Universidade de Aveiro
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Poluentes emergentes num ambiente em mudança: impactos nos bivalves estuarinos

Emerging pollutants in a changing environment: impacts to estuarine bivalves

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais, realizada sob a orientação científica da Doutora Rosa de Fátima Lopes de Freitas, Professora auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro, e da Doutora Maria Eduarda da Cunha Pereira, Professora associada do Departamento de Química da Universidade de Aveiro e da Doutora Montserrat Solé, Investigadora Sénior do Departamento de recursos marinhos renováveis do Intitut de Ciències del Mar de Barcelona.

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palavras-chave

Resíduos de equipamentos elétricos e eletrônicos (lixo eletrônico); Elementos de terras raras; Alterações Climáticas, Ecotoxicologia; Bivalves

resumo

Zonas costeiras, como estuários, enfrentam a ameaça de novos contaminantes oriundos não só da indústria, mas também de crescentes efluentes resultantes do aumento populacional no litoral. Com o avanço tecnológico e o desenvolvimento económico, multiplicaram-se as aplicações de diversos equipamentos elétricos e eletrónicos, e o aumento de resíduos elétricos e eletrónicos (REEE). Contudo, muitas vezes falta um tratamento e reciclagem adequados destes resíduos, levando à libertação de diversos elementos químicos, incluindo elementos das terras raras (ETRs), no ambiente. Em particular, ETRs com propriedades químicas semelhantes, como o Lantânio (La), Gadolínio (Gd), Ítrio (Y) e Térbio (Tb), são amplamente usados devido às suas características óticas e magnéticas, essenciais para muitas aplicações elétricas e eletrónicas. Contudo, os riscos ambientais resultantes destes ETRs são quase desconhecidos, sobretudo em ecossistemas marinhos, que podem igualmente ser afetados por alterações climáticas previstas, como o aumento da temperatura e variações na salinidade das águas. Assim, esta tese teve como objetivo usar o mexilhão *Mytilus galloprovincialis*, uma espécie ecológica e economicamente relevante reconhecida como um bom bioindicador global, para avaliar os efeitos fisiológicos e bioquímicos dos ETRs La, Gd, Y e Tb em cenários de mudanças climáticas reais e projetadas. Foi realizada uma investigação preliminar sobre as diferenças entre populações de mexilhões intertidais e subtidais da Ria de Aveiro face à exposição às marés e submersão. Ao comparar ambas as populações, a exposição às marés mostrou ser mais desafiante para os mexilhões subtidais, enquanto os intertidais não foram negativamente afetados durante o período de submersão. Ambas as populações apresentaram diferentes características de vida que influenciam as reservas energéticas e períodos de aclimatação, mas ambas estavam adaptadas ao stresse oxidativo, tornando-as adequadas para estudos ecotoxicológicos. Uma exposição *in vivo* a diferentes concentrações de Y e Tb foi considerada, dada a escassez de literatura sobre estes elementos. Ítrio causou alterações bioquímicas subtis principalmente em baixas concentrações, enquanto Tb mostrou uma resposta adaptativa em três etapas, mas nenhum dos elementos causou dano celular aparente. Alterações bioquímicas em baixas concentrações também foram verificadas para La e Gd por outros autores. Com base nos resultados obtidos, uma baixa concentração ambientalmente relevante dos quatro ETRs foi selecionada, e os mexilhões foram expostos a uma combinação de cada ETR, com aumento de temperatura e variações de salinidade, para avaliar se estes fatores alteram a toxicidade dos ETRs e a sensibilidade das espécies expostas. Os resultados mostraram que os organismos expostos a ETRs demonstraram uma suscetibilidade aos seus efeitos tóxicos, especialmente quando submetidos às variações de salinidade e ao aumento da temperatura. Estas condições causaram stresse osmótico e térmico intensificado nestes organismos. Entre os parâmetros relacionados com as mudanças climáticas,

uma diminuição da salinidade intensificou os impactos dos ETRs de forma mais significativa do que o aumento da salinidade ou da temperatura. Especificamente, a diminuição da salinidade intensificou os efeitos neurotóxicos do La e a atividade enzimática de defesa do Y, com organismos contaminados apresentando dano celular em comparação com organismos não contaminados. Já os mexilhões expostos a Tb mostraram maior resiliência entre os quatro elementos sob esta condição. O aumento da salinidade afetou os diferentes elementos de formas distintas, onde mexilhões expostos a Tb mostraram maior suscetibilidade com alterações metabólicas e dano celular. O aumento da temperatura produziu efeitos não lineares nos mexilhões contaminados, resultando em respostas bioquímicas tanto protetoras quanto prejudiciais. Esta tese apresenta contribuições significativas para a compreensão da contaminação e das respostas fisiológicas de uma espécie importante face a várias condições ambientais, através de uma recolha de dados e análise detalhada. As descobertas poderão ter implicações importantes para o desenvolvimento de estratégias eficazes de gestão e reciclagem de REEE e podem servir como base para a criação de diretrizes normativas.

keywords

Waste of electric and electronic equipment (e-waste); Rare-earth elements; Climate change; Ecotoxicology; Bivalves

abstract

Coastal zones, such as estuaries, face the eminence of new contaminants derived not just from the industry but also from higher volumes of effluents resulting from population growth in the littoral. With the technological advance and economic development, the multiplicity and wide variety of electrical and electronic equipment applications have increased, as well as the amount of electrical and electronic waste (e-waste). However, the proper treatment and recycling of e-waste are often lacking leading to the release of many chemical elements, including rare-earth elements (REEs), into the environment. Particularly, REEs possessing similar chemical properties, such as Lanthanum (La), Gadolinium (Gd), Yttrium (Y) and Terbium (Tb), are widely used due to their unique optical and magnetic characteristics, which are crucial for many electrical and electronic applications. Nevertheless, the environmental risks resulting from these REEs are almost unknown, especially considering marine ecosystems, which may be equally challenged by foreseen climate changes, such as increasing temperature and waters' salinity shifts. Therefore, the present thesis aimed to use the mussel *Mytilus galloprovincialis*, an ecological and economical relevant species considered a good bioindicator worldwide, to evaluate the physiological and biochemical effects of the REEs La, Gd, Y and Tb under actual and predicted climate change scenarios. Preliminary research on the differences between intertidal and subtidal mussel populations of Ria de Aveiro to both tidal exposure and submergence environments was performed. Comparing both populations, tidal exposure showed to be more challenging to subtidal mussels while intertidal mussels were not affected negatively when under the submersion period. Both populations presented different life-history traits influencing energy reserves and acclimation periods, but both were adapted to the oxidative stress, making them suitable for ecotoxicological studies. An *in vivo* exposure to different concentrations of Y and Tb, considering the lack of literature for these elements was considered as a starting point. Yttrium was found to cause subtle biochemical alterations mainly at lower concentrations, while Tb revealed a tri-step adaptive response, but neither element led to apparent cellular damage. Biochemical alterations at low concentrations were also verified for La and Gd by other authors. Based on the results obtained, a low and environmentally relevant concentration of the four REEs was selected, and mussels were exposed to a combination of each REE, with increasing temperature and salinity shifts, to evaluate if these factors alter the toxicity of REEs and the sensitivity of the species exposed to them. The findings revealed that organisms exposed to REEs displayed susceptibility to their toxic effects, especially when subjected to salinity shifts and increased temperature. These conditions caused intensified osmotic and heat stress in these organisms. Among the climate change related parameters, decreased salinity intensified the impacts of REEs more significantly than increased salinity or temperature. Specifically, decreased salinity intensified La neurotoxic effects and Y defense enzyme activity, with contaminated organism receiving

cellular damage compared to non-contaminated organisms. Meanwhile, Tb-exposed mussels showed more resilience among the four elements under this condition. Increased salinity affected the various elements differently, where Tb-exposed mussels exhibited higher susceptibility with metabolic changes and cellular damage. The increase of temperature produced nonlinear effects on the contaminated mussels, manifesting in both protective and detrimental biochemical responses. This thesis presents significant contributions to the understanding of contamination and the physiological responses of an important species to various environmental conditions, through detailed analysis and data collection. The findings may have important implications for the development of effective e-waste management and recycling strategies and can serve as a basis for creating normative guidelines.

LIST OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

ACh, Acetylcholine
AChE, Acetylcholinesterase
ADP, Adenosine diphosphate
ATChI, Acetylthiocholine iodide
ATP, Adenosine triphosphate
CAT, Catalase
CbEs, Carboxylesterases
CDNB, 1-chloro-2,4-dinitrobenzene
DNPH, Dinitrophenylhydrazine
DTNB, Ellman's reagent
DTPA, Diethylenetriaminepentaacetic acid
DTT, Dithiothreitol
DW, Dry weight
EDTA, Ethylenediaminetetraacetic acid
EE2, 17α-ethinylestradiol
EEE, Electrical and electronic equipment
ETS, Electron Transport System
E-waste, Electrical and electronic equipment waste
FAD, Flavin adenine dinucleotide
FMN, Flavin mononucleotide
FW, Fresh weight
GHGs, Greenhouse gases
GLY, Glycogen
GPx, Glutathione peroxidase
GR, Glutathione reductase
GSH, Reduced glutathione
GSSG, Oxidized glutathione
GSTP1, Glutathione *S*-transferases pi 1
GSTs, Glutathione *S*-transferases
IBR, Integrated Biomarker Response
IBRvs2, Integrated Biomarker Response version 2
ICP-MS, Inductively coupled mass spectroscopy
IS, Intertidal organisms always submersed
IT, Intertidal organisms under tidal exposure
LOOHs, Lipid hydroperoxides
LOQ, Limit of quantification
LPO, Lipid peroxidation
MTs, Metallothioneins
NAD^+, Nicotinamide adenine dinucleotide
NBT, Nitroblue tetrazolium
PC, Protein carbonylation
PCO, Principal coordinate analysis
***p*NPA,** *p*-nitrophenyl acetate
***p*NPB,** *p*-nitrophenyl butyrate
PROT, Protein
PVP, Polyvinylpyrrolidone
REEs, Rare-earth elements
ROS, Reactive oxygen species
SOD, Superoxide dismutase

SS, Subtidal organisms always submersed
SSP, Shared Socio-economic Pathway
ST, Subtidal organism under tidal exposure
TBA, Thiobarbituric acid
TCA, Trichloroacetic acid
WWTPs, Wastewater treatment plants

CHAPTER 1. INTRODUCTION

1.1. Estuaries Dynamics

Coastal systems, like estuaries, undergo constant and intricate changes, influenced by both natural and human-induced factors. These areas are defined as the interface or transitional zone between the sea and the land (Ward et al., 2020), making them highly susceptible to a diverse range of environmental factors. Ecosystems present in these areas play a critical role in supporting essential ecological functions, encompassing biological productivity, hydrologic flux regulation, biogeochemical cycling of metals and nutrients (Chilton et al., 2021; Wei et al., 2023). Additionally, these water bodies serve as vital habitats for a wide array of fish and shellfish species, holding significant economic importance for coastal communities (Whitfield et al., 2022).

Whitin the estuarine environments, physical and chemical parameters vary on a small spatial and temporal scale, a characteristic typical of transitional coastal ecosystems (*e.g.,* Mama et al., 2021; Nascimento et al., 2021). The hydrodynamic regime is known to encompass tidal cycles with a multitude of patterns, along with other processes related to the interaction of the varying bathymetry and geomorphology with other factors such as river and groundwater inflows, wind, waves, storm surges, atmospheric pressure, gravity, and Coriolis effect (Fotsi et al., 2023; Khojasteh et al., 2021, 2022). This complex interaction makes the estuaries one of the most challenging environments for organisms to endure.

Estuaries, similar to other coastal environments, can be characterized by two distinct zones influenced by tidal fluctuations. The intertidal zone that encompasses the area between high and low tides, and the subtidal zone that lies just below the intertidal zone and remains perpetually submerged, never exposed to air throughout the tidal cycle (Leeuwis and Gamperl, 2022) (Figure 1A). Because of this, organisms living in the intertidal zone confront the daily challenge of adapting to two contrasting habitats: submergence in the aquatic environment during high tide and exposure to the aerial environment when the tide recedes (Collins et al., 2023). The main abiotic challenges include the substantial temperature and salinity fluctuations, coupled with elevated desiccation risks and the dynamic challenge of managing oxygen availability between aquatic and aerial conditions (Collins et al., 2023; Horn et al., 2021; Leeuwis and Gamperl, 2022; Trevisan and Mello, 2024) (Figure 1B). Specifically, exposure to air during low tide implies coping with desiccation, osmotic stress, temperature stress, and UV radiation (Collins et al., 2023; Leeuwis and Gamperl, 2022), while frequent re-immergence during the tidal cycles adds further stress to intertidal organisms due to such fluctuations.

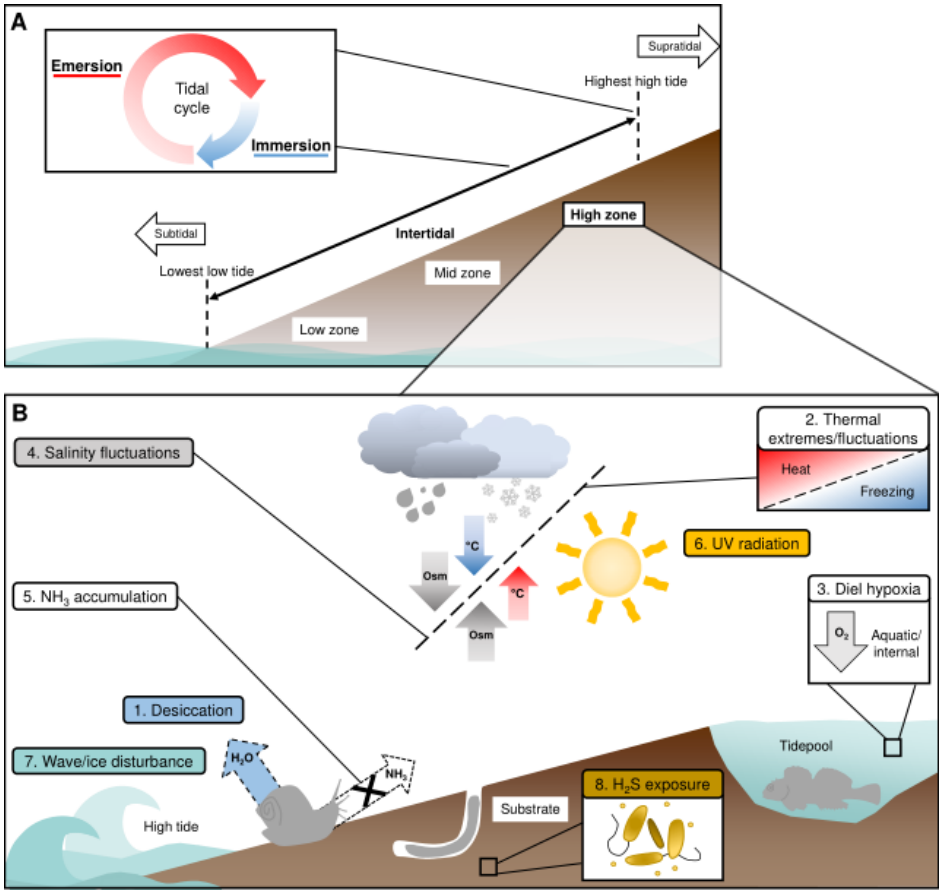

Figure 1. A: Location and definition of the different zones of coastal environments; B: Main abiotic challenges in the intertidal zone (retrieved from Leeuwis and Gamperl, 2022).

Nevertheless, the stability of estuaries is increasingly threatened by a convergence of other multifaceted environmental pressures. The simultaneous impact of three critical factors can be taking in account: the increasing urbanization and industrialization, the increasing use of resources, and the increasing effects of climate change (Defeo and Elliott, 2021). The interplay of these factors creates a precarious situation for estuarine environments, which are already complex and sensitive due to their transitional nature. Specifically, the increase of urbanization and industrialization brings with it several challenges, including pollution and habitat alteration. In fact, organisms inhabiting these systems may be subjected to the presence of different chemicals from industrial, domestic, and agricultural activities in the surrounding areas (Gavhane et al., 2021; Morin-Crini et al., 2022), leading to several biological and habitat effects. The demand for and consumption of physical and biological resources, may further strain these environments leading altogether to higher susceptibility to the effects of climate change, such as increased temperatures, changes in salinity, and more frequent extreme weather events.

These elements collectively impair the fragile balance of estuarine ecosystems, intensifying issues such as eutrophication, hypoxia, and coastal erosion (Defeo and Elliott, 2021). In this light, understanding these various pressures and their interplay becomes crucial to preserve the ecological integrity and functionality of these vital coastal systems.

1.2. Estuaries and Emerging Pressures: The Case of E-waste and Rare-Earth Elements

Among the environmental pressures threatening, pollution due to intensified human activities is one of the most menacing and rapidly growing threats. Around 40 % of the global population resides within 100 kilometers of coastal areas, driven by favorable economic prospects and livelihood opportunities (OECD, 2021). This high density of human activity emerges as a substantial contributor to environmental pollution, mainly due to the previously mentioned overexploitation of natural resources and the significant rise in waste generation resulting from the rapid urban-industrial and technological revolutions (Ajibade et al., 2021). Pollutants intricately intertwined with modern society's dynamics, are closely correlated with population growth and the continuous evolution of technology, encompassing the development of renewable energy sources and electronics (Mrozik et al., 2021; Silva et al., 2023; Wiedmann et al., 2020). Because of this, the rapid growth in electrical and electronic equipment (EEE), driven by technological advancements, represents a unique and escalating challenge.

This challenge is represented by the rising production of EEE waste (e-waste), characterized by an abundance of EEE with increasingly short lifespans, leading to a voluminous accumulation of discarded products (Forti et al., 2020). Simultaneously, as the longevity of such equipment diminishes, the scale of maintenance choices also constricts, leading to an accelerated cycle of equipment replacement and increasing the growth of e-waste (Sonego et al., 2022) (Figure 2). E-waste production reached approximately 53.6 million metric tons (Mt) in 2019, marking a substantial increase of 9.2 Mt since 2014 (Forti et al., 2020) (Figure 2). Projections indicated the continuity of this trend, with an expected quantity of roughly 74.7 Mt by 2030 (Forti et al., 2020). However, the management of e-waste currently lacks widespread recycling protocols and is hindered by expensive and underutilized legal mechanisms (Rautela et al., 2021; Thakur and Kumar, 2021). Additionally, a significant portion of e-waste does not undergo proper collection and recycling processes that adhere to sustainable environmental standards (Abalansa et al., 2021). This can result in the inadvertent release of hazardous chemical elements and compounds into the environment, such as mercury, lead, and cadmium, considered as priority pollutants of environmental concern, and which have well-documented impacts on both ecological resources and human health (Manikandan et al., 2023). However, beyond these well-known contaminants, there is growing concern about other elements, not often considered in mainstream environmental debate, such as rare-earth elements (REEs).

Rare-earth elements are released as tailings, byproducts, and waste during the production and use of EEE (Patel et al., 2023). Moreover, these elements have the potential to accumulate in various environmental compartments, such as soil, air, water, and biological organisms. Anthropogenic concentrations have already been detected in these areas, posing a multifaceted threat to ecological resources and human health, particularly in light of the rapid increase in e-waste production (Patel et al., 2023; Piarulli et al., 2021).

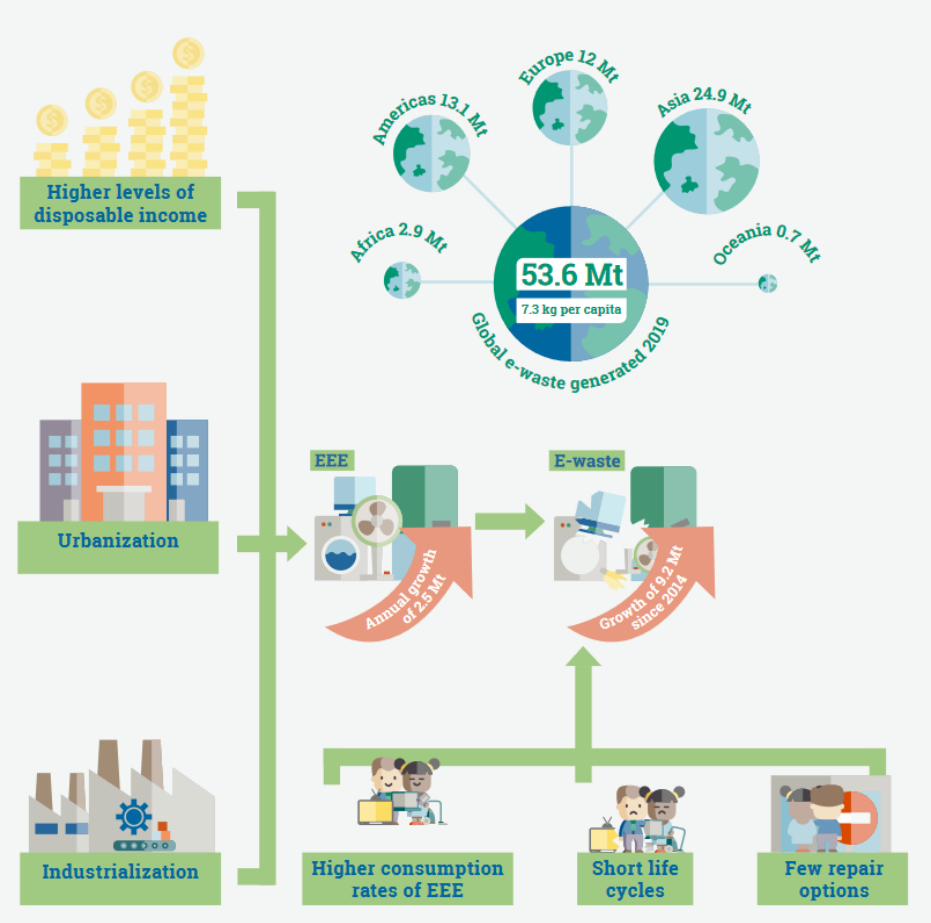

Figure 2. Factors contributing to the generation of global electrical and electronic equipment waste in 2019 (retrieved from Forti et al., 2020).

The significance of REEs in modern society cannot be overstated as they are crucial in an extensive range of products — from daily-used smartphones and laptops to advanced defense systems and green technologies like wind turbines and electric vehicles (Dias et al., 2020; Filho et al., 2023). These elements are particularly essential in the manufacturing of magnets, batteries, fluorescent lamps, and catalysts, thanks to their desirable physical and chemical attributes, such as great thermal stability, electrical conductivity, strong magnetism, and optical properties including fluorescence and the

ability to emit visible light (Dushyantha et al., 2020; Filho et al., 2023). Such unique optical and magnetic characteristics, combined with high reactivity, distinct from other elements in the periodic table (*e.g.,* Baranovskaya et al., 2021; Liang et al., 2022; Zhang and Zhang, 2022), have led to an increasing demand for REEs. The group comprises 17 elements, including scandium (Sc), yttrium (Y), and 15 lanthanides such as lanthanum (La), cerium (Ce), praseodymium (Pr), neodymium (Nd), promethium (Pm), samarium (Sm), europium (Eu), gadolinium (Gd), terbium (Tb), dysprosium (Dy), holmium (Ho), erbium (Er), thulium (Tm), ytterbium (Yb), and lutetium (Lu). Rare-earth elements typically exhibit higher abundances for elements with lower atomic numbers compared to those with higher atomic numbers. Consequently, they are often categorized into two primary groups according to the International Union of Pure and Applied Chemistry (IUPAC): the light rare earths (LREs), which include elements from La to Eu, and the heavy rare earths (HREs), encompassing Gd through Lu as well as Y. This classification, however, varies regionally. In Europe, REEs from La to Sm are usually considered LREs, whereas those from Eu to Lu plus Y are grouped as HREs. In contrast, the Chinese classification designates Sm as an HRE. The categorization of Gd is also not consistent, with its classification differing between IUPAC and the United States Geological Survey (USGS) (Filho et al., 2023). An alternative categorization can be also applied, often classifying them as LREs from La to Pm, medium REEs (Sm, Eu, and Gd), and HREs (Tb to Lu, along with Sc and Y) (*e.g.,* Neira et al., 2022).

Despite what the name "rare" suggests, REEs are actually abundant in the Earth's crust with the challenging lying in the fact that they are rarely found in economically viable concentrations (Dushyantha et al., 2020). However, there are also other significant challenges associated with REEs, such as China's control over a substantial portion of the global market (70% of production and 90% of processing) which leads to geopolitical tensions, especially given their critical role in global tech industries such as renewable energy applications (Park et al., 2023). Such geopolitical issues together with their increasing demand has given them the designation of “critical materials”, emphasizing the importance of efficient REE recycling from e-waste for both economic and environmental reasons. As technological advancements and digitalization progresses, the demand for REEs is projected to increase even more rapidly. In fact, compared to 2020 the production of critical materials is predicted to increase sixteen times by 2050 due to the rise in renewable energy technologies alone (Liang et al., 2023). Such escalating demand for REEs, coupled with the complexities of their production and geopolitical implications, underscores a pressing need for immediate and intensive efforts in sustainable

management and recycling of REEs to mitigate potential environmental risks and ensure a balanced and secure global supply. Given these challenges, it becomes essential to closely examine the environmental impacts of certain key REEs that are particularly influential in various applications.

Lanthanum, Gd, Y, and Tb are good examples of REEs with environmental impacts. Such elements present unique properties and widespread applications in diverse fields such as electronics, medical imaging, agriculture, and high-tech industries.

Lanthanum - with an atomic number of 57, is the first element among the lanthanides. This element is a silvery-white, ductile and soft metal relatively common in Earth's crust, ranking as one of the most abundant REEs, with estimates suggesting concentrations as high as 39 parts per million (Kloprogge et al., 2020). Lanthanum is largely utilized in electronics and optoelectronics, where it is employed in the production of LEDs, fluorescent lamps, lasers, sensors, semiconductors, catalysts, metal alloys, batteries, flat display electronics, among others (Dushyantha et al., 2020; Filho et al., 2023). Moreover, it has been utilized as a trace fertilizer to stimulate agricultural growth, being this practice mostly conducted in China (Banerjee and Roychoudhury, 2022; Iguchi et al., 2023). As a consequence, its occurrence in industrial wastewater and runoff from agricultural activities is anticipated, thereby affecting the nearby aquatic systems.

The natural concentrations of La in aquatic systems exhibit significant variability. In rivers, La levels range from 0.02 to 0.60 µg/L, in estuaries from 0.005 to 0.200 µg/L, and in coastal areas from 0.005 to 0.400 µg/L (Elderfield et al., 1990). While the natural abundance of La in seawater is estimated at approximately 0.0034 µg/L (Tai et al. 2010), there is still a lack of research on the quantification of anthropogenic La in seawater. Nevertheless, anthropogenic La has been measured in some contaminated freshwater systems. For instance, alluvial aquifers affected by acid mine drainages in Spain contained concentrations as high as 40.37 µg/L (Olías et al., 2005). Similarly, river samples near the São Domingos mine in Portugal reached concentrations of up to 177.4 µg/L (Gomes et al., 2022), and higher concentrations, up to 900 µg/L, have been detected in streams flowing from acid sulphate soils due to agricultural activities in Finland (Åström, 2001).

Notably, La has been detected (in dry weight) in various marine organisms from specific regions. In the South China Sea, among twenty-nine different fish species, La levels ranged from 0.001 to 0.009 µg/g, while five shellfish and four crustacean species exhibited concentrations of between 0.010-0.807 µg/g and 0.025-0.182 µg/g, respectively

(Li et al., 2016). In the Ligurian Sea, twelve species of algae contained between 0.29 and 4.30 µg/g of La (Squadrone et al., 2017). In Japan, clams such as *Ruditapes philippinarum* and *Mactra veneriformis* had concentrations of 0.317 and 0.522 µg/g, respectively, while mussels like *Mytilus galloprovincialis* and clams including *R. philippinarum*, *Phacosoma japonicum*, *M. veneriformis* had shells with La concentrations ranging from 0.024 to 0.103 µg/g (Akagi and Edanami, 2017). In Portugal, La has been detected in *M. galloprovincialis* along the coast, with medium concentrations ranging from 0.074 to 0.340 µg/g in the fall and 0.098 to 0.483 µg/g in the spring (Figueiredo et al., 2022b).

The presence of La in aquatic systems may significantly impact biological resources, including those of socio-economic and ecological importance, such as estuarine bivalves (Olivier et al., 2018). While the effects of La are not yet fully understood, recent studies have described its negative impact on bivalves, particularly in freshwater mussels like *Corbicula fluminea* and clams such as *Dreissena polymorpha*, where it has been shown to affect metabolic capacity and oxidative conditions (Hanana et al., 2017b; Zhao and Liu, 2018). In marine bivalves, La has been found to be embryotoxic to the oyster *Crassostrea gigas* (Moreira et al., 2020) and the mussel *M. galloprovincialis* (Mestre et al., 2019). Furthermore, in adults of *M. galloprovincialis*, it has been observed that the accumulation of La can cause changes in metabolic and oxidative stress-related defense mechanisms, ultimately resulting in cellular damage (Pinto et al. 2019).

Gadolinium - with an atomic number of 64, is a ductile and slightly malleable metal. It appears silvery-white when oxidation is prevented, however it reacts with atmospheric oxygen or moisture producing a black surface coating. It comprises approximately 6.2 parts per million in the Earth's crust being notable for its strong magnetism and the high neutron-absorption capability (Kloprogge et al., 2020). Although not so widely used in EEE as other REEs, Gd can be found in applications such as microwaves, TV tubes, batteries, alloys, catalysts, flat display electronics, HD drives, microphones, speakers, sensors and semiconductors (Filho et al. 2023, Patil et al., 2022). Additionally, Gd holds an important role in the medical field, particularly within magnetic resonance imaging (MRI), where it serves as a contrast-enhancing agent (Blomqvist et al., 2022; Iyad et al., 2023). These contrast agents are engineered as chelate molecules designed to withstand metabolic processes and facilitate easy excretion (Brünjes and Hofmann, 2020; Iyad et al., 2023). This unique property often results in their release into

the aquatic environment in an unaltered form, posing challenges for their removal in wastewater treatment plants (WWTPs) (Boester and Rüde, 2020; Oluwasola et al., 2022).

The presence of Gd reported in aquatic ecosystems is often linked to human activities, particularly the use of Gd-based contrast agents (*e.g.,* Al Momani et al., 2023; Kim et al., 2020; Liu et al., 2022). For instance, Gd-based contrast agents have been identified as sources of Gd in WWTP effluents, resulting in discharges ranging from 200-1100 µg/L of Gd into rivers in South Africa (Rogowska et al., 2018). While naturally occurring Gd concentrations in South African and German rivers typically range from 0.001-0.004 µg/L, anthropogenic Gd has been detected at levels as high as 0.181 µg/L in surface waters globally (Rogowska et al., 2018). Additionally, streams in Finland have recorded Gd concentrations between 0.032-207 µg/L, attributed to the leaching of REEs from acid sulfate soils used in agriculture (Åström et al., 2001). While the natural presence of Gd in seawater is estimated at approximately 0.0007 µg/L (Tai et al., 2010), some studies have identified traces of anthropogenic Gd in marine environments. Notably, concentrations ranging up to 0.027 µg/L have been observed in San Francisco Bay, United States, and from 0.14 to 0.41 µg/L at submarine outfalls in Brazil, suggesting associations with high industrialization and medical usage (Hatje et al., 2016; Pedreira et al., 2018).

Given the widespread presence of Gd in the environment, its occurrence in aquatic wildlife is not surprising. Mean Gd concentrations (measured in dry weight) have been identified in various marine species, including mollusks like *Moerella iridiescens* and *R. phillipinarum* (0.017 and 0.015 µg/g, respectively), as well as crustaceans such as *Scylla serrata* and *Penceus penicillatus* (0.022 and 0.001 µg/g, respectively) (Wang et al., 2019). Additionally, twelve species of algae from the Ligurian Sea contained Gd levels between 0.07 and 0.87 µg/g (Squadrone et al., 2017). Furthermore, mussels (*M. galloprovincialis*) in Spain exhibited Gd concentrations ranging from 0.004 to 0.092 µg/g (Costas-Rodríguez et al., 2010). In Portugal, Gd has also been detected in *M. galloprovincialis* along the coast, with medium concentrations ranging from 0.022 to 0.065 µg/g in the fall and 0.026 to 0.093 µg/g in the spring (Figueiredo et al., 2022b). Gadolinium concentrations have also been revealed in freshwater organisms such as clams (*C. fluminea*) and mussels (*D. rostiformis bugensis*) in France from 0.049 to 0.110 µg/g and 0.01 to 0.123 µg/g, respectively (Pereto et al., 2020; Perrat et al., 2017). It was also detected in *C. fluminea* shells (0.001-0.019 µg/g) from the European Rhine River (Merschel and Bau, 2015), as well as in marine scallops (*Pecten maximus*) in France, with concentrations reaching up to 0.019 µg/g (Le Goff et al., 2019).

Nevertheless, despite Gd's known adverse effects on human health (Rogosnitzky and Branch, 2016), its impact on marine organisms remains poorly understood, with limited available literature on the subject. Studies on marine mussels (*M. galloprovincialis*) have shown that Gd can bioaccumulate and lead to changes in their metabolic capacity, oxidative status, and neurotoxicity (Henriques et al., 2019; Trapasso et al., 2021). Other bivalves, including freshwater clams (*C. fluminea* and *Spisula solida*) and mussels (*D. rostriformis bugensis* and *D. polymorpha*), have also exhibited alterations in oxidative stress status following Gd accumulation (Figueiredo et al., 2022a; Hanana et al., 2017a; Perrat et al., 2017). Furthermore, Gd has been found to negatively impact the embryonic development of sea urchins such as *Paracentrotus lividus*, *Arbacia lixula*, *Heliocidaris tuberculata* and *Centrostephanus rodgersii* (Martino et al., 2017).

Yttrium - with an atomic number of 39, is a silvery metallic transition metal, chemically similar to the lanthanides, therefore often included in the REEs. It is moderately abundant in the Earth's crust, with an estimated concentration of 31 parts per million (Kloprogge et al., 2020). This element has diverse industrial applications, including its use in ceramics, metal alloys, lasers, satellite communications, temperature sensors, in phosphors for lamps and displays, extending to various microwave, radar, laser, and optical applications, and also it plays a significant role in surgical procedures in the medical and dentistry fields (Filho et al., 2023; Rajakumar et al., 2021; Sharma and Renu, 2022).

Concentrations of trace Y in global rivers range from 0.028 to 0.894 µg/L in North America, 0.05 to 1.4 µg/L in Europe, and 0.087 to 0.282 µg/L in Africa (Gaillardet et al., 2003). However, higher concentrations have also been observed, reaching up to 1.00 µg/L in North America, 0.46 µg/L in Africa, 0.45 µg/L in Asia, and up to 98 µg/L across Europe (Reimann and Caritat, 1998). Yttrium originating from known anthropogenic sources has been measured, with concentrations as high as 55.70 µg/L in an alluvial aquifer affected by acid mine drainage in Spain and as elevated as 266.2 µg/L in a river near the São Domingos mine in Portugal (Gomes et al., 2022; Olías et al., 2005). In contrast, the natural abundance of Y in seawater has been estimated to be approximately 0.013 µg/L (Tai et al., 2010). Although quantifications in seawater are limited, Y has been detected in the Mediterranean Sea, with concentrations ranging from 0.02 to 0.04 µg/L, and in the western South Pacific Ocean, where concentrations fall between 0.005 and 0.026 µg/L (Bau et al., 1997; Zhang and Nozaki, 1996).

Yttrium has been detected (in dry weight) in various marine organisms from different regions. In Australia, *Porites* spp. corals exhibited Y levels ranging from 0.066 to 0.176 µg/g (Leonard et al., 2019). Moreover, in China, mean Y concentrations were quantified in mollusks such as *M. iridiescens* and *R. phillipinarum* (0.058 and 0.046 µg/g, respectively), crustaceans such as *S. serrata* and *P. penicillatus* (0.006 and 0.003 µg/g, correspondingly), and fish such as *Pagrosomus major*, *Tilapia nilotica* and *Harengula zunasi* (0.0007, 0.0006 and 0.0004 µg/g, respectively) (Wang et al., 2019). In the Ligurian Sea, twelve species of algae encompassed between 0.2 and 3.4 µg/g of Y (Squadrone et al., 2017). Furthermore, in Spain, mussels (*M. galloprovincialis)* were found to have Y concentrations ranging from 0.013 to 0.281 µg/g (Costas-Rodríguez et al., 2010). In Portugal, Y has also been detected in *M. galloprovincialis* along the coast, with medium concentrations ranging from 0.101 to 0.413 µg/g in the fall and 0.094 to 0.282 µg/g in the spring (Figueiredo et al., 2022b).

Despite Y's commonly occurrence in aquatic environments, its impact on aquatic organisms remains poorly understood, with limited studies addressing its toxicological effects. Studies involving rainbow trout (*Oncorhynchus mykiss*) have revealed that Y influenced gene transcription associated to protein denaturation (Dubé et al., 2019). Similarly, hepatocytes from the same fish species exhibited altered biochemical activity when exposed to Y (Hanana et al., 2021a). In the case of the freshwater mussel *D. polymorpha,* exposure to Y resulted in anti-inflammatory and genotoxic effects, along with changes in the expression of genes related to antioxidant and biotransformation enzymes (Hanana et al., 2018). Furthermore, Y has been found to induce intermediate embryotoxicity in the marine oyster *C. gigas* (Moreira et al., 2020).

Terbium - possessing an atomic number of 65, is characterized by its soft, malleable, and ductile nature, along with a silver-white luster commonly found in metals (Spellman, 2022). It stands out as one of the scarcer lanthanides, with an average concentration of 0.65-2.5 ppm in the Earth's crust (Sinha et al., 2023). Terbium is characterized by unique photophysical characteristics including a significant difference between the excitation and emission wavelengths, a bright and long-lasting glow, and exceptional light production efficiency, position it as a valuable candidate for utilization in sensors, amplifiers, and other materials in electro-optical devices (Girotto et al., 2019). Although its primary application has traditionally revolved around phosphors for illumination and displays, including fluorescent lamps and emissive displays, it also plays an essential role in permanent magnets for various electrical and electronic components,

including computers, mobile phones, hard disk drives, hybrid car engines and wind turbines (Brown et al., 2023; München et al., 2021; Spellman, 2022; Shabaz, 2022; Sinha et al., 2023). Additionally, Tb contributes to the production of metal alloys that yield metallic films for magneto-optic data recording (Kumari, 2022).

Regarding the presence of Tb in aquatic environments, studies have revealed its occurrence in various concentrations. For instance, in the surface waters of the Terengganu River Basin in Malaysia, Tb was detected at concentrations ranging from 0.005 to 0.510 µg/L (Sultan and Shazili, 2009) and up to 0.0084 µg/L in stream water from Vosges in France (Aubert et al., 2002). The estimated abundance of Tb in surface seawater is approximately 0.00014 µg/L (Lide, 2004). While geogenic sources have contributed to concentrations of Tb as high as 0.00381 µg/L in open oceans and coastal areas, there remains a research gap in comprehending the distribution and fate of Tb from anthropogenic sources within marine environments (Piarulli et al., 2021). Nevertheless, Tb contamination stemming from different anthropogenic sources has been well-documented in freshwater systems. For instance, concentrations of Tb reaching up to 2.26 µg/L were detected in an alluvial aquifer polluted by acid mine drainage in Spain (Olías et al., 2005), and levels as high as 10.4 µg/L were found in river samples near a mine in Portugal (Gomes et al., 2022). Additionally, concentrations ranging from 0.007 to 28 µg/L have been identified in streams affected by acid sulfate soils resulting from agricultural activities in Finland (Åström, 2001). In the context of healthcare waste, French hospitals were found to discharge Tb concentrations of up to 0.0042 µg/L into the major European estuary of Garonne-Gironde (Lerat-Hardy et al., 2019).

Although present at relatively low concentrations, Tb has been detected in the tissue (in dry weight) of diverse marine organisms across different regions. In Australia, *Porites* spp. corals displayed Tb concentrations from 0.00009 to 0.0017 µg/g (Leonard et al., 2019). Similarly, in China, various species, including mollusks (*M. iridiescens* and *R. phillipinarum*), crustaceans (*S. serrata* and *P. penicillatus*), and fish (*P. major*, *T. nilotica*, and *H. zunasi*), exhibited mean Tb concentrations ranging from 0.00005 to 0.00334 µg/g (Wang et al., 2019). Within the Ligurian Sea, twelve algae species contained Tb levels between 0.01 and 0.22 µg/g (Squadrone et al., 2017). Additionally, in Portugal, *M. galloprovincialis* along the coast registered Tb with medium concentrations varying from 0.0019 to 0.0130 µg/g in the spring and 0.0021 to 0.0081 µg/g in the fall (Figueiredo et al., 2022b).

