-nelle-acque-prelevate-da-arpav

Avellán-Llaguno, R. D., Liu, X., Liu, L., Dong, S., & Huang, Q. (2020). Elevated bioaccumulation of PFAAs in *Oryzias melastigma* following the increase of salinity is associated with the up-regulated expression of PFAA-binding proteins. *Science of The Total Environment*, *725*, 138336. https://doi.org/10.1016/j.scitotenv.2020.138336

Bagchi, D., Bagchi, M., & Stohs, S. J. (2001). Chromium (vi)-induced oxidative stress, apoptotic Cell death and modulation of p53 tumor suppressor Gene. In X. Shi, V. Castranova, V. Vallyathan, & W. G. Perry (Eds.), *Molecular Mechanisms of Metal Toxicity and Carcinogenesis* (pp. 149–158). Springer US. https://doi.org/10.1007/978-1-4615-0793-2_18

Bal, A., Panda, F., Pati, S. G., Das, K., Agrawal, P. K., & Paital, B. (2021). Modulation of physiological oxidative stress and antioxidant status by abiotic factors especially salinity in aquatic organisms. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *241*, 108971. https://doi.org/10.1016/j.cbpc.2020.108971

Barrett, N. J., Thyrring, J., Harper, E. M., Sejr, M. K., Sørensen, J. G., Peck, L. S., & Clark, M. S. (2022). Molecular Responses to Thermal and Osmotic Stress in Arctic Intertidal Mussels (Mytilus edulis): The Limits of Resilience. *Genes*, *13*(1), Article 1. https://doi.org/10.3390/genes13010155

Bebianno, M. J., Lopes, B., Guerra, L., Hoarau, P., & Ferreira, A. M. (2007). Glutathione S-tranferases and cytochrome P450 activities in *Mytilus galloprovincialis* from the South coast of Portugal: Effect of abiotic factors. *Environment International*, *33*(4), 550–558. https://doi.org/10.1016/j.envint.2006.11.002

Bénit, P., Goncalves, J., El Khoury, R., Rak, M., Favier, J., Gimenez-Roqueplo, A.-P., & Rustin, P. (2022). Succinate Dehydrogenase, Succinate, and Superoxides: A Genetic, Epigenetic, Metabolic, Environmental Explosive Crossroad. *Biomedicines*, *10*(8), 1788. https://doi.org/10.3390/biomedicines10081788

Benzie, I. F. F., & Strain, J. J. (1996). The Ferric Reducing Ability of Plasma (FRAP) as a Measure of "Antioxidant Power": The FRAP Assay. *Analytical Biochemistry*, *239*(1), 70–76. https://doi.org/10.1006/abio.1996.0292

Bernardini, I., Matozzo, V., Valsecchi, S., Peruzza, L., Rovere, G. D., Polesello, S., Iori, S., Marin, M. G., Fabrello, J., Ciscato, M., Masiero, L., Bonato, M., Santovito, G., Boffo, L., Bargelloni, L., Milan, M., & Patarnello, T. (2021). The new PFAS C6O4 and its effects on marine invertebrates: First evidence of transcriptional and microbiota changes in the Manila clam Ruditapes

philippinarum. *Environment International*, *152*, 106484. https://doi.org/10.1016/j.envint.2021.106484

Bocchetti, R., Fattorini, D., Pisanelli, B., Macchia, S., Oliviero, L., Pilato, F., Pellegrini, D., & Regoli, F. (2008). Contaminant accumulation and biomarker responses in caged mussels, *Mytilus galloprovincialis*, to evaluate bioavailability and toxicological effects of remobilized chemicals during dredging and disposal operations in harbour areas. *Aquatic Toxicology*, *89*(4), 257–266. https://doi.org/10.1016/j.aquatox.2008.07.011

Bonato, M., Corrà, F., Bellio, M., Guidolin, L., Tallandini, L., Irato, P., & Santovito, G. (2020). PFAS Environmental Pollution and Antioxidant Responses: An Overview of the Impact on Human Field. *International Journal of Environmental Research and Public Health*, *17*(21), Article 21. https://doi.org/10.3390/ijerph17218020

Bordalo, D., Cuccaro, A., De Marchi, L., Soares, A. M. V. M., Meucci, V., Battaglia, F., Pretti, C., & Freitas, R. (2022). *In vitro* spermiotoxicity and *in vivo* adults' biochemical pattern after exposure of the Mediterranean mussel to the sunscreen avobenzone. *Environmental Pollution*, *312*, 119987. https://doi.org/10.1016/j.envpol.2022.119987

Cajaraville, M. P., Cancio, I., Ibabe, A., & Orbea, A. (2003). Peroxisome proliferation as a biomarker in environmental pollution assessment. *Microscopy Research and Technique*, *61*(2), 191–202. https://doi.org/10.1002/jemt.10329

Campos, A., Danielsson, G., Farinha, A. P., Kuruvilla, J., Warholm, P., & Cristobal, S. (2016). Shotgun proteomics to unravel marine mussel (*Mytilus edulis*) response to long-term exposure to low salinity and propranolol in a Baltic Sea microcosm. *Journal of Proteomics*, *137*, 97–106. https://doi.org/10.1016/j.jprot.2016.01.010

Cancio, I., Ibabe, A., & P. Cajaraville, M. (1999). Seasonal variation of peroxisomal enzyme activities and peroxisomal structure in mussels *Mytilus galloprovincialis* and its relationship with the lipid content. *Comparative Biochemistry and Physiology Part C: Pharmacology, Toxicology and Endocrinology*, *123*(2), 135–144. https://doi.org/10.1016/S0742-8413(99)00019-5

Capó, X., Tejada, S., Ferriol, P., Pinya, S., Mateu-Vicens, G., Montero-González, I., Box, A., & Sureda, A. (2020). Hypersaline water from desalinization plants causes oxidative damage in *Posidonia oceanica* meadows. *Science of The Total Environment*, *736*, 139601. https://doi.org/10.1016/j.scitotenv.2020.139601

Carella, F., Aceto, S., Mangoni, O., Mollica, M. P., Cavaliere, G., Trinchese, G., Aniello, F., & De Vico, G. (2018). Assessment of the Health Status of Mussels Mytilus galloprovincialis Along the Campania Coastal Areas: A Multidisciplinary Approach. *Frontiers in Physiology*, *9*. https://doi.org/10.3389/fphys.2018.00683

Carlberg, I., & Mannervik, B. (1985). [59] Glutathione reductase. In *Methods in Enzymology* (Vol. 113, pp. 484–490). Academic Press. https://doi.org/10.1016/S0076-6879(85)13062-4

Carregosa, V., Velez, C., Soares, A. M. V. M., Figueira, E., & Freitas, R. (2014). Physiological and biochemical responses of three Veneridae clams exposed to salinity changes. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *177–178*, 1–9. https://doi.org/10.1016/j.cbpb.2014.08.001

Casal, P., González-Gaya, B., Zhang, Y., Reardon, A. J. F., Martin, J. W., Jiménez, B., & Dachs, J. (2017). Accumulation of Perfluoroalkylated Substances in Oceanic Plankton. *Environmental Science & Technology*, *51*(5), 2766–2775. https://doi.org/10.1021/acs.est.6b05821