While several aquatic organisms have been studied for the biochemical and physiological effects of multiple REEs (Piarulli et al., 2021), there remains a gap in

research regarding the toxicological impact of Tb on aquatic biota. Nevertheless, Tb has been observed to induce a diverse array of sublethal effects in juvenile rainbow trout (*O. mykiss*), including anti-inflammatory responses and alterations in gene expression associated with metal homeostasis, protein denaturation, DNA repair, oxidative stress, calcium binding, hemoprotein activity, and protein folding and turnover (Hanana et al., 2021b). Additionally, in other marine bivalves such as clams *R. philippinarum* and *R. decussatus*, Tb has been shown to cause metabolic impairment, disrupt redox processes, and induce neurotoxicity (Lompré et al., 2021).

1.3. Climate Change and its Effects in Contaminants in Estuaries

Increase in Temperature

One of the most noticeable manifestations of climate change is the global increase in temperature. The consistent rise in emissions of greenhouse gases (GHGs), especially carbon dioxide (CO_2), stands as the primary driver behind this phenomenon. Greenhouse gases have the ability to capture infrared radiation emitted from the Earth's surface, reflecting it back and trapping energy within the atmosphere. This process contributes significantly to the warming of the Earth's surface, particularly when compared to periods of lower GHGs emission levels (IPCC, 2021). In fact, it is anticipated that the global mean surface temperature will increase within a range of 1.0-1.8 °C (Shared Socio-economic Pathway, SSP1-1.9) up to 3.3-5.7 °C (SSP5-8.5) by the end of this century, depending on various GHGs emission projections (IPCC, 2021) (Figure 3). Moreover, oceans, which absorb over 90 % of the excess heat from GHGs emissions, are also experiencing rising temperatures (Bindoff et al., 2019). Ocean temperatures are projected to rise within a range of 0.43-1.47 °C (SSP1-2.6) up to 2.01-4.07 °C (SSP5-8.5) (IPCC, 2021). This increase can be especially pronounced in estuarine areas due to their shallow nature, leading to enhanced vulnerability of marine life in these habitats (*e.g.,* Scanes et al., 2020). This has raised growing concerns about the potential impact of temperature increases and the associated sea-level rise on coastal wildlife (Marcot et al., 2023; Röthig et al., 2023).

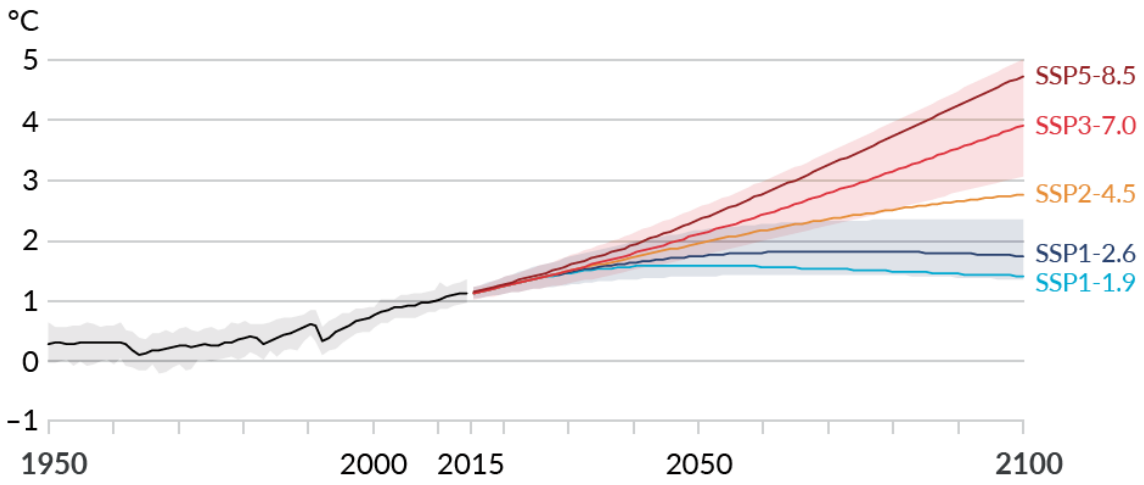

Figure 3. Simulated global surface temperature changes in °C relative to 1850–1900. The projections for each of the five scenarios are shown in color: very low greenhouse gases (GHGs) emission scenario (Shared Socio-economic Pathway, SSP1-1.9) represented in light blue; low GHGs emission scenario (SSP1-2.6) represented in dark blue; intermediate GHGs emission scenario (SSP2-4.5) represented in orange; high GHGs emission scenario (SSP3-7.0) represented in light red; very high GHGs emission scenario (SSP5-8.5) represented in dark red. Shades represent uncertainty ranges and the black curve represents the historical simulation (retrieved from IPCC, 2021).

The effects of global warming on marine ecosystems, biotic communities, and biodiversity have been extensively documented, including habitat loss and the displacement or decline of numerous species (Talukder et al., 2022). Various studies have also shown that temperatures exceeding the thermal tolerance range of marine organisms can lead to physiological and molecular disruptions, affecting growth and reproduction (*e.g.,* Masanja et al., 2023; Mugwanya et al., 2022). Moreover, elevated temperatures have been linked to alterations in aerobic capacity, metabolic rate, and respiratory capacity in these organisms (Eymann et al., 2020; Feidantsis et al., 2020; Pörtner et al., 2021). Furthermore, increased temperatures are known to facilitate the formation of cellular reactive oxygen species (ROS) (Benedetti et al., 2022; Sokolova, 2023), resulting in conditions of oxidative stress. Typically, intertidal organisms such as bivalves exhibit a pronounced sensitivity to variations in temperature. Small shifts outside their preferred thermal range can result in detrimental effects, such as cellular damage, increased oxidative stress, compromised immune responses, increased inflammatory reactions, alterations in microbial communities, and reduced growth (Masanja et al., 2023). Indeed, the projected temperature increase has already been demonstrated to influence the metabolism and oxidative status in different intertidal organisms, including bivalves (*e.g.*,

Bal et al., 2021; Pirone et al., 2019; Rahman et al., 2019). Taking marine mussels like *M. galloprovincialis* as an example, some studies have shown that these organisms reduce their metabolic, antioxidant, and biotransformation activities under thermal stress (Freitas et al., 2017a; Lopes et al., 2022; Morosetti et al., 2020). Conversely, other studies have identified an increase in these activities in the same mussel species (Andrade et al., 2019b; Pirone et al., 2019), suggesting the possibility of different adaptive strategies being employed to mitigate cellular damage. The effects of thermal stress were also evident in marine clams such as *R. philippinarum* and *R. decussatus*, as enzymatic defense mechanisms were enhanced, but there was decline in metabolic capacity or energy reserves (Almeida et al., 2021; Costa et al., 2020b).

Changes of Salinity

While in open oceans the salinity is remarkable stable, in transitional water bodies such as estuaries the salinity is easily influenced by tides, freshwater inputs and evaporation (Mills et al., 2021; Serrano et al., 2020). However, the global warming has been linked to the intensification of the global hydrological cycle, resulting in increased levels of precipitation, evaporation, atmospheric moisture, and more frequent extreme weather events, with far-reaching implications for salinity dynamics across various regions (IPCC, 2021; Yu et al., 2020) (Figure 4).

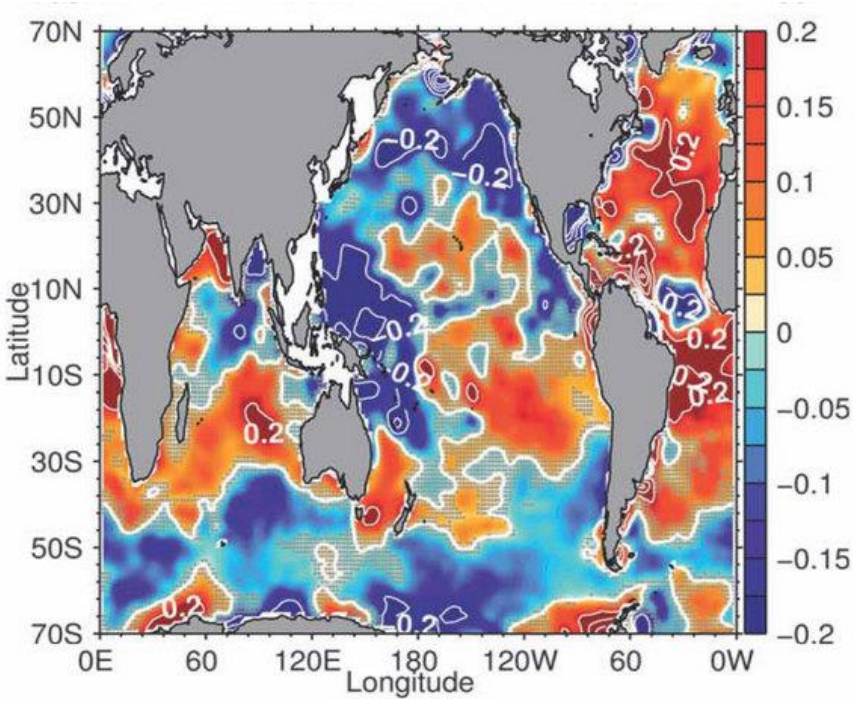

Figure 4. Alterations in surface salinity spanning the period from 1950 to 2000 are depicted, with the color red denoting areas experiencing an increase in salinity, while blue signifies regions undergoing a decrease in salinity (retrieved from Durack and Wijffels, 2010).

While organisms may possess a certain plasticity to salinity changes, alterations in their surrounding salinity levels can compromise their capacity to carry out essential biological processes, ultimately leading to consequences for community structure and species distribution boundaries (Röthig et al., 2023; Wernberg et al., 2024). Salinity shifts can directly affect the osmotic balance of marine organisms, leading to challenges in cellular processes and overall physiology. For bivalves, this is especially significant due to their osmoconformer nature. They generally exhibit a limited capacity for osmotic regulation in both diluted and concentrated media, often employing a strategy of intracellular isosmotic regulation, where their cells adapt to osmotic shifts by modifying levels of intracellular free amino acids and other small organic molecules (Medeiros et al., 2020). Consequently, they experience significant physiological challenges under fluctuating or unfavorable osmotic conditions (Medeiros et al., 2020). This sensitivity to salinity changes has been documented in various studies, showing its influence on the metabolic rate and oxidative status of bivalves (Bal et al., 2021, 2022; Pourmozaffar et al., 2019; Tan et al., 2023; Trevisan and Mello, 2024). For instance, in the mussel *M. galloprovincialis*, lower salinity has been linked to an increase in metabolic capacity. Concurrently, these mussels used their energy reserves, likely activating antioxidant defenses in response to hypotonic stress (Freitas et al., 2017b). Similarly, when exposed

to lower salinity, clams of the species *R. philippinarum* have been observed to augment their energy reserves and enhance their antioxidant defenses (Velez et al., 2016a,b; Freitas et al., 2016). Contrastingly, other bivalves like the clam *R. decussatus* and the oyster *C. angulata* have shown an increased metabolic capacity as salinity decreases (Velez er al., 2016b; Moreira et al., 2016).

Rising Sea Level and Hypoxia

Given the intense and varied challenges that organisms in the intertidal zone face, there is a crucial need to understand how they adapt and survive in such a dynamic environment. It is well-documented that organisms can adapt in distinctive ways when facing environmental stressors, including through structural, behavioral, and physiological adjustments. Notably, in intertidal habitats, a distinct distribution and vertical zonation of intertidal communities, particularly among sessile and sedentary organisms, have been observed (*e.g.,* Freitas et al., 2023; Garza et al., 2019; Ishida et al., 2021). However, the impact of climate change is altering these dynamics, with intertidal organisms facing the consequences of rising sea levels, while subtidal species are increasingly exposed to anthropogenically-induced hypoxic effects (Kaplanis et al., 2020; Khojasteh et al., 2021; Zhan et al., 2023).

Climate change exacerbates hypoxia in marine environments by increasing water temperature, which not only reduces oxygen solubility and heightens oxygen consumption by aquatic organisms, but also enhances stratification, restricting oxygen transfer to deeper waters and contributing to the expansion of low oxygen zones (Zhan et al., 2023). Furthermore, the extended exposure to air, a normal occurrence in these environments, can further lead to hypoxia or anoxia conditions (Falfushynska et al., 2020; Haider et al., 2020; Leeuwis and Gamperl, 2022; Resner et al., 2020), significantly impacting these organisms. For instance, organisms such as bivalves, may reduce their respiration and filtration rates and employ prolonged valve closure under air exposure, a strategy that conserves energy but limits oxygen intake (Anestis et al., 2007; Gosling, 2003; Leeuwis and Gamperl, 2022).

The rise in sea levels, similar to hypoxia conditions, is closely linked to an increase in seawater temperature, attributed to thermal expansion of the upper ocean layers and the melting of glaciers and ice sheets (Cheng et al., 2021; Swapna et al., 2020; Widlansky et al., 2020). Collectively, these changes contribute to a phenomenon known as “coastal squeeze”, where the physical reduction of intertidal habitats occurs due to

climate change. This phenomenon becomes particularly pronounced when coastal defenses prevent the natural inland migration of beaches (Silva et al., 2020). Consequently, both physical and biological parameters within these areas undergo transformation, impacting the vital thresholds of estuarine organisms, reducing their tolerance levels, and prompting shifts in species distribution and abundance (Williamson and Guinder, 2021).

Effects of Climate Change on Rare-Earth Elements Behavior

The behavior of REEs in marine environments may be significantly influenced by a range of abiotic factors. After entering marine environments, REEs can be significantly changed by factors such as pH, salinity, and the presence of organic and particulate matter (Piarulli et al., 2021), potentially altering their speciation, solubility, and bioavailability. These processes can lead to either their precipitation and sedimentation or dispersion in the water column. Marine organisms, such as filter feeders, might be especially vulnerable due to their direct interaction with suspended particulates containing these elements. This is particularly vital considering the extensive coastal human settlements and the reliance of billions of people on marine resources for sustenance and livelihood (Barceló et al., 2023).

The complexities associated with the environmental impacts caused by pollutants, and in particular by REEs, may become even more pronounced when considering the setting of climate change. Climate change is described as the gradual and accelerating alterations in Earth's climate patterns resulting from the cumulative impact of industrial and human activities in the last few decades (IPCC, 2021). The accelerated rate of these changes can primarily be traced back to the intensified release of GHGs emissions since the industrial revolution (Jones et al., 2023). As a consequence, marine environments and their wildlife may face greater abiotic alterations, including elevated temperatures, shifts in salinity, rising sea levels, and hypoxia, all of which carry the potential to disrupt food relationships, alter migration patterns, and challenge the adaptability of marine species to these changes (Muluneh, 2021; Ross et al., 2023). In particular, the increase of temperature and salinity alterations, influenced by climate change, could further influence the behavior, bioavailability, and toxicity of REEs in the marine environment. Such environmental alterations might intensify the effects on the solubility of REEs, potentially leading to greater exposure and altered bioaccumulation patterns in marine organisms. However, the exact nature of how these climate change factors modulate REEs behavior

remains largely unknown, presenting a significant gap in our understanding of the potential for both wildlife and humans who rely on them for food.

The profound impacts of climate change-driven environmental factors have originated an unprecedented need to develop and implement effective mitigation and adaptation strategies (Awuni et al., 2023; Kyprianou et al., 2023). Consequently, addressing climate change becomes not only an environmental urgency but also a socioeconomic one, as it is intrinsically links human well-being with the stability of ecosystems worldwide.

1.4. Biological Assessment of the Impact of Contaminants in Estuaries

1.4.1. Intertidal Organisms as Sentinels

To evaluate the sub-cellular effects and intricacies of intertidal environments in estuaries, as well as the combined impacts of contaminants and climate change, it becomes imperative to identify sentinels that can monitor and elucidate these complex interactions. Bioindicator species serve this purpose, defined as a species or a group of species that possesses the unique ability to reflect and reveal the repercussions of environmental changes on a given habitat, population, community or ecosystem (Hamza-Chaffai, 2014). To qualify as an ideal bioindicator, certain criteria have been outlined, including taxonomic simplicity, a broad or cosmopolitan distribution, limited mobility, well-documented ecological traits, numerical abundance, suitability for controlled laboratory experiments, high sensitivity to environmental stressors, and a high capacity for quantification and standardization (Sumudumali and Jayawardana, 2021). This criteria highlight the suitability of intertidal organisms, particularly filter-feeding sessile organisms such as bivalves, as ideal sentinel candidates.

Most bivalves are categorized as suspension-feeding or filter-feeding organisms, using their gills equipped with multiple ciliary tracts to filter particles from the surrounding water that is subsequently pumped through the mantle cavity. In their adult stages, most bivalves lead a sessile lifestyle and particular species like oysters and mussels, can attach themselves to solid surfaces through the utilization of byssal threads (Castro and Huber, 2023). This sessile and feeding behavior exposes them continuously to various environmental and biological stressors, reflecting the unique conditions of their respective habitats. In fact, intertidal bivalves consistently face abiotic stressors, including fluctuations in temperature, salinity, and oxygen availability, as well as alterations in food availability and the quality of their surrounding environment (Leeuwis and Gamperl, 2022; Trevisan and Mello, 2024). It is well-documented that these abiotic factors can have an impact in marine bivalve's physiology (*e.g.,* Masanja et al., 2023; Tan et al., 2023; Trevison and Mello, 2024). Furthermore, bivalves filter large volumes of water for feeding and respiration purposes that exposes them to ecotoxicological effects resulting from the xenobiotics present in the water column. In addition, bivalves are also organisms with high abundance and widespread distribution, and they are recognized for their capacity to endure high concentrations of xenobiotics (Baralla et al., 2021b; Vereycken and Aldridge, 2022). Because of such characteristics, bivalves have been regarded as good

bioindicators in both monitoring and ecotoxicological studies (Baralla et al., 2021a; Chahouri et al., 2023; Curpan et al., 2022; Strehse and Maser, 2020).

Intertidal invertebrates, including mussels, play a crucial role in maintaining the health and stability of coastal ecosystems (Whitaker et al., 2023; Salas et al., 2022). Mussels, such as *M. galloprovincialis* (Lamarck, 1819) (Figure 5), hold both economic significance (FAO, 2022) and ecological value by offering habitat for various species, contributing to water quality, and facilitating nutrient cycling within intertidal communities (Heckwolf et al., 2021; Suplicy, 2020). This species, commonly known as the Mediterranean mussel, exerts a dominant influence on rocky shore ecosystems in cooler regions of the northern and southern hemispheres, enhancing local biodiversity and providing suitable habitats for other organisms (Gosling, 2021; Veiga et al., 2022).

Figure 5. Biological shell features of *Mytilus galloprovincialis* (FAO, 2009).

Mytilus galloprovincialis thrives in diverse locations, encompassing the Mediterranean Sea, the British Isles, continental Europe, North and South Africa, southeastern Australia, and specific areas of the northern Pacific (Gosling, 2021; Ouagajjou et al., 2023; Springer and Crespi, 2007) (Figure 6). Although native to the Mediterranean, its range expansion over time can be attributed to geological changes during the Pleistocene era and the transport of shipping vessel ballast water, also leading to secondary contact with species like *M. edulis* and *M. trossulus*. This has resulted in hybrid zones, spanning from Spain to Britain in one instance and localized occurrences in California and Japan in the other (Springer and Crespi, 2007) (Figure 6).

Figure 6. Distribution of *Mytilus galloprovincialis* (yellow) and its hybrid populations with *Mytilus edulis* (orange) and *Mytilus trossulus* (green) (adapted from Springer and Crespi, 2007).

As with other bivalves, *M. galloprovincialis* serves as a valuable bioindicator sentinel species for environmental monitoring and toxicological assessments due to their capacity to accumulate contaminants and reflect surrounding toxic impacts effectively (*e.g.*, Abelouah et al., 2023; Curpan et al., 2022; Impellitteri et al., 2023; Provenza et al., 2022). They also display tolerance to abiotic changes, making them excellent indicators environmental shifts, including those linked to global climate change. In fact, the increase in temperature and the salinity alterations have been shown to impact *M. galloprovincialis*' metabolism and oxidative status, especially accentuated when combined with various contaminants (*e.g.*, Freitas et al., 2019b,c, 2020c; Morosetti et al., 2020; Lopes et al., 2022). While the combined effects of such physical parameters with REEs are unraveled, mussels constitute ideal candidate species for toxicological assays as pointed out in previous REEs studies (Freitas et al., 2020a,b; Henriques et al., 2019; Leite et al., 2023; Mestre et al., 2019; Pinto et al., 2019; Trapasso et al., 2021).

1.4.2. Biomarkers for Ecotoxicity Evaluation

From an environmental risk assessment perspective, the traditional approach of solely measuring lethal concentrations of pollutants, also in the case of REEs, reveals a significant limitation, particularly since environmental concentrations are relatively low. This approach may overlook the subtler, yet potentially significant, chronic impacts of low-level exposure. Chronic exposure to these low concentrations can result in sub-cellular effects, potentially escalating to impact higher biological levels such as population and community. This is especially critical when considering economically and ecologically

significant marine organisms. Thus, recognizing REEs potential as emerging pollutants necessitates a deeper, more comprehensive assessment of their sub-lethal effects in marine organisms.

Furthermore, the integration of climate change scenarios in the assessment of REEs has been largely neglected, failing to consider how future environmental changes could alter the behavior and impact of these elements. This oversight is particularly concerning given the projected alterations in marine conditions and their potential to exacerbate the risks associated with REEs. Understanding the bioaccumulation potential of these elements in marine organisms and their stability in aquatic environments are foundational steps, especially given not only its current lack of information in scientific literature but also a critical oversight in environmental policy and management. With the technological era intensifying our dependence on REEs, it becomes essential to balance the benefits of these elements with their potential marine ecological risks, particularly from a sub-lethal toxicity perspective. Such insights are indispensable, offering valuable information to environmental policymakers in determining the priority status of REEs as emerging contaminants.

With this broader perspective in mind, the choice and application of biomarkers in environmental monitoring becomes a focal point. A biomarker is a measurable indicator encompassing molecular, cellular, physiological, and behavioral changes reflecting deviations from an organism's normal condition in response to various stressors (Ahmad et al., 2023; Lemos, 2021). Thus, biomarkers are valuable tools for assessing the impacts of natural and anthropogenic factors on organisms' biochemical mechanisms and potential population consequences (Lomartire et al., 2021). Even when the source of a stressor is unclear or multiple stressors are present, carefully chosen biomarkers can detect early responses that may be further manifested at higher organizational levels and thus be of ecological relevance (Lemos, 2021). Additionally, biomarker responses can be evaluated in organisms collected or deployed in a field site, allowing the integration of effects from both chemical and non-chemical stressors, often overlooked in chemical analyses alone (Hook et al., 2014). Certain biomarkers can even identify specific contaminants or chemical groups within the ecosystem's complex array of environmental pollutants (*e.g.*, Lockridge and Schopfer, 2023).

Assessing the impact of stressors at the sub-individual or individual levels enables the prompt and effective recognition of sublethal effects compared to other approaches, such as morphological changes, being more informative of long-term consequences (Lomartire et al., 2021; Milinkovitch et al., 2019). Parameters relative to physiological and

biochemical performance are particularly useful for detecting early alterations well before visible signs of damage appear, enabling actions to mitigate stressor effects (Brosset et al., 2021; Lemos, 2021). To evaluate an organism's physiological status, biomarkers related to metabolic performance and energy reserves content are frequently employed (*e.g.,* Louis et al., 2020; Shang et al., 2021). Additionally, to gain insights at the cellular level, biomarkers informative of oxidative stress, including those associated with antioxidant and detoxification mechanisms, as well as those related to cellular damage and cellular redox status are encouraged (Kadim and Risjani, 2022; Lomartire et al., 2021; Regoli and Giuliano, 2014). Furthermore, the inhibition of acetylcholinesterase (AChE) as an indicator of potential neurotoxicity is commonly used (English and Webster, 2012). In fact, the combination of metabolism, oxidative stress and neurotoxicity-related biomarkers, have frequently been utilized in bivalves ecotoxicological studies (*e.g.*, Almeida et al., 2007; Bocchetti et al., 2008; Munari et al., 2018; Falfushynska et al., 2019; Rios-Fuster et al., 2022).

Metabolic Capacity and Energy Reserves

Cellular metabolism represents an interplay of numerous intricate biochemical reactions occurring within the cell, also known as metabolic pathways, which serve energy production to provide for the synthesis of vital biomolecules, and the maintenance of a homeostatic equilibrium among all biochemical constituents (Wilson and Matschinsky, 2021). This intricate network of metabolic processes plays a key role in an organism's survival and function, as well as in stress adaptation and tolerance (Sokolova et al., 2012).

In the context of cellular energy production, cells meet their energy needs by oxidizing energy reserves made of biomolecules such as lipids, proteins (PROT), and carbohydrates, including glycogen (GLY). Reduced equivalents are then transferred to coenzymes like nicotinamide adenine dinucleotide (NAD^+), flavin mononucleotide (FMN), or flavin adenine dinucleotide (FAD). These coenzymes facilitate the transfer of electrons through a series of coupled reduction-oxidation (redox) reactions within the inner mitochondrial membrane, collectively known as the electron transport system (ETS) (Bhagavan and Ha, 2015).

Marine bivalves may even thrive in environments where oxygen levels can be severely limited or even absent. Consequently, these organisms have evolved to employ anaerobic energy metabolism, equipping their mitochondria with the ability to endure anoxic conditions. Despite having both aerobic and anaerobic pathways, their enzymatic repertoire mirrors that of organisms specialized for aerobic respiration (Donaghy et al.,

2015). In both cases, the ETS comprises four enzyme complexes that facilitate electron transfer to oxygen, the ultimate electron acceptor (Figure 7). This electron flow creates an electrochemical gradient driving the synthesis of adenosine triphosphate (ATP) in a process referred to as oxidative phosphorylation. During oxidative phosphorylation, a portion of the free energy liberated during redox reactions is conserved in the terminal phosphoanhydride bond of ATP through the phosphorylation of adenosine diphosphate (ADP) to ATP (Bhagavan and Ha, 2015). This tightly coupled process is the primary mechanism for cellular ATP generation, the fundamental currency of cellular energy.

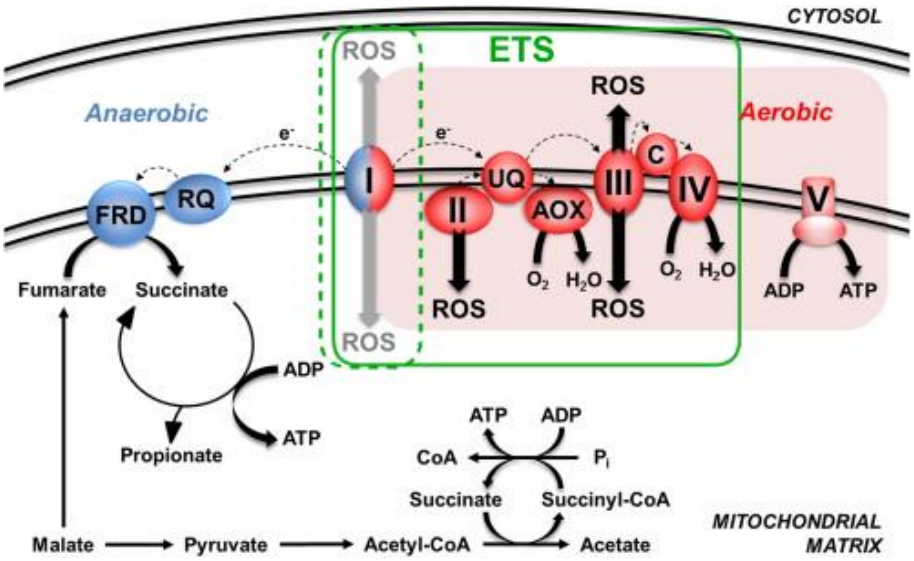

Figure 7. Anaerobically functioning mitochondria of marine bivalves and reactive oxygen species (ROS) production in the electron transport system (ETS) represented in green. The green dashed line represents the complex commonly analyzed following Packard (1974) methodology. The ETS and the ATP synthase complex employed during aerobic metabolism are marked in red and the membrane-bound enzymes preferentially employed during anaerobic metabolism are marked in blue. Known sites of ROS production are indicated by dark arrows with uncertain site of ROS production indicated by light grey arrows. Abbreviations: I to V, mitochondrial complexes I to V; AOX, alternative oxidase; C, cytochrome c; FRD, fumarate reductase; RQ, rhodoquinone; UQ, ubiquinone (retrieved from Donaghy et al., 2015).

It is well-established that environmental alterations can significantly impact an organism's energy metabolism, leading to the redirection of energy resources towards managing stress rather than fundamental functions such as growth, reproduction, and development (Sokolova et al., 2013). Thus, changes in ETS activity and fluctuations in energy reserves can serve as valuable indicators of shifts in cellular metabolism in various

organisms, including bivalves (*e.g.*, Bethke et al., 2023; Erk et al., 2011; Shang et al., 2023; Smolders et al., 2005). These indicators allow us to assess the overall health and physiological condition of organisms offering valuable insights into its adaptation and response to changing environmental conditions (*e.g.,* Louis et al., 2020; Shang et al., 2021).

Antioxidant and Biotransformation Capacity

Organisms naturally produce free radicals and reactive oxygen species (ROS) as byproducts of normal cellular metabolism involving oxygen. Common ROS include the superoxide anion ($O_2^{\bullet-}$), hydrogen peroxide (H_2O_2), hydroxyl radical (•OH), and singlet oxygen ($^1O_2$), as byproducts of normal cellular metabolism (Checa and Aran, 2020). The ETS is a well-known biological source of ROS, where leaked electrons from its complexes can react with molecular oxygen (O_2), leading to the production of $O_2^{\bullet-}$ and subsequently, H_2O_2 (Chenna et al., 2022; Nolfi-Donegan et al., 2020; Donaghy et al., 2015) (Figure 7). Additionally, excessive ROS can be generated because of abnormal reactions triggered by disease processes, metabolic dysregulation, or exposure to xenobiotics (Checa and Aran, 2020).

Exposure to stressful abiotic conditions can also lead to an overproduction of ROS, primarily affecting lipids, proteins and DNA, resulting in a state known as oxidative stress (Bal et al., 2021; Catalá, 2009; Regoli and Giuliani, 2014). Organisms exposed to oxidative stress may activate a cascade of defensive reactions to cope with ROS overproduction, protecting cells and tissues from severe pro-oxidative damage (Lushchak, 2011; Pisoschi et al., 2021; Regoli and Giuliani, 2014) (Figure 8). The first enzymatic defense mechanism to act is the antioxidant enzyme superoxide dismutase (SOD), which catalyzes the dismutation of $O_2^{\bullet-}$ into oxygen and H_2O_2 (Islam et al., 2021). However, H_2O_2 is also a potentially harmful byproduct and needs to be eliminated or degraded. To achieve this, the second antioxidant enzymatic mechanism, catalase (CAT), is activated to prevent further damage to cells and tissues by converting H_2O_2 into water (Andrés et al., 2022). A third mechanism involves the antioxidant enzyme glutathione peroxidase (GPx), which also neutralizes H_2O_2 utilizing hydrogen from two reduced glutathione (GSH) molecules, resulting in two water molecules and one oxidized glutathione (GSSG) (Pandey and Rizvi, 2010). Furthermore, GPx is involved in reducing lipid hydroperoxides (LOOHs), non-radical intermediates that pose a significant threat to cellular membranes (Lee et al. 2023; Regoli and Giuliani, 2014). Additionally, the enzyme glutathione reductase (GR) regenerates GSH from GSSG, utilizing nicotinamide adenine dinucleotide

phosphate (NADPH) as a hydrogen source (Pandey and Rizvi, 2010), ending up being a process more energy costly compared to CAT catalyzed reactions.

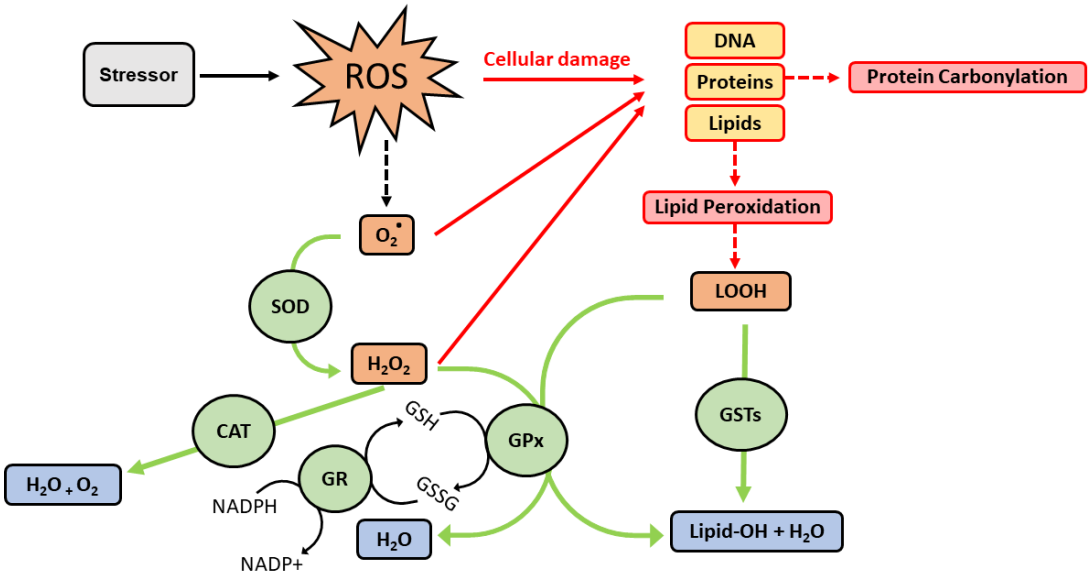

Figure 8. Schematic representation of the cascade of defensive reactions against reactive oxygen species (ROS) overproduction. Red arrows symbolize the possible cellular damage resulting from the action of ROS, and green arrows represent the ROS conversion/detoxification pathway. The colors of the boxes/circles are as follows: orange for key ROS species; green for enzymes involved in ROS defense; blue for final byproducts generated; and red for cellular damage resulting from ROS-mediated processes. Abbreviations: SOD, superoxide dismutase; CAT, catalase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, reduced glutathione; GSSG, oxidized glutathione; GSTs, glutathione *S*-transferases; LOOH, lipid hydroperoxide (created by Madalena Andrade).

When confronted with xenobiotics, organisms employ biotransformation enzymes as a crucial mechanism for detoxification and elimination (Regoli and Giuliani, 2014; Yan, 2014). This process involves two distinct phases. In Phase I, enzymes unveil or incorporate polar reactive groups (-OH, -COOH, -NH_2) into the xenobiotic molecules, catalyzing oxidation, reduction, and hydrolysis reactions. The products generated during Phase I reactions serve as substrates suitable for subsequent Phase II reactions. One important superfamily of Phase I biotransformation enzymes is the carboxylesterases (CbEs), known for their ability to hydrolyze ester- or amide-containing substrates, liberating free acids and alcohols (Cui and Li, 2018). Notably, CbEs play a pivotal role in the detoxification of organophosphorus, carbamate, and pyrethroid pesticides (Yan, 2014). Moreover, they possess the remarkable capability to irreversibly deactivate

metabolites of organophosphorus pesticides, making this non-catalytic interaction an efficient detoxification mechanism (Chambers et al., 2010). Nevertheless, the activity of these enzymes can be modulated by various factors, including the presence of metal ions (Shao et al., 2014; Stood et al., 2016).

In Phase II, a hydrophilic endogenous substrate conjugates with the xenobiotic, whether altered or unaltered, resulting in the formation of a water-soluble compound that can be readily excreted (Regoli and Giuliano, 2014; Testai, 2001). Similarly, glutathione *S*-transferases (GSTs), a superfamily of enzymes primarily found in the cytosol, belong to Phase II. Glutathione *S*-transferases catalyze the conjugation of foreign compounds or their metabolites, which possess electrophilic centers, with GSH. This process protects critical biomolecules, such as proteins and nucleic acids (Teslai, 2001). Some GSTs isoforms also contribute to the antioxidant defense system by reducing LOOHs to alcohols while oxidizing GSH to GSSG, similar to glutathione peroxidase (GPx) (Regoli and Giuliani, 2014). Both GSTs and CbEs activities have been demonstrated to be modulated by metal contamination (Dobritzsch et al., 2020; Hauser-Davis et al., 2021).

Oxidative Damage and Cellular Redox Status

It is known that if the enzymatic defenses are not efficient in eliminating excessive ROS production, cellular damage may take place such as the oxidation of lipids' membranes known as lipid peroxidation (LPO) (Hampel et al., 2016; Regoli and Giuliani, 2014) and proteins such as protein carbonylation (PC) (Cattaruzza and Hecker, 2008; Pisoschi et al., 2021). In particular, LPO is considered an irreparable event in a cell's life that can be divided into three stages: initiation, propagation, and termination (Ayala et al., 2014; Desai at al., 2005) (Figure 9). The initiation phase happens when a ROS steals a hydrogen atom from lipid molecules to yield free radicals of lipid, which are unstable molecules. A propagation phase starts when the now unstable lipid radicals react with O_2, creating lipid peroxyl radical, a highly unable species. This last removes a hydrogen from another lipid producing a LOOH and a new lipid radical which continues the cycle resulting in propagation (Desai et al., 2005; Regoli and Giuliani, 2014). As a consequence of this chain reaction, other substances such as conjugated dienes and malondialdehyde (MDA), are generated (Ayala et al., 2014; Finaud et al., 2012). The termination phase happens when antioxidants or other protective molecules step in to stop this chain reaction, neutralizing the highly reactive molecules and breaking the cycle (Desai et al., 2005; Regoli and Giuliani, 2014).

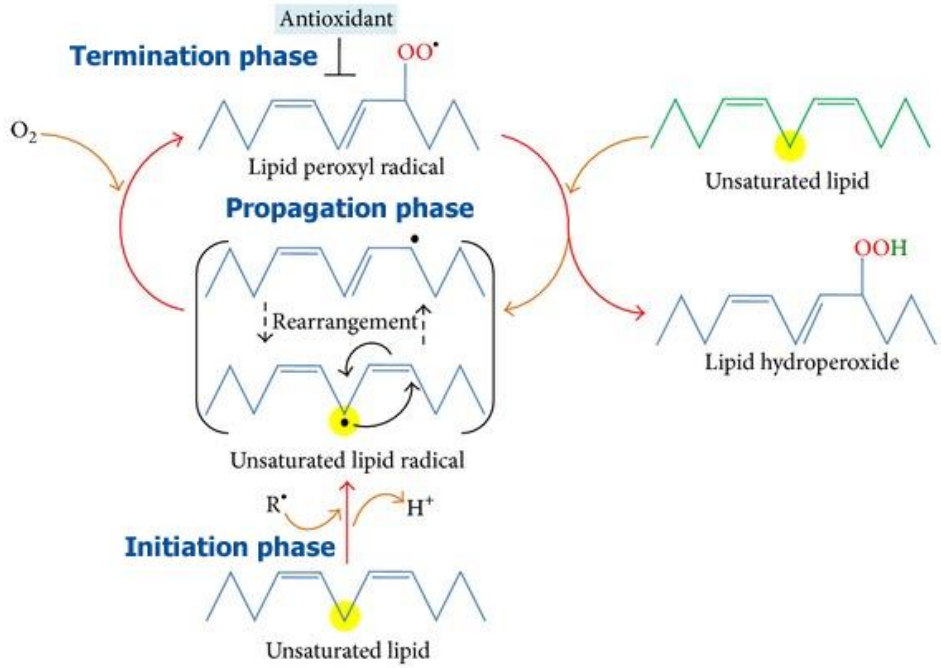

Figure 9. Lipid peroxidation mechanism. Initiation phase: reactive oxygen species initiate the process by removing a hydrogen atom, leading to the formation of a lipid radical that usually undergoes a molecular rearrangement. Propagation phase: lipid radical reacts rapidly with O_2, giving rise to a lipid peroxyl radical, which, in turn, abstracts a hydrogen from another lipid molecule, generating both a new lipid radical and lipid hydroperoxide. Termination phase: antioxidants donate a hydrogen atom to the lipid peroxyl radical species, resulting in the production of nonradical end products (retrieved and modified from Ayala et al., 2014).

The PC is a common oxidative modification of proteins induced by ROS (Cattaruzza and Hecker, 2008). It involves the addition of carbonyl groups to specific amino acid residues in proteins, which can impair their function and potentially lead to their degradation (Dalle-Done et al., 2006). Protein Carbonylation can be directly achieved through the oxidation of amino acid side chains with metals and H_2O_2, leading to the formation of semialdehyde amino acids. Notably, these reactions often target lysine, arginine, and proline residues. Alternatively, PC can result from an indirect mechanism involving the oxidation of lipids by •OH. During this process, polyunsaturated acyl chains of phospholipids or polyunsaturated fatty acids can undergo breakdown, giving rise to various lipid-derived aldehydes and ketones. These LPO products can diffuse across membranes and covalently modify proteins localized throughout the cell (Grimsrud et al., 2008).

An indicator of cellular redox status is often used in conjunction with biomarkers of cellular damage. One example is the balance between GSH and GSSG, known as the GSH:GSSG ratio. Glutathione, a tripeptide comprising cysteine, glycine, and glutamate, exists in two forms in the cell: the reduced form (GSH) and the oxidized form (GSSG). Reduced glutathione, which is the predominant form, act as a substrate for specific enzymes, such as GPx and GSTs, aiding in the ROS neutralization and preventing potential damage (Pandey and Rizvi, 2010; Regoli and Giuliani, 2014). As GSH gets oxidized to GSSG during this defensive mechanism, the GSH:GSSG ratio emerges as an accurate representation of the cell's oxidative state, underlining its importance in preserving redox homeostasis (Ishkaeva et al., 2022). A high GSH:GSSG ratio indicates that the cell possesses ample antioxidant capacity, with a predominance of GSH ready to counteract ROS. In contrast, a decreased GSH:GSSG ratio suggests a more oxidized state of the cell, pointing to the possibility that its antioxidant defenses might be compromised, thereby elevating the risk of oxidative stress (Franco et al., 2007; Rahman et al., 2006). This balance is crucial in regulating various cellular processes, including proliferation, differentiation, senescence, and programmed cell death (Ishkaeva et al., 2022).

Neurotoxicity

The enzyme acetylcholinesterase (AChE) plays a pivotal role in ensuring the proper transmission of nervous signals across cholinergic synapses and neuromuscular junctions (Beiras et al., 2018; Metzler, 2003). This enzyme possesses two distinct binding sites: one responsible for hydrolysis and another that accommodates the quaternary ammonium group of the neurotransmitter acetylcholine (ACh). Its primary function is to tightly regulate ACh levels by rapidly breaking it down into choline and acetate, inert constituents, through enzymatic hydrolysis. Acetylcholinesterase also features a peripheral binding site at the entrance to its active gorge, serving as the initial point of contact for substrates and acting as a binding site for certain inhibitors (Morrison, 2021). Notably, synthetic chemicals such as organophosphates and carbamates selectively bind to AChE, rendering it inactive (Colović et al., 2013; English and Webster, 2012). In organisms exposed to these chemicals, this effect can disrupt nerve signals, leading to sustained muscle contractions, paralysis, and, in severe cases, death (Beiras et al., 2018). AChE activity can also be inhibited by other chemicals, including metals, surfactants, and polycyclic aromatic hydrocarbons, making it adequate for detecting a broader range of neurotoxic contaminants (Beiras et al., 2018). Studies on marine

invertebrates have evidenced that metals and nanoparticles can modulate AChE activity (*e.g.*, Brown et al., 2004; Perić et al., 2017; Roma et al., 2020).

Approaches for Biomarker Integration and Analysis

The evaluation of multiple biomarkers associated with these previously mentioned mechanisms has proven valuable in assessing the impacts of both human-induced and natural factors (Lomartire et al., 2021). When evaluating the combined effects of exposure to chemical contaminants and other environmental stressors, it is recommended practice to employ a battery of biochemical parameters. Using a broad range of biomarkers in whole tissue samples, as opposed to specific organs, provides on an integrative response, is cost-effective, and informs on organism's overall health. However, challenges often arise, including data acquisition issues and the complexity of identifying patterns and relationships within the data. Moreover, when organisms face simultaneous exposure to multiple stressors, interpreting individual data can be overwhelming. In such cases, the use of multivariate statistical analysis methods becomes highly recommended (Beliaeff and Burgeot, 2002). To address these challenges, researchers have increasingly employed multivariate analysis techniques such as Principal Coordinates (PCO) analysis and biomarker response indexes like the Integrated Biomarker Response (IBR) index.

Principal Coordinates analysis is a multivariate statistical method whose its primary advantage lies in its ability to reduce the dimensionality of complex datasets. By doing so, it condenses the data into fewer dimensions, thus simplifying the visualization and interpretation of patterns without losing significant information. It's particularly helpful in cases where the dataset includes large number of biomarkers or where relationships between variables aren't immediately apparent (Wang, 2012). On the other hand, the IBR is an index specifically developed to provide a comprehensive representation of the health status of an organism or ecosystem based on the collective response of multiple biomarkers. The IBR index is derived by integrating data from different biomarkers, thereby offering a summarized, yet nuanced, indication of health or stress. Its advantage is in its holistic approach: instead of relying on individual biomarkers, which might provide a biased or limited view, the IBR encapsulates the broader picture, combining different measures into a singular value (Beliaeff and Burgeot, 2002).

While PCO assists in data interpretation by simplifying and showcasing patterns, IBR acts as a holistic measure, capturing the cumulative effects of stressors on an organism or ecosystem. Numerous studies have used PCO and/or the IBR model to

evaluate risks in bivalves (*e.g.*, Cao et al., 2022; Chahouri et al., 2022; Cunha et al., 2023; Khan et al., 2021), demonstrating their effectiveness.

1.5. Quantification of Rare-Earth Elements in Estuarine Matrices

Given the diverse roles and impacts of REEs in various environmental matrices, a thorough understanding of the analytical methods used to quantify their presence and effects becomes essential. Advanced analytical techniques are indispensable for the quantitative assessment of REEs concentrations, not only in intertidal environments but also within the tissues of marine organisms. An exemplary technique in this context is mass spectrometry, which identifies and quantifies chemical substances based on their mass-to-charge ratio. In this process, a sample is first ionized, converting its molecules or atoms into charged ions. These ions are then channeled into a mass analyzer, where they are separated based on their specific mass-to-charge ratios. The resulting spectrum of ions provides a unique pattern that can be used to determine the elemental or isotopic signature of the sample (Kaklamanos et al., 2020). Due to its sensitivity and precision, mass spectroscopy is widely employed in various fields, ranging from chemistry and biology to environmental science and medicine, offering insights into the molecular constitution of samples (Bakir et al., 2022; Torregrosa et al., 2021; Zhang et al., 2020).

Among various mass spectrometry techniques, inductively coupled plasma mass spectrometry (ICP-MS) stands out as a highly sensitive and precise method, enabling the detection of trace metals at extremely low concentrations. This technique plays a crucial role in environmental monitoring and ecotoxicology, providing valuable data on the bioaccumulation of metals and their potential effects on marine organisms and ecosystems. Such technique contributes to a comprehensive understanding of their bioavailability, toxicity, and the overall health of marine habitats.

In particular, ICP-MS represents a sophisticated analytical technique that necessitates a comprehensive understanding of its intricate components and operational principles to fully use its capabilities for elemental and isotopic analysis. At its core, the ICP-MS system integrates a high-temperature argon plasma source with a mass spectrometer, meticulously designed to achieve precise and accurate measurements at trace levels. Initially, the sample, often in a liquid state, is pumped with a peristaltic pump into a nebulizer, where is transformed into a fine aerosol with argon gas. The fine droplets of the aerosol are separated from the larger through a spray chamber. The fine aerosol is then transported via a sample injector into the plasma torch, consisting of a quartz tube with argon gas flowing through it, which is ionized by a radio-frequency coil to generate the plasma. The resultant argon plasma, reaching temperatures up to 10,000 K, effectively atomizes and ionizes the sample constituents. Precision in ICP-MS is partly

controlled by the careful management of gas flows, including the plasma gas, auxiliary gas, and nebulizer gas. The optimal adjustment of these gas flows is vital, as it influences the efficiency of sample introduction, ionization, and the overall stability of the plasma (Cazes, J., 2004; Thomas, 2008).

The instrument also features a series of cones, typically made of nickel or platinum, with the most common types being the sampler cone and the skimmer cone. The sampler cone acts as an interface between the plasma and the mass spectrometer, allowing ions to pass through a small orifice while maintaining the vacuum conditions necessary for mass spectrometry. Following this, the skimmer cone further focuses the ion beam, ensuring that only ions of interest reach the detector. Once the ions navigate through the cones, they enter the mass spectrometer, where they are separated based on their specific mass-to-charge ratios. A series of lenses and quadrupoles are employed to focus and filter the ions, ensuring that only the ions with the desired mass-to-charge ratio reach the detector. The detector then quantifies the ions, translating the ion counts into concentration values for the elements present in the sample (Cazes, J., 2004; Thomas, 2008).

The main advantage of ICP-MS is its ability to detect elements at very low concentrations, often down to ng/L. Furthermore, multiple elements can be quickly measured simultaneously in a single run. It also presents a wide linear dynamic range allowing for the quantification of both major and trace elements in a single analysis (ThermoElemental, 2001). However, the analysis of seawater samples presents some challenges. For instance, seawater possesses a complex matrix with high salt concentrations that can lead to matrix interferences suppressing or enhancing the signal of certain elements. Because this can affect the sensitivity and accuracy of measurements, a dilution is normally required, which can lead to concentrations that are too low of the elements to be detected (Søndergaard et al., 2015).

When dealing with solid or semi-solid samples, such as bivalves' tissues, a digestion process is employed to break down the sample matrix, converting the analyte into a form amenable to ICP-MS analysis. This ensures that the target elements are fully extracted and available for quantification (Barnes et al., 2014). A common method involves using a strong acid, such as nitric acid (HNO_3), in conjunction with hydrogen peroxide (H_2O_2), which acts as a powerful oxidizing agent (*e.g.*, Henriques et al., 2019; Pinto et al., 2019). To enhance efficiency, microwave-assisted digestion can be utilized, considering that microwave energy can heat the samples in strong acids within closed vessels, ensuring rapid and efficient digestion (Mello et al., 2014).

To ensure the quality in ICP-MS analysis several components are considered. Calibration curves are developed using standards that have known concentrations of the elements of interest, which are involved in determining the concentration of these elements in unknown samples. Typically, a set of at least five standards is employed, striving for a correlation coefficient that exceeds 0.995. Another essential component is the use of blanks (samples that contain all the reagents used in a specific analytical process but lack the sample matrix and the analyte) that helps identifying and quantifying any contamination that might be introduced during sample preparation or analysis. For the findings to be valid, the quantification of the blank should fall below the limit of quantification (LOQ). Certified reference materials are also crucial, offering samples with known concentrations of the elements of interest that can validate the accuracy of the analytical process (Barnes et al., 2014). In this particular case, the analytical procedure is validated using the difference between the measured value and the certified value of the certified reference material (expressed as a percentage) often referred to as the recovery. The commonly accepted recovery ranges fall between 80-120 %. Furthermore, employing both sample and standard duplicates is fundamental to determining the precision of the entire analytical procedure. A coefficient of variation that remains below 10 % is indicative of high precision.

1.6. Thesis Aims

The main aim of the present thesis was to provide novel information on the effects of four REEs (La, Gd, Y, Tb) at environmental relevant concentrations on economically and ecologically relevant marine sentinel species, the mussel *M. galloprovincialis*. The potentially toxicological effects were also evaluated in a context of climate change, particularly salinity shifts and temperature increase, as a more realistic scenario. While there is some information available regarding the responses to REEs and impacts of climate change-related factors on this species separately, there is limited knowledge regarding both factors together. Thus, to fill this gap of information and achieve this overarching goal, three specific research objectives have been defined:

1. To comprehensively investigate the oxidative pathways and mechanisms employed by intertidal and subtidal populations of *M. galloprovincialis* when exposed to air and prolonged submergence. This preliminary background research was motivated by the scarcity of comparative studies between intertidal and subtidal populations of the same species, despite the intertidal group being commonly employed in ecotoxicological assessments, typically under submerged conditions. We believe characterization these basal responses will set the grounds for better understanding the consequences of REEs and climate change responses. To achieve this partial goal, the biochemical changes, including energy metabolism and oxidative stress, induced after 28 days of submersion for intertidal mussels and tidal exposure for subtidal mussels were evaluated to better characterize the tolerance mechanisms to submersion and natural aerobic/anaerobic shifts of these two populations.
2. To evaluate the impact of Y and Tb in organisms, at a range of environmentally relevant concentrations. While former studies have already explored the effects of La and Gd on estuarine species, limited information exists regarding the impacts of others such as Y and Tb, especially on *M. galloprovincialis*. To expand this knowledge, biochemical markers related to energy metabolism, oxidative stress, and neural status were evaluated in organisms exposed separately to different concentrations of Y and Tb for 28 days.
3. To investigate how climate change predicted changes such as extreme salinities and increased temperature may influence the biochemical effects associated to elements such as La, Gd, Y, and Tb in *M. galloprovincialis*, at environmentally relevant concentrations. For this, energy metabolism, oxidative stress, and neuro-

related biomarkers were analyzed after 28 days of exposure to 10 µg/L of each REE under different salinities (20, 30, and 40) or temperatures (17 °C and 22 °C) in a realistic climate-change context.

1.7. List of Publications

The three research objectives defined in this thesis will be discussed in Chapters 2 to 5, organized as follows: materials and methods (Chapter 2), results (Chapter 3), discussion and conclusions (Chapter 4), and concluding remarks and future perspectives (Chapter 5). The content presented in these chapters is based on several international peer-reviewed articles, which are listed below.

Intertidal *versus* subtidal mussels' exposure:

- Andrade, M., Rivera-Ingraham, G., Soares, A.M.V.M., Rocha, R.J.M, Pereira, E., Solé, M., Freitas, R., 2021. How do life-history traits influence the fate of intertidal and subtidal *Mytilus galloprovincialis* in a changing climate? Environ. Res. 196, 110381. https://doi.org/10.1016/j.envres.2020.110381.

Single rare-earth element exposure:

- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Threats of Pollutants Derived from Electronic Waste to Marine Bivalves: The Case of the Rare-Earth Element Yttrium. Environ. Toxicol. Chem. 42(1), 166-177. https://doi.org/10.1002/etc.5508.
- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Assessing the impact of terbium on *Mytilus galloprovincialis*: Metabolic and oxidative stress responses. Chemosphere 337, 139299. https://doi.org/10.1016/j.chemosphere.2023.139299.

Rare-earth element exposure under a climate change scenario:

- 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. Sci. Total Environ. 794, 148512. https://doi.org/10.1016/j.scitotenv.2021.148512.
- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2022. Will climate changes enhance the impacts of e-waste in aquatic systems? Chemosphere 288, 132264. https://doi.org/10.1016/j.chemosphere.2021.132264.
- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2022. Do climate change related factors modify the response of *Mytilus galloprovincialis* to lanthanum?

The case of temperature rise. Chemosphere 307, 135577. https://doi.org/10.1016/j.chemosphere.2022.135577.

- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Gadolinium accumulation and its biochemical effects in *Mytilus galloprovincialis* mussels under a scenario of global warming. Environ. Sci. Pollut. Res. 30, 116120-116133. https://doi.org/10.1007/s11356-023-30439-2.
- Andrade, M., Pinto, J., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Yttrium effects on the Mediterranean mussel under a scenario of salinity shifts and increased temperature. Mar. Environ. Res. https://doi.org/10.1016/j.marenvres.2024.106365.
- Andrade, M., Pinto, J., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. How predicted climate changes will modulate the impacts induced by terbium in bivalves? Chemosphere. https://doi.org/10.1016/j.chemosphere.2024.141168.

CHAPTER 2. MATERIALS AND METHODS

2.1. Sampling Area

Situated in northwestern Portugal, the coastal region of Ria de Aveiro stands as a remarkable estuarine system within the country. This unique and shallow lagoon is characterized by a multitude of distinct features and ecosystems, creating an ideal habitat for a diverse array of species, such as salt marshes (Sousa et al., 2017a), *Zostera noltei* seagrass meadows (Azebedo et al., 2013; Sousa et al., 2017b), and dune systems (Lopes et al., 2007). Ria de Aveiro is renowned for its diverse intertidal zones, including mud flats, salt marshes, and a network of narrow channels (Dias et al., 2000; Dias and Picado, 2011). Covering an area of 66 to 83 km^2 between low and high spring tides, it extends over 45 km in length and 10 km in width, being intricately connected to the ocean through a single inlet (Dias and Lopes, 2006). This inlet, in turn, gives rise to four primary branches: Mira, S. Jacinto, Ílhavo, and Espinheiro channels (Picado et al., 2010) (Figure 10). Notably, among these channels, the Mira channel stands out as the least affected (Castro et al., 2006), being considered an uncontaminated site.

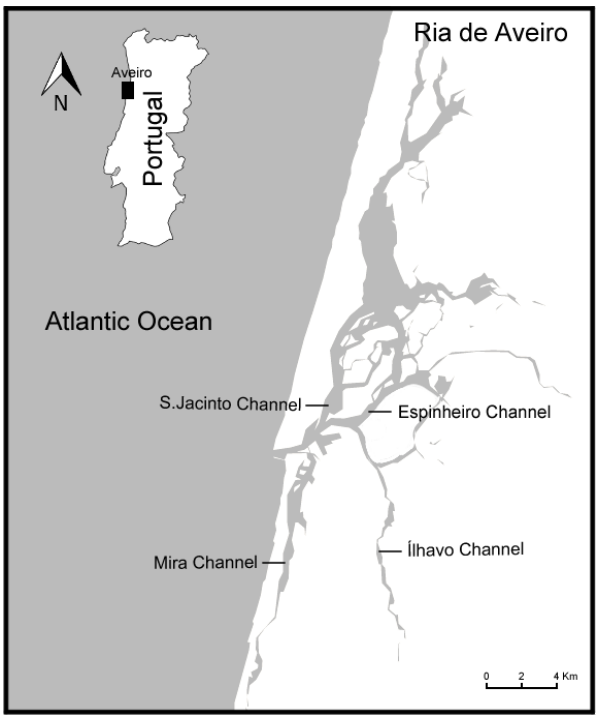

Figure 10. The Ria of Aveiro coastal lagoon in the northwest coast of Portugal (created by Madalena Andrade).

In the context of the present thesis, adult *Mytilus galloprovincialis* mussels of similar sizes (length: 59.1 ± 5.2 mm; width: 34.5 ± 3.2 mm) were collected from the Mira Channel (coordinates: 40° 38′ 31.7″N, 8° 44′ 10.9″W) during low tide.

2.1.1. Intertidal Versus Subtidal Mussels' Exposure

For the intertidal versus subtidal mussel's exposure, organism were collected during low tide from intertidal and subtidal zones of the same contiguous population and transported to the laboratory where they were allowed to acclimate to laboratory conditions and depurate for 7 days. During the acclimation period, aquaria were filled with artificial seawater (salinity 35 ± 1), adding artificial salt (Tropic Marin® SEA SALT, Wartenberg, Germany) to deionized water. Furthermore, organisms from the intertidal and subtidal areas were maintained separately at 18 ± 1.0 °C (control temperature) and pH 8.0 ± 0.1 (control pH) under continuous aeration during a natural photoperiod, resembling the original estuarine conditions where the animals were collected. For this, subtidal organisms were maintained submerged while intertidal organisms were exposed to tides induced by an automatic system mimicking the estuarine tidal regime of the habitat where the animals were collected (5 hours air exposure, followed by a submerged period of 7 hours) (Figure 11).

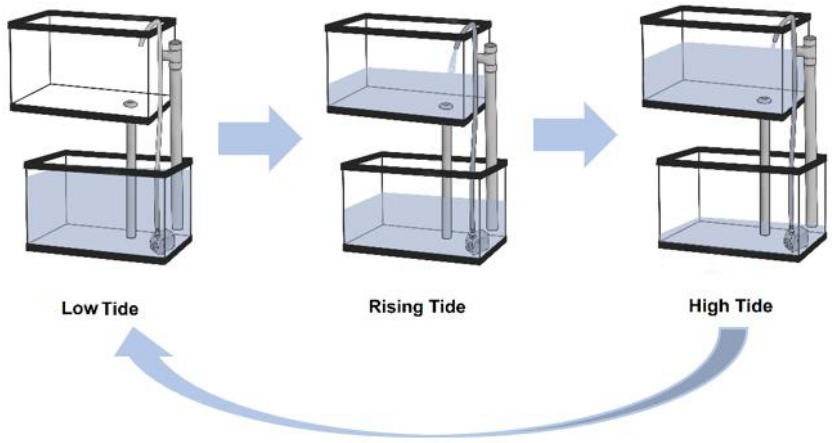

Figure 11. Schematic representation of the automated system designed for simulating the tidal cycle, involving 5 hours of air exposure to simulate low tide, followed by a 7-hour period mimicking high tide.

After the acclimation period, mussels were distributed into different aquaria (20 L seawater, salinity 35), with three individuals per aquarium and three aquaria per treatment (total of nine individuals per treatment). The treatments tested were: intertidal organisms maintained always under tidal exposure (IT); intertidal organisms maintained submersed (IS); subtidal organisms maintained always submersed (SS); subtidal organisms under tidal exposure (ST) (Figure 12). The tidal simulation was performed as previously described. The experimental period lasted 28 days, during which organisms were fed

three times per week with AlgaMac Protein Plus (Aquafauna BioMarine®, CA, USA) at a concentration of 150,000 cells/ animal per day. Seawater was renewed at the end of every week, with reestablishment of water conditions.

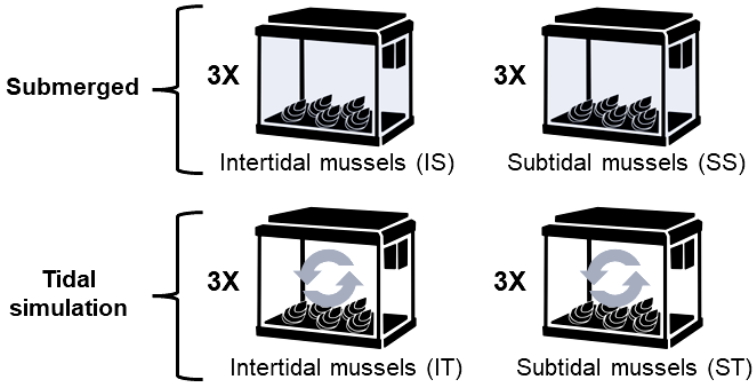

Figure 12. Experimental design for the intertidal *versus* subtidal *Mytilus galloprovincialis* exposure of 28 days. IS: Intertidal mussels always submerged; SS: Subtidal mussels always submerged; IT: Intertidal mussels under tidal exposure; ST: Subtidal mussels under tidal exposure.

At the end of the experimental period and during the first minutes after low tide, organisms were immediately frozen (without further manipulation to avoid any interference with oxidative stress parameters) by immersion in liquid nitrogen. Samples were maintained at -80 °C until further analysis. Prior to analysis, shells of the frozen organisms (three per aquarium, nine per treatment) were removed and the frozen whole soft tissues were manually homogenized under liquid nitrogen. For each organism, the resulting homogenate was carefully separated in 0.5 g fresh weight (FW) aliquots and stored at -80 °C for further biochemical analysis.

2.1.2. Single Rare-Earth Element Exposures

For the single rare-earth element exposures, mussels collected from the intertidal were brought to the laboratory for acclimation for 14 days in synthetic seawater, which was made with deionized water and Tropic Marin® SEA SALT. The mussels were kept under controlled conditions, including aeration, temperature (17.0 ± 1.0 °C), pH (8.0 ± 0.1), salinity (30 ± 1) and natural photoperiod. The water was changed twice in the first week and once in the second week. After five days of acclimation, the mussels were fed with 150,000 cells/mussel/day of AlgaMac Protein Plus (Aquafauna BioMarine®, CA, USA) every other day.

After the two-week acclimation period, mussels were separated into different aquaria with five mussels per aquarium and three aquaria per treatment. Two additional aquaria without mussels were used as positive controls for chemistry quality control. Each aquarium contained 3 L of artificial seawater. A 28-day experimental period was chosen, and ambient conditions were kept identical to those used during acclimation. Five different treatments were applied, each with different concentrations of either Yttrium (Y) or Terbium (Tb): 0 (control), 5, 10, 20, and 40 µg/L (Figure 13). Stock solutions were prepared by mixing commercial Y (1,000 mg/L from Inorganic Ventures) or Tb (1,000 mg/L from Inorganic Ventures) solutions with ultrapure water to achieve a final concentration of 10 mg/L.

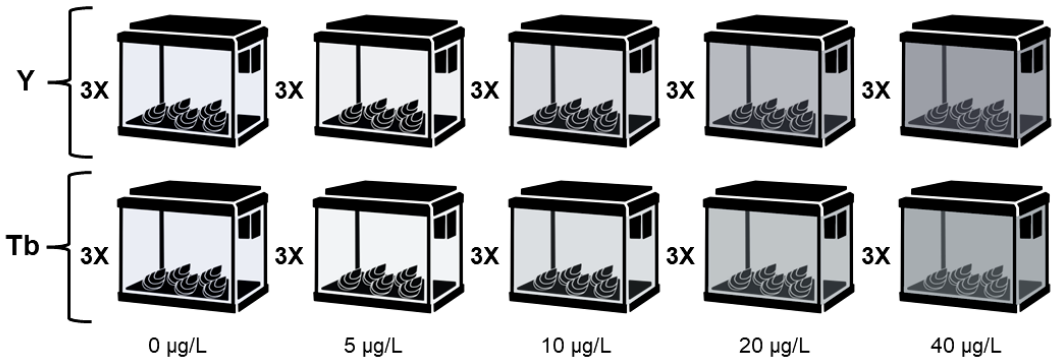

Figure 13. Experimental design for the single rare-earth element exposure of 28 days. *Mytilus galloprovincialis* mussels were exposed to five different concentrations (0, 5, 10, 20 and 40 µg/L) of yttrium (Y) and terbium (Tb).

The concentrations chosen for both elements were based on levels reported from pristine and contaminated aquatic systems (Åström, 2001; Bau et al., 1997; Gaillardet et al., 2003; Gomes et al., 2022; Olías et al., 2005; Reimann and Caritat, 1998; Sultan and

Shazili, 2009; Zhang and Nozaki, 1996); and previous research involving these contaminants (Hanana et al., 2018, 2021b; Lompré et al., 2021). As for the acclimation period, during the experiment, organisms were fed with a consistent amount of Algamac Protein Plus, three times a week. Aquaria seawater was changed once a week re-establishing desired Y and Tb concentrations and physicochemical parameters.

Seawater samples were taken immediately after spiking (introduction of the element in the medium) from all aquaria including positive controls during the first two weeks to assess the real aquaria rare-earth elements (REEs) concentrations. To confirm that the selected REEs concentrations were stable, additional samples were collected from the positive control aquaria right before renewing the water. At the end of the 28-day exposure period, organisms were collected, promptly frozen in liquid nitrogen and stored at -80 °C for analysis. For biochemical measures, three mussels per aquarium (nine per treatment) were chosen and one of them (three per treatment) was used for Y and Tb quantification. The entire soft tissue of each mussel was first homogenized using a pestle, mortar, and liquid nitrogen, and then divided into 5 aliquots of 0.5 g of FW and stored at -80 °C for biochemical determinations.

2.1.3. Rare-Earth Elements Exposure Under a Climate Change Scenario

For the rare-earth elements exposure under a climate change scenario, mussels were transported to air-acclimated rooms upon collection from the intertidal, where they underwent a two-week period of depuration and acclimation in synthetic seawater (prepared using deionized water and Tropic Marin® SEA SALT from Tropic Marine Center). Throughout the first week, the mussels were kept under conditions resembling those of the collection site, including stable temperature (17.0 ± 1.0 °C), salinity (30 ± 1), pH (8.0 ± 0.1), and natural photoperiod. During the second week, mussels were divided in groups and gradually acclimated to the experimental treatments, which involved different temperatures (17.0 ± 1.0 °C and 22.0 ± 1.0 °C) and salinity levels (20 ± 1, 30 ± 1 and 40 ± 1). To achieve this, a daily temperature increment of 1 °C and a daily salinity adjustment of 2 units were applied until the desired temperature (22.0 ± 1.0 °C) and salinity levels (20 ± 1 and 40 ± 1) were reached. Starting from the fifth day of this period, the mussels were fed with AlgaMac Protein Plus (Aquafauna BioMarine®, CA, USA) at a concentration of 150,000 cells per mussel per day. Seawater conditions were maintained by replacing it twice in the first week and once in the second week, with the exception of organisms acclimated to different salinities, which experienced gradual adjustments by renewing the seawater up to five times until reaching the desired conditions.

During the 28-day experimental period, fifteen mussels per treatment were distributed across three aquaria replicates with five organisms in each. Additionally, to assess the stability of lanthanum (La), gadolinium (Gd), Y and Tb over a one-week period under the various tested conditions, two positive controls per treatment were included, consisting of aquaria without mussels but subjected to the same conditions. The control conditions established during the initial two weeks acclimation were maintained throughout the experimental period. The exposure to each element, considering different salinities and temperatures, was evaluated in eight treatment groups: 1) uncontaminated mussels at 17 °C and salinity 20; 2) uncontaminated mussels at 17 °C and salinity 30 (control); 3) uncontaminated mussels at 17 °C and salinity 40; 4) uncontaminated mussels at 22 °C and salinity 30; 5) contaminated mussels at 17 °C and salinity 20; 6) contaminated mussels at 17 °C and salinity 30; 7) contaminated mussels at 17 °C and salinity 40 and 8) contaminated mussels at 22 °C and salinity 30 (Figure 14).

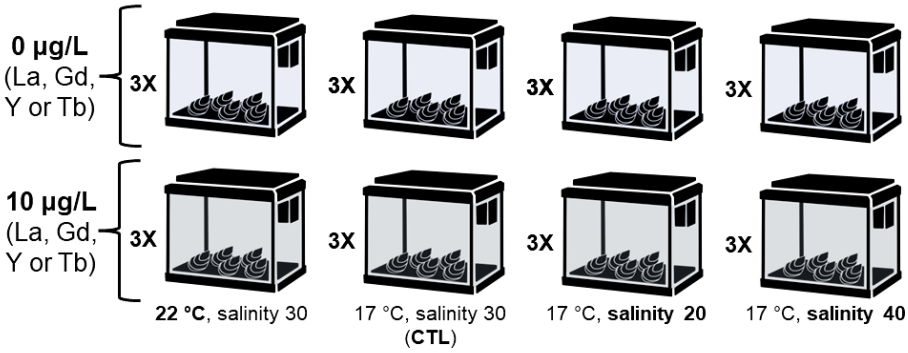

Figure 14. Experimental design for the rare-earth element exposure under a climate change scenario of 28 days. *Mytilus galloprovincialis* mussels were exposed to lanthanum (La), gadolinium (Gd), yttrium (Y) and terbium (Tb) concentrations (0 and 10 µg/L), each at different salinities (20, 30, and 40) and temperatures (17 °C and 22 °C).

A 10 µg/L concentration for REEs was selected based on prior studies involving La and Gd (Hanana et al., 2017a; 2017b; Moreira et al., 2020; Perrat et al., 2017), as well as the findings discussed in Chapter 4.2. for Y and Tb exposures in the same species, and actual environmental concentrations in aquatic settings (Åström, 2001; Gaillardet et al., 2003; Gomes et al., 2022; Olías et al., 2005; Reimann and Caritat, 1998; Rogowska et al., 2018; Sultan and Shazili, 2009). Initial stock solutions of La, Gd, Y, and Tb were prepared by diluting 1,000 mg/L commercial solutions (Inorganic Ventures) to 15 mg/L for La and Gd and 10 mg/L for Y and Tb using ultrapure water. These solutions were then added in predetermined volumes to each aquarium to achieve the desired concentrations. Throughout the experimental period, the mussels were fed with AlgaMac preparation, at a consistent concentration of 150,000 cells per mussel per day. Additionally, water renewal was conducted once a week to restore and maintain optimal conditions, including pH, REE concentration, and salinity.

Seawater collection took place from each exposure aquarium and positive control groups immediately after spiking of each REE into the medium, as well as right before water renewal in the positive control groups. Seawater samples were also collected from positive controls at 24, 48, 72, 144 and 168 hours after La and Tb spiking and at 0, 24, 48, 72, and 144 hours after Gd spiking. At the end of the experimental period, the organisms were collected and promptly frozen in liquid nitrogen, and storage at -80 °C. For the biochemical analysis, three frozen mussels per aquarium (nine per treatment) were homogenized using a mortar and pestle in liquid nitrogen. The homogenized tissue was divided into five aliquots of 0.5 g of FW each. From the three selected mussels per aquarium, the remaining tissue of one mussel per aquaria (3 per treatment) was used for

chemical analyses. In both cases, when not analyzed immediately, the samples were stored at -80 °C.

2.2. Rare-Earth Elements Quantification in Seawater and Mussels Soft Tissues

For REEs quantification, seawater samples for each element (La, Gd, Y and Tb) were diluted and acidified with HNO_3 2 % to pH < 2, and measured through ICP-MS, on a Thermo ICP-MS XSeries equipped with a 3-channel peristaltic pump, Peltier Nebulizing Camera, Burgener Nebulizer, and nickel cones.

Total REE concentration in the mussels' tissues was also determined by ICP-MS, after microwave assisted acid digestion. About 200 mg per freeze-dried samples were weighted in a Teflon vessel being added afterwards 1 mL of HNO_3 65 % (v/v), 2 mL of H_2O_2 and 1 mL of ultrapure water. Subsequently, the samples were processed using either a CEM MARS microwave, with a temperature increase up to 170-180 °C over 10-15 minutes and an additional 5 minutes at this temperature. After cooling, samples were transferred to polyethylene flasks which were filled with ultrapure water up to a final volume 25 mL and stored at room temperature until quantification.

Quality control measures were implemented during the analytical process of La, Gd, Y and Tb, including the use of digested blanks (microwave vessels without samples), duplicates, and the certified reference material BCR-668, which consisted of mussel tissue with known concentrations of these elements (La: 80 ± 6 µg/kg, Gd: 13.0 ± 0.6 µg/kg, Y: 59 ± 5 µg/kg, and Tb: 1.62 ± 0.12 µg/kg). Samples of each element for both seawater and tissues were analyzed separately, and minimum limit of quantification (LOQ), determined by the lowest standard concentration used in the calibration curve, was established at 0.01 µg/L for both seawater and tissue samples. Concentrations of all elements in digested blanks were found to be below the LOQ, indicating the absence of contamination. The validation of the digestion process and quantification protocols was accomplished by assessing recovery values from the analysis of the reference material, which was conducted in triplicate. The recovery values among all elements fell within the range of 90-119 %, validating the accuracy of the digestion process and quantification protocols.

The bioconcentration factor (BCF) of each REE in mussels was determined using the equation introduced by Arnot and Gobas (2006), which calculates the ratio between the total concentration quantified in the mussel tissues at the end of the exposure period and the exposure medium concentration. The concentrations measured after spiking were considered in the assumption of remaining relatively constant throughout the duration of the experiments.

2.3. Biochemical Parameters

To evaluate the biological impacts under each exposure's treatment, a set of biochemical parameters were adopted as biomarkers. These parameters encompassed measurements of: 1) Metabolic capacity and energy reserves, such as the electron transport system (ETS) activity, and glycogen (GLY) and total protein (PROT) contents; 2) Activities of antioxidant enzymes, such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), and glutathione reductase (GR); 3) Activities of detoxification defense enzymes, such as glutathione *S*-transferases (GSTs) and carboxylesterases (CbEs); 4) Indicators of cellular damage and cellular redox status, including lipid peroxidation (LPO) and protein carbonylation (PC) levels, as well as the balance between reduced (GSH):oxidized (GSSG) glutathione; 5) a biomarker indicative of neurotoxicity, such as acetylcholinesterase (AChE) activity. Each treatment involved the analysis of nine individual samples, corresponding to three mussels per aquarium and three aquaria per treatment. Sample analyses in duplicates were performed for each parameter. To obtain the analytical samples, tissue homogenization was carried in the adequate buffer for each assay at a ratio of 1:2 (w/v) by using a TissueLyzer II (Qiagen) set at frequency 20 1/s, during 90 seconds. For most of the parameters, including GLY, PROT, SOD, CAT, GPx, GR, GSTs, CbEs, and AChE, analytical samples were extracted using a buffer composed of 50 mmol/L potassium phosphate buffer containing 1 mmol/L ethylenediaminetetraacetic acid (EDTA), 1 % Triton X-100, and 1 mmol/L dithiothreitol at pH 7.0 and extracted after a centrifugation step at 4 °C for 20 minutes at 10,000 *g*. For ETS a buffer containing 100 mmol/L Tris-HCl, 15 % (w/v) polyvinylpyrrolidone, 0.153 mmol/L magnesium sulfate, and 0.2 % (v/v) Triton X-100 at pH 8.5 was used and centrifugation was also performed at 4 °C for 20 minutes but at 3,000 *g*. For LPO, a 20 % (w/v) solution of trichloroacetic acid (TCA) served as the extracting buffer and centrifugation was done at 4 °C for 20 minutes at 10,000 *g*. For the analysis of GSH and GSSG content, a buffer containing 100 mmol/L potassium phosphate buffer with 5 mmol/L EDTA, 0.6 % (w/v) sulfosalicylic acid and 0.1 % (v/v) Triton X-100 at pH 7.5 was used and centrifugation was completed at 4 °C for 10 minutes at 10,000 *g*. The respective supernatants, obtained after the centrifugation steps, were collected for subsequent analysis. If not immediately used, supernatants were stored at -80 °C until analysis.

The biochemical parameters were measured using a microplate reader (Synergy™HT, Biotek Instruments, Inc.), with readings classified as either punctual or kinetic. For punctual readings, the principle of the enzyme activity assay was predominantly utilized, which detects color changes from enzymatic reactions. An essential aspect of these assays is the use of standards and blanks. Standards, which are solutions with known concentrations of the substance being examined, are vital for creating a calibration curve. By plotting the absorbance values of these standards against their known concentrations, the concentration of the substance can be inferred in the test samples based on their respective absorbance readings. Blanks, which typically contain all reagents except the enzyme, are essential in ensuring that readings obtained are due solely to the enzyme's activity and not from other confounding factors. In kinetic readings, the activity is inferred by tracking the rate of absorbance change over a set period. Here, the importance is on the slope of the change, revealing the reaction's speed, rather than the absolute absorbance value. This rate can describe the quantity of substrate consumed or product generated *per* unit of time.

For some parameters where standards are unsuitable, or when the reading is more about activity than concentration, the extinction coefficient can be employed to deduce the compound's concentration or activity directly from its absorbance, guided by the Lambert-Beer Law. This law is mathematically expressed as $A=\varepsilon \times c \times l$, where A is the solution's absorbance, ε is the extinction coefficient of the analyzed substance, c is the solute's concentration in the solution, and l denotes the path length of the sample cell through which light travels. For certain protocols, the methodology can diverge slightly from the standard approach. For instance, in some assays designed to evaluate the influence of specific substances on enzyme activity, the difference in absorbance between a microplate exposed to the substance for a short duration and one not exposed is measured. These differential readings help elucidate the specific activity or effect of the substance on the parameter being assessed.

2.3.1. Metabolic Capacity and Energy Reserves

The ETS activity was measured using Packard (1974) methodology, with modifications implemented by De Coen and Janssen (1997). The principle behind this method is based on the reduction of a tetrazolium salt, specifically *p*-Iodonitrotetrazolium (INT), when exposed to the ETS in a given sample, leading to the formation of a colored compound, formazan. The intensity of the color change is directly proportional to the ETS activity. In this procedure, the sample was combined with buffered substrate solution (composed of Tris-HCl buffer with Triton X-100, pH 8.5) to permeabilize cellular membranes and provide access to the ETS. Additionally, NAD(P)H (comprising NADH and NADPH) was added as the primary electron donors for the ETS. The reaction started after INT was added, as in the presence of an active ETS, the INT accepts electrons and gets reduced. The final reaction product was read at an absorbance of 490 nm over a 10-minute period at 25-second intervals. The amount of formazan formed was calculated using an extinction coefficient (ε) of 15,900 $(mol/L)^{-1}$ cm^{-1} and results were expressed in nmol minute *per* g FW.