Coelho, J. P., Pereira, M. E., Duarte, A., & Pardal, M. A. (2005). Macroalgae response to a mercury contamination gradient in a temperate coastal lagoon (Ria de Aveiro, Portugal). *Estuarine, Coastal and Shelf Science*, *65*(3), 492–500. https://doi.org/10.1016/j.ecss.2005.06.020

Conley, J. M., Lambright, C. S., Evans, N., McCord, J., Strynar, M. J., Hill, D., Medlock-Kakaley, E., Wilson, V. S., & L Earl Gray, J. (2020). Hexafluoropropylene oxide-dimer acid (HFPO-DA or GenX) alters maternal and fetal glucose and lipid metabolism and produces neonatal mortality, low birthweight, and hepatomegaly in the Sprague-Dawley rat. *Environment International*, *146*, 106204. https://doi.org/10.1016/j.envint.2020.106204

Costanza, R., d'Arge, R., de Groot, R., Farber, S., Grasso, M., Hannon, B., Limburg, K., Naeem, S., O'Neill, R. V., Paruelo, J., Raskin, R. G., Sutton, P., & van den Belt, M. (1998). The value of the world's ecosystem services and natural capital. *Ecological Economics*, *25*(1), 3–15. https://doi.org/10.1016/S0921-8009(98)00020-2

Cravo, A., Lopes, B., Serafim, Â., Company, R., Barreira, L., Gomes, T., & Bebianno, M. J. (2009). A multibiomarker approach in Mytilus galloprovincialis to assess environmental quality. *Journal of Environmental Monitoring*, *11*(9), 1673–1686. https://doi.org/10.1039/B909846A

Cruz, P., Cuccaro, A., Pretti, C., He, Y., Soares, A. M. V. M., & Freitas, R. (2023). Comparative subcellular responses to pharmaceutical exposures in the mussel *Mytilus galloprovincialis*: An *in vitro* study. *Environmental Toxicology and Pharmacology*, *104*, 104314. https://doi.org/10.1016/j.etap.2023.104314

Cuccaro, A., De Marchi, L., Oliva, M., Sanches, M. V., Freitas, R., Casu, V., Monni, G., Miragliotta, V., & Pretti, C. (2021). Sperm quality assessment in *Ficopomatus enigmaticus* (Fauvel, 1923): Effects of selected organic and inorganic chemicals across salinity levels. *Ecotoxicology and Environmental Safety*, *207*, 111219. https://doi.org/10.1016/j.ecoenv.2020.111219

Cuccaro, A., Freitas, R., De Marchi, L., Monni, G., Meucci, V., Oliva, M., Fumagalli, G., & Pretti, C. (2023). Multi-biomarker approach for the (eco)toxicity

of UV-filter environmental pollution on the Mediterranean mussel *Mytilus galloprovincialis* in a multiple stressor context. The case of 4-MBC under salinity shifts. *Environmental Pollution*, *336*, 122490. https://doi.org/10.1016/j.envpol.2023.122490

Cunha, I., Hoff, P., Van de Vijver, K., Guilhermino, L., Esmans, E., & De Coen, W. (2005). Baseline study of perfluorooctane sulfonate occurrence in mussels, *Mytilus galloprovincialis*, from north-central portuguese estuaries. *Marine Pollution Bulletin*, *50*(10), 1128–1132. https://doi.org/10.1016/j.marpolbul.2005.06.046

Curpan, A.-S., Impellitteri, F., Plavan, G., Ciobica, A., & Faggio, C. (2022). Review: *Mytilus galloprovincialis*: An essential, low-cost model organism for the impact of xenobiotics on oxidative stress and public health. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *256*, 109302. https://doi.org/10.1016/j.cbpc.2022.109302

David, E., Tanguy, A., & Moraga, D. (2007). Peroxiredoxin 6 gene: A new physiological and genetic indicator of multiple environmental stress response in Pacific oyster *Crassostrea gigas*. *Aquatic Toxicology*, *84*(3), 389–398. https://doi.org/10.1016/j.aquatox.2007.06.017

De Coen, W. M., & Janssen, C. R. (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*(1), 43–55. https://doi.org/10.1023/A:1008228517955

Denny, M. W., Dowd, W. W., Bilir, L., & Mach, K. J. (2011). Spreading the risk: Small-scale body temperature variation among intertidal organisms and its implications for species persistence. *Journal of Experimental Marine Biology and Ecology*, *400*(1), 175–190. https://doi.org/10.1016/j.jembe.2011.02.006

DeRivera, C. E., Ruiz, G. M., Hines, A. H., & Jivoff, P. (2005). Biotic resistance to invasion: Native predator limits abundance and distribution of an introduced crab. *Ecology*, *86*(12), 3364–3376.

Dias, J. M., Lopes, J., & Dekeyser, I. (1999). Hydrological characterisation of Ria de Aveiro, Portugal, in early summer. *Oceanologica Acta*, *22*(5), 473–485. https://doi.org/10.1016/S0399-1784(00)87681-1

Dias, J. M., & Lopes, J. F. (2006). Implementation and assessment of hydrodynamic, salt and heat transport models: The case of Ria de Aveiro Lagoon (Portugal). *Environmental Modelling & Software*, *21*(1), 1–15. https://doi.org/10.1016/j.envsoft.2004.09.002

Dobal, V., Suárez, P., Ruiz, Y., García-Martín, O., & San Juan, F. (2022). Activity of antioxidant enzymes in *Mytilus galloprovincialis* exposed to tar: Integrated response of different organs as pollution biomarker in aquaculture areas. *Aquaculture*, *548*, 737638. https://doi.org/10.1016/j.aquaculture.2021.737638

Drago, L., Perin, G., Santovito, G., & Ballarin, L. (2023). The stress granule component TIAR during the non-embryonic development of the colonial ascidian *Botryllus schlosseri*. *Fish & Shellfish Immunology*, *141*, 108999. https://doi.org/10.1016/j.fsi.2023.108999

DuBois, Michel., Gilles, K. A., Hamilton, J. K., Rebers, P. A., & Smith, Fred. (1956). Colorimetric Method for Determination of Sugars and Related Substances. *Analytical Chemistry*, *28*(3), 350–356. https://doi.org/10.1021/ac60111a017

EFSA. (2008). Perfluorooctane sulfonate (PFOS), perfluorooctanoic acid (PFOA) and their salts scientific opinion of the panel on contaminants in the food chain. *EFSA Journal*, *6*(7), 653.

Ellman, G. L., Courtney, K. D., Andres, V., & Featherstone, R. M. (1961). A new and rapid colorimetric determination of acetylcholinesterase activity. *Biochemical Pharmacology*, *7*(2), 88–95. https://doi.org/10.1016/0006-2952(61)90145-9

Escartín, E., & Porte, C. (1997). The use of cholinesterase and carboxylesterase activities from Mytilus galloprovincialis in pollution monitoring. *Environmental Toxicology and Chemistry*, *16*(10), 2090–2095. https://doi.org/10.1002/etc.5620161015

EUR-Lex. (n.d.). *Directive—2006/122—EN - EUR-Lex*. Retrieved 23 October 2024, from https://eur-lex.europa.eu/eli/dir/2006/122

Fabbri, R., Montagna, M., Balbi, T., Raffo, E., Palumbo, F., & Canesi, L. (2014). Adaptation of the bivalve embryotoxicity assay for the high throughput screening of emerging contaminants in *Mytilus galloprovincialis*. *Marine Environmental Research*, *99*, 1–8. https://doi.org/10.1016/j.marenvres.2014.05.007

Fabrello, J., Ciscato, M., Masiero, L., Finos, L., Valsecchi, S., Polesello, S., Bernardini, I., Rovere, G. D., Bargelloni, L., Massimo, M., Patarnello, T., Marin, M. G., & Matozzo, V. (2021). New compounds, old problems. The case of C6O4—A substitute of PFOA - and its effects to the clam *Ruditapes philippinarum*. *Journal of Hazardous Materials*, *420*, 126689. https://doi.org/10.1016/j.jhazmat.2021.126689

FAO. (2009). *Mytilus galloprovincialis. In Cultured aquatic species fact sheets. Text by Figueras, A. Edited and compiled by Valerio Crespi and Michael New*.