The GLY content was quantified by following the sulfuric acid method (Dubois et al., 1956). This method is based on the principle that, when carbohydrates (such as glycogen) are exposed to sulfuric acid, they undergo hydrolysis to release simpler sugars. These sugars, upon reacting with phenol under acidic conditions, form a colored compound that is quantifiable. For the assay, the sample and phenol were mixed. Subsequently, sulfuric acid was added to induce the hydrolysis. After a 30-minute incubation, the intensity of the color formed, which is proportional to the GLY content, was read at an absorbance of 492 nm. To relate the absorbance values to actual GLY concentrations, a calibration curve was plotted using standards of glucose (0–5 mg/mL), a monomer of glycogen. The results were reported as mg *per* g FW.

The PROT content was measured by following the Biuret method (Robinson and Hogden, 1940). The core principle of the Biuret test is that peptides and proteins, having peptide bonds, react with copper ions in an alkaline solution (Biuret reagent). This reaction yields a violet-colored complex, the intensity of which is directly proportional to the protein concentration in the sample. For the assay, the sample was mixed with the Biuret reagent. The mixture was then incubated for 10 minutes at 30 °C and the absorbance was subsequently measured at 540 nm. A calibration curve created using bovine serum albumin standards (0–40 mg/mL), a well-characterized protein, was considered. The results were expressed as mg *per* g FW.

2.3.2. Antioxidant and Biotransformation Capacity

The SOD activity was quantified using two distinct methods, each aiming to assess the capability of the enzyme to inhibit specific oxidative reactions. For the first method, derived from Beauchamp and Fridovich (1971), the sample was mixed with a reaction buffer containing Tris–HCl (pH 8.0) with diethylenetriaminepentaacetic acid (DTPA) to chelate metal ions that might interfere with the reaction, hypoxanthine as a superoxide generator, and NBT as the indicator molecule. To catalyze the reaction, converting hypoxanthine to xanthine and then to uric acid (which produces superoxide radicals in the process), xanthine oxidase was added. These radicals would typically reduce NBT, forming a colored compound. However, in the presence of SOD, this reduction is inhibited. After a 20-minute incubation with agitation, the extent of NBT reduction was measured at 560 nm. A calibration curve was considered through the preparation of SOD standards ranging from 0 to 60 U/mL. The SOD activity was expressed in units *per* g FW, where one unit of enzyme activity represents a reduction of 50 % of NBT.

For the second method by Magnani et al. (2000), the sample was mixed with Tris-EDTA buffer (Tris base with EDTA, pH 8.2) designed to maintain an optimal pH and chelate metal ions. The mixture's absorbance was read at 420 nm. The reaction was initiated by adding pyrogallol solution (in 0.01 mmol/L HCl). After waiting for 1 minute, the absorbance was read again at 420 nm. By comparing these two absorbance readings, the rate at which SOD inhibits pyrogallol autoxidation can be deduced, taking into account that the autoxidation of pyrogallol produces a colored compound. Results were also expressed in units *per* g FW, where one unit of enzyme activity represents the inhibition of 50 % of pyrogallol autoxidation.

The CAT activity was determined using the method described by Johansson and Borg (1988), a procedure based in the enzymatic breakdown of H_2O_2 by CAT to produce water and molecular oxygen. However, in this specific assay, the decomposition of H_2O_2 by CAT also leads to the formation of formaldehyde in the presence of methanol. For the assay, the sample was mixed with potassium phosphate buffer at pH 7.0 to ensure enzyme stability. To initiate the reaction, methanol and H_2O_2 were added and samples were incubated at room temperature for 20 minutes. After the reaction, potassium hydroxide was added to stop the reaction. Detection of formaldehyde was facilitated by introducing purpald, a chromogen agent that changes color upon reacting with formaldehyde. After agitating for 10 minutes, potassium periodate was added to enhance

the coloration. The CAT reaction was measured after an additional 5 minutes of agitation at 540 nm. To quantify the formaldehyde produced, a calibration curve was established using formaldehyde standards ranging from 0 to 150 µmol/L. The CAT activity was expressed as nmol *per* minute *per* g FW.

The GPx activity was determined using the method outlined by Paglia and Valentine (1967). This method is based on the enzymatic ability of GPx to reduce hydroperoxides, to their corresponding alcohols. This reduction is facilitated by the oxidation of GSH to GSSG. The formed GSSG is then reduced back to GSH with the help of GR and NADPH. During this, NADPH gets oxidized to NADP+, and this change in NADPH concentration serves as an indirect measure of GPx activity. For the assay, the sample was combined with a dilution buffer containing Tris-HCl at pH 7.6 with EDTA (to maintain an optimal pH and chelate metal ions). Subsequently, GSH and cumene hydroperoxide were added. To ensure the reduction of formed GSSG, GR was added. The reaction was initiated by adding NADPH, the cofactor essential for the conversion of GSSG back to GSH. The reaction was monitored at 340 nm (ε=6,220 $(mol/L)^{-1}$ cm^{-1}) over a 5-minute period at 15-second intervals. The results were expressed as µmol *per* minute *per* g FW.

The GR activity was determined according to the method described by Carlberg and Mannervik (1985). This method is based around the primary function of GR to catalyze the reduction of GSSG back to GSH using NADPH as an electron donor. As GR facilitates this process, the concentration of NADPH decreases due to its conversion to its oxidized form, NADP+, and this decline in NADPH serves as an indirect measure of GR activity. For the assay, the sample was mixed with buffer consisted of potassium phosphate buffer at pH 7.0 with EDTA (to maintain the required pH and chelate metal ions). The substrate for the enzyme, GSSG, was then added to the mixture. For volume adjustment and to maintain the desired reaction conditions, distilled water was incorporated. The reaction was initiated by adding NADPH prepared in Tris-HCl, serving as the electron donor for the reduction process. The reaction was monitored at 340 nm (ε=6,220 $(mol/L)^{-1}$ cm^{-1}) over a 5-minute period at 15-second intervals. The results were expressed as µmol *per* minute *per* g FW.

The GSTs activity was determined following the method originally outlined by Habig et al. (1974) with modifications by Carregosa et al. (2014). The principle behind this method centers on the role of GSTs in catalyzing the conjugation of GSH with electrophilic centers on a variety of substrates, including 1-chloro-2,4-dinitrobenzene (CDNB). When

GSH reacts with CDNB, a thioether compound is formed. The increase in the concentration of this thioether is indicative of the GST activity. In this assay, the sample was mixed with the reaction solution, consisting of potassium phosphate buffer at pH 6.5, containing CDNB and GSH. The kinetic reaction of the thioether formation was monitored at 340 nm over a 5-minute period at 10-second intervals. The amount of thioether formed was calculated using the extinction coefficient (Ɛ) of 9,600 $(mol/L)^{-1}$ cm^{-1}. The results were expressed in µmol *per* minute per g FW.

The CbEs activity was determined following the method as originally performed by Hosokawa and Satoh (2002) and subsequently adapted by Solé et al. (2018). The principle of this method revolves around the enzyme's capacity to hydrolyze ester substrates, releasing *p*-nitrophenol in the process. In this particular assay, two commercial colorimetric substrates, *p*-nitrophenyl acetate (*p*NPA) and *p*-nitrophenyl butyrate (*p*NPB), were chosen. For the assay, the sample was mixed with reaction buffer, consisting of potassium phosphate buffer at pH 7.0 with *p*NPA/*p*NPB. The release of *p*-nitrophenol produces a yellow color that intensifies with the progression of the reaction. The rate of *p*-nitrophenol formation, indicative of CbEs activity, was monitored at 405 nm over a 5-minute period at 25-second intervals. For activity quantification, the formation of *p*-nitrophenol was considered, utilizing an extinction coefficient (Ɛ) of 18,000 $(mol/L)^{-1}$ cm^{-1}. The results were expressed in µmol *per* minute *per* g FW.

2.3.3. Cellular Damage and Cellular Redox Status

The LPO levels were measured following the method originally described by Ohkawa et al. (1979) but with modifications by Carregosa et al. (2014). This method measures the amount of malondialdehyde (MDA), a product of lipid peroxidation, by reacting it with thiobarbituric acid (TBA) under high temperature to produce a pink-red chromogen, which is quantified spectrophotometrically. For this assay, the samples were prepared in microtubes. To this, TCA was added, which aids in protein precipitation and enhances the visibility of the MDA-TBA complex. Additionally, TBA solution (0.5 % thiobarbituric acid in 20 % (v/v) TCA) was incorporated. The mixtures were then incubated at 96 °C for 25 minutes and the reaction was halted by transferring the samples to ice. Subsequently, each sample was transferred to a microplate, and the absorbance was measured at 535 nm. The concentration of MDA in the sample was deduced using an extinction coefficient (ε) of 156,000 $(mol/L)^{-1}$ cm^{-1}. Results expressed in nmol of MDA equivalents formed *per* g FW.

The PC levels were quantified using the method outlined by Mesquita et al. (2014). The principle of this method revolves around the chemical reaction between protein carbonyl groups in oxidized proteins and 2,4-dinitrophenylhydrazine (DNPH). This reaction results in the formation of a stable dinitrophenyl hydrazone product, which is distinctively quantifiable through its spectrophotometric absorbance. For this assay, the sample was mixed with DNPH prepared in HCl creating an acidic environment conducive for the derivatization of protein carbonyls to dinitrophenyl hydrazones. After incubating for 10 minutes at room temperature, sodium hydroxide was added forming an alkaline environment that aids in the stabilization and color enhancement of the dinitrophenyl hydrazone product. After a 10-minute incubation the final absorbance was measured at 450 nm. Utilizing the specific extinction coefficient (ε) of 22,308 $(mol/L)^{-1}$ cm^{-1} for the hydrazone derivatives, the concentration of protein carbonyls in the sample was calculated. The results were expressed in µmol of protein carbonyls groups formed *per* g FW.

The GSH and GSSG contents were measured according to the method described by Rahman et al. (2006). This method is based on the ability of Ellman's reagent (DTNB) to react with thiol groups present in GSH to produce a yellow-colored compound, which can be spectrophotometrically detected. For the determination of GSH, the sample was combined with a buffer containing potassium phosphate buffer (adjusted to pH 7.5) with EDTA, ensuring optimal pH and the chelation of metal ions which could interfere with the reaction. Following buffer addition, DTNB was incorporated, facilitating the reaction between DTNB and thiol groups in GSH. For the GSSG assay, the samples were pipetted into microtubes. To derivatize and protect free thiol groups from reacting with DTNB, 2-vinylpyridine was added, and samples were thoroughly mixed. After a 1-hour incubation period, triethanolamine was added to neutralize excess 2-vinylpyridine. Samples were vortexed once more, and after 10 minutes, they were transferred to a microplate. For the reaction, buffer (the same as used for GSH) and a mixture containing GR and DTNB were incorporated. To catalyze the conversion of GSSG to GSH in the presence of GR, NADPH was added after 30 seconds. Both final reactions of GSH and GSSG levels were measured at 412 nm. Standards of GSSG and GSH were employed within a range of 0–90 µmol/L to generate a calibration curve. The GSH:GSSG ratio was determined by dividing the GSH content by two times the GSSG content, and results were expressed in µmol *per* g FW.

2.3.4. Neurotoxicity

The activity of AChE was determined following the methodology originally described by Ellman et al. (1961) with modifications by Mennillo et al. (2017). This method is based on the hydrolysis of acetylthiocholine iodide (ATChI) by AChE to produce thiocholine. Thiocholine then reacts with DTNB to generate a yellow-colored product, 5-thio-2-nitrobenzoate, which can be spectrophotometrically measured due to its distinct absorbance. For the assay, the sample was mixed with K_2HPO_4 at pH 8.0. This was followed by the addition of DTNB, and ATChI serving as the substrate for AChE. The kinetic reaction was monitored at 412 nm over a 5-minute period at 25-second intervals. For quantification, an extinction coefficient (Ɛ) 13,600 $(mol/L)^{-1}$ cm^{-1} was used representing the molar absorptivity of the 5-thio-2-nitrobenzoate product. The results were expressed in nmol per minute *per* g FW.

2.4. Statistical and Multivariate Analyses

2.4.1. Hypothesis Testing Using PERMANOVA

To conduct hypothesis testing on the REEs concentration, BCFs, and the biochemical data related to energy metabolism, oxidative status, and neurotoxicity obtained from each exposure assay, a non-parametric permutational analysis of variance was carried out using PRIMER v6 software with the PERMANOVA add-on (Anderson et al., 2008). The data underwent a square root transformation, and a resemblance matrix was constructed using the Euclidean distance, considering its effectiveness in representing dissimilarity in multidimensional space. The created data matrix was evaluated using type III sums of squares and unrestricted permutation of raw data (9,999 permutations). Significance testing for the main tests in the PERMANOVA analysis was carried out by examining the pseudo-*F* values. In cases where the main test indicated statistically significant differences ($p < 0.05$), subsequent pairwise comparisons were performed. The *t*-statistic was used to assess the pairwise comparisons, and results with *p*-values less than 0.05 were deemed to be statistically significant. The specific null hypotheses tested were as follows:

1. For the comparison of intertidal *versus* subtidal mussels' exposure, the null hypothesis tested was: "no significant differences exist among treatments (IT, IS, SS, ST)." Statistically significant differences were represented by different letters in the figures.
2. For the single rare-earth element exposure, the null hypothesis for each element (Y and Tb) tested was: "mussels exposed to different concentrations have similar biochemical responses". Significant differences were indicated by different letters in the figures.
3. For the rare-earth elements exposure under a climate change scenario, three distinct hypotheses for each element (La, Gd, Y, Tb) were tested: "in uncontaminated mussels, there were no significant differences among different salinities (30, 20, 40, at temperature 17 °C) or temperatures (17 and 22 °C, at salinity 30)"; "in contaminated mussels, there were no significant differences among different salinities (30, 20, 40, at temperature 17 °C) or temperatures (17 and 22 °C, at salinity 30)"; and "there were no significant differences between uncontaminated and contaminated mussels at each salinity and temperature tested". In the figures, significant differences among different salinities in uncontaminated mussels were labeled with different lowercase letters, whereas in

contaminated mussels were indicated using uppercase letters. Moreover, the symbol # was utilized to represent the differences among different temperatures between uncontaminated and contaminated organisms, while an asterisk was used to denote the differences between uncontaminated and contaminated mussels at each salinity or temperature.

4. In the specific case of La and Gd under a climate change scenario, the hypothesis "there were no significant differences between contaminated mussels at 22 °C and uncontaminated ones at control temperature of 17 °C" was also tested and *p*-values were represented in a table.

2.4.2. Principal Coordinates Analysis

When considered appropriate, ordination analysis using Principal Coordinates (PCO) was conducted using the obtained biochemical data from the exposures. In cases where not all data was included (*e.g.*, for temperature and salinity variations), relevant subsets of the data were utilized. For this, the Euclidean distance similarity matrix was simplified by using the different treatments to calculate the distance between centroids. Vectors relating to biochemical descriptors were placed as new variables superimposed on the PCO graph using the Spearman correlation with a correlation > 75 %, considering its robustness to outliers and non-normally distributed data often included in biochemical data.

2.4.3. Integrated Biomarker Response Index

When deemed relevant, the Integrated Biomarker Response version 2 (IBRvs2) index, proposed by Beliaeff and Burgeot (2002) and later modified by Sanchez et al. (2013), was adopted as a tool to consolidate data obtained from various biochemical parameters. The integration process involved comparing the responses of biochemical parameters within treatment groups to those in the control group.

To calculate the IBRvs2 index, a log transformation (Y_i) was initially applied to minimize variance, where $Y_i = \log (X_i / X_0)$. Here, X_i represented individual biomarker data from each treatment, and X_0 denoted the mean reference data from the control treatment. Subsequently, Y_i values were standardized using $Z_i = (Y_i - \mu) / \sigma$, considering the general mean (μ) and standard deviation (σ) of Y_i. The biomarker deviation index ($A = Z_i - Z_0$) was then calculated, establishing a baseline centered on 0 to visualize variations in

parameters relative to this baseline. Finally, IBRvs2 was derived as IBRvs2 = Σ |*A*|, providing a quantified representation of the integrated biomarker response.

For most cases, the biomarker deviation index results were visualized using a star plot, where the region outside of the REF line (representing the control treatment, used as reference data) indicated biomarker induction, while the area inside the REF line signified biomarker inhibition. The overall response provided by the final IBRvs2 model was discussed in terms of these values, with higher IBRvs2 scores reflecting a greater degree of biochemical responsiveness in mussels.

CHAPTER 3. RESULTS

3.1. Intertidal *Versus* Subtidal Mussels' Exposure

The present subchapter presents the findings on the metabolic capacity, energy reserves, antioxidant capacity, oxidative damage, and cellular redox status of mussels from intertidal and subtidal environments. It details the observed differences in response to environmental conditions between these two groups. The data provide a comparative view of the physiological state of *Mytilus galloprovincialis* in differing habitats, contributing to our understanding of their adaptive responses.

The results presented in this section are published in:

- Andrade, M., Rivera-Ingraham, G., Soares, A.M.V.M., Rocha, R.J.M, Pereira, E., Solé, M., Freitas, R., 2021. How do life-history traits influence the fate of intertidal and subtidal *Mytilus galloprovincialis* in a changing climate? Environ. Res. 196, 110381. https://doi.org/10.1016/j.envres.2020.110381.

Metabolic Capacity and Energy Reserves

The electron transport system (ETS) activity in intertidal organisms did not differ between those maintained under the periodic influence of tides (IT) and those transferred to submerged conditions (IS). For subtidal organisms, however, those exposed to tidal influence (ST) significantly increased their ETS activity compared to their controls (SS) and both intertidal groups (IT and IS) (Figure 15A).

The glycogen (GLY) content in intertidal organisms exposed to tides (IT) was significantly lower than in their submersed conspecifics (IS). For submersed subtidal organisms (SS) this content was higher than in those exposed to tides (ST) (Figure 15B). Submerged subtidal organisms (SS) showed also significantly higher GLY content when compared with organisms from the intertidal (IT), while organisms from the subtidal exposed to tides (ST) had significantly lower GLY content when compared to submersed intertidal organisms (IS) (Figure 15B).

The protein (PROT) content in intertidal organisms exposed to tides (IT) was significantly higher than those maintained submersed (IS). Subtidal organisms maintained submersed (SS) also displayed higher PROT content than the two intertidal groups (IT and IS) (Figure 15C).

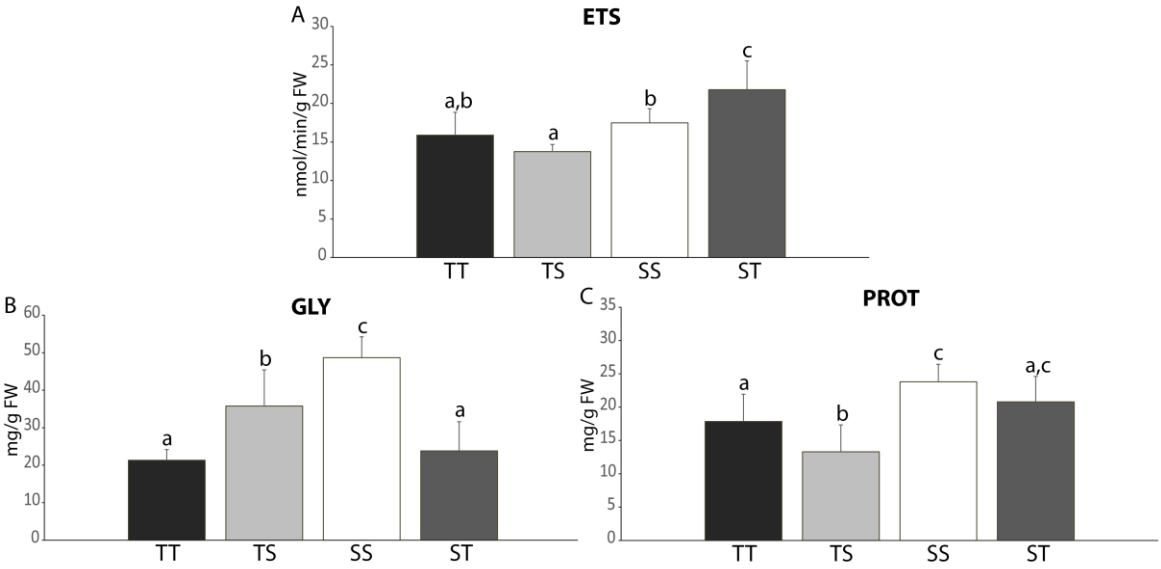

Figure 15. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Protein (PROT) content, in intertidal (IT) and subtidal (SS) *Mytilus galloprovincialis* exposed to different treatments (submergence, IS and tidal exposure, ST) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among treatments.

Antioxidant Capacity

The superoxide dismutase (SOD) activity in intertidal organisms exposed to tides (IT) was significantly lower than their submersed conspecifics (IS), the latter being the one displaying the highest values. Also, subtidal organisms maintained submerged (SS) had significantly lower activities than those kept under tidal exposure (ST) (Figure 16A).

The catalase (CAT) activity in the intertidal organisms groups (IT and IS) was similar despite them being under different treatments. Subtidal organisms under submersion (SS) showed significantly lower CAT activities than the group under tidal exposure (ST) being this mussel group the one to show the highest CAT activity values (Figure 17B). Submerged intertidal organisms (IS) presented significantly lower CAT activity compared with subtidal organisms exposed to tides (ST) (Figure 16B).

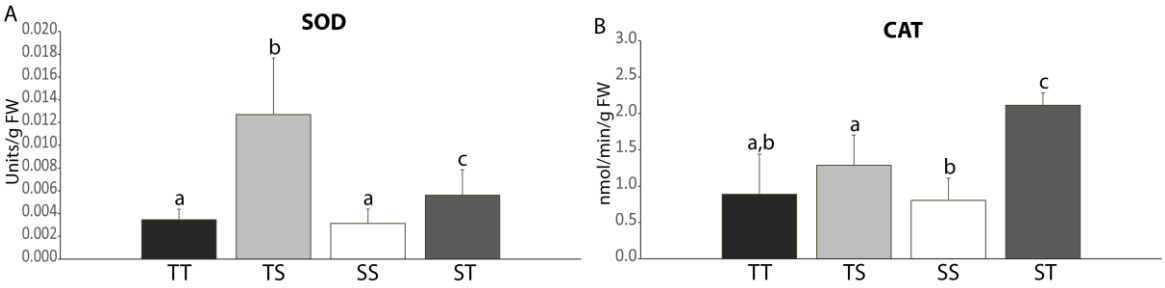

Figure 16. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity, in intertidal (IT) and subtidal (SS) *Mytilus galloprovincialis* exposed to different treatments (submergence, IS and tidal exposure, ST) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among treatments.

Oxidative Damage and Cellular Redox Status

The lipid peroxidation (LPO) levels in submerged subtidal organisms (SS) were significantly higher than in subtidal mussels maintained under tidal exposure (ST) and those from intertidal origin (IT and IS) (Figure 17A).

The protein carbonylation (PC) levels did not change significantly among any of the treatments (Figure 17B).

The reduced (GSH):oxidized (GSSG) glutathione ratio in intertidal organisms exposed to tides (IT) displayed significant lower values compared to the submersed group (IS), while submersed subtidal organisms (SS) expressed an elevated ratio in respect to their tide-exposed conspecifics (ST) (Figure 17C). Submerged subtidal organisms (SS) had significantly higher ratios than the intertidal mussels maintained under tides (IT), while subtidal organisms exposed to tides (ST) had significantly lower ratios than submersed intertidal organisms (IS) (Figure 17C). In general, organisms exposed to the tidal regime (IT, ST) showed lower GSH:GSSG values than organisms maintained always submersed (IS, SS) (Figure 17C).

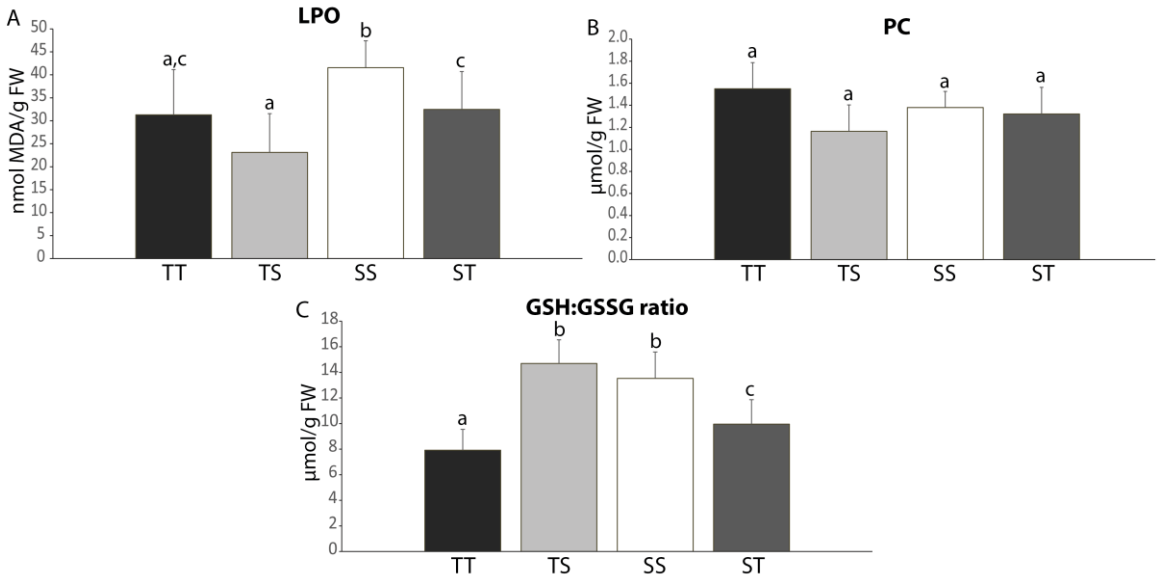

Figure 17. A: Lipid peroxidation (LPO) levels; B: Protein carbonylation (PC) levels; C: Reduced (GSH) and oxidized (GSSG) glutathione ratio (GSH:GSSG ratio), in intertidal (IT) and subtidal (SS) *Mytilus galloprovincialis* exposed to different treatments (submergence, IS and tidal exposure, ST) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among treatments.

3.2. Single Rare-Earth Element Exposures

Building on the understanding of metabolic and physiological responses in different tidal environments, the present subchapter presents the findings from the exposure of intertidal mussels to specific rare-earth elements (REEs), namely yttrium (Y) and terbium (Tb). The section provides data on how these elements impact the physiology of intertidal mussels.

3.2.1. Yttrium Exposure

This section presents the results related to the concentration of Y in mussel tissues, the bioconcentration factor, and various biomarkers. These biomarkers include those related to metabolic capacity, energy reserves, antioxidant and biotransformation capacity, oxidative damage, and neurotoxicity following exposure to Y. Additionally, results from the integrated biomarker response index are also detailed.

The results presented in this section corresponding to Y exposure are published in:

- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Threats of Pollutants Derived from Electronic Waste to Marine Bivalves: The Case of the Rare-Earth Element Yttrium. Environ. Toxicol. Chem. 42(1), 166-177. https://doi.org/10.1002/etc.5508.

Yttrium Concentration in Mussels' Tissue and Bioconcentration Factor

The concentration of Y in seawater samples from the control (0 µg/L) treatment was consistently below the limit of quantification (LOQ). Concentrations in the water right after spiking were very close to the desired nominal concentrations (shown in brackets) in each treatment: 6.80 ± 0.65 µg/L (5 µg/L), 10.28 ± 0.85 µg/L (10 µg/L), 20.53 ± 1.83 µg/L (20 µg/L), and 43.14 ± 5.27 µg/L (40 µg/L).

The levels of Y in the seawater measured from positive controls right after spiking and right before water renewal showed values varying between 6.45 ± 0.64 and 5.25 ± 0.41 µg/L (5 µg/L), 10.10 ± 0.26 and 10.20 ± 1.56 µg/L (10 µg/L), 21.30 ± 1.4 and 20.50 ± 1.92 µg/L (20 µg/L), and 46.67 ± 4.62 and 41.50 ± 3.42 µg/L (40 µg/L). Thus, the Y presence in the water, with coefficients of variation below 20 %, was considered stable for at least one week under the actual experimental conditions.

The Y content in mussels' tissue (in dry weight) confirmed an increasing accumulation trend with exposure gradient, as follows: 0.07 ± 0.01 µg/g for 0 µg/L, 0.21 ± 0.06 µg/g for 5 µg/L, 0.32 ± 0.07 µg/g for 10 µg/L, 0.40 ± 0.11 µg/g for 20 µg/L and 0.64 ± 0.03 µg/g for 40 µg/L. Yttrium concentrations were significantly lower in control mussels (0 µg/L) but significantly higher in those exposed to 40 µg/L in relation to the intermediate treatments. Of all treatments, only mussels exposed to 10 µg/L showed no significant differences from mussels exposed to 5 and 20 µg/L (Table 1). The bioconcentration factor (BCF) showed a decreasing trend with increasing Y water concentrations. However, no significant differences were reached among BCF values obtained at the lowest (5 µg/L) and the intermediate (10 and 20 µg/L) Y concentrations. Furthermore, BCF values at the highest dosage (40 µg/L) did not show any significant difference from those at 20 µg/L (Table 1).

Table 1. Yttrium (Y) concentration (µg/g dry weight) in mussels' soft tissues and bioconcentration factor (L/Kg, BCF) after 28 days of exposure to spiked seawater. Values are means with standard deviations (±) of (n = 3) measures. Different lowercased letters for Y in mussels' tissue or uppercased letters for BCF represent significant differences among tested concentrations in the respective columns.

[Y] (µg/L)	Mussel tissues (µg/g DW)	BCF (L/Kg)
0	0.07 ± 0.01^{a}	-
5	0.21 ± 0.06^{b}	$37 \pm 12^{A,B}$
10	$0.32 \pm 0.07^{b,c}$	33 ± 7^{A}
20	0.40 ± 0.11^{c}	$20 \pm 4^{B,C}$
40	0.64 ± 0.03^{d}	15 ± 1^{C}

Metabolic Capacity and Energy Reserves

The ETS activity was significantly lower at the lowest Y exposure (5 µg/L), with no significant differences among the remaining treatments (Figure 18A).

The GLY content was significantly lower at the 5, 10, and 20 µg/L treatments, with the lowest values observed at 5 µg/L (Figure 18B).

The PROT content did not change significantly among all the treatments (Figure 18C).

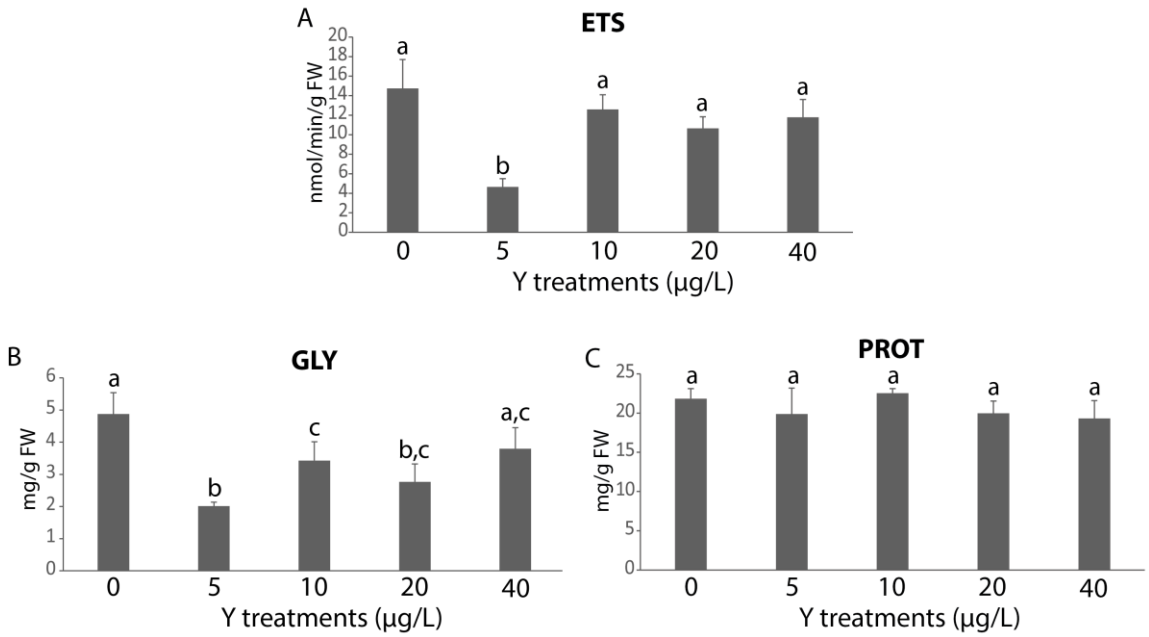

Figure 18. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Protein (PROT) content, in *Mytilus galloprovincialis* exposed to yttrium at different concentrations (0, 5, 10, 20 and 40 µg/L) after 28 days. Results are means with standard deviations and significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters.

Antioxidant and Biotransformation Capacity

The SOD activity was significantly higher at the 5 and 10 µg/L treatments, with no significant differences between 0 µg/L (control) and the other concentrations (20 and 40 µg/L; Figure 19A).

The CAT activity was significantly lower at the 5 and 10 µg/L treatments, in contrast to the other treatments (20 and 40 µg/L; Figure 19B), albeit they did not differ significantly from the control.

The glutathione reductase (GR) activity was significantly lower at the 5, 10, and 20 µg/L treatments, with no significant differences between the control and the highest exposure (40 µg/L; Figure 19C).

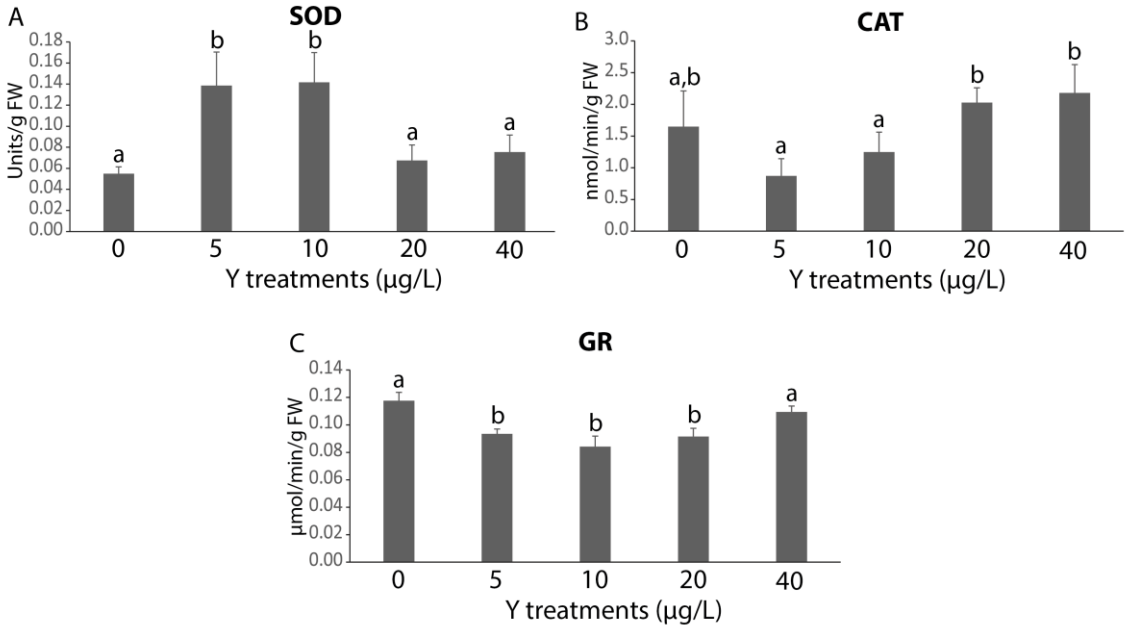

Figure 19. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione reductase (GR) activity, in *Mytilus galloprovincialis* exposed to yttrium at different concentrations (0, 5, 10, 20 and 40 µg/L) after 28 days. Results are means with standard deviations and significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters.

The glutathione *S*-transferases (GSTs) activity was significantly lower in the 5 and 40 µg/L treatments, in contrast to the remaining ones (0, 10, and 20 µg/L), with the lowest value seen at 5 µg/L (Figure 20A).

The carboxylesterases (CbEs) activity was significantly lower at the 5 and 10 µg/L treatments and higher at 40 µg/L with respect to the control (Figure 20B).

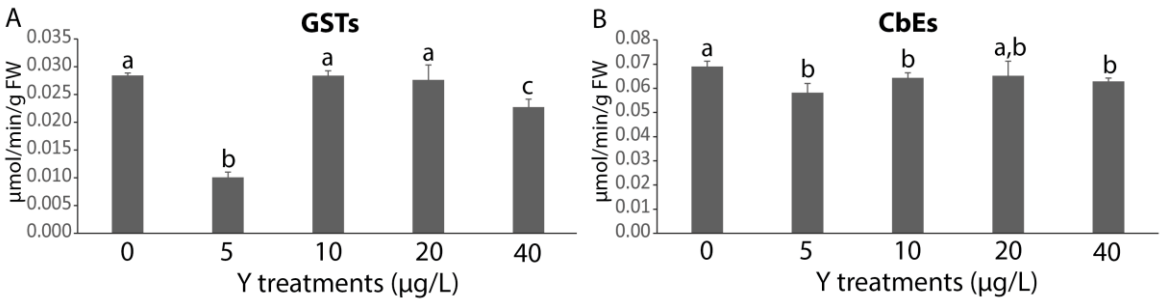

Figure 20. A: Glutathione *S*-transferases (GSTs) activity; B: Carboxylesterases (CbEs) activity, in *Mytilus galloprovincialis* exposed to yttrium at different concentrations (0, 5, 10, 20 and 40 µg/L) after 28 days. Results are means with standard deviations and significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters.

Oxidative Damage

The LPO levels were significantly lower in the 5 and 20 µg/L treatments with respect to the 10 µg/L treatment, with no significant differences between these two intermediate treatments (5 and 20 µg/L) and mussels exposed to 40 µg/L or the control (Figure 21A).

The PC levels were significantly higher at the lowest Y concentration (5 µg/L), in contrast to the remaining treatments (Figure 21B) but not the control.

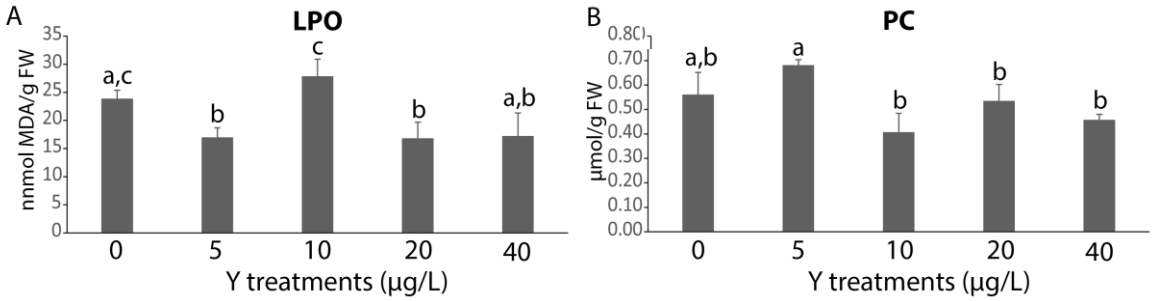

Figure 21. A: Lipid peroxidation (LPO) levels; B: Protein carbonylation (PC) levels, in *Mytilus galloprovincialis* exposed to yttrium at different concentrations (0, 5, 10, 20 and 40 µg/L) after 28 days. Results are means with standard deviations and significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters.

Neurotoxicity

The acetylcholinesterase (AChE) activity was significantly higher only in the 20 µg/L treatment in comparison with the other treatments (Figure 22).

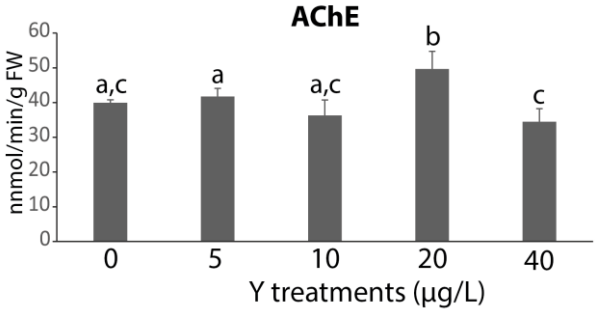

Figure 22. Acetylcholinesterase (AChE) activity, in *Mytilus galloprovincialis* exposed to yttrium at different concentrations (0, 5, 10, 20 and 40 µg/L) after 28 days. Results are means with standard deviations and significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters.