FAO. (2024). *The State of World Fisheries and Aquaculture 2024. Blue Transformation in action. Rome*. FAO ; https://openknowledge.fao.org/handle/20.500.14283/cd0683en

Fenoglio, S., Bo, T., Cucco, M., Mercalli, L., & Malacarne, G. (2010). Effects of global climate change on freshwater biota: A review with special emphasis on the Italian situation. *Italian Journal of Zoology*, *77*(4), 374–383. https://doi.org/10.1080/11250000903176497

Fisher, M., Arbuckle, T. E., Wade, M., & Haines, D. A. (2013). Do perfluoroalkyl substances affect metabolic function and plasma lipids?—Analysis of the 2007–2009, Canadian Health Measures Survey (CHMS) Cycle 1. *Environmental Research*, *121*, 95–103. https://doi.org/10.1016/j.envres.2012.11.006

Flohé, L. (2012). Glutathione Peroxidases. In J. Liu, G. Luo, & Y. Mu (Eds.), *Selenoproteins and Mimics* (pp. 1–25). Springer. https://doi.org/10.1007/978-3-642-22236-8_1

Forman, H. J., Zhang, H., & Rinna, A. (2009). Glutathione: Overview of its protective roles, measurement, and biosynthesis. *Molecular Aspects of Medicine*, *30*(1–2), 1–12. https://doi.org/10.1016/j.mam.2008.08.006

Franco, L., Romero, D., García-Navarro, J. A., Teles, M., & Tvarijonaviciute, A. (2016). Esterase activity (EA), total oxidant status (TOS) and total antioxidant capacity (TAC) in gills of *Mytilus galloprovincialis* exposed to pollutants: Analytical validation and effects evaluation by single and mixed heavy metal exposure. *Marine Pollution Bulletin*, *102*(1), 30–35. https://doi.org/10.1016/j.marpolbul.2015.12.010

Fransen, M., Nordgren, M., Wang, B., & Apanasets, O. (2012). Role of peroxisomes in ROS/RNS-metabolism: Implications for human disease. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1822*(9), 1363–1373. https://doi.org/10.1016/j.bbadis.2011.12.001

Freire, C. A., Welker, A. F., Storey, J. M., Storey, K. B., & Hermes-Lima, M. (2011). Oxidative Stress in Estuarine and Intertidal Environments (Temperate and Tropical). In *Oxidative Stress in Aquatic Ecosystems* (pp. 41–57). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781444345988.ch3

Freitas, R., De Marchi, L., Bastos, M., Moreira, A., Velez, C., Chiesa, S., Wrona, F. J., Figueira, E., & Soares, A. M. V. M. (2017). Effects of seawater acidification and salinity alterations on metabolic, osmoregulation and oxidative stress markers in *Mytilus galloprovincialis*. *Ecological Indicators*, *79*, 54–62. https://doi.org/10.1016/j.ecolind.2017.04.003

Freitas, R., Leite, C., Pinto, J., Costa, M., Monteiro, R., Henriques, B., Di Martino, F., Coppola, F., Soares, A. M. V. M., Solé, M., & Pereira, E. (2019a). The influence of temperature and salinity on the impacts of lead in *Mytilus galloprovincialis*. *Chemosphere*, *235*, 403–412. https://doi.org/10.1016/j.chemosphere.2019.05.221

Freitas, R., Silvestro, S., Coppola, F., Meucci, V., Battaglia, F., Intorre, L., Soares, A. M. V. M., Pretti, C., & Faggio, C. (2019b). Biochemical and physiological responses induced in *Mytilus galloprovincialis* after a chronic exposure to salicylic acid. *Aquatic Toxicology*, *214*, 105258. https://doi.org/10.1016/j.aquatox.2019.105258

Freitas, R., Silvestro, S., Coppola, F., Meucci, V., Battaglia, F., Intorre, L., Soares, A. M. V. M., Pretti, C., & Faggio, C. (2020). Combined effects of salinity changes

and salicylic acid exposure in *Mytilus galloprovincialis*. *Science of The Total Environment*, *715*, 136804. https://doi.org/10.1016/j.scitotenv.2020.136804

Funes, V., Alhama, J., Navas, J. I., López-Barea, J., & Peinado, J. (2006). Ecotoxicological effects of metal pollution in two mollusc species from the Spanish South Atlantic littoral. *Environmental Pollution*, *139*(2), 214–223. https://doi.org/10.1016/j.envpol.2005.05.016

Gadelha, J. R., Rocha, A. C., Camacho, C., Eljarrat, E., Peris, A., Aminot, Y., Readman, J. W., Boti, V., Nannou, C., Kapsi, M., Albanis, T., Rocha, F., Machado, A., Bordalo, A., Valente, L. M. P., Nunes, M. L., Marques, A., & Almeida, C. M. R. (2019). Persistent and emerging pollutants assessment on aquaculture oysters (*Crassostrea gigas*) from NW Portuguese coast (Ria De Aveiro). *Science of The Total Environment*, *666*, 731–742. https://doi.org/10.1016/j.scitotenv.2019.02.280

Gebbink, W. A., van Asseldonk, L., & van Leeuwen, S. P. J. (2017). Presence of Emerging Per- and Polyfluoroalkyl Substances (PFASs) in River and Drinking Water near a Fluorochemical Production Plant in the Netherlands. *Environmental Science & Technology*, *51*(19), 11057–11065. https://doi.org/10.1021/acs.est.7b02488

Gebbink, W. A., & van Leeuwen, S. P. J. (2020). Environmental contamination and human exposure to PFASs near a fluorochemical production plant: Review of historic and current PFOA and GenX contamination in the Netherlands. *Environment International*, *137*, 105583. https://doi.org/10.1016/j.envint.2020.105583

Geng, Q., Zou, L., Guo, M., Peng, J., Li, F., Bi, Y., Jiang, S., Qin, H., & Tan, Z. (2024). Insights into the combined toxicity and mechanisms of BDE-47 and PFOA in marine blue mussel: An integrated study at the physiochemical and molecular levels. *Aquatic Toxicology*, *273*, 106999. https://doi.org/10.1016/j.aquatox.2024.106999

Giannessi, J., De Marchi, L., Meucci, V., Intorre, L., Monni, G., Baratti, M., & Pretti, C. (2023). Subcellular tissue-specific responses of *Mytilus galloprovincialis* to fluoroquinolone antibiotics. *Environmental Toxicology and Pharmacology*, *104*, 104306. https://doi.org/10.1016/j.etap.2023.104306

Grimmelpont, M., Payton, L., Lefrançois, C., & Tran, D. (2024). Molecular and behavioural responses of the mussel *Mytilus edulis* exposed to a marine heatwave. *Marine Environmental Research*, *196*, 106418. https://doi.org/10.1016/j.marenvres.2024.106418

Habig, W. H., Pabst, M. J., & Jakoby, W. B. (1974). Glutathione S-Transferases: THE FIRST ENZYMATIC STEP IN MERCAPTURIC ACID FORMATION. *Journal of Biological Chemistry*, *249*(22), 7130–7139. https://doi.org/10.1016/S0021-9258(19)42083-8

He, A., Dean, J. M., & Lodhi, I. J. (2021). Peroxisomes as cellular adaptors to metabolic and environmental stress. *Trends in Cell Biology*, *31*(8), 656–670. https://doi.org/10.1016/j.tcb.2021.02.005

Helmstetter, A., Gamerdinger, A. P., & Pruell, R. J. (1996). Acute toxicity of methanol to Mytilus edulis. *Bulletin of Environmental Contamination and Toxicology*, *57*(4), 675–681. https://doi.org/10.1007/s001289900243

Herbert, E. R., Boon, P., Burgin, A. J., Neubauer, S. C., Franklin, R. B., Ardón, M., Hopfensperger, K. N., Lamers, L. P. M., & Gell, P. (2015). A global perspective on wetland salinization: Ecological consequences of a growing threat to freshwater wetlands. *Ecosphere*, *6*(10), art206. https://doi.org/10.1890/ES14-00534.1

Heydebreck, F., Tang, J., Xie, Z., & Ebinghaus, R. (2015). Correction to Alternative and Legacy Perfluoroalkyl Substances: Differences between European and Chinese River/Estuary Systems. *Environmental Science & Technology*, *49*(24), 14742–14743. https://doi.org/10.1021/acs.est.5b05591

Hosokawa, M., & Satoh, T. (2001). Measurement of Carboxylesterase (CES) Activities. *Current Protocols in Toxicology*, *10*(1), 4.7.1-4.7.14. https://doi.org/10.1002/0471140856.tx0407s10

Hou, J., & Zhang, M. (2024). Chapter 8—Toxicological impacts of perfluorinated compounds on marine bivalves. In G. Liu (Ed.), *Marine Bivalve Mollusks and Emerging Pollutants* (pp. 167–191). Academic Press. https://doi.org/10.1016/B978-0-443-19117-6.00002-7

Houde, M., Wells, R. S., Fair, P. A., Bossart, G. D., Hohn, A. A., Rowles, T. K., Sweeney, J. C., Solomon, K. R., & Muir, D. C. G. (2005). Polyfluoroalkyl Compounds in Free-Ranging Bottlenose Dolphins (Tursiops truncatus) from the Gulf of Mexico and the Atlantic Ocean. *Environmental Science & Technology*, *39*(17), 6591–6598. https://doi.org/10.1021/es0506556

Hutchinson, T. H., Shillabeer, N., Winter, M. J., & Pickford, D. B. (2006). Acute and chronic effects of carrier solvents in aquatic organisms: A critical review. *Aquatic Toxicology*, *76*(1), 69–92. https://doi.org/10.1016/j.aquatox.2005.09.008

IPCC. (2023). *Climate Change 2023: Synthesis Report. Contribution of Working Groups I, II and III to the Sixth Assessment Report of the Intergovernmental Panel on Climate Change [Core Writing Team, H. Lee and J. Romero (eds.)].* doi: 10.59327/IPCC/AR6-9789291691647.

Islam, N., Garcia da Fonseca, T., Vilke, J., Gonçalves, J. M., Pedro, P., Keiter, S., Cunha, S. C., Fernandes, J. O., & Bebianno, M. J. (2021). Perfluorooctane sulfonic acid (PFOS) adsorbed to polyethylene microplastics: Accumulation and ecotoxicological effects in the clam *Scrobicularia plana*. *Marine Environmental Research*, *164*, 105249. https://doi.org/10.1016/j.marenvres.2020.105249

Itoh, S., Araki, A., Mitsui, T., Miyashita, C., Goudarzi, H., Sasaki, S., Cho, K., Nakazawa, H., Iwasaki, Y., Shinohara, N., Nonomura, K., & Kishi, R. (2016). Association of perfluoroalkyl substances exposure in utero with reproductive hormone levels in cord blood in the Hokkaido Study on Environment and Children's Health. *Environment International*, *94*, 51–59. https://doi.org/10.1016/j.envint.2016.05.011

Jahngen-Hodge, J., Obin, M. S., Gong, X., Shang, F., Nowell, T. R., Gong, J., Abasi, H., Blumberg, J., & Taylor, A. (1997). Regulation of Ubiquitin-conjugating Enzymes by Glutathione Following Oxidative Stress*. *Journal of Biological Chemistry*, *272*(45), 28218–28226. https://doi.org/10.1074/jbc.272.45.28218

Ji, C., Wu, H., Wei, L., & Zhao, J. (2014). iTRAQ-based quantitative proteomic analyses on the gender-specific responses in mussel *Mytilus galloprovincialis* to tetrabromobisphenol A. *Aquatic Toxicology*, *157*, 30–40. https://doi.org/10.1016/j.aquatox.2014.09.008

Joerss, H., Apel, C., & Ebinghaus, R. (2019). Emerging per- and polyfluoroalkyl substances (PFASs) in surface water and sediment of the North and Baltic Seas. *Science of The Total Environment*, *686*, 360–369. https://doi.org/10.1016/j.scitotenv.2019.05.363

Joerss, H., Xie, Z., Wagner, C. C., von Appen, W.-J., Sunderland, E. M., & Ebinghaus, R. (2020). Transport of Legacy Perfluoroalkyl Substances and the Replacement Compound HFPO-DA through the Atlantic Gateway to the Arctic Ocean—Is the Arctic a Sink or a Source? *Environmental Science & Technology*, *54*(16), 9958–9967. https://doi.org/10.1021/acs.est.0c00228

Johansson, L. H., & Håkan Borg, L. A. (1988). A spectrophotometric method for determination of catalase activity in small tissue samples. *Analytical Biochemistry*, *174*(1), 331–336. https://doi.org/10.1016/0003-2697(88)90554-4

Kaviraj, A., Bhunia, F., & Saha, N. C. (2004). Toxicity of Methanol to Fish, Crustacean, Oligochaete Worm, and Aquatic Ecosystem. *International Journal of Toxicology*, *23*(1), 55–63. https://doi.org/10.1080/10915810490265469

King, F. D., & Packard, T. T. (1975). Respiration and the activity of the respiratory electron transport system in marine zooplankton1. *Limnology and Oceanography*, *20*(5), 849–854. https://doi.org/10.4319/lo.1975.20.5.0849

Kroeker, K. J., Kordas, R. L., & Harley, C. D. G. (2017). Embracing interactions in ocean acidification research: Confronting multiple stressor scenarios and context dependence. *Biology Letters*, *13*(3), 20160802. https://doi.org/10.1098/rsbl.2016.0802