Integrated Biomarker Response Index

The highest values reported by the Integrated Biomarker Response version 2 (IBRvs2) model were those seen at the 5 µg/L dosage, indicating a greater impact in organisms subjected to this Y treatment. The star plot clearly showed that most biochemical parameter values were lower at this treatment, with the exception of SOD, PC, and AChE, which were higher, contributing to this high score (Figure 23). The results also showed that organisms exposed to the other Y treatments scored similar IBRvs2 values. As determining factors, variations in SOD and GR were primarily responsible for the score in the 10 µg/L treatment, whereas variations in AChE and GR accounted for those at 20 µg/L. The biomarker variations experienced at the 40 µg/L treatment were noticeably lower, with CbEs, LPO, and PROT modifications being the highest contributors to the IBRvs2 score (Figure 23).

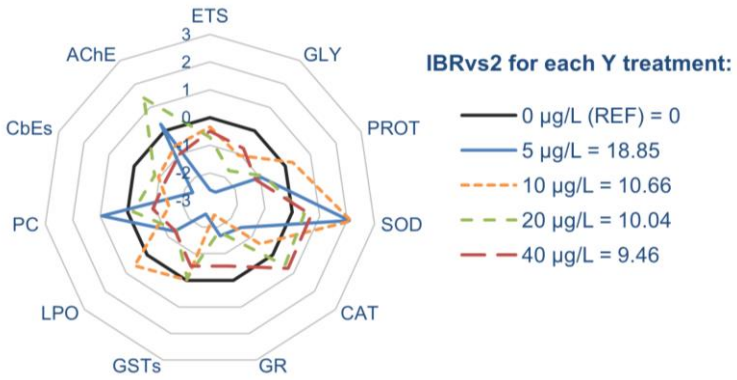

Figure 23. Integrated Biomarker Response version 2 (IBRvs2) index considering all biochemical parameters used on *Mytilus galloprovincialis* after 28-day exposure to yttrium at different concentrations: 0 (reference), 5, 10, 20, and 40 µg/L. ETS = Electron transport system; AChE = Acetylcholinesterase; GLY = Glycogen; CbEs = Carboxylesterases; PROT = Protein; PC = Protein carbonylation; SOD = Superoxide dismutase; LPO = Lipid peroxidation; CAT = Catalase; GSTs = Glutathione *S*-transferases; GR = Glutathione reductase.

3.2.2. Terbium Exposure

This section presents a comparison of mussel responses to Tb exposure, offering an opportunity to contrast the impacts of different rare-earth elements. It presents the results related to the concentration of Tb in mussel tissues, the bioconcentration factor, and various biomarkers. These biomarkers include those related to metabolic capacity, energy reserves, antioxidant and biotransformation capacity, oxidative damage, and neurotoxicity following exposure to Tb.

The results presented in this section corresponding to Tb exposure are published in:

- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Assessing the impact of terbium on Mytilus galloprovincialis: Metabolic and oxidative stress responses. Chemosphere 337, 139299. https://doi.org/10.1016/j.chemosphere.2023.139299.

Terbium Concentration in Mussels' Tissue and Bioconcentration Factor

The concentration of Tb in seawater samples from the control (0 µg/L) treatment was consistently below the LOQ. Concentrations in the water right after spiking were very close to the desired nominal concentrations (shown in brackets) in each treatment: 5.0 ± 0.5 µg/L (5 µg/L), 9.3 ± 0.7 µg/L (10 µg/L), 18.0 ± 1.1 µg/L (20 µg/L), and 38 ± 4 µg/L (40 µg/L).

The levels of Tb in the seawater measured from positive controls right after spiking and right before water renewal showed values varying between: 5.1 ± 0.6 and 4.6 ± 0.6 µg/L (5 µg/ L), 9.8 ± 0.6 and 9.4 ± 0.4 µg/L (10 µg/L), 18.4 ± 1.5 and 17 ± 1 µg/L (20 µg/L), and 37 ± 2 and 39 ± 1 µg/L (40 µg/L). Thus, the Tb presence in the water, with coefficients of variation below 9 %, was considered stable for at least one week under the actual experimental conditions.

The Tb content in mussels' tissue (in dry weight) for the control treatment was below the LOQ while concentrations for the other treatments were: 0.13 ± 0.03 µg/g for 5 µg/L, 0.27 ± 0.03 µg/g for 10 µg/L, 0.45 ± 0.04 µg/g for 20 µg/L and 1.26 ± 0.08 µg/g for 40 µg/L. Concentration in the tissues was in agreement with the exposure medium since there was a significant step-wise increase in concentration with each treatment (Table 2). However, the BCF was stable and didn't show any significant differences among treatments (Table 2).

Table 2. Terbium (Tb) concentration (µg/g dry weight) in mussels' soft tissues and bioconcentration factor (BCF, L/Kg) after 28 days of exposure to spiked seawater. Values are means with standard deviations (±) of (n = 3) measures. Different lowercased letters for Tb in mussels' tissue or uppercased letters for BCF represent significant differences among tested concentrations in the respective columns.

[Tb] (µg/L)	Mussel tissues (µg/g DW)	BCF (L/Kg)
0	< LOQ	-
5	0.13 ± 0.03^{a}	26 ± 6^{A}
10	0.27 ± 0.03^{b}	29 ± 3^{A}
20	0.45 ± 0.04^{c}	24 ± 2^{A}
40	1.26 ± 0.08^{d}	33 ± 2^{A}

Metabolic Capacity and Energy Reserves

The ETS activity tended to decrease with Tb concentration but was only significantly lower at the highest exposure (40 µg/L); although, in this case, not differing from the lowest Tb condition (5 µg/L; Figure 24A).

The GLY content was significantly lower under all Tb treatments (5, 10, 20 and 40 µg/L) in relation to control (0 µg/L), but with no significant differences among them (Figure 24B).

The PROT content remained similar in all the treatments (Figure 24C).

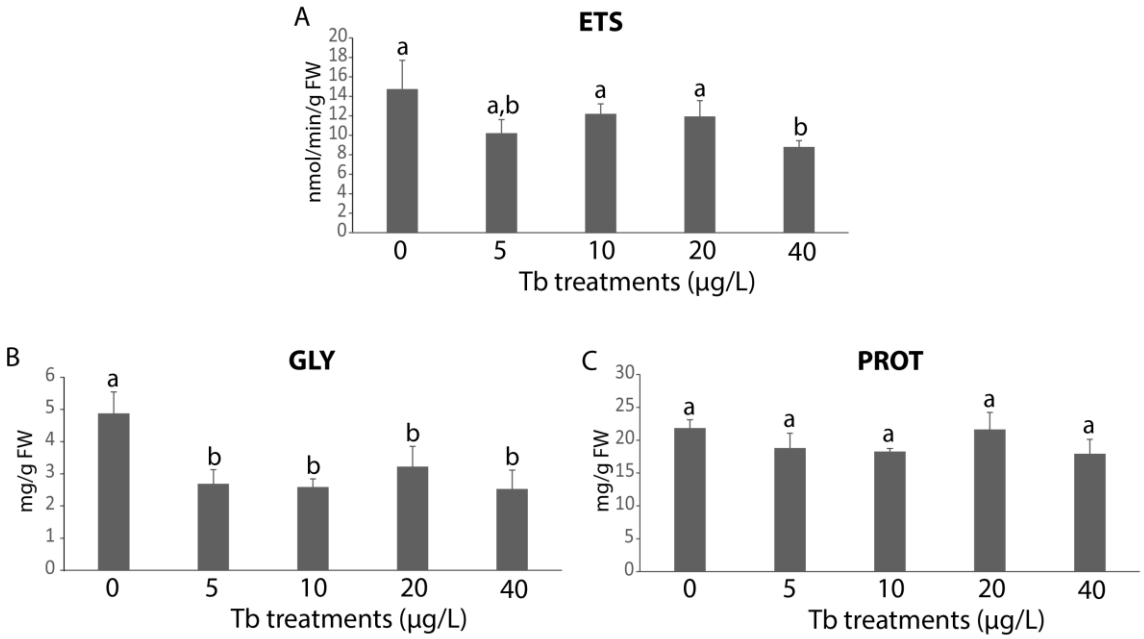

Figure 24. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Protein (PROT) content, in *Mytilus galloprovincialis* exposed to terbium at different concentrations (0, 5, 10, 20 and 40 µg/L) after 28 days. Results are means with standard deviations and significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters.

Antioxidant and Biotransformation Capacity

The SOD activity increased significantly with increasing Tb, reaching the highest activity at 10 µg/L, and decreasing then after, although it still remained significantly different from controls at the highest treatment (40 µg/L; Figure 25A).

The CAT activity did not change significantly among all the treatments (Figure 25B).

The GR activity was significantly lower in mussels for all Tb treatments except for the 20 µg/L condition. Mussels in the 10 µg/L treatment displayed the lowest GR activity but it did not significantly differ from the highest Tb dose (40 µg/L; Figure 25C).

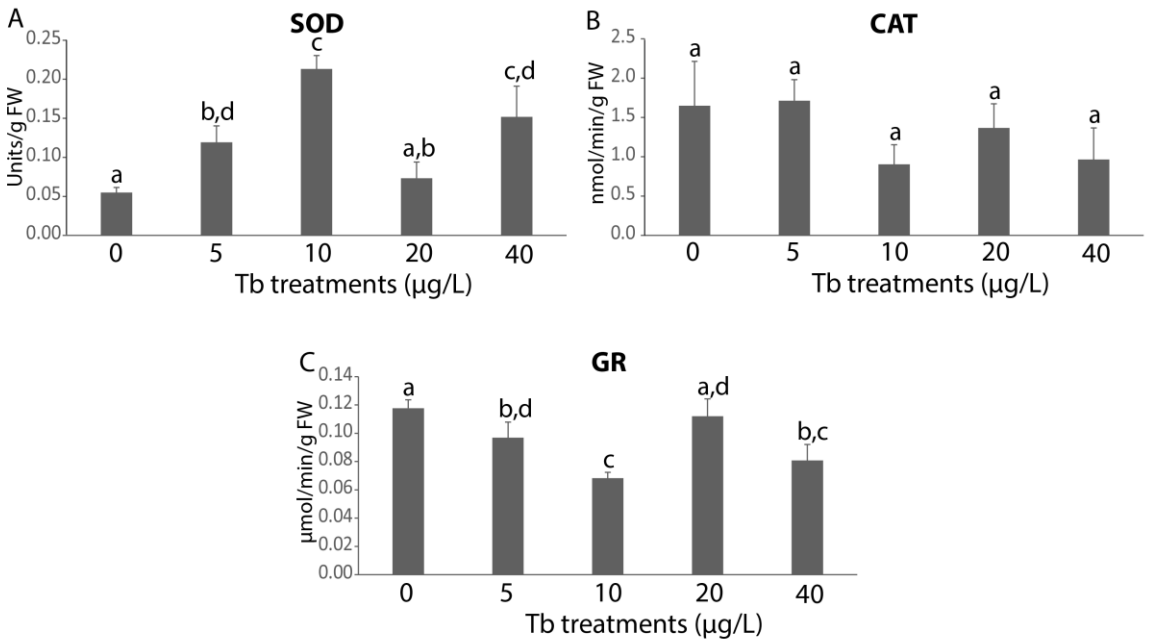

Figure 25. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione reductase (GR) activity, in *Mytilus galloprovincialis* exposed to terbium at different concentrations (0, 5, 10, 20 and 40 µg/L) after 28 days. Results are means with standard deviations and significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters.

The GSTs activity was significantly lower under the 10 and 40 µg/L treatments but it not differed between them (Figure 26A).

The CbEs activity significantly decreased with increasing Tb exposure, with only the highest two Tb treatments (20 and 40 µg/L) remaining similar to each other (Figure 26B).

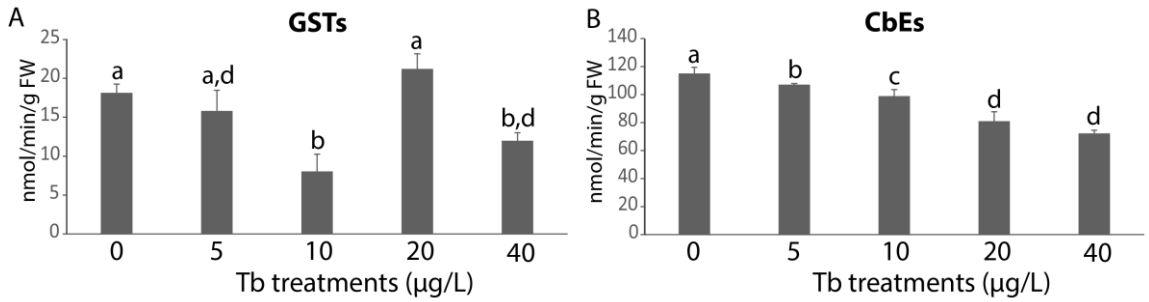

Figure 26. A: Glutathione *S*-transferases (GSTs) activity; B: Carboxylesterases (CbEs) activity, in *Mytilus galloprovincialis* exposed to terbium at different concentrations (0, 5, 10, 20 and 40 µg/L) after 28 days. Results are means with standard deviations and significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters.

Oxidative Damage

The LPO levels did not vary significantly among all treatments (Figure 27).

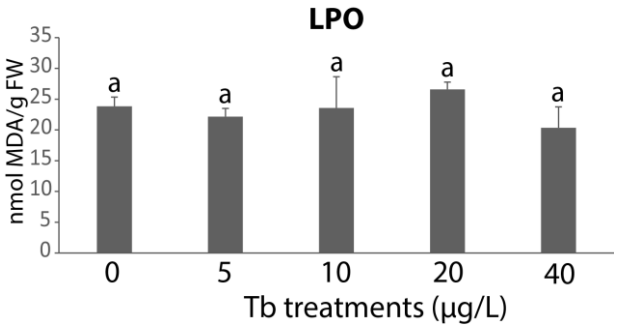

Figure 27. Lipid peroxidation (LPO) levels, in *Mytilus galloprovincialis* exposed to terbium at different concentrations (0, 5, 10, 20 and 40 µg/L) after 28 days. Results are means with standard deviations and significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters.

Neurotoxicity

The AChE activity remained similar in all the treatments (Figure 28).

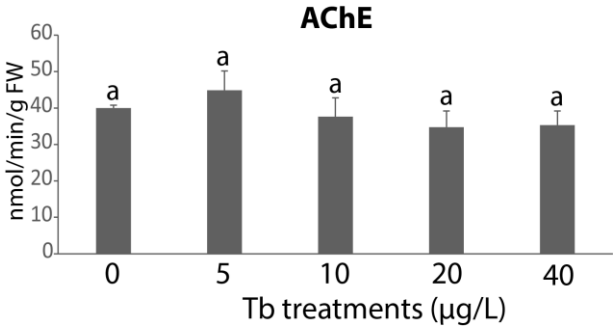

Figure 28. Acetylcholinesterase (AChE) activity, in *Mytilus galloprovincialis* exposed to terbium at different concentrations (0, 5, 10, 20 and 40 µg/L) after 28 days. Results are means with standard deviations and significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters.

3.3. Rare-Earth Elements Under a Climate Change Scenario

The next subchapter presents data on the effects of REEs such as lanthanum (La), gadolinium (Gd), Y and Tb on mussel physiology under climate change conditions. Following the detailed observations of individual rare-earth elements, this subchapter aims to document the influence of climate change-related stressors in the impact of these elements on mussels.

3.3.1. Lanthanum Exposure

This section presents a comparison of mussel responses to La exposure under a climate change scenario. It presents the results related to the concentration of La in mussel tissues, the bioconcentration factor, and various biomarkers. These biomarkers include those related to metabolic capacity, energy reserves, antioxidant and biotransformation capacity, oxidative damage, and neurotoxicity following exposure to La under changes of salinity or increased temperature. Additionally, results from the principal coordinates analyses are also detailed.

The results presented in this section are published in two publications:

- 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. Sci. Total Environ. 794, 148512. https://doi.org/10.1016/j.scitotenv.2021.148512.
- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2022. Do climate change related factors modify the response of *Mytilus galloprovincialis* to lanthanum? The case of temperature rise. Chemosphere 307, 135577. https://doi.org/10.1016/j.chemosphere.2022.135577.

Lanthanum Concentration in Mussels' Tissue and Bioconcentration Factor

The concentration of La in uncontaminated seawater samples was below LOQ regardless of temperature and salinity conditions. The concentration of seawater samples taken immediately after La introduction in the aquaria was around 10.6 ± 0.7 µg/L at 22 °C and salinity 30, of 10.3 ± 0.3 µg/L at 17 °C and salinity 20, of 10.5 ± 0.5 µg/L at 17 °C and salinity 30, and of 10.8 ± 0.5 µg/L at 17 °C and salinity 40, thus, being close to the adopted nominal concentration (10 µg/L).

The levels of La measured in the seawater from positive controls at 0, 24, 48, 72, 144 and 168 h after spiking (Table 3), showed coefficients of variation that were lower than 20 % regardless of the temperature and salinity levels. For this reason, La concentration was considered stable for at least a week under the adopted conditions.

Table 3. Lanthanum concentration (µg/L) measured in seawater from positive controls at 0, 24, 48, 72, 144 and 168 h after its introduction in the medium along the experimental period at two different temperatures and three different salinities.

Sampling time (h)	22 ºC, salinity 30	17 ºC, salinity 30	17 ºC, salinity 20	17 ºC, salinity 40
0	10.50	10.34	10.66	9.61
24	9.24	8.98	8.12	8.81
48	8.98	8.32	9.70	8.17
72	8.84	8.00	8.96	7.02
144	9.02	8.52	7.41	6.09
168	11.40	11.34	6.96	5.98

At the end of the 28 days experimental period, La was detected in the mussels' tissues regardless of the treatment. For both uncontaminated and La-exposed mussels, the La content was significantly higher at salinity 20, while no significant differences were observed between the other two salinities levels (30 and 40) and between the two tested temperatures (17 and 22 °C) (Table 4). Nevertheless, La concentration values in contaminated mussels were between 16-24 times higher than the ones found in uncontaminated mussels (Table 4). The BCF was significantly higher at 17 °C and salinity 20, with no significant differences among the remaining treatments (Table 4).

Table 4. Lanthanum (La) concentration (µg/g dry weight) in mussels' soft tissues and bioconcentration factor (BCF, L/Kg) after 28 days of exposure at two different temperatures, three salinities and two different concentrations of La (0 and 10 µg/L). Values are means with standard deviations (±) of (n = 3) measures. Different lowercased letters for La in mussels' tissue or uppercased letters for BCF represent significant differences among tested concentrations in the respective columns.

[La] (µg/L)	Temperature (ºC)	Salinity	Mussels tissues (µg/g DW)	BCF (L/Kg)
0	17	20	0.094 ± 0.034^{a}	-
		30	0.032 ± 0.001^{b}	-
		40	0.029 ± 0.006^{b}	-
	22	30	0.04 ± 0.01^{b}	-
10	17	20	1.60 ± 0.41^{c}	155 ± 35^{A}
		30	0.75 ± 0.15^{d}	75 ± 15^{B}
		40	0.47 ± 0.23^{d}	46 ± 16^{B}
	22	30	0.78 ± 0.17^{d}	77 ± 15^{B}

Metabolic Capacity and Energy Reserves

At a control temperature of 17 °C, ETS activity of uncontaminated mussels was significant higher at salinity 20 and lower at salinity 40, while La-exposed mussels showed significantly higher activity only at salinity 20 (Figure 29A). At control salinity of 30, La-exposed organisms at 22 °C experienced significantly lower ETS activity comparison with the ones maintained at 17 °C (Figure 29A). Between uncontaminated and contaminated organisms, those La-exposed under salinity 30 and 17 °C demonstrated significantly lower ETS activity (Figure 29A). La-exposed mussels at 22 °C showed significantly lower ETS activity in relation to uncontaminated mussels at 17 °C (Table 5, Figure 29A).

At a control temperature of 17 °C, GLY content of uncontaminated and La-exposed organisms did not differ significantly among salinities, although it tended to decrease with increasing salinities (Figure 29B). At control salinity of 30, the GLY content was noticeably lower in uncontaminated organisms at 22 °C when compared to those kept at 17 °C, whereas La-exposed organisms exhibited a significantly higher GLY content at 22 °C compared to those at 17 °C (Figure 29B). Between uncontaminated and contaminated mussels, those La-exposed mussels under salinity 30 and 22 °C exhibited significantly higher GLY content (Figure 29B). La-exposed mussels at 22 °C showed

significantly higher GLY content in relation to uncontaminated mussels at 17 °C (Table 5, Figure 29B).

At a control temperature of 17 °C, the PROT content of uncontaminated and La-exposed organisms was significantly lower at the salinity of 20 and significantly higher at a salinity of 40 (Figure 29C). At control salinity of 30, uncontaminated and contaminated organisms did not show significant differences according to temperatures (Figure 29C).

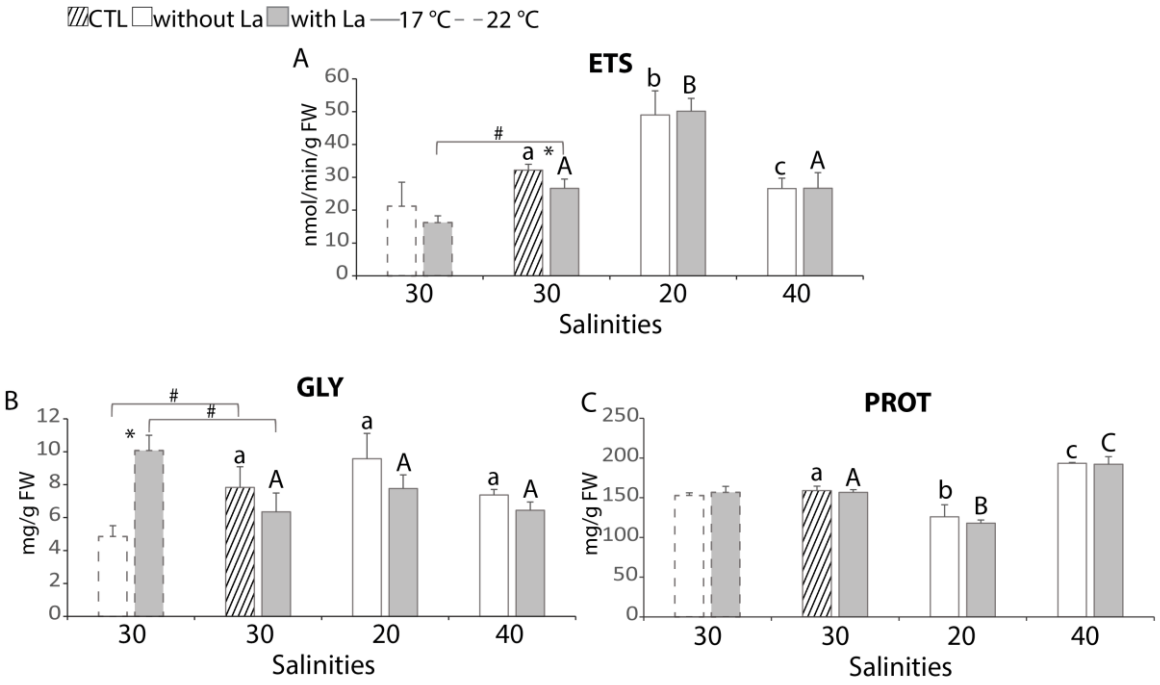

Figure 29. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Protein (PROT) content, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and lanthanum (La)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and La-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Table 5. Significance levels between La-exposed mussels at 22 °C and uncontaminated at 17 °C. Significant differences ($p < 0.05$) are highlighted in bold.

ETS	GLY	PROT	SOD	CAT	GR	GSTs	CbEs	LPO	PC	AChE
0.0009	**0.0469**	>0.05	**0.0064**	>0.05	>0.05	**0.0480**	>0.05	**0.0184**	**0.0177**	>0.05

Antioxidant and Biotransformation Capacity

At a control temperature of 17 °C, SOD activity of uncontaminated mussels was significantly higher at salinity 20 compared to salinity 30, while La-exposed organisms showed significantly higher activity at the lowest salinity compared to values registered at salinities 30 and 40 (Figure 30A). At the control salinity of 30, SOD activity was significantly lower in uncontaminated mussels at 22 °C when compared to those kept at 17 °C (Figure 30A). Between uncontaminated and contaminated organisms, significant differences were only recorded at salinity 40 and 17 °C, with the lowest SOD values at those contaminated (Figure 30A). La-exposed mussels at 22 °C showed significantly lower SOD activity in relation to uncontaminated mussels at 17 °C (Table 5, Figure 30A).

At the control temperature of 17 °C, CAT activity of uncontaminated mussels decreased from salinity 20 to 40 with significant differences among all salinities, while in La-exposed mussels a similar trend was observed with significant differences between the lowest (20) and the other two salinities (Figure 30B). At the control salinity of 30, CAT activity was significantly lower in uncontaminated mussels at 22 °C when compared to those kept at 17 °C (Figure 30B). Between uncontaminated and contaminated mussels, significant differences were found at control salinity (30) at 17 °C, with lower CAT activity in those contaminated (Figure 30B).

In terms of GR activity, this activity remained similar across treatments and was unaffected by La exposure or physical parameters of temperature and salinity (Figure 30C).

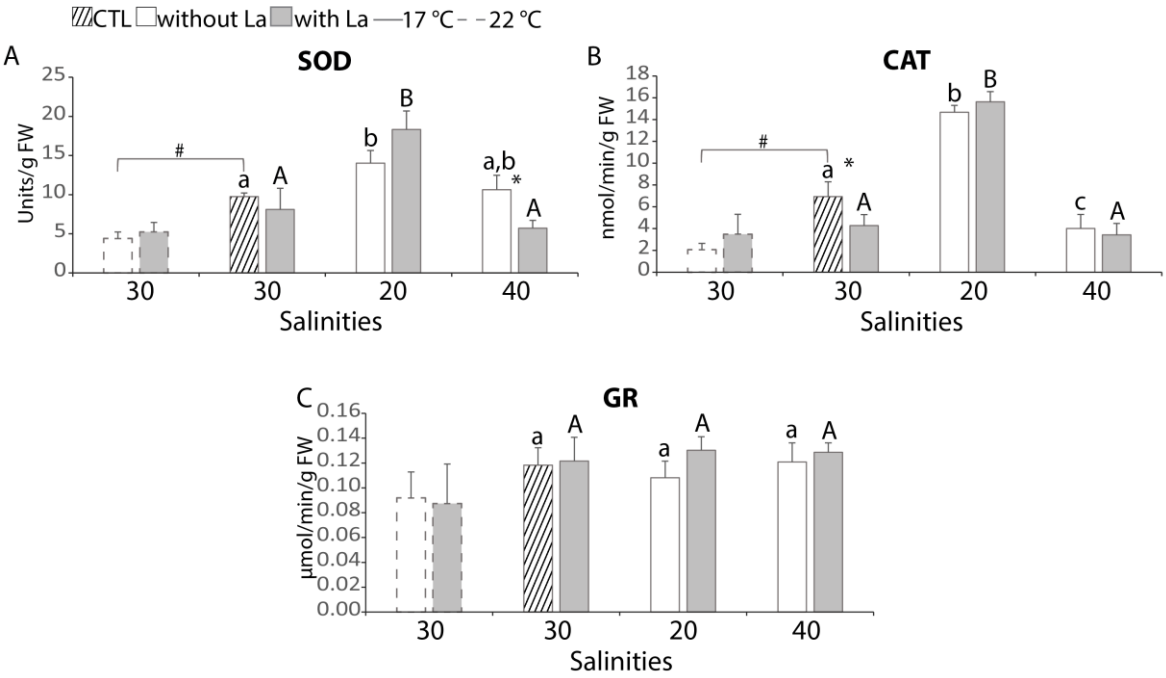

Figure 30. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione reductase (GR) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and lanthanum (La)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and La-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

At the control temperature of 17 °C, the GSTs activity in uncontaminated mussels was significantly higher at salinity 20, while in La-exposed mussels this activity was significantly different at all salinity levels, with higher values at salinity 20 and lower values at salinity 40 (Figure 31A). At control salinity of 30, uncontaminated mussels at 22 °C showed significantly lower GSTs activity that those kept at 17 °C (Figure 31A). Between uncontaminated and contaminated mussels, significant differences were found at salinity 30, with higher GSTs activity in contaminated organisms regardless of the tested temperature (Figure 31A). La-exposed mussels at 22 °C showed significantly higher GSTs activity in relation to uncontaminated mussels at 17 °C (Table 5, Figure 31A).

At the control temperature of 17 °C, the CbEs activity of uncontaminated and La-exposed organisms was significantly higher at the salinity of 20 (Figure 31B). At control

salinity of 30, uncontaminated mussels at 22 °C showed significantly lower CbEs activity that those kept at 17 °C (Figure 31B).

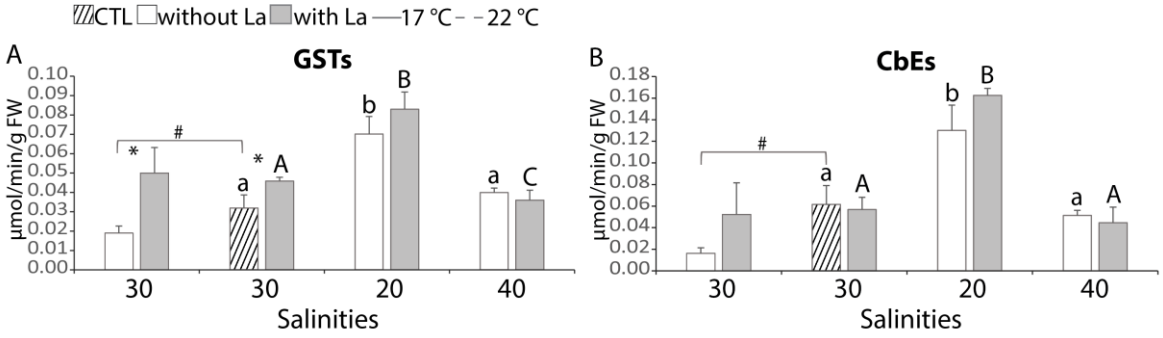

Figure 31. A: Glutathione *S*-transferases (GSTs) activity; B: Carboxylesterases (CbEs) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and lanthanum (La)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and La-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Oxidative Damage

At a control temperature of 17 °C, the LPO levels of uncontaminated and La-exposed organisms did not differ significantly among salinities, although there was a decreasing trend with the increase in salinity in contaminated organisms (Figure 32A). At control salinity of 30, uncontaminated and contaminated organisms did not show significant differences between temperatures (Figure 32A). Between uncontaminated and contaminated mussels, those La-exposed under salinity of 30 at both tested temperatures as well under salinity 20 at 17 °C showed significantly higher LPO levels (Figure 32A). La-exposed mussels at 22 °C showed significantly higher LPO levels in relation to uncontaminated mussels at 17 °C (Table 5, Figure 32A).

At a control temperature of 17 °C, the PC levels observed in uncontaminated organisms did not differ significantly among different salinities; however La-exposed mussels showed significantly higher activity at salinity 20 (Figure 32B). At control salinity of 30, uncontaminated mussels at 22 °C showed significantly lower PC levels that those kept at 17 °C (Figure 32B). Between uncontaminated and contaminated mussels, those

La-exposed under salinity of 20 at 17 °C showed significantly higher PC levels (Figure 32B). La-exposed mussels at 22 °C showed significantly lower PC levels in relation to uncontaminated mussels at 17 °C (Table 5, Figure 32B).

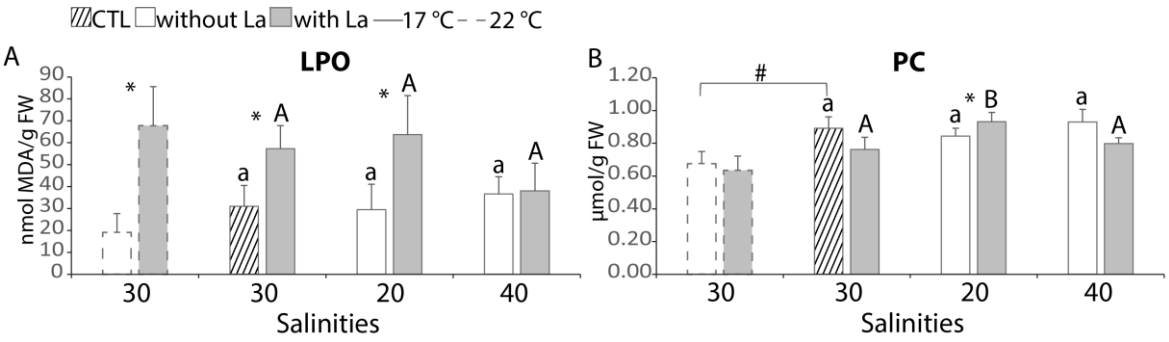

Figure 32. A: Lipid peroxidation (LPO) levels; B: Protein carbonylation (PC) levels, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and lanthanum (La)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and La-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Neurotoxicity

At a control temperature of 17 °C, the AChE activity observed in uncontaminated organisms did not differ significantly among different salinities; however La-exposed mussels showed significantly lower activity at salinity 20 (Figure 33). At control salinity of 30, uncontaminated and contaminated organisms did not show significant differences between temperatures (Figure 33).

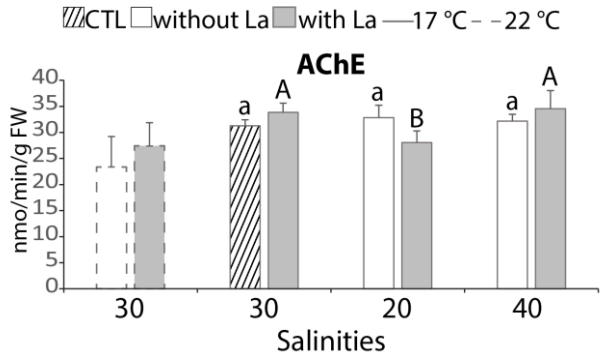

Figure 33. Acetylcholinesterase (AChE) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and lanthanum (La)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and La-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Principal Coordinates Analyses

At a control temperature of 17 °C, the Principal Coordinates (PCO) analysis (Figure 34) revealed that the first principal component (PCO1) explained 71.7 % of total variance among treatments, clearly separating the uncontaminated and La-exposed mussels at the lowest salinity in the positive side and the organisms under the two other salinities (30 and 40) in the negative side. PCO2 explained 14.5 % of total variation, separating the La-exposed mussels at all salinities in the positive side from the uncontaminated mussels under all salinities in the negative side. The biochemical descriptors superimposed on the PCO1 showed that ETS, SOD, CAT, GSTs and CbEs were highly correlated ($r > 0.75$) with the lowest salinity (20) since higher values of these parameters were achieved in all mussels at this salinity. On the other hand, PROT and AChE were highly correlated with the other two salinities (30 and 40) in the negative side of the axis. LPO and GR were associated to La exposure since higher values were attained in mussels exposed at all salinities.

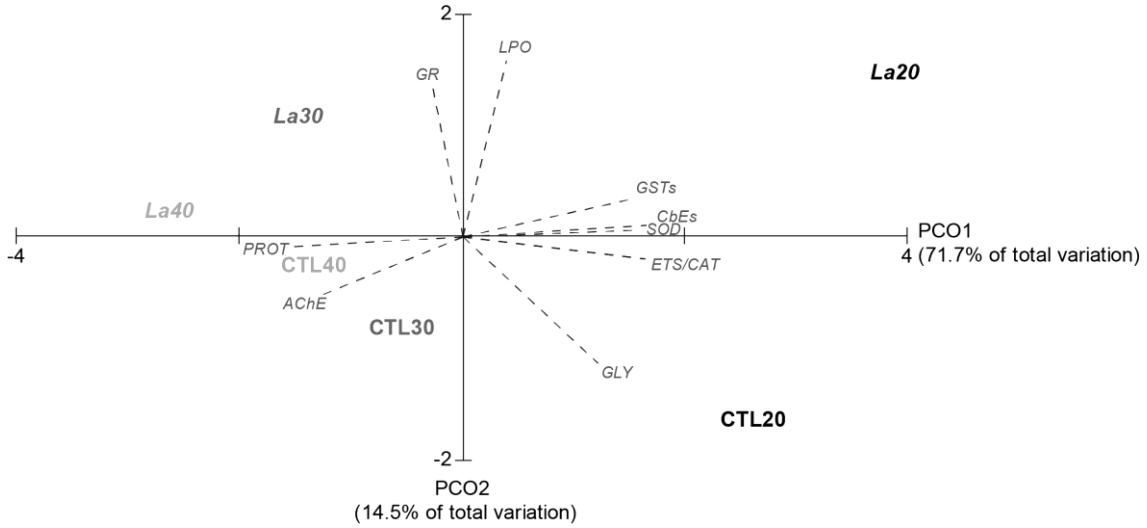

Figure 34. Principal Coordinates (PCO) analysis based on the tested conditions and biochemical markers measured, in *Mytilus galloprovincialis* at control temperature of 17 °C. The following conditions are presented: CTL20, CTL30, CTL40 (uncontaminated organisms under different salinities of 20, 30 and 40) and La20, La30, La40 (La-exposed organisms under different salinities of 20, 30 and 40). Spearman correlation vectors are superimposed as supplementary variables, namely biochemical data (r > 0.75): ETS, PROT, GLY, SOD, CAT, GR, GSTs, CbEs, LPO, AChE.

At a control salinity of 30, PCO1 of the PCO analysis (Figure 35) accounted for 57.8 % of total variation among treatments, mostly differentiating mussels from the control temperature on the negative side of the axis from mussels under increased temperature on the positive side. PCO2 explained 35.3 % of total variation by excluding organisms exposed to both La and increased temperature on the positive side from non-contaminated organisms on the negative side. The biochemical parameters overlaid on the PCO1 revealed a strong negative correlation (r < -0.75) especially between antioxidant and biotransformation enzymes and the uncontaminated organisms under 22 °C and, at to a lower extent, with the organisms exposed to the combination of increased temperature and La (corresponding to lower activities on these parameters). In terms of PCO2, the organisms under the combination of increased temperature and exposure to La, showed a strong positive correlation with LPO (r = 0.8) and GSTs (r = 0.8) and a negative one with PC (r = -0.8) and ETS (r = -0.8), showing under this condition higher and lower values, respectively.

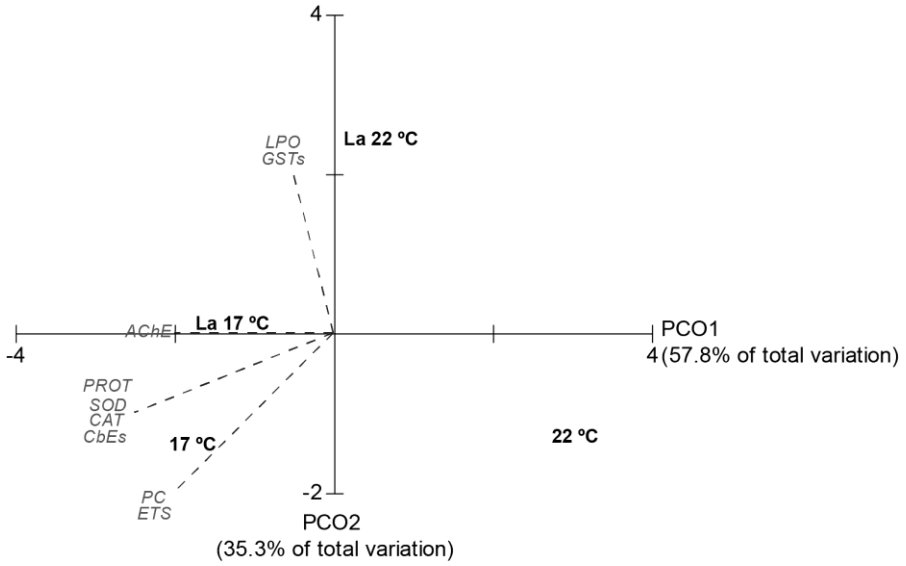

Figure 35. Principal Coordinates (PCO) analysis based on the tested conditions and biochemical markers measured, in *Mytilus galloprovincialis* at control salinity of 30. The following conditions are presented: 17 °C and 22 °C (non-contaminated organisms under 17 °C and 22 °C) and La 17 °C and La 22 °C (organisms exposed to 10 µg/L of La under 17 °C and 22 °C). Spearman correlation vectors are superimposed as supplementary variables, namely biochemical data (r > 0.75): ETS, PROT, SOD, CAT, GSTs, CbEs, LPO, PC, AChE.