Kube, S., Gerber, A., Jansen, J. M., & Schiedek, D. (2006). Patterns of organic osmolytes in two marine bivalves, Macoma balthica, and Mytilus spp., along their European distribution. *Marine Biology*, *149*(6), 1387–1396. https://doi.org/10.1007/s00227-006-0303-7

Kudo, N., Suzuki, E., Katakura, M., Ohmori, K., Noshiro, R., & Kawashima, Y. (2001). Comparison of the elimination between perfluorinated fatty acids with different carbon chain length in rats. *Chemico-Biological Interactions*, *134*(2), 203–216. https://doi.org/10.1016/S0009-2797(01)00155-7

Kwok, K. Y., Wang, X.-H., Ya, M., Li, Y., Zhang, X.-H., Yamashita, N., Lam, J. C. W., & Lam, P. K. S. (2015). Occurrence and distribution of conventional and new classes of per- and polyfluoroalkyl substances (PFASs) in the South China Sea. *Journal of Hazardous Materials*, *285*, 389–397. https://doi.org/10.1016/j.jhazmat.2014.10.065

Lamarck, J.-B. M. de. (1819). *Histoire naturelle des animaux sans vertèbres. Tome 6(1)*. Published by the author. http://www.biodiversitylibrary.org/item/47441

Lee, J. W., Choi, K., Park, K., Seong, C., Yu, S. D., & Kim, P. (2020). Adverse effects of perfluoroalkyl acids on fish and other aquatic organisms: A review. *Science of The Total Environment*, *707*, 135334. https://doi.org/10.1016/j.scitotenv.2019.135334

Leite, C., Russo, T., Cuccaro, A., Pinto, J., Polese, G., Soares, A. M. V. M., Pretti, C., Pereira, E., & Freitas, R. (2023). Can temperature rise change the impacts induced by e-waste on adults and sperm of *Mytilus galloprovincialis*? *Science of The Total Environment*, *902*, 166085. https://doi.org/10.1016/j.scitotenv.2023.166085

Letendre, J., Chouquet, B., Rocher, B., Manduzio, H., Leboulenger, F., & Durand, F. (2008). Differential pattern of Cu/Zn superoxide dismutase isoforms in relation to tidal spatio-temporal changes in the blue mussel *Mytilus edulis*. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *148*(3), 211–216. https://doi.org/10.1016/j.cbpc.2008.05.012

Li, Y., Liu, X., Zheng, X., Yang, M., Gao, X., Huang, J., Zhang, L., & Fan, Z. (2021). Toxic effects and mechanisms of PFOA and its substitute GenX on the photosynthesis of *Chlorella pyrenoidosa*. *Science of The Total Environment*, *765*, 144431. https://doi.org/10.1016/j.scitotenv.2020.144431

Liberatore, H. K., Jackson, S. R., Strynar, M. J., & McCord, J. P. (2020). Solvent Suitability for HFPO-DA (“GenX” Parent Acid) in Toxicological Studies. *Environmental Science & Technology Letters*, *7*(7), 477–481. https://doi.org/10.1021/acs.estlett.0c00323

Liu, C., Gin, K. Y. H., & Chang, V. W. C. (2014). Multi-biomarker responses in green mussels exposed to PFCs: Effects at molecular, cellular, and physiological levels. *Environmental Science and Pollution Research*, *21*(4), 2785–2794. https://doi.org/10.1007/s11356-013-2216-6

Livingstone, D. R., Lips, F., Martinez, P. G., & Pipe, R. K. (1992). Antioxidant enzymes in the digestive gland of the common mussel Mytilus edulis. *Marine Biology*, *112*(2), 265–276. https://doi.org/10.1007/BF00702471

Lockwood, B. L., & Somero, G. N. (2011). Transcriptomic responses to salinity stress in invasive and native blue mussels (genus Mytilus). *Molecular Ecology*, *20*(3), 517–529. https://doi.org/10.1111/j.1365-294X.2010.04973.x

Lopes, C. B., Lillebø, A. I., Dias, J. M., Pereira, E., Vale, C., & Duarte, A. C. (2007). Nutrient dynamics and seasonal succession of phytoplankton assemblages in a Southern European Estuary: Ria de Aveiro, Portugal. *Estuarine, Coastal and Shelf Science*, *71*(3), 480–490. https://doi.org/10.1016/j.ecss.2006.09.015

Lukić Bilela, L., Matijošytė, I., Krutkevičius, J., Alexandrino, D. A. M., Safarik, I., Burlakovs, J., Gaudêncio, S. P., & Carvalho, M. F. (2023). Impact of per- and polyfluorinated alkyl substances (PFAS) on the marine environment: Raising awareness, challenges, legislation, and mitigation approaches under the One Health concept. *Marine Pollution Bulletin*, *194*, 115309. https://doi.org/10.1016/j.marpolbul.2023.115309

Magnani, L., Gaydou, E. M., & Hubaud, J. C. (2000). Spectrophotometric measurement of antioxidant properties of flavones and flavonols against superoxide anion. *Analytica Chimica Acta*, *411*(1), 209–216. https://doi.org/10.1016/S0003-2670(00)00717-0

Mahmoudnia, A. (2023). The role of PFAS in unsettling ocean carbon sequestration. *Environmental Monitoring and Assessment*, *195*(2), 310. https://doi.org/10.1007/s10661-023-10912-8

Mahoney, H., Xie, Y., Brinkmann, M., & Giesy, J. P. (2022). Next generation per- and poly-fluoroalkyl substances: Status and trends, aquatic toxicity, and risk assessment. *Eco-Environment & Health*, *1*(2), 117–131. https://doi.org/10.1016/j.eehl.2022.05.002

Manduzio, H., Monsinjon, T., Galap, C., Leboulenger, F., & Rocher, B. (2004). Seasonal variations in antioxidant defences in blue mussels *Mytilus edulis* collected from a polluted area: Major contributions in gills of an inducible isoform of Cu/Zn-superoxide dismutase and of glutathione *S*-transferase. *Aquatic Toxicology*, *70*(1), 83–93. https://doi.org/10.1016/j.aquatox.2004.07.003

Manduzio, H., Monsinjon, T., Rocher, B., Leboulenger, F., & Galap, C. (2003). Characterization of an inducible isoform of the Cu/Zn superoxide dismutase in the blue mussel *Mytilus edulis*. *Aquatic Toxicology*, *64*(1), 73–83. https://doi.org/10.1016/S0166-445X(03)00026-2

Martín, J., Hidalgo, F., García-Corcoles, M. T., Ibáñez-Yuste, A. J., Alonso, E., Vilchez, J. L., & Zafra-Gómez, A. (2019). Bioaccumulation of perfluoroalkyl substances in marine echinoderms: Results of laboratory-scale experiments with *Holothuria tubulosa* Gmelin, 1791. *Chemosphere*, *215*, 261–271. https://doi.org/10.1016/j.chemosphere.2018.10.037

Matozzo, V., Boscolo, A., & Marin, M. G. (2013a). Seasonal and gender-related differences in morphometric features and cellular and biochemical parameters of

Carcinus aestuarii from the Lagoon of Venice. *Marine Environmental Research*, *89*, 21–28.

Matozzo, V., Chinellato, A., Munari, M., Bressan, M., & Marin, M. G. (2013b). Can the combination of decreased pH and increased temperature values induce oxidative stress in the clam *Chamelea gallina* and the mussel *Mytilus galloprovincialis*? *Marine Pollution Bulletin*, *72*(1), 34–40. https://doi.org/10.1016/j.marpolbul.2013.05.004

Melzer, D., Rice, N., Depledge, M. H., Henley, W. E., & Galloway, T. S. (2010). Association between Serum Perfluorooctanoic Acid (PFOA) and Thyroid Disease in the U.S. National Health and Nutrition Examination Survey. *Environmental Health Perspectives*, *118*(5), 686–692. https://doi.org/10.1289/ehp.0901584

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 & Pharmacology*, *191*, 152–159. https://doi.org/10.1016/j.cbpc.2016.10.007

Molavian, H., Madani Tonekaboni, A., Kohandel, M., & Sivaloganathan, S. (2015). The Synergetic Coupling among the Cellular Antioxidants Glutathione Peroxidase/Peroxiredoxin and Other Antioxidants and its Effect on the Concentration of H2O2. *Scientific Reports*, *5*, 13620. https://doi.org/10.1038/srep13620

Monserrat, J., Letts, R. E., Ferreira, J. L. R., Ventura-Lima, J., Amado, L. L., Rocha, A. M., Gorbi, S., Bocchetti, R., Benedetti, M., & Regoli, F. (2011). Biomarkers of Oxidative Stress: Benefits and Drawbacks for their Application in Biomonitoring of Aquatic Environments. In *Oxidative Stress in Aquatic Ecosystems* (pp. 317–326). John Wiley & Sons, Ltd. https://doi.org/10.1002/9781444345988.ch23

Monserrat, J. M., Martínez, P. E., Geracitano, L. A., Lund Amado, L., Martinez Gaspar Martins, C., Lopes Leães Pinho, G., Soares Chaves, I., Ferreira-Cravo, M., Ventura-Lima, J., & Bianchini, A. (2007). Pollution biomarkers in estuarine animals: Critical review and new perspectives. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *146*(1), 221–234. https://doi.org/10.1016/j.cbpc.2006.08.012

Mullin, L., Katz, D. R., Riddell, N., Plumb, R., Burgess, J. A., Yeung, L. W. Y., & Jogsten, I. E. (2019). Analysis of hexafluoropropylene oxide-dimer acid (HFPO-DA) by liquid chromatography-mass spectrometry (LC-MS): Review of current approaches and environmental levels. *TrAC Trends in Analytical Chemistry*, *118*, 828–839. https://doi.org/10.1016/j.trac.2019.05.015

Niu, Z., Na, J., Xu, W., Wu, N., & Zhang, Y. (2019). The effect of environmentally relevant emerging per- and polyfluoroalkyl substances on the

growth and antioxidant response in marine *Chlorella* sp. *Environmental Pollution*, *252*, 103–109. https://doi.org/10.1016/j.envpol.2019.05.103

OECD. (2018a). *Summary report on the new comprehensive global database of Per- and Polyfluoroalkyl Substances (PFASs)*. https://www.oecd-ilibrary.org/content/publication/1a14ad6c-en

OECD. (2018b). *Test No. 319B: Determination of in vitro intrinsic clearance using rainbow trout liver S9 sub-cellular fraction (RT-S9). OECD Guidelines for the Testing of Chemicals, Section 3, OECD Publishing, Paris*. https://doi.org/10.1787/9789264303232-en.

OECD. (2023). *Endocrine Disrupting Chemicals in Freshwater: Monitoring and Regulating Water Quality: Vol. OECD Studies on Water*. OECD Publishing. https://doi.org/10.1787/5696d960-en

Ohkawa, H., Ohishi, N., & Yagi, K. (1979). Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction. *Analytical Biochemistry*, *95*(2), 351–358. https://doi.org/10.1016/0003-2697(79)90738-3

Ohoro, C. R., Amaku, J. F., Conradie, J., Olisah, C., Akpomie, K. G., Malloum, A., Akpotu, S. O., Adegoke, K. A., Okeke, E. S., & Omotola, E. O. (2024). Effect of physicochemical parameters on the occurrence of per- and polyfluoroalkyl substances (PFAS) in aquatic environment. *Marine Pollution Bulletin*, *208*, 117040. https://doi.org/10.1016/j.marpolbul.2024.117040

Orbea, A., Ortiz-Zarragoitia, M., & Cajaraville, M. P. (2002). Interactive effects of benzo(a)pyrene and cadmium and effects of di(2-ethylhexyl) phthalate on antioxidant and peroxisomal enzymes and peroxisomal volume density in the digestive gland of mussel Mytilus galloprovincialis Lmk. *Biomarkers*, *7*(1), 33–48. https://doi.org/10.1080/13547500110066119

Paciello, S., Russo, T., De Marchi, L., Soares, A. M. V. M., Meucci, V., Pretti, C., He, Y., Della Torre, C., & Freitas, R. (2023). Sub-lethal effects induced in *Mytilus galloprovincialis* after short-term exposure to sodium lauryl sulfate: Comparison of the biological responses given by mussels under two temperature scenarios. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *270*, 109644. https://doi.org/10.1016/j.cbpc.2023.109644

Piva, E., Nicorelli, E., Pacchini, S., Schumann, S., Drago, L., Vanzan, G., Tolomeo, A. M., Irato, P., Bakiu, R., Gerdol, M., & Santovito, G. (2024). Unravelling stress granules in the deep cold: Characterisation of TIA-1 gene sequence in Antarctic fish species. *Fish & Shellfish Immunology*, *154*, 109903. https://doi.org/10.1016/j.fsi.2024.109903

Podbielski, I., Schmittmann, L., Sanders, T., & Melzner, F. (2022). Acclimation of marine invertebrate osmolyte systems to low salinity: A systematic review & meta-analysis. *Frontiers in Marine Science*, *9*. https://doi.org/10.3389/fmars.2022.934378

Podlipaeva, Y. I., & Berger, V. Ya. (2012). The effect of environmental salinity on the level of heat shock proteins in gill epithelium of Mytilus edilis L. mussel. *Cell and Tissue Biology*, *6*(5), 498–502. https://doi.org/10.1134/S1990519X12050100

Rahman, I., Kode, A., & Biswas, S. K. (2006). Assay for quantitative determination of glutathione and glutathione disulfide levels using enzymatic recycling method. *Nature Protocols*, *1*(6), 3159–3165. https://doi.org/10.1038/nprot.2006.378

Rai, Y., Pathak, R., Kumari, N., Sah, D. K., Pandey, S., Kalra, N., Soni, R., Dwarakanath, B. S., & Bhatt, A. N. (2018). Mitochondrial biogenesis and metabolic hyperactivation limits the application of MTT assay in the estimation of radiation induced growth inhibition. *Scientific Reports*, *8*(1), 1531. https://doi.org/10.1038/s41598-018-19930-w

Regoli, F., Benedetti, M., Giuliani, M. E., & others. (2011a). Antioxidant defenses and acquisition of tolerance to chemical stress. *Tolerance to Environmental Contaminants*, *1*, 153–173.