3.3.2. Gadolinium Exposure

This section presents a comparison of mussel responses to Gd exposure under climate change scenario. It presents the results related to the concentration of Gd in mussel tissues, the bioconcentration factor, and various biomarkers. These biomarkers include those related to metabolic capacity, energy reserves, antioxidant and biotransformation capacity, oxidative damage, and neurotoxicity following exposure to Gd under changes of salinity or increased temperature. Additionally, results from the principal coordinates analyses and integrated biomarker response index are also detailed.

The results presented in this section are published in two publications:

- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2022. Will climate changes enhance the impacts of e-waste in aquatic systems? Chemosphere 288, 132264. https://doi.org/10.1016/j.chemosphere.2021.132264.
- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Gadolinium accumulation and its biochemical effects in *Mytilus galloprovincialis* mussels under a scenario of global warming. Environ. Sci. Pollut. Res. 30, 116120-116133. https://doi.org/10.1007/s11356-023-30439-2.

Gadolinium Concentration in Mussels' Tissue and Bioconcentration Factor

The concentration of Gd in uncontaminated seawater samples was below LOQ regardless of temperature and salinity conditions. The concentration of seawater samples taken immediately after Gd introduction in the aquaria was around 10.10 ± 0.55 µg/L at 22 °C and salinity 30, of 9.2 ± 0.3 µg/L at 17 °C and salinity 20, of 9.7 ± 0.2 µg/L at 17 °C and salinity 30, and of 9.9 ± 0.3 µg/L at 17 °C and salinity 40, thus, being close to the adopted nominal concentration (10 µg/L).

The levels of Gd measured in the seawater from positive controls at 0, 24, 48, 72 and 144 h after spiking (Table 6) varied less than 10 % along the experimental period. Thus, Gd concentration was considered stable for at least a week under the adopted conditions.

Table 6. Gadolinium concentration (µg/L) measured in seawater from positive controls at 0, 24, 48, 72 and 144 h after its introduction in the medium along the experimental period at two different temperatures and three different salinities.

Sampling time (h)	22 ºC, salinity 30	17 ºC, salinity 30	17 ºC, salinity 20	17 ºC, salinity 40
0	10.18	10.04	9.80	9.75
24	9.28	8.72	8.95	9.67
48	9.04	8.48	8.91	9.29
72	8.96	8.60	8.73	9.56
144	8.72	8.36	8.40	9.05

At the end of the 28 days experimental period, Gd was detected in the mussels' tissues regardless of the treatment. For contaminated organisms, Gd content was significantly higher salinity 20, compared to those at salinities 30 and 40 at control temperature (Table 7). At control salinity, Gd-exposed mussels demonstrated significant higher Gd concentration values at 22 °C compared to the ones present at 17 °C (Table 7). Additionally, Gd concentration values in contaminated mussels were between 2-8 times higher than the ones found in uncontaminated mussels (Table 7). The BCF was significantly higher at salinity 20 compared to the other two salinities levels (30 and 40), with no significant differences between these (Table 7). At control salinity of 30, mussel under 22 °C showed significantly higher BCF than at 17 °C (Table 7).

Table 7. Gadolinium (Gd) concentration (µg/g dry weight) in mussels' soft tissues and bioconcentration factor (BCF, L/Kg) after 28 days of exposure at two different temperatures, three salinities and two different concentrations of Gd (0 and 10 µg/L). Values are means with standard deviations (±) of (n = 3) measures. Different lowercased letters for Gd in mussels' tissue or uppercased letters for BCF represent significant differences among tested concentrations in the respective columns.

[Gd] (µg/L)	Temperature (ºC)	Salinity	Mussels tissues (µg/g DW)	BCF (L/Kg)
0	17	20	0.035 ± 0.001^{a}	-
		30	0.015 ± 0.002^{b}	-
		40	0.077 ± 0.015^{c}	-
	22	30	0.045 ± 0.012^{a}	-
10	17	20	0.151 ± 0.012^{d}	16.2 ± 1.2^{A}
		30	0.126 ± 0.008^{e}	13.0 ± 0.57^{B}
		40	0.112 ± 0.009^{f}	11.3 ± 1.13^{B}
	22	30	0.150 ± 0.008^{d}	15.6 ± 0.5^{A}

Metabolic Capacity and Energy Reserves

At a control temperature of 17 °C, the ETS activity of uncontaminated mussels was significant higher at salinity 20, while Gd-exposed mussels showed significantly higher activity at salinity 20 but significantly lower activity at salinity 40 (Figure 36A). At control salinity of 30, uncontaminated and contaminated organisms did not show significant differences between temperatures (Figure 36A). Between uncontaminated and contaminated organisms, those Gd-exposed under salinity 20 and 17 °C demonstrated significantly higher ETS activity (Figure 36A).

At a control temperature of 17 °C, the GLY content of uncontaminated and Gd-exposed organisms did not differ significantly among salinities (Figure 36B). At control salinity of 30, the GLY content was noticeably lower in uncontaminated organisms at 22 °C when compared to those kept at 17 °C (Figure 36B). Between uncontaminated and contaminated mussels, significant differences were found at salinities 30 and 40 and 17 °C with higher GLY content in those Gd-contaminated (Figure 36B).

At a control temperature of 17 °C, the PROT content of uncontaminated and Gd-exposed organisms was significant lower at salinity 20 and higher at salinity 40 (Figure 36C). At control salinity of 30, PROT content was significantly higher in Gd-exposed mussels at 22 °C when compared to those kept at 17 °C (Figure 36C). Between

uncontaminated and contaminated mussels, PROT content of Gd-exposed organisms was significantly lower at salinity 40 and 17 °C, and higher at salinity 30 and 22 °C (Figure 36C).

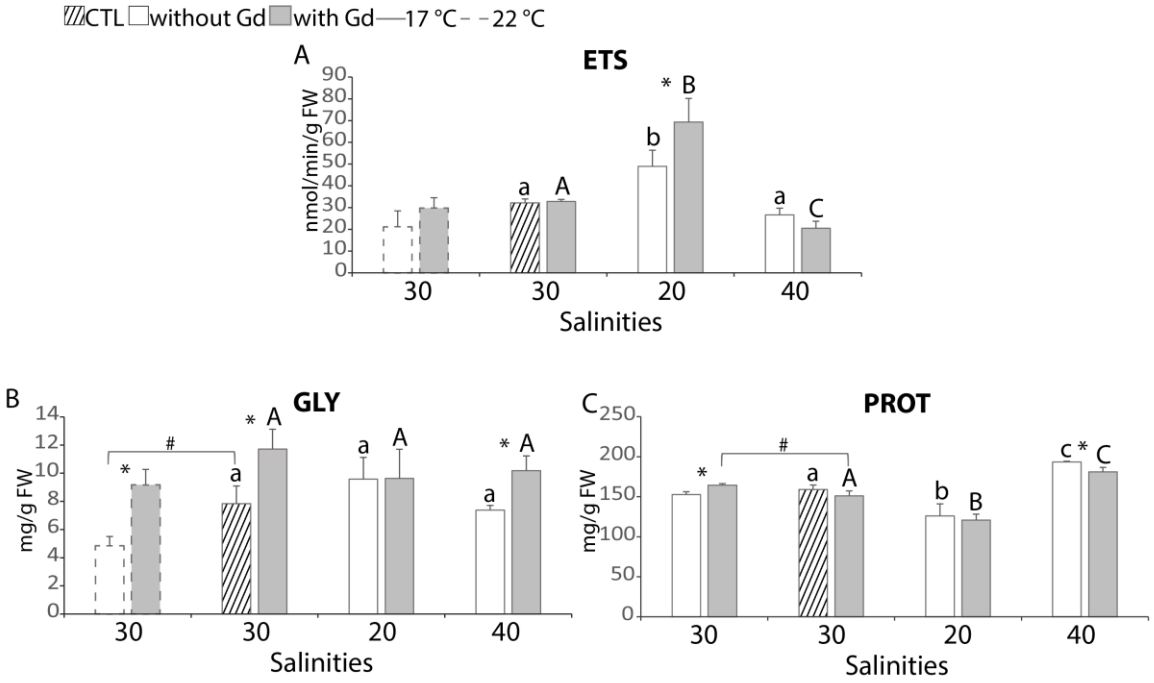

Figure 36. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Protein (PROT) content, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and gadolinium (Gd)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Gd-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Antioxidant and Biotransformation Capacity

At a control temperature of 17 °C, the SOD activity of uncontaminated mussels was significantly higher at salinity 20 compared to salinity 30 (Figure 37A). At the control salinity of 30, SOD activity was significantly lower in uncontaminated mussels at 22 °C when compared to those kept at 17 °C (Figure 37A).

At the control temperature of 17 °C, the CAT activity in uncontaminated mussels decreased along an increasing salinity gradient, with significant higher activity at salinity 20 compared to salinities 30 and 40. In Gd-exposed mussels a similar trend was observed

with significant differences reached at the highest salinity (40) (Figure 37B). At the control salinity of 30, CAT activity displayed a similar pattern to SOD with significantly lower activity in both uncontaminated and Gd-exposed mussels at 22 °C when compared to those kept at 17 °C (Figure 37B). Between uncontaminated and contaminated mussels, significant differences were found at salinity 30, with higher CAY activity in contaminated organisms regardless of the tested temperature (Figure 37B).

In terms of GR activity, this activity remained similar across treatments and was unaffected by Gd exposure or physical parameters of temperature and salinity (Figure 37C).

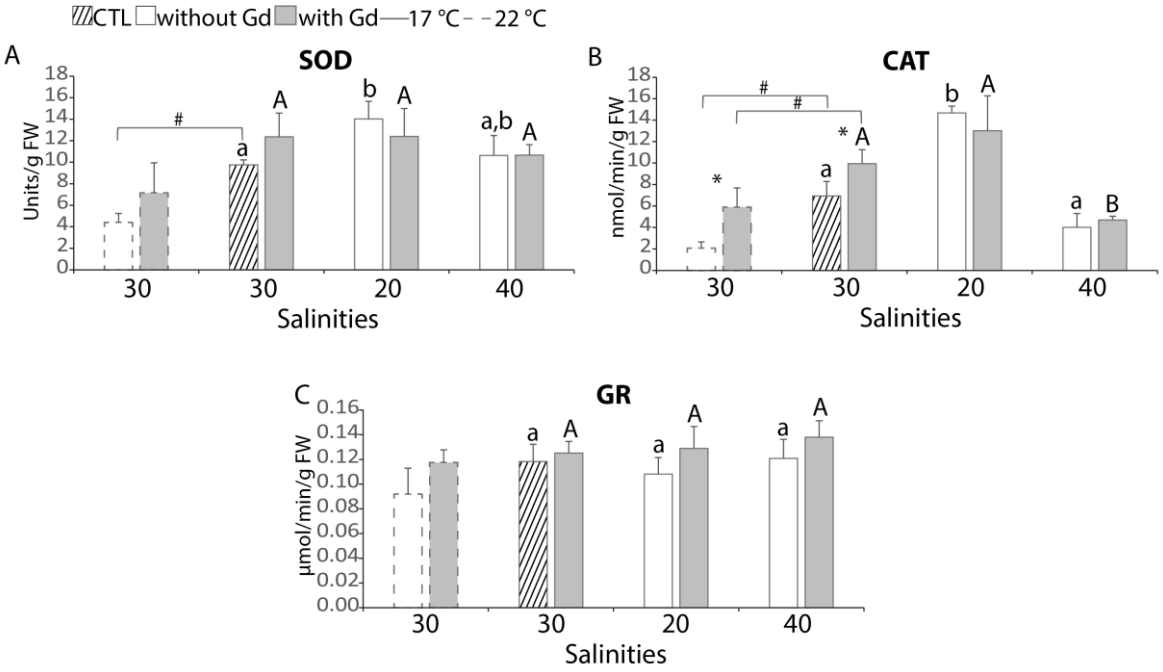

Figure 37. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione reductase (GR) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and gadolinium (Gd)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Gd-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

At the control temperature of 17 °C, the GSTs activity in uncontaminated mussels was significantly higher at salinity 20, while in Gd-exposed mussels this activity was significantly lower at salinity 40 (Figure 38A). At control salinity of 30, uncontaminated

mussels at 22 °C showed significantly lower GSTs activity that those kept at 17 °C (Figure 38A). Between uncontaminated and contaminated mussels, significant differences were found at salinity 30, with higher GSTs activity in contaminated organisms regardless of the tested temperature (Figure 38A).

At the control temperature of 17 °C, the CbEs activity of Gd-exposed organisms showed a decrease trend with significantly lower activity at the salinity of 40 compared to those at the salinity 20 (Figure 38B). At control salinity of 30, CbEs activity displayed a similar pattern to GSTs, with significantly lower activity in uncontaminated organisms at 22 °C when compared to those kept at 17 °C (Figure 38B). Between uncontaminated and contaminated mussels, those Gd-exposed under salinity of 30 at 22 °C showed significantly higher CbEs activity (Figure 38B).

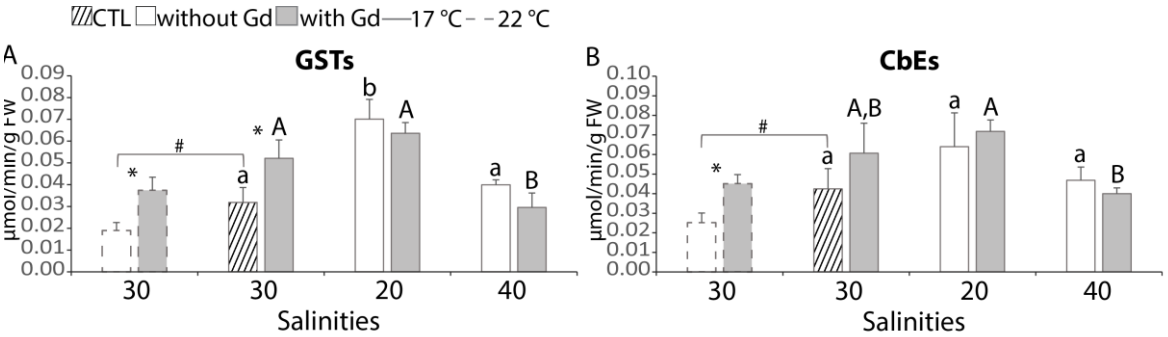

Figure 38. A: Glutathione *S*-transferases (GSTs) activity; B: Carboxylesterases (CbEs) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and gadolinium (Gd)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Gd-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Oxidative Damage

At a control temperature of 17 °C, the LPO levels of Gd-exposed organisms were significantly higher at salinity 40 compared to salinity 30 (Figure 39A). At control salinity of 30, Gd-exposed organisms at 22 °C showed significantly higher LPO levels that those kept at 17 °C (Figure 39A). Between uncontaminated and contaminated mussels, Gd-exposed mussels showed significantly higher LPO levels regardless of the temperature

and salinity tested (Figure 39A). Gd-exposed mussels at 22 °C showed significantly higher LPO levels in relation to uncontaminated mussels at 17 °C (Table 8, Figure 39A).

At a control temperature of 17 °C, the PC levels in uncontaminated organisms did not differ significantly among different salinities at the temperature of 17 °C; however Gd-exposed mussels showed significantly higher activity at salinity 40 compared to salinity 20 (Figure 39B). At control salinity of 30, uncontaminated mussels at 22 °C showed significantly lower PC levels that those kept at 17 °C (Figure 39B).

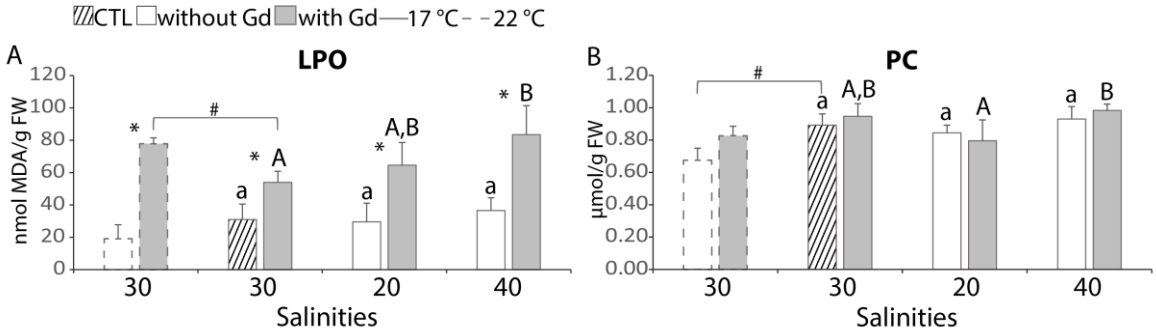

Figure 39. A: Lipid peroxidation (LPO) levels; B: Protein carbonylation (PC) levels, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and gadolinium (Gd)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Gd-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Table 8. Significance levels between Gd-exposed mussels at 22 °C and uncontaminated at 17 °C. Significant differences ($p < 0.05$) are highlighted in bold.

ETS	GLY	PROT	SOD	CAT	GR	GSTs	CbEs	LPO	PC	AChE
>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	>0.05	**0.0036**	>0.05	>0.05

Neurotoxicity

At a control temperature of 17 °C, the AChE activity of uncontaminated and Gd-exposed organisms did not differ significantly among salinities (Figure 40). At the control salinity of 30, uncontaminated and contaminated organisms did not show significant differences between temperatures (Figure 40). However, contrasts between uncontaminated and contaminated organisms, indicated that Gd-exposed mussels had

significantly higher AChE activity under control salinity of 30 at elevated temperature (22 °C) (Figure 40).

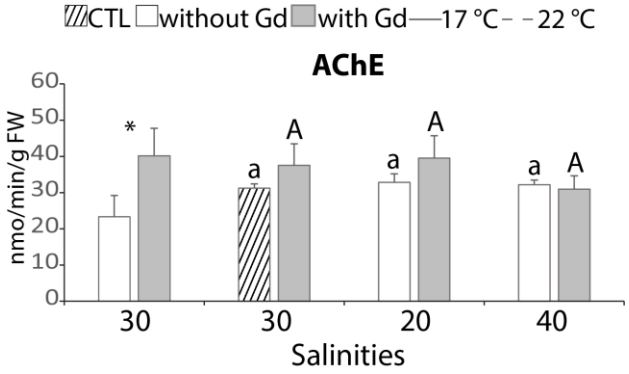

Figure 40. Acetylcholinesterase (AChE) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and gadolinium (Gd)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Gd-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Principal Coordinates Analyses

At a control temperature of 17 °C, the PCO analysis (Figure 41) revealed that the PCO1 explained 66.2 % of total variance among treatments, separating mussels exposed to the lowest salinity (CTL20, Gd20) in the negative side and the mussels at the highest salinity (CTL40, Gd40) and CTL30 in the positive side. PCO2 explains 20.2 % of total variation, clearly separating the mussels exposed to Gd at all salinities in the negative side from those non-contaminated mussels in the positive side. The biochemical descriptors superimposed on the PCO1 showed a high correlation ($r > 0.75$) among ETS, CAT, GSTs, CbEs and AChE and the lowest salinity (20) since the highest values of these parameters were achieved in mussels at this salinity (CTL20, Gd20). LPO, GR and GLY were associated to Gd exposure (PCO2 negative side) since higher values were observed in contaminated mussels regardless of salinity.

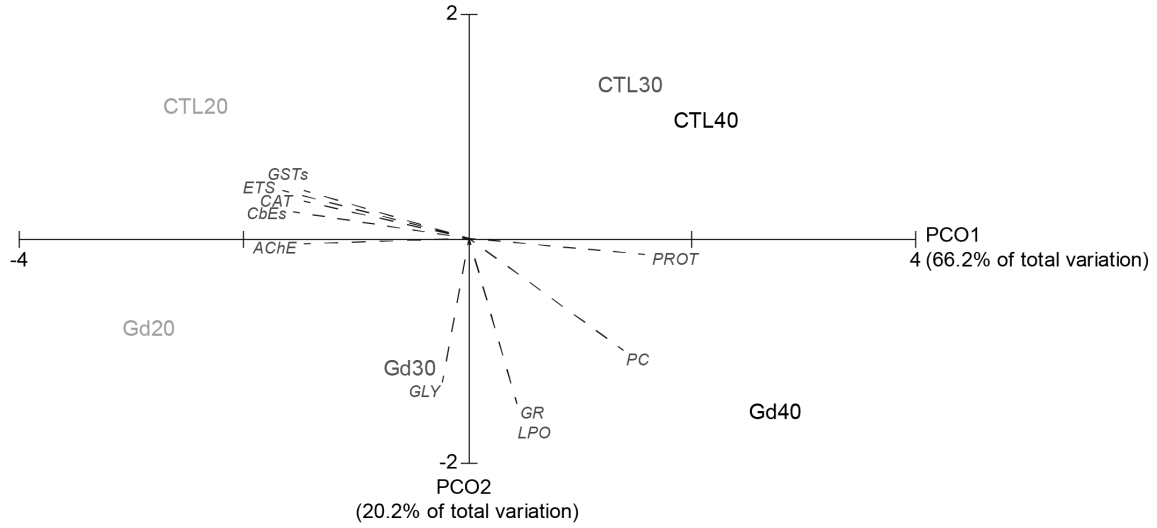

Figure 41. Principal Coordinates (PCO) analysis based on the tested conditions and biochemical markers measured, in *Mytilus galloprovincialis* at control temperature of 17 °C. The following conditions are presented: CTL20, CTL30, CTL40 (uncontaminated organisms under different salinities of 20, 30 and 40) and Gd20, Gd30, Gd40 (Gd-exposed organisms under different salinities of 20, 30 and 40). Spearman correlation vectors are superimposed as supplementary variables, namely biochemical data (r > 0.75): ETS = Electron transport system, GLY = Glycogen, PROT = Protein, CAT = Catalase, GR = Glutathione reductase, GSTs = Glutathione *S*-transferases, CbEs = Carboxylesterases, LPO = Lipid peroxidation, PC = Protein carbonylation, AChE = Acetylcholinesterase.

At a control salinity of 30, the PCO1 of the PCO analysis (Figure 42) represented 81 % of the total variance among treatments, it primarily separated contaminated mussels at the lowest temperature (Gd 17 °C) on the positive side of the axis from uncontaminated mussels at the increased temperature on the negative side (22 °C). The second component, PCO2 explained 14.2 % of the total variance distinguishing mussels exposed to Gd and increased temperature (Gd 22 °C) on the positive side from the ones exposed to Gd at control temperature (Gd 17 °C) on the negative side. The biochemical descriptors superimposed on the PCO1 revealed a strong positive correlation (r > 0.75) among all parameters with the exception of PROT and the contaminated organisms under control temperature (Gd 17 °C). This was a direct result of increased activities and levels at this treatment and the lower activities of these parameters in higher temperature (22 °C) which was on the opposite side of the axis. As for PCO2, the organisms under the combination of increased temperature and exposure to Gd, showed an especially strong positive correlation with PROT (r = 0.8) with higher values at this treatment, while also negatively correlating with the antioxidant enzymes, ETS and PC (r = -0.8).

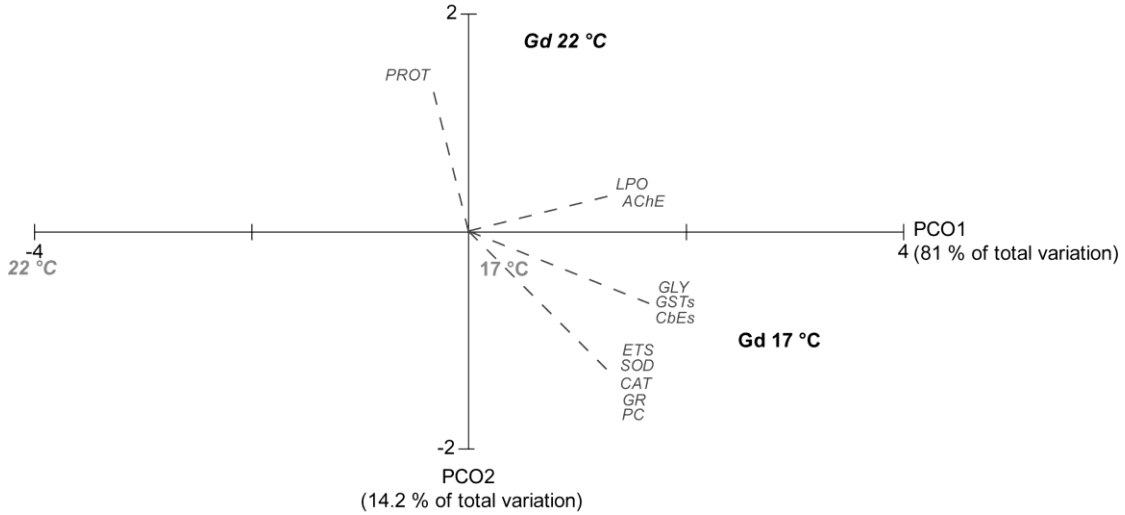

Figure 42. Principal Coordinates (PCO) analysis based on the tested conditions and biochemical markers measured in *Mytilus galloprovincialis* at control salinity of 30. The following conditions are presented: 17 °C and 22 °C (non-contaminated organisms under 17 °C and 22 °C) and Gd 17 °C and Gd 22 °C (organisms exposed to 10 µg/L of Gd under 17 °C and 22 °C). Spearman correlation vectors are superimposed as supplementary variables, namely biochemical data (r > 0.75): ETS = Electron transport system, GLY = Glycogen, PROT = Protein, SOD = Superoxide dismutase, CAT = Catalase, GR = Glutathione reductase, GSTs = Glutathione *S*-transferases, CbEs = Carboxylesterases, LPO = Lipid peroxidation, PC = Protein carbonylation, AChE.= Acetylcholinesterase.

Integrated Biomarker Response Index

The IBRvs2 index was considered at control salinity of 30. The greater values (IBRvs2 = 15) reached were those seen in uncontaminated organisms at the increased temperature (22 °C), indicating a greater impact at this temperature. The star plot clearly illustrated that this treatment resulted in lower values for all biochemical indicators, which contributed to this high score (Figure 43). The results also showed that Gd-exposed organisms at both temperatures (Gd 17 °C and Gd 22 °C) scored similar IBRvs2 values (IBRvs2 = 8 and 6, respectively). As determining features, variations in LPO showed to be a common factor being especially relevant at the increased temperature treatment. Nevertheless, Gd-exposed organisms at control temperature showed a slightly higher IBRv2 value associated to PROT, GLY and GSTs as these parameters were more determinant (higher inhibition or increase) at this treatment (Figure 43). Between Gd treatments at the two tested temperatures, most of the enzymes analyzed showed a greater positive contribution, indicating a higher level of influence, under the control condition (17 °C) (Figure 43).

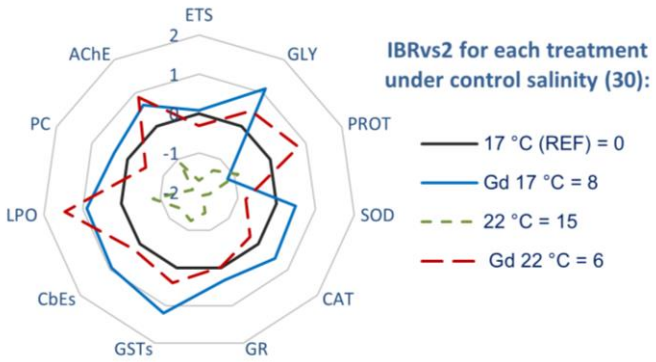

Figure 43. Integrated Biomarker Response (IBRvs2) index considering all biochemical parameters used on *Mytilus galloprovincialis* after exposure to different temperatures (17 ºC and 22 ºC) in the absence and presence (0 and 10 µg/L) of Gd for 28 days. The biomarker quantified for uncontaminated organisms under 17 ºC were considered for the reference line (REF). ETS = Electron Transport System; GLY = Glycogen; PROT = Protein; SOD = Superoxide dismutase; CAT = Catalase; GR = Glutathione reductase; GSTs = Glutathione S-transferases; CbEs = Carboxylesterases; LPO = Lipid peroxidation; PC = Protein carbonylation; AChE = Acetylcholinesterase.

3.3.3. Yttrium Exposure

This section presents a comparison of mussel responses to Y exposure under climate change scenario. It presents the results related to the concentration of Y in mussel tissues, the bioconcentration factor, and various biomarkers. These biomarkers include those related to metabolic capacity, energy reserves, antioxidant and biotransformation capacity, oxidative damage, and neurotoxicity following exposure to Y under changes of salinity or increased temperature. Additionally, results from the integrated biomarker response index are also detailed.

The results presented in this section are published as:

- Andrade, M., Pinto, J., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Yttrium effects on the Mediterranean mussel under a scenario of salinity shifts and increased temperature. Mar. Environ. Res. https://doi.org/10.1016/j.marenvres.2024.106365.

Yttrium Concentration in Mussels' Tissue and Bioconcentration Factor

The concentration of Y in uncontaminated seawater samples was below LOQ regardless of the temperature and salinity tested. The concentration of seawater samples taken immediately after Y introduction in the aquaria was around 7.00 ± 0.35 µg/L at salinity 30 and 22 °C, 6.89 ± 0.85 µg/L at salinity 20 and 17 °C, 6.40 ± 0.13 at salinity 30 and 17 °C, and 6.76 ± 0.41 at salinity 40 and 17 °C, being slight lower than the chosen nominal concentration (10 µg/L).

The levels of Y measured in the seawater from positive controls at 0 and 168 h after spiking indicated less than 9 % losses regardless of the temperature and salinity tested. For this reason, Y concentration was considered stable for at least a week under the adopted conditions.

At the end of the 28 days experimental period, Y was detected in the mussels' tissues regardless of the treatment. For uncontaminated organisms, Y content was significantly lower under salinity 40 compared to those under salinity 20 (Table 7). Additionally, Y-exposed mussels exhibited significantly higher (approximately 2-5 fold) Y content than uncontaminated ones (Table 9). Nevertheless, no significant differences in Y content were observed among the different treatments for Y-exposed mussels (Table 9). The BCF of Y-exposed mussels ranged from 26 to 34 L/kg, with no significant differences among the tested treatments.

Table 9. Yttrium (Y) concentration (µg/g dry weight) in mussels' soft tissues and bioconcentration factor (BCF, L/Kg) after 28 days of exposure at two different temperatures, three salinities and two different concentrations of Y (0 and 10 µg/L). Values are means with standard deviations (±) of (n = 3) measures. Different lowercased letters for Y in mussels' tissue or uppercased letters for BCF represent significant differences among tested concentrations in the respective columns.

[Y] (µg/L)	Temperature (ºC)	Salinity	Mussels tissues (µg/g DW)	BCF (L/Kg)
0	17	20	0.045 ± 0.005^{a}	-
		30	$0.056 \pm 0.037^{a,b}$	-
		40	0.025 ± 0.008^{b}	-
	22	30	$0.073 \pm 0.037^{a,b}$	-
10	17	20	0.210 ± 0.061^{c}	30.3 ± 8.8^{A}
		30	0.226 ± 0.040^{c}	34.2 ± 6.1^{A}
		40	0.179 ± 0.025^{c}	25.8 ± 3.6^{A}
	22	30	0.217 ± 0.068^{c}	31.4 ± 9.8^{A}

Metabolic Capacity and Energy Reserves

At a control temperature of 17 °C, the ETS activity of uncontaminated organisms was significantly lower at salinity 40, while no significant changes were observed in Y-exposed mussels (Figure 44A). At control salinity of 30, uncontaminated organisms under 22 °C exhibited significantly lower ETS activity compared to those kept at 17 °C, while Y-exposed mussels displayed the opposite response (Figure 44A). Between uncontaminated and contaminated organisms, Y-exposed mussels at control salinity (30) and 22 °C displayed significantly higher ETS activity, showing the opposite response at 17 °C under the same salinity (30) (Figure 44A).

At a control temperature of 17 °C, the GLY content of uncontaminated organisms was significantly lower at salinities of 20 and 40. Similarly, Y-exposed mussels also exhibited significantly reduced GLY content at these extreme salinities, with organisms at a salinity of 20 displaying a significantly lower content than those at a salinity of 40 (Figure 44B). At control salinity of 30, uncontaminated organisms under 22 °C demonstrated significantly lower GLY content, while Y-exposed mussels showed the opposite response (Figure 44B). Additionally, between uncontaminated and contaminated organisms, Y-exposed mussels at salinity 30 and 22 °C displayed significantly higher GLY

content, showing the opposite response at 17 °C under the same salinity (30) (Figure 44B).

In terms of PROT content, it remained similar across treatments and was unaffected by Y exposure or physical parameters of temperature and salinity (Figure 44C).

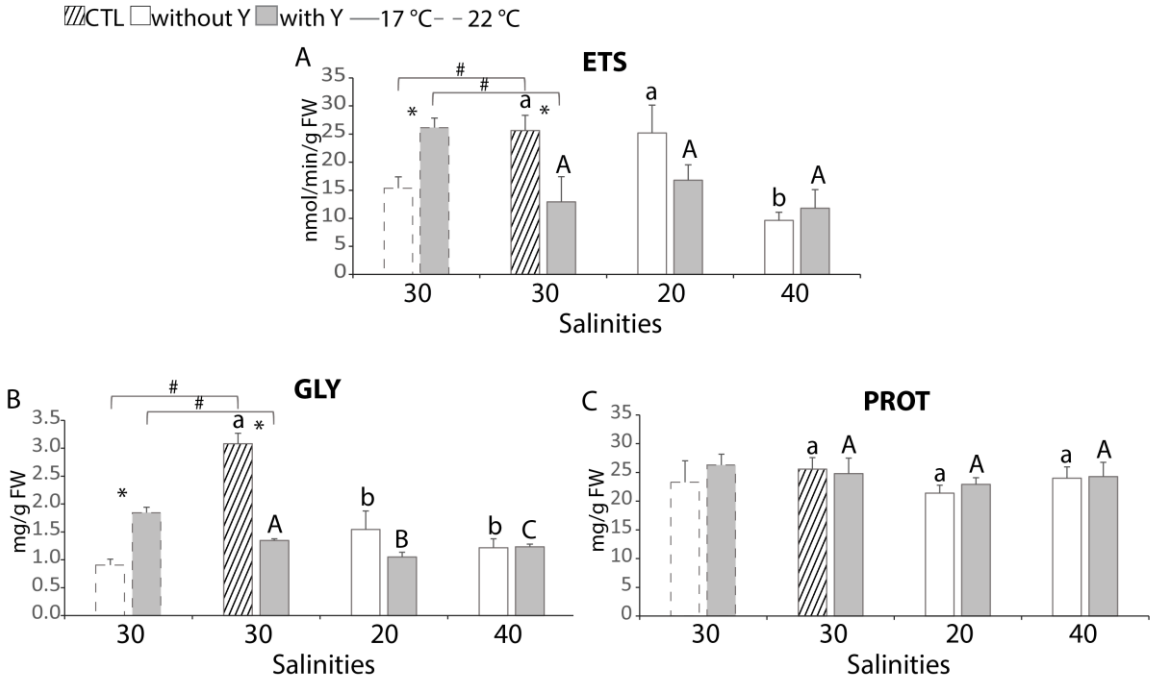

Figure 44. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Protein (PROT) content, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and yttrium (Y)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Y-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Antioxidant and Biotransformation Capacity

At control temperature of 17 °C, the SOD activity of uncontaminated organisms was significantly higher at salinity 20 and significantly lower at salinity 40, while Y-exposed organisms showed only significantly higher activity at salinity 20 (Figure 45A). At control salinity of 30, uncontaminated and contaminated organisms did not show significant

differences between temperatures (Figure 45A). Between uncontaminated and contaminated organisms, Y-exposed mussels showed a significant increase in SOD activity regardless of the temperature and salinity tested (Figure 45A).

At control temperature of 17 °C, the CAT activity of uncontaminated mussels did not show significant differences, while Y-exposed mussels showed significantly higher activity under salinity 20 (Figure 45B). At control salinity of 30, uncontaminated and contaminated organisms did not show significant differences between temperatures (Figure 45B). Nevertheless, between uncontaminated and contaminated organisms, Y-exposed mussels showed a significant increase in CAT activity regardless of the temperature and salinity tested (Figure 45B).

At control temperature of 17 °C, the GR activity of uncontaminated organisms was significantly higher at salinity 20 compared to those at salinity 40, while Y-exposed mussels exhibited significantly lower values at salinity 40 (Figure 45C). At control salinity of 30, uncontaminated and contaminated organisms did not show significant differences between temperatures (Figure 45C). Between uncontaminated and contaminated organisms, Y-exposed mussels under salinity 40 at 17 °C displayed significantly higher GR activity (Figure 45C).

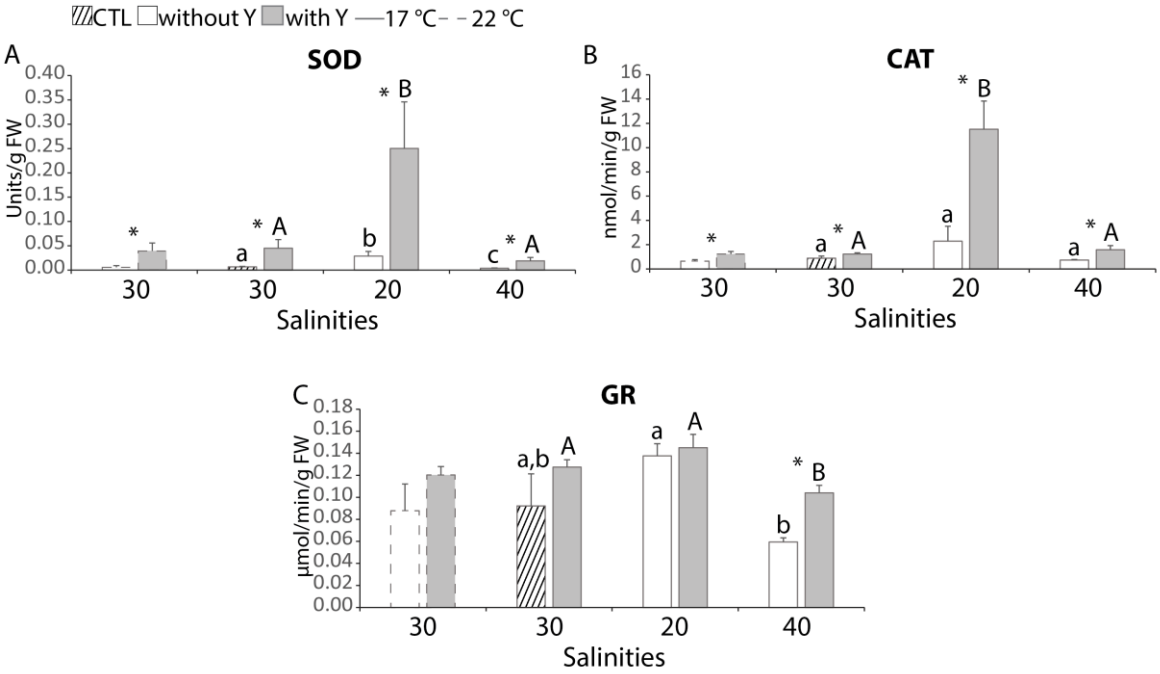

Figure 45. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione reductase (GR) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and yttrium (Y)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Y-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

At control temperature of 17 °C, the GSTs activity of both uncontaminated and Y-exposed organisms was significantly lower at salinity 40 with contaminated mussels also displaying significantly higher activity at the salinity 20 (Figure 46A). At control salinity of 30, uncontaminated organisms at 22 °C exhibited significantly lower GSTs activity compared to those kept at 17 °C, while Y-exposed mussels displayed the opposite response (Figure 46A). Between uncontaminated and contaminated organisms, Y-exposed mussels at salinity 30 and 22 °C as well as the ones at salinity 20 and 17 °C significantly increased their GSTs activity (Figure 46A).

At control temperature of 17 °C, the CbEs activity of uncontaminated organisms was significantly higher at salinity 20 compared to those at salinity 40, while Y-exposed mussels displayed significantly higher activity at salinity 20 and significantly lower at salinity 40 (Figure 46B). At control salinity of 30, uncontaminated and contaminated organisms did not show significant differences between temperatures (Figure 46B).

Between uncontaminated and contaminated organisms, only Y-exposed mussels under salinity 20 and 17 °C increased significantly their CbEs activity (Figure 46B).