Regoli, F., & Giuliani, M. E. (2014). Oxidative pathways of chemical toxicity and oxidative stress biomarkers in marine organisms. *Marine Environmental Research*, *93*, 106–117. https://doi.org/10.1016/j.marenvres.2013.07.006

Regoli, F., Giuliani, M. E., Benedetti, M., & Arukwe, A. (2011b). Molecular and biochemical biomarkers in environmental monitoring: A comparison of biotransformation and antioxidant defense systems in multiple tissues. *Aquatic Toxicology*, *105*(3, Supplement), 56–66. https://doi.org/10.1016/j.aquatox.2011.06.014

Regoli, F., & Principato, G. (1995). Glutathione, glutathione-dependent and antioxidant enzymes in mussel, Mytilus galloprovincialis, exposed to metals under field and laboratory conditions: Implications for the use of biochemical biomarkers. *Aquatic Toxicology*, *31*(2), 143–164. https://doi.org/10.1016/0166-445X(94)00064-W

Richardson, S. J., Bai, A., Kulkarni, A. A., & Moghaddam, M. F. (2016). Efficiency in Drug Discovery: Liver S9 Fraction Assay As a Screen for Metabolic Stability. *Drug Metabolism Letters*, *10*(2), 83–90. https://doi.org/10.2174/1872312810666160223121836

Robinson, H. W., & Hogden, C. G. (1940). THE BIURET REACTION IN THE DETERMINATION OF SERUM PROTEINS: I. 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*(2), 707–725. https://doi.org/10.1016/S0021-9258(18)73134-7

Rodrigues, C. M., Bio, A., Amat, F., & Vieira, N. (2011). Artisanal salt production in Aveiro/Portugal—An ecofriendly process. *Saline Systems*, *7*, 3. https://doi.org/10.1186/1746-1448-7-3

Rodrigues, M., Oliveira, A., Queiroga, H., & Brotas, V. (2012). Seasonal and diurnal water quality and ecological dynamics along a salinity gradient (Mira channel, Aveiro lagoon, Portugal). *Procedia Environmental Sciences*, *13*, 899–918. https://doi.org/10.1016/j.proenv.2012.01.084

Röthig, T., Trevathan-Tackett, S. M., Voolstra, C. R., Ross, C., Chaffron, S., Durack, P. J., Warmuth, L. M., & Sweet, M. (2023). Human-induced salinity changes impact marine organisms and ecosystems. *Global Change Biology*, *29*(17), 4731–4749. https://doi.org/10.1111/gcb.16859

Rutter, J., Winge, D. R., & Schiffman, J. D. (2010). Succinate Dehydrogenase—Assembly, Regulation and Role in Human Disease. *Mitochondrion*, *10*(4), 393–401. https://doi.org/10.1016/j.mito.2010.03.001

Sakurai, T., Kobayashi, J., Kinoshita, K., Ito, N., Serizawa, S., Shiraishi, H., Lee, J.-H., Horiguchi, T., Maki, H., Mizukawa, K., Imaizumi, Y., Kawai, T., & Suzuki, N. (2013). Transfer kinetics of perfluorooctane sulfonate from water and sediment to a marine benthic fish, the marbled flounder (Pseudopleuronectes yokohamae). *Environmental Toxicology and Chemistry*, *32*(9), 2009–2017. https://doi.org/10.1002/etc.2270

Santovito, G., Piccinni, E., Cassini, A., Irato, P., & Albergoni, V. (2005). Antioxidant responses of the Mediterranean mussel, *Mytilus galloprovincialis*, to environmental variability of dissolved oxygen. *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology*, *140*(3), 321–329. https://doi.org/10.1016/j.cca.2005.02.015

Savoca, D., & Pace, A. (2021). Bioaccumulation, Biodistribution, Toxicology and Biomonitoring of Organofluorine Compounds in Aquatic Organisms. *International Journal of Molecular Sciences*, *22*(12), Article 12. https://doi.org/10.3390/ijms22126276

Scott, J. W., Gunderson, K. G., Green, L. A., Rediske, R. R., & Steinman, A. D. (2021). Perfluoroalkylated Substances (PFAS) Associated with Microplastics in a Lake Environment. *Toxics*, *9*(5), Article 5. https://doi.org/10.3390/toxics9050106

Shi, W., Zhang, Z., Li, X., Chen, J., Liang, X., & Li, J. (2024). GenX Disturbs the Indicators of Hepatic Lipid Metabolism Even at Environmental Concentration in Drinking Water via PPARα Signaling Pathways. *Chemical Research in Toxicology*, *37*(1), 98–108. https://doi.org/10.1021/acs.chemrestox.3c00342

Smolders, R., De Boeck, G., & Blust, R. (2003). Changes in cellular energy budget as a measure of whole effluent toxicity in zebrafish (Danio rerio). *Environmental Toxicology and Chemistry*, *22*(4), 890–899. https://doi.org/10.1002/etc.5620220429

Sokolova, I. M., Sukhotin, A. A., & Lannig, G. (2012). *Stress effects on metabolism and energy budgets in mollusks* [Inbook]. Oxidative Stress in Aquatic Ecosystems; Wiley-Blackwell. https://epic.awi.de/id/eprint/25426/

Solé, M., Bonsignore, M., Rivera-Ingraham, G., & Freitas, R. (2018). Exploring alternative biomarkers of pesticide pollution in clams. *Marine Pollution Bulletin*, *136*, 61–67. https://doi.org/10.1016/j.marpolbul.2018.08.062

Solé, M., Freitas, R., & Rivera-Ingraham, G. (2021). The use of an *in vitro* approach to assess marine invertebrate carboxylesterase responses to chemicals of environmental concern. *Environmental Toxicology and Pharmacology*, *82*, 103561. https://doi.org/10.1016/j.etap.2020.103561

Song, X., Vestergren, R., Shi, Y., Huang, J., & Cai, Y. (2018). Emissions, Transport, and Fate of Emerging Per- and Polyfluoroalkyl Substances from One of the Major Fluoropolymer Manufacturing Facilities in China. *Environmental Science & Technology*, *52*(17), 9694–9703. https://doi.org/10.1021/acs.est.7b06657

Steffani, C. N., & Branch, G. M. (2003). Growth rate, condition, and shell shape of Mytilus galloprovincialis: Responses to wave exposure. *Marine Ecology Progress Series*, *246*, 197–209. https://doi.org/10.3354/meps246197

Stoichev, T., Coelho, J. P., De Diego, A., Valenzuela, M. G. L., Pereira, M. E., de Chanvalon, A. T., & Amouroux, D. (2020). Multiple regression analysis to assess the contamination with metals and metalloids in surface sediments (Aveiro Lagoon, Portugal). *Marine Pollution Bulletin*, *159*, 111470. https://doi.org/10.1016/j.marpolbul.2020.111470

Sun, M., Arevalo, E., Strynar, M., Lindstrom, A., Richardson, M., Kearns, B., Pickett, A., Smith, C., & Knappe, D. R. U. (2016). Legacy and Emerging Perfluoroalkyl Substances Are Important Drinking Water Contaminants in the Cape Fear River Watershed of North Carolina. *Environmental Science & Technology Letters*, *3*(12), 415–419. https://doi.org/10.1021/acs.estlett.6b00398