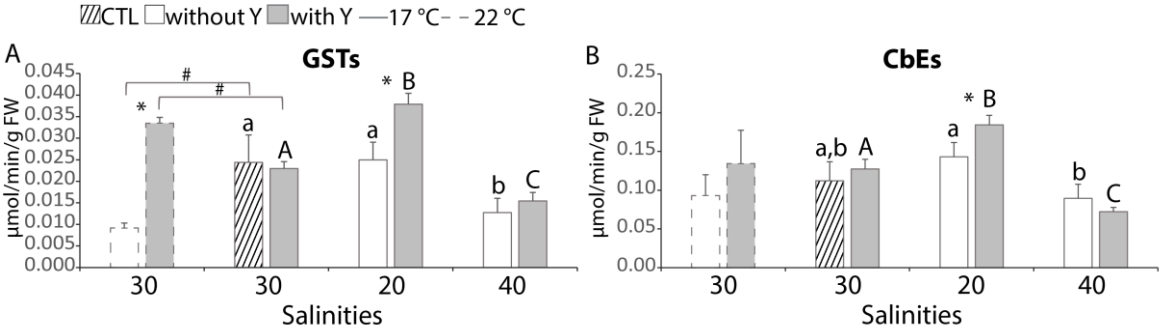

Figure 46. A: Glutathione *S*-transferases (GSTs) activity; B: Carboxylesterases (CbEs) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and yttrium (Y)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Y-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Oxidative Damage

At control temperature of 17 °C, the LPO levels of uncontaminated organisms were significantly lower at salinity 20 than those at salinity 30, while no significant changes were observed in Y-exposed mussels (Figure 47A). At control salinity of 30, uncontaminated organisms at 22 °C showed significantly lower LPO levels than those kept at 17 °C (Figure 47A). Between uncontaminated and contaminated organisms, Y-exposed mussels under salinity 30 regardless of the temperature and the ones at salinity 20 at 17 °C showed significantly higher LPO levels (Figure 47A).

At control temperature of 17 °C, the PC levels of uncontaminated organisms were significantly higher at salinity 40, while Y-exposed mussels did not display significant alterations (Figure 47B). At control salinity of 30, Y-exposed mussels at 22 °C exhibited significantly lower PC levels than those kept at 17 °C (Figure 47B).

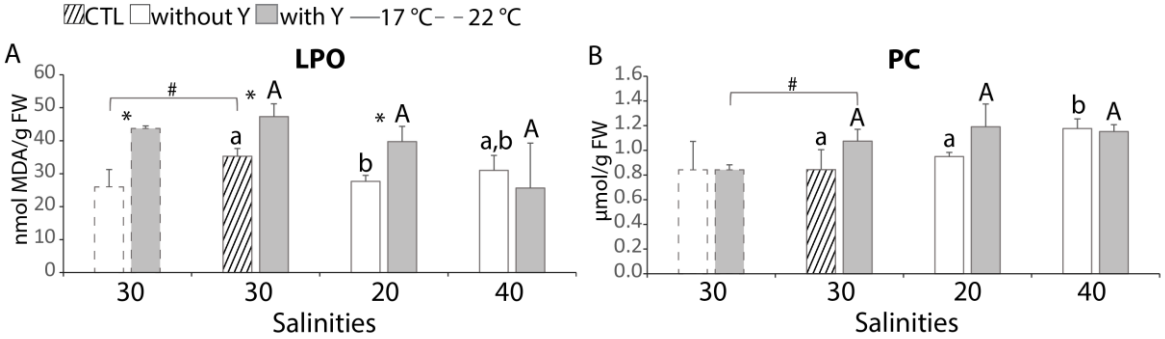

Figure 47. A: Lipid peroxidation (LPO) levels; B: Protein carbonylation (PC) levels, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and yttrium (Y)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Y-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Neurotoxicity

At control temperature of 17 °C, the AChE activity of uncontaminated organisms was significantly higher at salinity 20 compared to those at salinity 40, while Y-exposed mussels exhibited significantly higher activity at salinity 20 than those at salinities 30 and 40 (Figure 48). At control salinity of 30, Y-exposed mussels at 22 °C displayed higher AChE activity than those kept at 17 °C (Figure 48). Between uncontaminated and contaminated organisms, Y-exposed mussels at salinity 30 and 22 °C as well at salinities 20 and 40 under 17 °C showed significantly higher AChE activity (Figure 48).

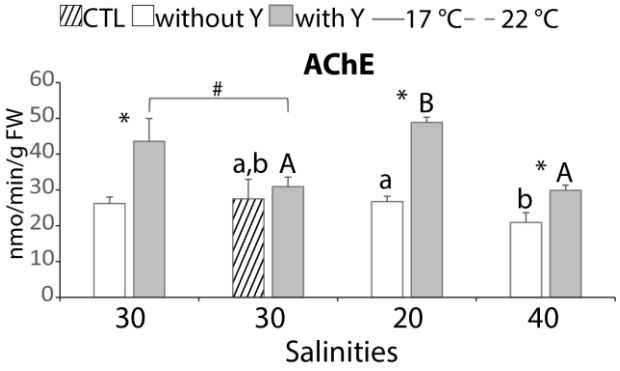

Figure 48. Acetylcholinesterase (AChE) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and yttrium (Y)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Y-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Integrated Biomarker Response Index

The IBRvs2 index was considered at both control temperature (17 °C) and control salinity (30). The highest IBRvs2 values were observed at 17 °C in Y-exposed organisms under decreased salinity (17Y20, IBRvs2 = 18.8), followed by both uncontaminated and Y-exposed mussels under increased salinity (17CTL40, IBRvs2 = 12.6 and 17Y40, IBRvs2 = 12.4), indicating a more pronounced impact on organisms under reduced and elevated salinity, particularly in the presence of Y (Figure 49). In the case of decreased salinity in Y-exposed mussels, the reduction of GLY reserves, induction of antioxidant and biotransformation enzymes, increased PC levels and AChE activity were the determining factors for such a high score. For increased salinity in both non-contaminated and Y-exposed mussels, the factors contributing for its score were mainly the reduction of ETS activity and GLY reserves, as well as, the increase of PC levels. The results also showed that the remaining treatments scored similar and low IBRvs2 values, such as at salinity 30, with non-contaminated and Y-exposed organisms under increased temperature (22CTL30, IBRv2= 10.0 and 22Y30, IBRv2 = 7.9) or with Y-exposed mussels at 17 °C (17Y30, IBRv2 = 10.4), as well non-contaminated mussels at salinity 20 under 17 °C (17CTL20, IBRvs2 = 9.5), resulted of small variations among the biochemical indicators (Figure 49).

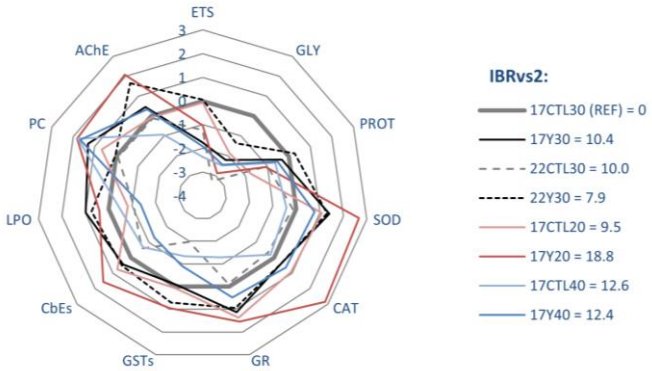

Figure 49. Integrated Biomarker Response (IBRvs2) index considering all biochemical parameters used on *Mytilus galloprovincialis* after 28 days under different treatments: uncontaminated mussels under 17 °C at salinities 30 (17CTL30, REF), 20 (17CTL20) and 40 (17CTL40); yttrium (Y)-exposed mussels under 17 °C at salinities 30 (17Tb30), 20 (17Tb20) and 40 (17CTL40); uncontaminated mussels under salinity 30 at 22 °C (22CTL30); and Y-exposed mussels salinity 30 at 22 °C (22Tb30). ETS = Electron transport system; GLY = Glycogen; PROT = Protein; SOD = Superoxide dismutase; CAT = Catalase; GR = Glutathione reductase; GSTs = Glutathione *S*-transferases; CbEs = Carboxylesterases; LPO = Lipid peroxidation; PC = Protein carbonylation; AChE = Acetylcholinesterase.

3.3.4. Terbium Exposure

This section presents a comparison of mussel responses to Tb exposure under climate change scenario. It presents the results related to the concentration of Tb in mussel tissues, the bioconcentration factor, and various biomarkers. These biomarkers include those for metabolic capacity, energy reserves, antioxidant and biotransformation capacity, oxidative damage and cellular redox status, and neurotoxicity following exposure to Tb under changes of salinity or increased temperature. Together with the previously data of the present subchapter, it offers an opportunity to contrast the effects of salinity or temperature in the impacts of different rare-earth elements.

The results presented in this section are published as:

- Andrade, M., Pinto, J., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. How predicted climate changes will modulate the impacts induced by terbium in bivalves? Chemosphere. https://doi.org/10.1016/j.chemosphere.2024.141168.

Terbium Concentration in Mussels' Tissue and Bioconcentration Factor

The concentration of Tb in uncontaminated seawater samples was below LOQ regardless of temperature and salinity conditions. The concentration of seawater samples taken immediately after Tb introduction in the aquaria was around 9.38 ± 1.39 µg/L at 22 °C and salinity 30, of 9.63 ± 0.49 µg/L at 17 °C and salinity 20, of 9.70 ± 1.89 at 17 °C and salinity 30, and of 9.33 ± 0.81 at 17 °C and salinity 40, thus, being close to the adopted nominal concentration (10 µg/L).

The levels of Tb measured in the seawater from positive controls at 0, 24, 48, 72, 144 and 168 h after spiking (Table 10), showed coefficients of variation that were lower than 14 % regardless of the temperature and salinity levels. For this reason, Tb concentration was considered stable for at least a week under the adopted conditions.

Table 10. Terbium concentration (µg/L) measured in seawater from positive controls at 0, 24, 48, 72, 144 and 168 h after its introduction in the medium along the experimental period at two different temperatures and three different salinities.

Sampling time (h)	22 ºC, salinity 30	17 ºC, salinity 30	17 ºC, salinity 20	17 ºC, salinity 40
0	9.30	7.95	10.20	7.95
24	8.50	10.95	9.40	9.90
48	8.70	9.90	8.20	9.15
72	8.40	10.50	8.20	10.05
144	7.00	8.55	7.60	8.25
168	6.90	7.95	9.40	8.70

At the end of the 28 days experimental period, Tb was detected in the mussels' tissues under all conditions except for uncontaminated mussels at salinity 20. No significant differences were seen among uncontaminated organisms nor among those exposed to Tb at the different experimental treatments, nevertheless, the concentrations found in Tb-exposed mussels were significantly higher (about 38-47 fold) than those uncontaminated (Table 11). The BCF varied between 29 and 44 L/Kg, with no significant differences among tested conditions either (Table 11).

Table 11. Terbium (Tb) concentration (µg/g dry weight) in mussels' soft tissues and bioconcentration factor (BCF, L/Kg) after 28 days of exposure at two different temperatures, three salinities and two different concentrations of Tb (0 and 10 µg/L). Values are means with standard deviations (±) of (n = 3) measures. Different lowercased letters for Tb in mussels' tissue or uppercased letters for BCF represent significant differences among tested concentrations in the respective columns.

[Tb] (µg/L)	Temperature (ºC)	Salinity	Mussels tissues (µg/g DW)	BCF (L/Kg)
0	17	20	< LOQ	-
		30	0.006 ± 0.003^{a}	-
		40	0.007 ± 0.002^{a}	-
	22	30	0.009 ± 0.002^{a}	-
10	17	20	$0.420 \pm 0.143^{b,c}$	44.3 ± 15.7^{A}
		30	$0.274 \pm 0.026^{b,c}$	29.5 ± 3.8^{A}
		40	0.264 ± 0.017^{b}	28.8 ± 1.9^{A}
	22	30	0.398 ± 0.080^{c}	42.9 ± 14.1^{A}

Metabolic Capacity and Energy Reserves

At a control temperature of 17 °C, the ETS activity of uncontaminated organisms did not differ significantly among different salinities; however, Tb-exposed mussels showed significantly higher activity at salinity 40 compared to salinity 30 (Figure 50A). At salinity 30, both uncontaminated and Tb-exposed organisms at 22 °C experienced significantly higher ETS activity in comparison with the ones maintained at 17 °C (Figure 50A).

At a control temperature of 17 °C, the GLY content among uncontaminated organisms was significantly lower at salinity 20, while Tb-exposed mussels showed significantly higher GLY levels at extreme salinities of 20 and 40 (Figure 50B). At control salinity of 30, uncontaminated organisms at elevated 22 °C presented significantly lower GLY reserves in comparison with the ones at 17 °C (Figure 50B). Between uncontaminated and contaminated organisms, those Tb-exposed under salinity 30 demonstrated significantly lower GLY reserves while the opposite trend was seen under salinity 20 (Figure 50B).

At a control temperature of 17 °C, the PROT content of uncontaminated and Tb-exposed organisms was significantly lower at the salinity of 20 and significantly higher at a salinity of 40 (Figure 50C). At salinity control of 30, Tb-exposed organisms at 22 °C showed significantly higher PROT content in comparison with the ones maintained at 17 °C (Figure 50C).

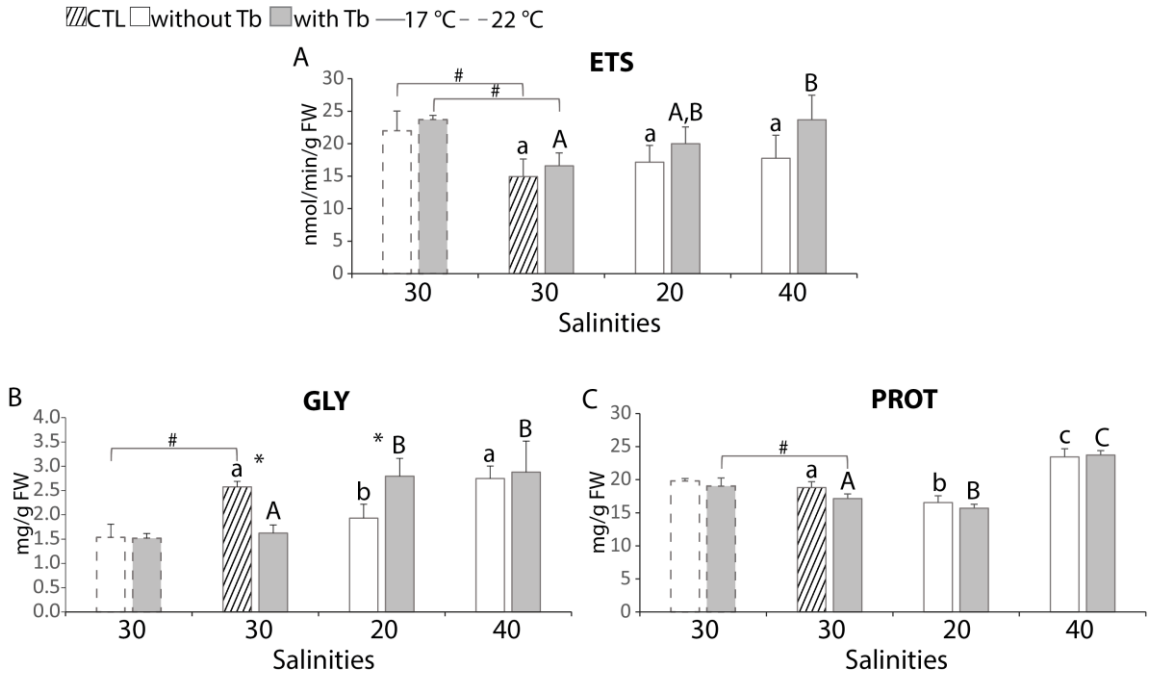

Figure 50. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Protein (PROT) content, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and terbium (Tb)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Y-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Antioxidant and Biotransformation Capacity

At a control temperature of 17 °C, the SOD activity of uncontaminated and Tb-exposed organisms did not differ significantly among salinities (Figure 51A). At the control salinity of 30, uncontaminated and contaminated organisms did not show significant differences between temperatures (Figure 51A). However, contrasts between uncontaminated and contaminated organisms, indicated that Tb-exposed mussels had significantly lower SOD activity under control salinity of 30 at elevated temperature (22 °C) but also under enhanced salinity of 40 at the control temperature of 17 °C (Figure 51A).

At a control temperature of 17 °C, the CAT activity observed in uncontaminated organisms did not differ significantly among different salinities at the temperature of 17 °C; however Tb-exposed mussels showed significantly higher activity at salinity 20 compared

to control salinity of 30 (Figure 51B). At salinity 30, Tb-exposed organisms at 22 °C had significantly higher CAT activity in comparison with the ones at 17 °C (Figure 51B). Between uncontaminated and contaminated organisms, Tb-exposed mussels under salinity 30 showed significantly higher CAT activity at 22 °C and significantly lower activity at 17 °C (Figure 51B).

At a control temperature of 17 °C, the glutathione peroxidase (GPx) activity in uncontaminated organisms did not differ significantly among salinities, while in those Tb-exposed higher activity was observed at salinities 20 and 40 (Figure 51C). At a control salinity of 30, Tb-exposed organisms at 22 °C had significantly higher GPx activity in comparison with the ones at 17 °C (Figure 51C). Tb-exposed mussels under control conditions of salinity 30 at temperature 17 °C had significantly decreased GPx activity (Figure 51C).

At a control temperature of 17 °C, the GR activity in uncontaminated mussels did not differ significantly among salinities; however, Tb-exposed mussels showed significantly higher activity at salinities 20 and 40 (Figure 51D). At salinity 30, uncontaminated and Tb-exposed organisms at 22 °C had significantly lower GR activity in comparison with the ones at the control temperature of 17 °C (Figure 51D). Between uncontaminated and contaminated mussels, those exposed under salinity of 30 at both tested temperatures showed significantly lower GR activity values while under salinity of 40 this activity was significantly enhanced (Figure 51D).

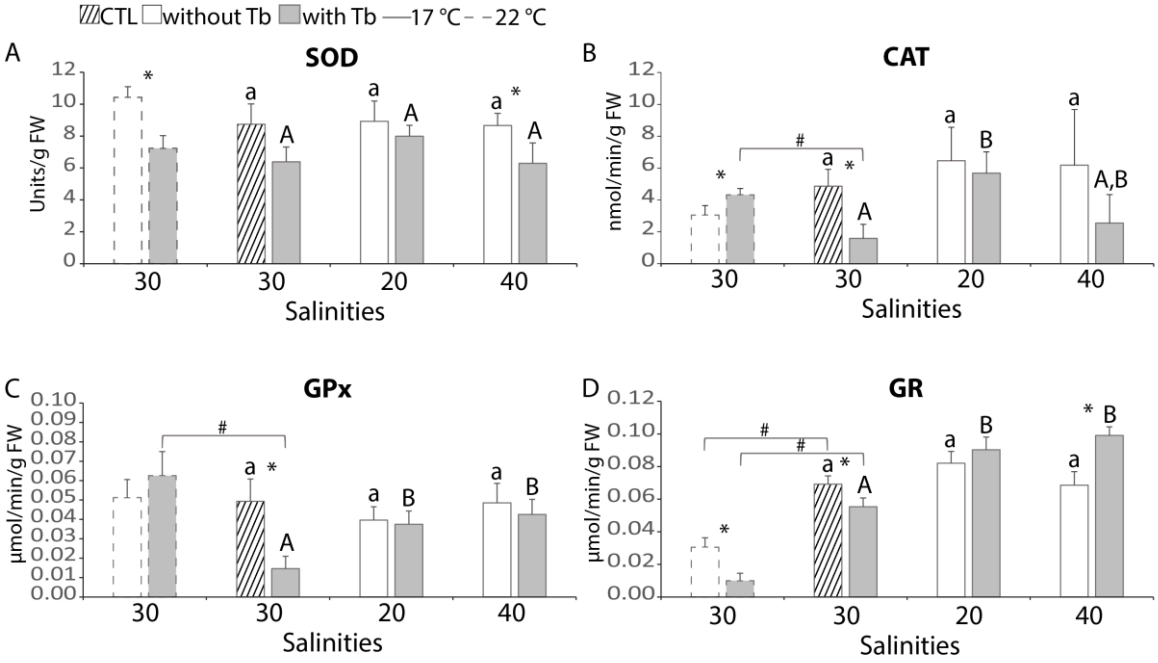

Figure 51. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C: Glutathione peroxidase (GPx) activity; Glutathione reductase (GR) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and terbium (Tb)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Y-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

At a control temperature of 17 °C, the GSTs activity in uncontaminated organisms did not differ significantly among salinities at the control temperature of 17 °C, while in Tb-exposed mussels significantly higher activity was registered at the lower salinity of 20 compared to 40 but not in respect to the control salinity of 30 (Figure 52A). At this salinity of 30, uncontaminated and contaminated organisms did not show significant differences according to temperatures (Figure 52A). However, Tb-exposed mussels under salinity 30 at 22 °C as well as the ones under salinity 40 at 17 °C presented significantly higher GSTs activity (Figure 52A).

In terms of CbEs activity, this activity remained similar across conditions and was unaffected by Tb exposure or physical parameters of temperature and salinity (Figure 52B).

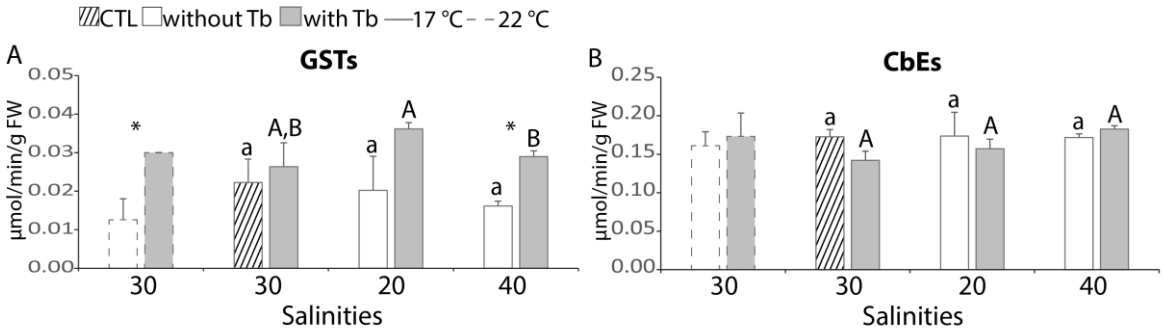

Figure 52. A: Glutathione *S*-transferases (GSTs) activity; B: Carboxylesterases (CbEs) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and terbium (Tb)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Y-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Oxidative Damage and Cellular Redox Status

At the temperature of 17 °C, the LPO levels in both uncontaminated and Tb-exposed organisms at salinity 40 were significantly higher than those at salinity 20 and 30 (Figure 53A). At the control salinity of 30, Tb-exposed organisms at 22 °C also showed significantly higher LPO levels in comparison with the ones at the control temperature of 17 °C (Figure 53A). Tb-exposed mussels under 22 °C exhibited significantly higher LPO levels than their non-exposed counterpart (Figure 53A).

At the temperature of 17 °C, the PC levels in uncontaminated mussels were significantly higher at salinities 20 and 40; however, no significant changes were observed in Tb-exposed mussels at the different salinities tested (Figure 53B). At salinity of 30, uncontaminated and Tb-exposed organisms at elevated temperature (22 °C) exhibited significantly lower PC levels in comparison with the ones at the control temperature of 17 °C (Figure 53B). Tb-exposed mussels under the 22 °C treatment significantly increased their PC levels while decreasing in those exposed under salinity of 40 at 17 °C (Figure 53B).

At the temperature of 17 °C, the GSH:GSSG ratio in uncontaminated organisms was significantly higher ratio at the salinity of 20 compared to those maintained the salinity of 40. Tb-exposed mussels showed a significantly lower ratio under the salinity of 40 compared to the control of 30 (Figure 53C). Uncontaminated and contaminated mussels

did not show significant differences between both tested temperatures regardless of the salinity (Figure 53C).

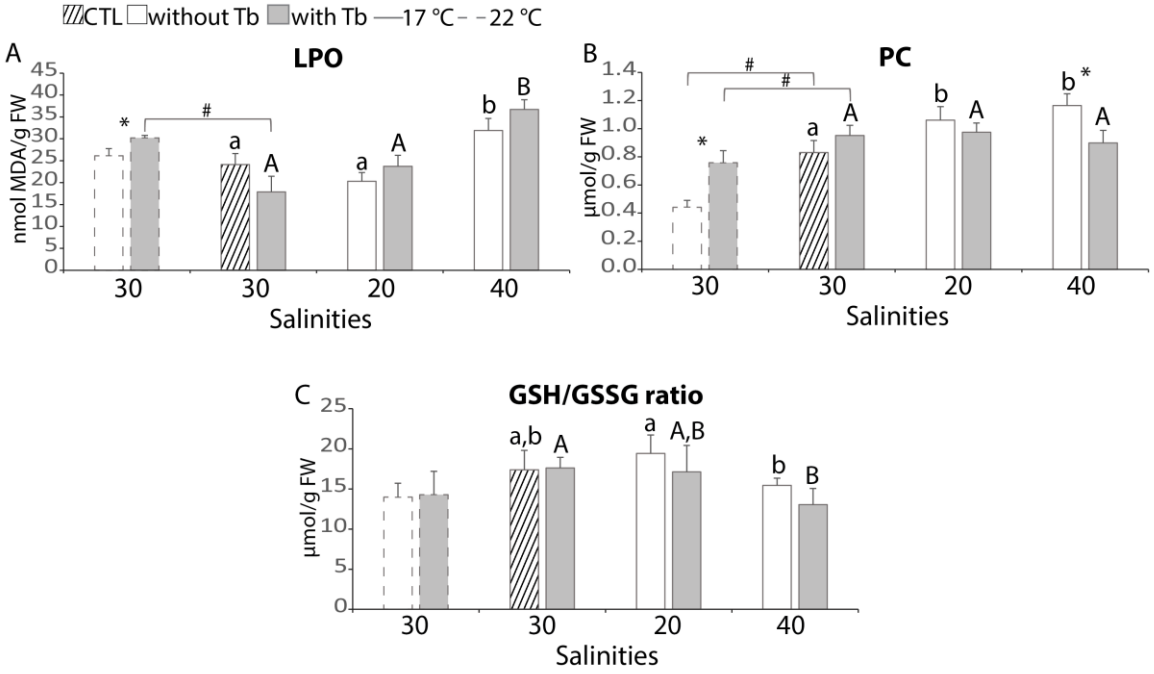

Figure 53. A: Lipid peroxidation (LPO) levels; B: Protein carbonylation (PC) levels; C: Reduced (GSH) and oxidized (GSSG) glutathione ratio (GSH:GSSG ratio), in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and terbium (Tb)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Y-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

Neurotoxicity

At the temperature of 17 °C, the AChE activity in both uncontaminated and Tb-exposed organisms was significantly higher at the salinity of 40 (Figure 54). At salinity control (30), uncontaminated and Tb-exposed mussels under increased temperature (22 °C) displayed significantly higher AChE activity in comparison with the ones at 17 °C (Figure 54). Tb-exposed mussels under salinity 30 at 17 °C exhibited significantly lower AChE activity than the non-exposed group (Figure 54).

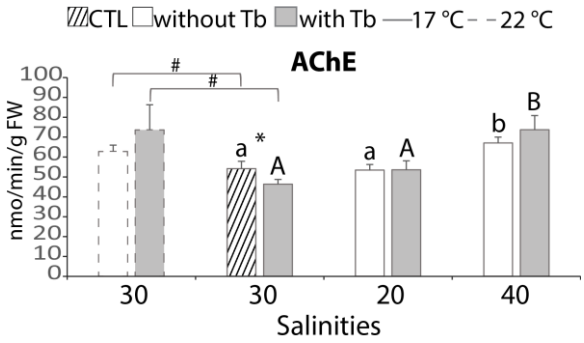

Figure 54. Acetylcholinesterase (AChE) activity, in *Mytilus galloprovincialis* under different conditions for 28 days: uncontaminated and terbium (Tb)-exposed mussels at salinities 30, 20 and 40 at 17 °C, uncontaminated and Y-exposed mussels at temperature 22 °C under salinity 30. Results are means with standard deviations. Significant differences ($p < 0.05$) among salinity treatments at 17 °C are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). Differences among temperature treatments at salinity 30 are identified with the symbol #. For each treatment, differences between contaminated and non-contaminated mussels are identified by an asterisk.

CHAPTER 4. DISCUSSION AND CONCLUSIONS

4.1. Intertidal *Versus* Subtidal Mussels' Exposure

The discussion presented in this section corresponds to the main findings of the following publication:

- Andrade, M., Rivera-Ingraham, G., Soares, A.M.V.M., Rocha, R.J.M, Pereira, E., Solé, M., Freitas, R., 2021. How do life-history traits influence the fate of intertidal and subtidal *Mytilus galloprovincialis* in a changing climate? Environ. Res. 196, 110381. https://doi.org/10.1016/j.envres.2020.110381.

4.1.1. Responses of Intertidal Mussels to Submersion

The results obtained suggested that intertidal organisms would not be negatively impacted by longer submersions periods as a consequence of the predicted climate-change scenario. These findings although not surprising are confirmatory, since permanent submerged conditions are likely less stressful than variable tidal regimes. This conclusion is supported by the higher reduced (GSH): oxidized (GSSG) glutathione ratio (a good indication of the organisms' redox homeostasis, *e.g.*, Pinto et al., 2019; Freitas et al., 2019a,d) shown by intertidal organisms when maintained submersed (IS), confirming a lower oxidative status in this group. This was further supported by the absence of oxidative damage (lipid peroxidation, LPO and protein carbonylation, PC) in the two intertidal groups (IS and IT). Intertidal conditions are specially challenging, not only because of the reactive oxygen species (ROS)-burst caused by reperfusion (as it will be further discussed below), but also because of hypoxia, which can also cause through other non-aerobic pathways oxidative stress by itself, as well. Under air exposure, an increase of LPO was observed in *Mytilus galloprovincialis* (Andrade et al., 2018) and in *Perna perna* (Almeida et al., 2005). Furthermore, an increase of PC levels was also observed in the same species of mussels under hypoxia (Woo et al., 2011). This has been attributed to the increase of ROS signals mediating the enhancement of the antioxidant defenses to counteract the deleterious effects of reoxygenation in hypoxia tolerant species (Hermes-Lima et al., 2015). Nevertheless, the most challenging event of a hypoxic period is the reoxygenation: the return to submerged (or normoxic) conditions, which increases ROS production and results, when the defense mechanisms are overwhelmed, in the oxidation of lipids (Catalá, 2009; Regoli and Giuliani, 2014) and proteins (Cattaruzza and Hecker, 2008; Suzuki et al., 2010). These results may further explain the reduced protein (PROT) content in the submerged group which, on the other hand, was increased under tidal conditions in an attempt to prevent cellular damage. This agrees with the study by

Andrade et al. (2018), in which air-exposed *M. galloprovincialis* showed higher PROT content than those maintained always submerged. In addition, higher glycogen (GLY) reserves under submersion could further indicate that this energy source was not used to fuel up the oxidation of GSH. Nevertheless, activities of superoxide (SOD) and catalase (CAT, to a lower extend) increased under submersion. This suggests that despite being submerged, intertidal mussels may, to a certain extent, still maintain a durable preparative defense. Similarly, Clark et al. (2018) observed that intertidal limpets acclimated during longer periods (9 months) to submersed conditions, still maintained several “classical” stress genes upregulated.

4.1.2. Responses of Subtidal Mussels to Emersion

An increased frequency of hypoxic events in the context of global climate change will certainly challenge subtidal organisms. It is well known that subtidal organisms manifest lower tolerance to air exposure or hypoxia in comparison with their intertidal conspecifics. This trend has been proved in *M. galloprovincialis* (Tagliarolo et al., 2012) and *Mytilus edulis* (Altieri, 2006; Tagliarolo et al., 2012) but also in the oyster *Crassostrea gigas* (Meng et al., 2018). Nevertheless, the present results suggest that subtidal mussels will likely be able to successfully cope with a future increase in anoxia/hypoxia events and avoid oxidative damage, at least within the time scale tested in our experimental trial. The challenge will be mainly of an energetic nature, as evidenced by the fact that submersed organisms exposed to tides enhanced significantly their electron transport system (ETS) activity. Similar results were seen formerly by Andrade et al. (2018, 2019b) who suggested that mussels increased their metabolic capacity as an adaptation to re-establish their physiological and biochemical performance after enduring anoxia. Additionally, the metabolic capacity of subtidal organisms exposed to tides (ST) seemed to rely on GLY reserves in a greater extent than intertidal ones, as evidenced by a larger difference in GLY content between ST and SS than between IT and IS mussels. Energy reserves may be affected by general physiological stressors (Scott-Fordsmand and Weeks, 2000), but it is also known that bivalve’s ability to survive under prolonged anoxia is largely dependent on the use of GLY in anaerobic metabolic pathways (De Zwaan, 1983; De Zwaan and Zandee, 1972; Hochachka and Mustafa, 1972; Korycan and Storey, 1983). Meng et al. (2018) found that subtidal and intertidal oysters would initiate anaerobic glycolysis metabolism under air exposure. This would explain the high GLY content present in submerged organisms and their consumption when exposed to a tidal regime.

These hypoxia-induced energetic expenditures may have been intended, at least in part, i) to fuel up the GSH oxidation through glutathione peroxidase (GPx) activity, observed by lower GSH:GSSG ratio, and ii) to enhance antioxidant SOD and CAT activities. Such strategies may confer protection against the ROS formation derived from the hypoxia/reoxygenation events given the lower LPO levels shown by the subtidal mussels exposed to tides. Similarly, Teixeira et al. (2013) observed that when emerged, soft corals *Veretillum cynomorium* increased their antioxidant defenses which likely helped maintaining LPO levels low. When under anoxic conditions, Pannunzio and Storey (1998) also demonstrated that periwinkles *Littorina littorea* increased their antioxidant defenses and strongly suppressed the formation of lipid hydroperoxides while, during recovery, they increased their GSH:GSSG ratio.

4.1.3. Influence of Mussels Life-History Traits on Energy Budget and Acclimation Strategies

The observation of mussels' tidal groups under their respective original natural conditions (IT and SS) allows confirmation that life-history traits influence animal energy allocation. While both groups expressed similar ETS activity (which probably reflects the natural background activity for the species), energy reserves and their use clearly discriminates between them. Subtidal organisms maintained always submersed (SS) had higher contents of GLY and PROT reserves than intertidal organisms under a tidal regime (IT). Even if the expenditure of GLY in IT mussels may be related to anaerobic respiration, life-history may still play a role on accounting for these differences, given that IS had lower GLY and PROT content than SS. This difference in the energy budget may also be explained by a greater feeding capacity in organisms permanently submersed, contributing also to a natural increase of in their stored energy reserves. Arranz et al. (2016) observed that subtidal *M. galloprovincialis* possessed a greater energy budget than intertidal ones, with the latter showing reduced growth efficiency due to reduced feeding time. Furthermore, and in accordance with the observed differences in the energy budget, Freites et al. (2002) reported differences in shell morphology, physiological energetics, and fatty acid profiles between *M. galloprovincialis* from two habitats. Having more energetic resources available positively influenced redox homeostasis in terms of a better antioxidant status (*i.e.*, high GSH:GSSG ratio) in the subtidal group although it did not affect the enzymatic antioxidant defenses (SOD and CAT). Despite this, the subtidal organisms could not prevent the occurrence of oxidative damage, while the intertidal

mussels did. This could also be attributed to the more regular feeding of the subtidal mussels, which could influence tissue lipid composition, as suggested by elevated PROT and GLY reserves in the subtidal group. Anyhow, the differences in the biochemical background between intertidal and subtidal mussels may contribute to their distinct response upon a challenge of similar nature.

4.1.4. Strategies in Intertidal and Subtidal Mussels to Deal with Hypoxia

Altogether, the present findings showed that both intertidal and subtidal mussels are capable of dealing with the challenges of hypoxia, despite their different particular backgrounds. One common feature in both intertidal and subtidal mussels is the lower GSH:GSSG ratio under tidal conditions (IT and ST) than those under submergence (IS and SS). This suggests the usage of defense enzymes that require GSH (*i.e.*, glutathione reductase, GR, glutathione *S*-transferases, GSTs and GPx) under hypoxia/air exposure, as indicated by a depleted GSH content. Various authors have demonstrated that hypoxic conditions can increase the antioxidant enzyme defenses in mussels such as *M. galloprovincialis* (Andrade et al., 2018; Woo et al., 2011) and *P. perna* (Almeida and Bainy, 2006); and in barnacles such as *Balanus amphitrite* (Desai and Prakash, 2009). However, other metabolic strategies used to achieve a successful response to hypoxia varied between intertidal and subtidal mussels, probably due, as previously discussed, to the differences in energy budget between these two groups under submergence (IS and SS). For the case of intertidal organisms, they are capable of avoiding oxidative damage, likely due to the induction of antioxidants (possibly in SOD, as seen in the IS). However, the response of antioxidant defenses (presumably aiming to prevent oxidative damage) was not registered for the IT organisms in the present study probably due to the fact that mussels were sacrificed immediately after air exposure. It is known that hypoxia-tolerant animals (such as intertidal mussels) are often capable of upregulating certain antioxidants during the first hours of hypoxic exposure, despite a likely decrease in metabolic rates. This has been interpreted as an anticipative response to the ROS burst occurring upon re-oxygenation (Hermes-Lima et al., 2015) and has been termed as the “Preparation for Oxidative Stress” (POS) theory (Hermes-Lima et al., 2015; Giraud-Billoud et al., 2019). This is a mechanism usually associated to (environmental or functional) hypoxia-tolerant organisms and it may be here applicable to the intertidal group as supported by a lower ETS activity in IT that in ST. In contrast, subtidal organisms faced the intertidal challenge not just by increasing SOD but also CAT activities, which would presumably be more

energetically costly, as confirmed by an increased ETS activity and a drop in GLY content. Also, unlike intertidal organisms, these changes occurred well before the tidal exposure, hence the observed increase in antioxidants, despite animals being sacrificed during the first minutes of air exposure. Altogether, the obtained results suggest that subtidal organisms also prepare for the air-exposure challenge but probably not through the same pathways that the POS-theory proposes. In fact, the present study shows a clear link between habitat and adaptation strategy, and despite their differences, these strategies allow both groups to successfully deal with the challenges here tested. This shows an interesting phenotypic plasticity in these two groups which were collected from the same area, but which experienced different conditions (tidal vs subtidal) throughout their lifetime.

Conclusions

Overall, the present study demonstrates that both intertidal and subtidal *M. galloprovincialis* will likely be able to cope with sea-level rise and the increasing frequency of hypoxic events predicted to occur in the context of climate change. However, while the performance of intertidal mussels did not seem to be compromised by submergence, tidal exposure was energetically costly to subtidal organisms, altogether suggesting the influence of former life-history traits on the acclimation processes. Furthermore, the physiological and biochemical mechanisms and their timing differ among tidal groups. That is, to counteract the ROS-burst upon reoxygenation, intertidal organisms would probably increase their antioxidant defenses during the hypoxic transgression. Subtidal organisms, on the other hand, would use their energy reserves to deploy antioxidant defenses before the hypoxia-induced metabolic shutdown. Hypoxic events will increasingly affect water bodies in the context of global warming and increased anthropogenic impact, bringing additional challenges to coastal organisms. Moreover, the different strategies that subtidal and intertidal mussels set to counteract such hypoxic events are efficient and demonstrate good plasticity between groups. This may however still act as a confounding factor with other environmental variables and thus, should be considered in laboratory experimental designs aimed to test the toxicity of targeted chemicals such as REEs.

4.1.5. Final Considerations

The presented section (4.1.) provides a better understanding of the biochemical modifications that long-term (twenty-eight days) submerged conditions and tidal exposure have on intertidal and subtidal groups of *M. galloprovincialis*. The results allowed inferring on the specific challenges that climate change factors alone may exert on each of these groups. For instance, intertidal mussels respond to longer submersions periods by maintaining a durable preparative mechanisms defense under such conditions. Subtidal mussels, on the other hand, are faced with an energetic challenge to avoid oxidative damage when increasingly exposed to hypoxic events. Overall, both intertidal and subtidal mussels are capable to deal with similar stressors although by setting a different array of energetic, acclimation and metabolic strategies, suggesting that both groups have the same ecological origin.