Suzuki, Y. J., Carini, M., & Butterfield, D. A. (2010). Protein carbonylation. *Antioxidants & Redox Signaling*, *12*(3), 323–325. https://doi.org/10.1089/ars.2009.2887

van der Schatte Olivier, A., Jones, L., Vay, L. L., Christie, M., Wilson, J., & Malham, S. K. (2020). A global review of the ecosystem services provided by bivalve aquaculture. *Reviews in Aquaculture*, *12*(1), 3–25. https://doi.org/10.1111/raq.12301

van Meerloo, J., Kaspers, G. J. L., & Cloos, J. (2011). Cell Sensitivity Assays: The MTT Assay. In I. A. Cree (Ed.), *Cancer Cell Culture: Methods and Protocols* (pp. 237–245). Humana Press. https://doi.org/10.1007/978-1-61779-080-5_20

Vargas, C. I. C., Vaz, N., & Dias, J. M. (2017). An evaluation of climate change effects in estuarine salinity patterns: Application to Ria de Aveiro shallow water system. *Estuarine, Coastal and Shelf Science*, *189*, 33–45. https://doi.org/10.1016/j.ecss.2017.03.001

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. https://doi.org/10.1016/j.marenvres.2016.11.010

Viarengo, A., Burlando, B., Cavaletto, M., Marchi, B., Ponzano, E., & Blasco, J. (1999). Role of metallothionein against oxidative stress in the musselMytilus galloprovincialis. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *277*(6), R1612–R1619. https://doi.org/10.1152/ajpregu.1999.277.6.R1612

Vieira Sanches, M., Oliva, M., Mezzetta, A., Guazzelli, L., De Marchi, L., Freitas, R., & Pretti, C. (2023). *In vitro* screening of imidazolium and pyrrolidinium based ionic liquids toxicity on subcellular fractions of the Mediterranean mussel *Mytilus galloprovincialis*. *Environmental Toxicology and Pharmacology*, *104*, 104305. https://doi.org/10.1016/j.etap.2023.104305

Wang, F., Xiang, L., Leung, K. S.-Y., Elsner, M., Zhang, Y., Guo, Y., Pan, B., Sun, H., An, T., Ying, G., Brooks, B. W., Hou, D., Helbling, D. E., Sun, J., Qiu, H., Vogel, T. M., Zhang, W., Gao, Y., Simpson, M. J., … Tiedje, J. M. (2024). Emerging contaminants: A One Health perspective. *The Innovation*, *5*(4). https://doi.org/10.1016/j.xinn.2024.100612

Wang, Q., Ning, X., Pei, D., Zhao, J., You, L., Wang, C., & Wu, H. (2013). Molecular cloning, characterization and expression profiles of thioredoxin 1 and thioredoxin 2 genes in Mytilus galloprovincialis. *Chinese Journal of Oceanology and Limnology*, *31*(3), 493–503. https://doi.org/10.1007/s00343-013-2234-8

Wang, Y., Jiang, S., Wang, B., Chen, X., & Lu, G. (2023). Comparison of developmental toxicity induced by PFOA, HFPO-DA, and HFPO-TA in zebrafish embryos. *Chemosphere*, *311*, 136999. https://doi.org/10.1016/j.chemosphere.2022.136999

White, N. D., Balthis, L., Kannan, K., De Silva, A. O., Wu, Q., French, K. M., Daugomah, J., Spencer, C., & Fair, P. A. (2015). Elevated levels of perfluoroalkyl substances in estuarine sediments of Charleston, SC. *Science of The Total Environment*, *521–522*, 79–89. https://doi.org/10.1016/j.scitotenv.2015.03.078

Wootton-Beard, P. C., Moran, A., & Ryan, L. (2011). Stability of the total antioxidant capacity and total polyphenol content of 23 commercially available vegetable juices before and after *in vitro* digestion measured by FRAP, DPPH, ABTS and Folin–Ciocalteu methods. *Food Research International*, *44*(1), 217–224. https://doi.org/10.1016/j.foodres.2010.10.033

Wu, Y., Jin, R., Chen, Q., Du, X., Yang, J., & Liu, M. (2023). Organic contaminants of emerging concern in global estuaries: Environmental occurrence, fate, and bioavailability. *Critical Reviews in Environmental Science and Technology*, *53*(4), 550–575. https://doi.org/10.1080/10643389.2022.2077062

Xu, P., Junaid, M., Liu, Y., Jiang, X., Chen, Y., Bi, C., Wang, J., & Xu, N. (2022). Nanoplastics influence the perfluorooctane sulfonate (PFOS) mediated toxicity on marine mussel *Perna viridis*: Single and mixture exposure study. *Gondwana Research*, *108*, 144–157. https://doi.org/10.1016/j.gr.2021.12.001

Yang, L.-H., Yang, W.-J., Lv, S.-H., Zhu, T.-T., Adeel Sharif, H. M., Yang, C., Du, J., & Lin, H. (2022). Is HFPO-DA (GenX) a suitable substitute for PFOA? A comprehensive degradation comparison of PFOA and GenX via electrooxidation. *Environmental Research*, *204*, 111995. https://doi.org/10.1016/j.envres.2021.111995

Yeldandi, A. V., Rao, M. S., & Reddy, J. K. (2000). Hydrogen peroxide generation in peroxisome proliferator-induced oncogenesis. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, *448*(2), 159–177. https://doi.org/10.1016/S0027-5107(99)00234-1

Yin, C., Pan, C.-G., Xiao, S.-K., Wu, Q., Tan, H.-M., & Yu, K. (2022). Insights into the effects of salinity on the sorption and desorption of legacy and emerging per-and polyfluoroalkyl substances (PFASs) on marine sediments. *Environmental Pollution*, *300*, 118957. https://doi.org/10.1016/j.envpol.2022.118957

Yuan, W., Song, S., Lu, Y., Shi, Y., Yang, S., Wu, Q., Wu, Y., Jia, D., & Sun, J. (2024). Legacy and alternative per-and polyfluoroalkyl substances (PFASs) in the Bohai Bay Rim: Occurrence, partitioning behavior, risk assessment, and emission scenario analysis. *Science of The Total Environment*, *912*, 168837. https://doi.org/10.1016/j.scitotenv.2023.168837

Zhang, C., McElroy, A. C., Liberatore, H. K., Alexander, N. L. M., & Knappe, D. R. U. (2022). Stability of Per- and Polyfluoroalkyl Substances in Solvents Relevant to Environmental and Toxicological Analysis. *Environmental Science & Technology*, *56*(10), 6103–6112. https://doi.org/10.1021/acs.est.1c03979

Zhang, J., Wang, X., Vikash, V., Ye, Q., Wu, D., Liu, Y., & Dong, W. (2016). ROS and ROS-Mediated Cellular Signaling. *Oxidative Medicine and Cellular Longevity*, *2016*, 4350965. https://doi.org/10.1155/2016/4350965

Zhao, Z., Cheng, X., Hua, X., Jiang, B., Tian, C., Tang, J., Li, Q., Sun, H., Lin, T., Liao, Y., & Zhang, G. (2020). Emerging and legacy per- and polyfluoroalkyl substances in water, sediment, and air of the Bohai Sea and its surrounding rivers. *Environmental Pollution*, *263*, 114391. https://doi.org/10.1016/j.envpol.2020.114391

Zhao, Z., Xie, Z., Möller, A., Sturm, R., Tang, J., Zhang, G., & Ebinghaus, R. (2012). Distribution and long-range transport of polyfluoroalkyl substances in the Arctic, Atlantic Ocean and Antarctic coast. *Environmental Pollution*, *170*, 71–77. https://doi.org/10.1016/j.envpol.2012.06.004