While our study offers significant insights into the biochemical adaptations of *M. galloprovincialis* along the dynamic Atlantic coast of Portugal, it also prompts consideration of its applicability to non-tidal regions like the Mediterranean, where tidal influences are minimal. This highlights the need for caution in extrapolating our results to non-tidal settings and underscores the importance of context-specific research in environmental studies. Nevertheless, choosing intertidal organisms for further exploring the effects of xenobiotics, even in experiments lacking tidal fluctuations, can be considered a wise choice. These organisms possess innate adaptability to a wide range of environmental conditions and are well-prepared for submersion as demonstrated here. With this perspective, the subsequent section investigates the impact of two rare-earth elements, yttrium (Y) and terbium (Tb), on intertidal mussels. This approach contributes to broaden in the context of environmental research the importance of understanding the specific natural life history adaptations of intertidal organisms in the context of pollution, even when tidal fluctuations are not directly relevant.

4.2. Single Rare-Earth Element Exposures

4.2.1. Yttrium Exposure

The discussion presented in this section corresponds to the main findings of the following publication:

- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Threats of Pollutants Derived from Electronic Waste to Marine Bivalves: The Case of the Rare-Earth Element Yttrium. Environ. Toxicol. Chem. 42(1), 166-177. https://doi.org/10.1002/etc.5508.

Bioconcentration of Yttrium in Mussels

The present study confirmed that higher Y exposure concentrations resulted in a greater accumulation of Y in mussel tissue. However, the bioaccumulation factor (BCF) diminished at increasing Y water concentrations, indicating that the mussels tried to limit the accumulation of this element. Available studies on Y accumulation have focused on freshwater, rather than on estuarine and marine environments. For instance, previous studies conducted by Hanana et al. (2018) revealed that when the freshwater mussel *Dreissena polymorpha* was exposed to waterborne Y at 10 µg/L, Y reached a concentration of 0.37 µg/g dry weight (DW), which is very similar to the 0.32 µg/g DW reported in the present study for the same water concentration. However, the accumulation registered in the freshwater species was nearly five times higher (3.43 µg/g DW) at a waterborne Y concentration of 50 µg/L, which contrasts with our results with marine mussels where the 40 µg/L treatment yielded bioconcentration of 0.64 µg/g DW, likely due to Y behavior in two media with different ionic strength. Regarding the ability of mussels to restrict the accumulation of Y, the marine species *M. galloprovincialis* has repeatedly been shown to display this ability for other rare-earth elements (REEs) such as lanthanum (La; Pinto et al., 2019), gadolinium (Gd; Henriques et al., 2019), and neodymium (Nd; Freitas et al., 2020b). In the present study, limited Y accumulation may result from mussels' adaptive physiological responses, such as reducing filtration rate and/or respiration capacity. Although it was not monitored in the present study, it is well known that mussels may close their valves under unfavorable conditions (Anestis et al., 2007; Gosling, 2003), which would restrain the entrance of Y into the organism. In this situation, biomarkers related to respiratory metabolism could suggest the use of these adaptive responses, which would imply a change from aerobic to anaerobic metabolism.

Bivalves are also recognized to sequester foreign chemicals into their shells (see Akagi and Edanami, 2017; Merschel and Bau, 2015; Valdés-Vilchis et al., 2021), and proteins, such as metallothioneins (MTs), are particularly related to metal binding and sequestration. Although MTs are not so investigated in aquatic organisms in terms of REEs exposures, MTs levels were modulated by Gd in the freshwater *mussel D. polymorpha* (Hanana et al., 2017a) and increased in rainbow trout hepatocytes exposed to several REEs including Y (Hanana et al., 2021a). Because Y content was measured in the soft part of the mussels, the first hypothesis on metal storage in the shell could not be confirmed. As for the role of MTs, it cannot justify the decrease in BCF but it may account for the lack of biochemical effects at higher Y concentrations in the present study.

Metabolic Capacity and Energy Reserves

In terms of metabolic capacity, organisms under Y exposure either reduced ETS activity (5 µg/L) or maintained it (10–40 µg/L). This result may indicate that at low Y concentrations organisms can cope with the situation by reducing their aerobic respiration and, thus, restrain Y accumulation. In fact, besides being used as a marker of metabolic activity in marine macrofauna (Cammen et al., 1990), it has been repeatedly proven that a decrease in ETS activity was associated with a reduction in filtration and/or respiration rate, to prevent/limit chemical accumulation in bivalves (see Almeida et al., 2015). In the present study, at higher Y concentrations, ETS activity was maintained at levels similar to control. This is also a mechanism to avoid Y accumulation because enhanced aerobic respiration is expected to face energetic needs during stress. However, a sound explanation for the decrease of BCF observed based solely on ETS results can only be speculative; other biochemical and/or physiological processes, not considered in the present study, may have contributed to this decrease. Nevertheless, an association between ETS activity and BCF was previously outlined in mussels with respect to other REEs. For instance, Pinto et al. (2019) found that *M. galloprovincialis* experienced a significant drop in the BCF of La as the concentration increased (0.1–10mg/L), and this drop in BCF was associated with a decrease in ETS activity. Similar observations were registered in the same mussel species exposed to more comparable Gd concentrations (15–120 µg/L) by Henriques et al. (2019).

In terms of energy reserves, in the present study, GLY content was lowered in contaminated organisms, while PROT reserves were maintained. These results suggest that, despite efforts to avoid Y accumulation by lowering or maintaining aerobic

respiration, the presence of Y resulted in greater energetic usage, probably associated with demands in antioxidant and detoxification defenses. Likewise, a decline of glycogen was formerly seen in mussels, *M. galloprovincialis*, exposed to other REEs such as Nd and dysprosium (Dy), which was also indicated to provide protection (Freitas et al., 2020a,b). In the particular case of intertidal mussels, the decrease in GLY was also associated with its use in anaerobiosis (De Zwaan, 1983; Hochachka and Mustafa, 1972). This would be further supported by the decrease (and/or maintenance) of ETS observed because organisms may not only limit their aerobic respiration but also shift it to an anaerobic pathway. All of these findings may be of physiological relevance because reproduction and growth could be compromised by reduced respiration/filtration rates and enhanced GLY expenditure at long-term ambient Y exposures.

Antioxidant and Biotransformation Capacity

The fast response of SOD at relevant Y concentrations (5 and 10 µg/L) indicated that mussels were able to activate this first defense line without the need to enhance their metabolic energy supplies. A fast response by SOD to La (0.1 and 1mg/L) was described by Pinto et al. (2019) in *M. galloprovincialis*, whereas another antioxidant defense (CAT activity) remained constant. Likewise, Freitas et al. (2020b) reported that mussels exposed to Nd increased SOD activity at the lowest concentration (2.5 µg/L), whereas CAT only responded at the upper ones (5, 10, and 40 µg/L). Similarly, mussels exposed to Dy enhanced SOD activity in the range of 5, 10, 20, and 40 µg/L, whereas CAT was only activated at the two highest exposures (Freitas et al., 2020a). Particularities between these former responses may be associated with specific REE toxicity, but all coincided with the fact that SOD was a fast response. The Y treatments responsible for a significant SOD activity increase were parallel to CAT activity reduction that could reflect higher H_2O_2 production, which in turn caused CAT inhibition. In fact, the increase in intracellular reactive ROS due to H_2O_2 overproduction was linked to a reduction in CAT expression (Venkatesan et al., 2007). In addition, the decrease or maintenance of ETS activity observed in the present study under Y exposure could have constrained the responses of GR and even CAT, to a certain extent, because energetic (GLY) reserves would be shifted to maintain anaerobic metabolism. However, inhibition of these antioxidant enzymes by an excess of ROS could be the most likely event because SOD was enhanced. Decreased GR defenses were also reported in freshwater mussels, *Unio*

tumidus and *Unio pictorum*, transplanted to metal-polluted sites (Cossu et al., 1997; Guidi et al., 2010).

The coincident responses of both biotransformation enzymes (carboxylesterases, CbEs, and glutathione *S*-transferases, GSTs), with a decrease at the lowest Y dosage (5 µg/L) support their role as Phase I and II steps in detoxification. The positive relationship between the two enzymes in bivalves has been studied by Solé et al. (2020) in polycyclic aromatic hydrocarbon–contaminated environments, and they suggested CbEs as a good indicator of Phase I metabolism in bivalves. In fact, in the present study, these two biomarkers have also shown a significant positive Pearson correlation value ($r = 0.679$, $p < 0.05$), supporting this proposal. There is also a possible link between GSTs and other antioxidant defenses in mussels, at least in the case of 5 µg/L of Y. Under this treatment, the enhanced production of H_2O_2 (likely due to the rise in SOD activity and lack of CAT removal action) may have contributed to the prevention of other antioxidant responses, including GSTs, because ROS are known to interact with cytosolic GSTs, leading to their inactivation (Letelier et al., 2010). Morosetti et al. (2020) observed that in *M. galloprovincialis* exposed to mercury (Hg) or a mixture of Hg and nanoparticles of cerium (CeO_2), the GSTs activity decrease was associated with a lack of GR response.

Oxidative Damage

In terms of oxidative damage, mussels in the 5 µg/L Y exposure showed the lowest LPO levels, which could be partly due to the efficiency of SOD at preventing cellular damage but also to decreased ETS activity, which would generate less ROS in the mitochondrial electron transport chain (Phaniendra et al., 2015). The study of Pinto et al. (2019) also revealed a decrease in LPO and PC levels in *M. galloprovincialis* subjected to La, which was correlated with an enhancement of antioxidant enzymes, such as SOD and glutathione peroxidase, and lower ETS activity. Nonetheless, the freshwater mussel *D. polymorpha* exposed to Y (10–250 µg/L) exhibited unaltered LPO levels, despite the fact that these organisms manifested anti-inflammatory and genotoxic responses (Hanana et al., 2018). It is also likely that the freshwater mussels from that study used other not considered mechanisms to avoid damage.

Neurotoxicity

Instead of acetylcholinesterase (AChE) inhibition, as a sign of neurotoxic action, only a rise in AChE activity at 20 µg/L of Y was noticeable. Likewise, in a fish study,

Figueiredo et al. (2018) observed that the REE La caused enhanced AChE activity in glass eels, *Anguilla anguilla*, which the authors linked to La binding to the acetylcholine receptors, replacing calcium. In fact, Y has also been shown to interfere with calcium-dependent processes in vertebrates (Shemarova et al., 2014), which could explain the increase of AChE at particular Y concentrations in mussels.

Integrated Biomarker Response Index

The application of the Integrated Biomarker Response version 2 (IBRvs2) model further confirmed that the lowest Y concentration (5 µg/L) had the greatest effect, which is due to its negative impact on most of the biomarkers evaluated (including CbEs and GSTs decreases). A reduced metabolism was related to a switch to anaerobic respiration (lower ETS and GLY), potentially due to valve closure and reduced filtration rate that refrained Y accumulation. At the same time, the increase in SOD activity at this particular concentration prevented LPO. Moreover, a decrease in the IBRvs2 values (despite an increase in Y water concentrations) was seen, which was in line with less marked biomarker responses and a tendency to return to control values. This trend may be due to the use of other physiological adaptive responses which were not addressed in the present study. Furthermore, because most oxidative stress and neurotoxicity indicators were not affected in the range of selected Y concentrations, it is likely that mussels can cope with these exposures. However, because the overall IBR results suggest that a maximum response could be reached at <5 µg/L and this gains in environmental relevance, further lower realistic exposures deserve investigation.

Conclusions

In the present study, the effects of Y exposure on the biochemical and physiological performance of the mussel *M. galloprovincialis* were described for the first time, demonstrating their ability to successfully deal with this rare-earth element. Overall, organisms were most affected at the lowest concentration, when ETS, CAT, GR, CbEs, and GSTs activities were reduced, forcing organisms to use their energy reserves (GLY) and increase SOD activity to avoid cellular damage. Most of the inhibitions observed may have been a consequence of metabolic depression, with a shift to anaerobiosis, possibly due to valve closure. However, because at higher Y concentrations, the biomarker responses were less evident and the uptake rate of Y was lowered, the use of physiological adaptive processes not evaluated in the present study could be speculated.

The long-term risks of Y presence in coastal systems cannot be ignored because the levels that caused more significant biochemical alterations were those of environmental relevance.

4.2.2. Terbium Exposure

The discussion presented in this section corresponds to the main findings of the following publication:

- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Assessing the impact of terbium on *Mytilus galloprovincialis*: Metabolic and oxidative stress responses. Chemosphere 337, 139299. https://doi.org/10.1016/j.chemosphere.2023.139299.

Bioconcentration of Terbium in Mussels

The present study confirmed mussels' Tb accumulation capacity from water in a dose-dependent way reaching a BCF higher than the bioaccumulation threshold of 1 L/kg (24–33 L/kg). Previous studies have reported similar BCF values in *M. galloprovincialis* mussels for other rare-earth elements. For example, BCFs between 13 and 37 L/kg were observed in the range of 10–120 µg/L of Gd (Andrade et al., 2022b; Henriques et al., 2019; Trapasso et al., 2021), approximately 75 L/kg at 10 µg/L of La (Andrade et al., 2021), from 40 to 50 L/kg at 2.5–40 µg/L of Nd (Freitas et al., 2020b), and from 15 to 37 L/kg in exposures from 5 to 40 µg/L of Y (Andrade et al., 2023b). Nevertheless, BCF values in the presented study also indicated that the ratio between the concentration of Tb in the mussels' tissue and the concentration in the water was relatively stable over the range of concentrations tested. Similarly, Henriques et al. (2019) and Freitas et al. (2020b) observed very similar BCF values for a range of 15–120 µg/L of Gd and 2.5–40 µg/L of Nd, respectively, being attributed to the mussels' efforts for preventing accumulation as exposure concentrations increased. In addition, Andrade et al. (2023b) observed a decrease in BCF with increasing Y concentration (5–40 µg/L), indicating that mussels also limit the accumulation of this element. Thus, the results observed in the present work suggest that there is a limit to the amount of Tb that the mussels can accumulate, beyond which, further uptake may be prevented. Several factors limiting bioaccumulation can be taken into consideration such as the initiation of detoxification processes that may regulate the accumulation of Tb in their tissues and/or other physiological adaptive strategies. In fact, enzymes involved in the detoxification, such as GSTs and CbEs per example, may modify the chemical compounds to which organisms are exposed, to be less harmful and more easily excrete them from the body (dos Santos et al., 2022). However, in the present work, the activity of these enzymes tended to decrease with Tb exposure. On the other hand, physiological responses related to

adaptation traits such as the well-known valve closure under unfavorable conditions (Anestis et al., 2007; Gosling, 2003), may lead to changes in the filtration and respiration rates that can prevent chemical accumulation. In this case, biomarkers related to respiratory metabolism such as changes in ETS activity, GLY and PROT reserves may inform on the use of these adaptive traits and be traced as changes between aerobic and anaerobic metabolism. In the present work, ETS activity tended to decrease with the usage of GLY under Tb exposure, possibly indicating a switch to anaerobic metabolism which will be further discussed.

Metabolic Capacity and Energy Reserves

In the present study, ETS activity decreased alongside Tb increasing concentration but only reached significance at the highest concentration (40 µg/L), possibly explaining the maintenance of mussels' BCF, and limited Tb accumulation. Previous research has suggested that a decrease in ETS activity could be associated with a reduction in filtration and/or respiration rate as a strategy to prevent the accumulation of chemicals (*e.g.,* Almeida et al., 2015). Furthermore, former studies with other REEs such as Gd and La (Henriques et al., 2019; Pinto et al., 2019) give support to our observations and backup this hypothesis in mussels.

In terms of energy reserves, mussels maintained their PROT reserves while increasing GLY consumption with exposure to Tb, regardless of the concentration tested. These results suggest that organisms may have prioritized using GLY to support metabolic needs related to stress responses such as cellular defenses and/or physiological ones. Similarly, Freitas et al. (2020a,b) observed that mussels *M. galloprovincialis* exposed to a similar range of concentrations with Nd and Dy also used GLY as the main fuel under metabolic needs. Moreover, in intertidal mussels, the rise in GLY consumption has also been linked to its use for anaerobic respiration (De Zwaan, 1983; Hochachka and Mustafa, 1972). The present findings with decreased ETS activity, use of GLY reserves and BCF maintenance along the increasing Tb concentrations, may suggest that organisms switched to an anaerobic pathway, possibly linked to valve closure during stressful conditions as mentioned earlier. This shift to a less efficient pathway could have impact on the mussels' physiology, reproduction and development if the exposure to Tb, or another stressing agent, persist over time.

Antioxidant and Biotransformation Capacity

In the present study, a distinct pattern could be seen in the activity of the enzymes related to defense mechanisms. That is, there was an increasing (SOD activity)/decreasing (GR and GSTs activity) trend up to 10 µg/L of Tb, a return to control levels at 20 µg/L and then renewing the first response at 40 µg/L. Thus, a complex response was seen with increasing Tb concentrations, which can be considered in three steps (see Figure 55). The first step, corresponding to 5 and 10 µg/L treatments, where organisms showed concentration-dependent responsiveness in parallel with Tb concentration, characterized by changes in SOD, GR and GSTs activities. The second step corresponded to changes at 20 µg/L, where enzymatic activity returned to control values. The third step corresponded to the highest dose 40 µg/L, where a response similar to the initial step was regained.

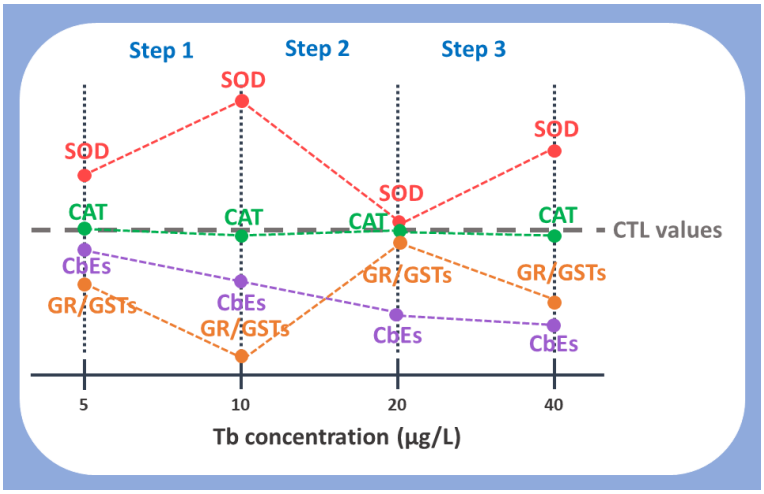

Figure 55. Scheme illustrating a tri-step dose-response of enzymatic activity in *Mytilus galloprovincialis* after 28 days exposure to terbium at different concentrations (5, 10, 20 and 40 µg/L) compared to control (CTL) levels. SOD: superoxide dismutase activity; CAT: catalase activity; GR: glutathione reductase activity; GSTs: glutathione *S*-transferases activity; CbEs: carboxylesterases activity.

Under steps 1 and 3 (5, 10 and 40 µg/L Tb treatments), the steady increase in SOD activity (almost 4-fold) together with the maintenance of CAT activity, were sufficient to compensate for the decrease in GR activity, by scavenging ROS and preventing Tb accumulation and consequent damage. In this case, the maintenance and decrease in CAT and GR activities may be associated with the maintenance and decrease in ETS activity observed previously. This could potentially impact the activation of these enzymes,

making it more difficult for them to perform their normal functions. Nevertheless, this response pattern has been formerly described in other studies involving REEs and mussels *M. galloprovincialis*. For instance, Andrade et al. (2023) observed that low concentrations of Y (5 and 10 µg/L), caused an increase of SOD activity while other antioxidant enzymes decreased. Likewise, Pinto et al. (2019) found that concentrations of 0.1 and 1 mg/L of La increased SOD activity while CAT activity remained unchanged. Moreover, Freitas et al. (2020a) reported that SOD activity increased at concentrations of 5, 10, 20, and 40 µg/L of Dy, with CAT activity only responding at the two highest concentrations. Nonetheless, a significant response in SOD activity could result in a consequent increase in H_2O_2, which may in turn greatly increase the activity of other enzymes or led to its inhibition. Although this inhibition has not yet been fully investigated in bivalves, in mammals H_2O_2 has been reported to have inhibitory effects in cytosolic GSTs (Letelier et al., 2010) and CAT (Lardinois et al., 1996). An inhibition of GR activity may have a direct consequence on GSTs activity since the latter requires GSH as a cofactor to form conjugates with xenobiotics in order to excrete them from organisms (Blanchette et al., 2007). In the present study, both GR and GSTs exhibited the same pattern, most likely as a result of a reduction in the amount of GSH in the cell. Similarly, Morosetti et al. (2020) noticed that the activity of GSTs was reduced in *M. galloprovincialis* after being exposed to Hg or a combination of Hg and CeO_2 and it was associated with the lack of GR response. Lompré et al. (2021) observed that clams *Ruditapes philippinarum* exposed to 50 µg/L of Tb, also increased SOD and glutathione peroxidase (GPx) activities while GR did not change. In the present work, the response of CbEs, as another metabolic defense in detoxification, was consistent along all Tb exposures. This activity decreased with increasing Tb concentration, indicating a clear inhibitory effect of Tb which was dose-dependent and could compromise the detoxification potential in Tb-exposed mussels. Recently, Andrade et al. (2023b) also observed that CbEs activity decreased in mussels *M. galloprovincialis* exposed to Y although, in that case, the inhibitory effect was not so dose-dependent.

The particular response observed for step 2 (20 µg/L Tb treatment), was characterized by the fact that antioxidant defenses and GSTs activity aimed to return to control values. This condition possibly indicates that mussels were no longer impacted by Tb due to either physiological adaptions or other mechanistic responses not evaluated in the present study. One possible adaptive strategy could be the upregulation of heat shock proteins, which are known to protect cells from various stressors, including oxidative stress caused by metals (Ivanina et al., 2008; Liu et al., 2014). Nonetheless, as indicated

before, at step 3 (40 µg/L Tb treatment), the mussels' behavior in most biochemical responses was similar to that seen in step 1, leading to an enhancement in the organism's enzymatic responses.

Overall, the present results suggest that at the range of Tb concentrations assayed (between 5 and 40 µg/L) mussels adopted different strategies to deal with increasing exposure to Tb and it could be regarded as a tri-step response. Additionally, the lower antioxidant and biotransformation response in mussels observed under 40 µg/L compared to those at 10 µg/L could have been not only due to enzyme inhibition, as discussed previously, but also as a consequence of a decreased metabolic capacity (lower ETS activity). In this sense, the study by Henriques et al. (2019) evidenced that mussels *M. galloprovincialis* exposed to Gd concentrations of 60 and 120 µg/L were not able to proportionally increase their antioxidant defenses capacity and it was associated with a reduction of the metabolic capacity, an enzymatic inhibition likely due to increased ROS and to other not considered defense mechanisms, all aiming to avoid Gd toxicity.

Oxidative Damage and Neurotoxicity

In the present work, regardless of the concentration tested, no cellular damage on the lipids were evidenced and mussel's antioxidant defenses, and potentially other adaptive mechanisms, were able to counteract ROS formation. Likewise, Lompré et al. (2021) observed that in clams, Tb caused activation of GPx in *Ruditapes decussatus* and of SOD, CAT and GPx in *R. philippinarum*, increases that were sufficient to avoid cellular damages in both specimens. Moreover, Pinto et al. (2019) observed that the same mussel species even decreased their LPO levels when exposed to La, associating it with the increase of SOD, GPx and GSTs enzymes.

Although AChE inhibition has mostly been associated with pesticides, several studies have shown that metals may also compromise this activity (*e.g.,* Brown et al., 2004; Perić et al., 2017). Nevertheless, in the present work, AChE activity was not affected by exposure to Tb at concentrations up to 40 µg/L for 28 days. Likewise, Lompré et al. (2021) observed that clams *R. philippinarum* did not change this activity when exposed to 50 µg/L of Tb for 28 days.

Conclusions

Terbium was accumulated on *M. galloprovincialis* although biochemical responses regulated the intake. Antioxidant enzymes and GSTs showed a dose response in Tb

exposed mussels. The first step implied an increase in SOD activity, maintenance of CAT, and a decrease in GR and GSTs. The second step (20 µg/L) implied somehow a return to control values in antioxidant defenses, possibly due to other adaptive mechanisms being employed. In the third step, organisms responded similarly to the first step, but to a lesser extent. Out of this tri-step pattern, CbEs activity was steadily and gradually inhibited at increasing Tb concentration. Overall, the different strategies adopted by mussels at the range of Tb concentrations tested were sufficient to prevent cellular damage and neurotoxicity. Nevertheless, Tb presence implied certain biochemical responses in mussels that need to be further considered under long-term and at more ecologically relevant concentrations. Given the ecosystem services provided by mussels, and other bivalves, and the likely increase of REEs and Tb worldwide, their ecological role in coastal ecosystems may be compromised.

4.2.3. Final Considerations

In the present section (4.2.) the evaluation of the effects of Y and Tb on the mussel *M. galloprovincialis* has provided in-depth understanding of the adaptive biochemical responses of these marine organisms. Both studies revealed the mussels' remarkable ability to adapt to the presence of REEs either through subtle physiological changes in response to Y or the tri-step pattern of biochemical reactions in response to Tb. In both cases, the mussels employed diverse strategies to mitigate cellular damage and maintain homeostasis. Notably, lower concentrations of these elements invoked more pronounced biochemical alterations, emphasizing the importance of monitoring low and environmentally relevant levels in marine habitats.

The present findings also highlighted the potential long-term risks associated with the continuous presence of these elements. Even though immediate cellular damage may not be evident, the biochemical alterations observed, especially under persistent exposure, can have cascading effects. Energy allocation in these organisms can be disrupted, prioritizing survival over other vital functions, which can eventually affect mussel populations at large. Considering the complexity of marine ecosystems, teeming with various stressors, a comprehensive approach becomes indispensable when assessing the impact of such contaminants. In light of these findings, the upcoming section will focus on the effects of REEs, including La, Gd, Y, and Tb, within the context of two foremost climate change scenarios: the changes of salinity and the increase of temperature. An environmentally relevant concentration of 10 µg/L for each element was

determined, based on the insights of the current studies and corroborating literature. As global temperatures rise and salinity patterns change, understanding the synergistic effects of these elements and environmental stressors on coastal ecosystems becomes crucial.

4.3. Rare-Earth Elements Under a Climate Change Scenario

4.3.1. Lanthanum Exposure

The discussion presented in this section is based on the results from the following publication:

- 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. Sci. Total Environ. 794, 148512. https://doi.org/10.1016/j.scitotenv.2021.148512.
- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2022. Do climate change related factors modify the response of *Mytilus galloprovincialis* to lanthanum? The case of temperature rise. Chemosphere 307, 135577. https://doi.org/10.1016/j.chemosphere.2022.135577.

Bioconcentration of Lanthanum in Mussels

In the present study, *M. galloprovincialis* mussels accumulated La in their tissues. While salinity did influence this accumulation, with mussels exposed to the lowest salinity (20) showing higher La presence and BCF, increased temperature (22 °C) as well higher salinity (40) had no evident effect on the accumulation. Previous studies testing the impacts of La in mussels showed that this REE was accumulated in soft tissues of freshwater species such as *D. polymorpha* and seawater *M. galloprovincialis* (Hanana et al., 2017b; Pinto et al., 2019). However, to the best of our knowledge, the bioaccumulation of La under different salinities and increased temperature in bivalves has not yet been explored. Marine mussels tend to uptake certain trace metals more as salinity decreases (Ali and Taylor, 2010; Blackmore and Wang, 2003; Moreira et al., 2016; Wright, 1995). This is attributed to a decrease in chloride complexation, which results in an increased concentration of free metal ions, considered the primary form absorbed by marine invertebrates (Campbell, 1995). La^{3+} ions can displace Ca^{2+} from the cell membrane, affecting cell functions (Evans, 1983) and, in mussels *M. edulis* and algae *Chara corallina*, the uptake of La^{3+} into cells has been evidenced (Chassard-Bouchard and Hallegot, 1984; Li et al., 2008). As for temperature effects, former experiments demonstrated varied outcomes. While Figueiredo et al. (2020) demonstrated that warming enhanced the accumulation of La in the eels *A. anguilla*, other studies indicated that bivalves such as *R. philippinarum* and *M. galloprovincialis* might maintain or reduce other chemicals

accumulation under warming stress (Almeida et al., 2021; Morosetti et al., 2020; Pirone et al., 2019).

Such results are also often associated to physiological responses of bivalves, including metabolism and contaminant uptake, that may be significantly influenced by salinity and temperature (Wright, 1995). Recent studies showed that low salinity levels were responsible for higher metabolism, potentially favoring contaminant accumulation, such as the case of lead (Pb, Freitas et al., 2019c). Conversely, bivalves may reduce contaminant uptake at higher temperatures by decreasing metabolic activity, often closing their valves for prolonged duration (Anestis et al., 2007; Gosling, 2003; Lopes et al., 2021; Ortmann, 2003). In the present study, La-exposed mussels exhibited greater metabolism at salinity 20 and control temperature (17 ºC) but reduced it at control salinity (30) and 22 °C. This suggests that factors beyond just medium properties, such as metabolic rates, influence accumulation. This relationship will be explored further in subsequent sections.

Metabolic Capacity and Energy Reserves

In terms of energy metabolism, the present study evidenced that low salinity induced greater alterations than higher salinity or the presence of La, with the highest increase on ETS activity at the lowest salinity regardless of La presence. These findings indicate higher energy consumption at the mitochondrial level (De Coen and Janssen, 1997) in mussels at this condition, which was concomitant to the activation of defense mechanisms such as antioxidant and biotransformation enzymes. The present findings also revealed that in non-contaminated mussels the lowest ETS activity was reached at the highest salinity, which is in agreement with Freitas et al. (2017b), reporting that *M. galloprovincialis* specimens presented lower ETS under salinity 35 in respect to those maintained at 28. Other bivalves, such as the clam *R. decussatus* and the oyster *Crassostrea angulata*, also showed higher ETS at lower salinities, phenomena associated to hypotonic stress (Velez et al., 2016b; Moreira et al., 2016). In fact, this hypotonic stress was previously demonstrated in *M. galloprovincialis* when recovering from low salinity conditions, which showed a decreased in the proteins involved in energy metabolism and ROS scavenging capacity confirming higher energy requirements at low salinity (Tomanek et al., 2012). Although low salinity was the main factor affecting ETS, at control salinity (30) and temperature (17 °C) ETS activity was lower in La-exposed mussels compared to non-contaminated ones. Such results may indicate a physiological trait to prevent La accumulation by reducing their metabolism. It has been repeatedly demonstrated that

when under stressful conditions, bivalves may close their valves and reduce their metabolism to avoid the negative consequences of an unfavorable environment (Anestis et al., 2007; Gosling, 2003; Ortmann, 2003). In particular, former studies in bivalves already revealed that in the presence of rare-earth elements (Gd and La) as well as others such as mercury (Hg) and arsenic (As), to decrease ETS activity and/or associated filtration capacity, was an strategy adopted to prevent contaminant accumulation (Henriques et al., 2019; Pinto et al., 2019; Coppola et al., 2017, 2018).

Although at control temperature the consumption of GLY was not affected by salinity or La, the results here presented suggest that non-contaminated and contaminated organisms increased their energy consumption by mobilizing PROT as a source of energy (at salinity 20 with high ETS values), and further supporting the hypothesis of hypotonic stress at low salinities. The relationship between the reduced PROT reserves and lower salinities has been observed previously in *R. philippinarum* (Velez et al., 2016a; Freitas et al., 2016) and *M. galloprovincialis* (Freitas et al., 2017b). High energy demand observed in the present study is likely related to higher ROS production typical of hypotonic stress, with the energy reserves being used to fuel up defense mechanisms and, as well, to repair cellular damage at the lowest salinities. Regarding the presence of La, the results obtained showed no significant influence of this element on mussels' energy consumption at 17 °C, and salinity was the main driving factor. Other studies also demonstrated that the exposure to REEs was either not accompanied by changes on bivalves' energy content or by its increase (Pinto et al., 2019; Henriques et al., 2019).

At control salinity, a decrease in energy metabolism (lower ETS activity) was evidenced in mussels under La exposure, which was supported by lower energy fuel consumption, especially when La was combined with increased temperature. Similarly, Morosetti et al. (2020) reported that mussels in the presence of cerium nanoparticles (CeO_2) at enhanced temperature had their metabolic capacity reduced, limiting their ability to activate antioxidant defenses. Furthermore, warming was demonstrated to negatively influence *M. galloprovincialis* metabolic capacity and energetic expenditure when subjected to Hg and As (Coppola et al., 2017, 2018) which was explained by valves closure and other behavioral adaptations. In this way, the greatest metabolic depression observed in the present study when both stressors were combined indicates the most stressful scenario at control salinity. In fact, in contrast to this particular condition, un-exposed mussels under the increase of temperature alone maintained their ETS activity,

while using GLY reserves. Likewise, Andrade et al. (2019b) showed that *M. galloprovincialis* under warming stress maintained its metabolic capacity while spending this energy fuel. These findings in bivalves could be associated with the consumption of GLY under anaerobic conditions in order to face prolonged anoxia (De Zwaan, 1983; Hochachka and Mustafa, 1972). In the present case, mussels under stress may have not only limited their aerobic respiration (as observed by ETS activity) but also started to use the anaerobic pathway linked to a possible physiological/behavior adaptation such as valve closure. Accordingly, organisms from all treatments in the present study may have developed a physiological trait to prevent either La accumulation or heat stress. Environmental stress has been shown to impact energy allocation in organisms, with the energy required for survival taking preference over other vital activities like reproduction, growth, and development (Sokolova, 2013). Hence, in mussels, this type of response might have long-term consequences on energy expenditure during such critical physiological events, which in turn, could evolve into effects at higher biological levels (population) with an economic impact on aquaculture.

Antioxidant and Biotransformation Capacity

Higher antioxidant enzymes activity (SOD and CAT) was equally observed at lower salinity in noncontaminated and contaminated organisms. Similarly, Freitas et al. (2017b) observed higher CAT and SOD activities in mussels at low salinity, which was also related with higher metabolic capacity and lower energy reserves. In particular, higher antioxidant defenses at lower salinities have been observed in the clams *R. philippinarum* and in the oysters *C. angulata* (Velez et al., 2016b; Moreira et al., 2016), thus further supporting that hypotonic stress is associated with the activation of antioxidant defenses probable related to increased ROS production and to prevent cellular damage. The particular effect of La at studied salinities took place differently: at salinity 40 for SOD and at salinity 30 for CAT, in both cases being the antioxidant capacity lowered in La-exposed mussels. Similarly, CAT inhibition was observed in eels *A. anguilla* (Figueiredo et al., 2018) and in goldfish *Carassius auratus* (Chen et al., 2000) exposed to La. Overall, as demonstrated by metabolism-related biomarkers, it seems that salinity plays a major role on mussels' antioxidant defenses than La acting alone (*i.e.*, under salinity 30 at 17 °C).

In the present study, both biotransformation enzymes displayed higher activities at the lowest salinity for noncontaminated as well as La-exposed organisms. Former studies

have already demonstrated that shifts in abiotic factors, such as temperature, pH and salinity, often induce multifactorial effects on GSTs activity (Park et al., 2020). As an example, Rivera-Ingraham et al. (2016) observed an upregulation of glutathione *S*-transferases pi 1 (GSTP1) in marine intertidal flatworms at low salinity, justifying that ROS formation under hypoosmotic shock could have triggered GSTs upregulation. Furthermore, these authors also hypothesized this response as a "preparation for oxidative stress", as a mechanism to overcome upcoming challenges when returning to seawater (*i.e.*, submerged), representing a shift to hypersalinity. The results observed in the present study for GSTs could be related to ROS formation under hypotonic stress and an upregulation of these enzymes to counterattack the effects of salinities shifts. At control temperature, the presence of La caused an increase in GSTs at salinity 30, supporting former evidences by other metabolic enzymes in which salinity had a stronger impact on mussels' biotransformation capacity than the presence of La alone. This is probably due to low concentration tested and, on the other hand, suggesting that when salinity is not acting as a confounding factor the presence of La is responsible for the activation of GSTs activity. This last role was recently pointed out by Pinto et al. (2019) assessing the effects on these enzymes in mussels at wider range of La (0.1–10 mg/L). Mussels increased GSTs activity up to 1.0 mg/L of La exposure, while similar values to control were observed in organisms under the highest concentration tested (10 mg/L), being suggested a possible inhibition of this enzyme under higher concentrations. Similarly, Hanana et al. (2017b) showed a tendency of GSTs to increase in the presence of the lowest concentration of La (10 µg/L) while being inhibited at the highest tested concentration (1250 µg/L).

In the case of CbEs, at control temperature no differences were observed between noncontaminated and contaminated mussels at each of the tested salinities, while a significant increase in activity was observed in mussels exposed to the lowest salinity, regardless the presence of La. As seen for GSTs, the present results on CbEs activity highlight the influence of low salinity in metabolic detoxification measures but, so far, studies on CbEs modulation by salinity or La are mostly nonexistent.

In the present experimental set-up, organisms decreased most antioxidant enzyme activities at increased temperature (with and without La compared to control condition). The decrease of an overall antioxidant capacity might be associated to the mussels' strategy to reduce metabolic capacity. However, a reduction of this overall antioxidant capacity could also evolve into a higher stress condition, with increased intracellular ROS production that, consequently, could inactivate the enzymatic defenses augmenting cellular damage (Venkatesan et al., 2007). Other studies have demonstrated that warming alone may lead to reduced antioxidant activity in mussels *M. galloprovincialis* (Freitas et al., 2017a; Lopes et al., 2022; Morosetti et al., 2020). The interaction of contaminants and warming conditions has yielded a similar pattern. As an example, exposure to 17α-ethinylestradiol (EE2) and warming led to depressed antioxidant defenses in these same mussel species (Lopes et al., 2022) and Morosetti et al. (2020) observed GR inhibition in mussels under warming conditions treated with CeO_2 as well as with Hg with CeO_2. These former studies associated the decrease in the antioxidant capacity due to warming with a loss of metabolic performance.

In the current study, both enzymes at control salinity evolved similarly but differences between treatments were more accentuated in the case of GSTs. In fact, La exposure led to an increase in GSTs activity in mussels at both temperatures. Other studies confirmed that the same mussel species was capable to activate GSTs in the occurrence of other REEs such as Gd (Andrade et al., 2022b; Henriques et al., 2019), Dy (Freitas et al., 2020a) and Nd (Freitas et al., 2020b), although at particular concentrations. Such results reveal the capacity of mussels to activate GSTs as a detoxification pathway when exposed to REEs. To our knowledge, few studies investigated the response of CbEs in the presence of REEs. In agreement with the present results, Andrade et al. (2022b) observed that the REE Gd did not influence CbEs activity in the same mussel species. Likewise, Rivera-Ingraham et al. (2021) showed that aluminum oxide (Al_2O_3) did not lead to any changes in this enzyme in the freshwater shrimp *Macrobrachium jelskii*. However, under more unfavorable conditions, the combination Al_2O_3 with lower pH caused inhibition of these enzymes. In the present study, when mussels were under warming conditions alone, the temperature was responsible for a reduction in the activity of these two biotransformation enzymes (GSTs and CbEs). Similar results were found by other authors when evaluating the influence of temperature in *M. galloprovincialis* (Andrade et al., 2019a; Morosetti et al., 2020). Reduced activity in GSTs and CbEs at higher temperatures was also seen in fish and explained as an enzyme compensatory mechanism strategy (Solé et al., 2014). Thus, the activation of GSTs appears to be

closely associated with the presence of La, as a detoxification pathway; warming also influenced the outcome of the combined exposure (La and temperature) since a greater GSTs response was achieved at this combined condition. Similarly, Coppola et al. (2018) showed in *M. galloprovincialis* that GSTs were enhanced in response to As, being this response greater also at a higher temperature.

Oxidative Damage

In the present study no cellular damage was observed due to salinity, most probably due to the activation of antioxidant defense mechanisms. However, La-exposed organisms displayed at the lowest salinity, increased LPO levels and significantly enhanced PC levels with significantly higher values in contaminated mussels, which may be due to the fact that antioxidant enzymes were not significantly activated in La exposed mussels in comparison to non-contaminated ones. These results further indicate that noncontaminated mussels would be able to better cope with the hypotonic stress than the La-exposed ones as two stressing factors may overwhelm their defense mechanisms. Nevertheless, in the freshwater mussel *D. polymorpha*, Hanana et al. (2017b) demonstrated a nonsignificant increase of lipid peroxidation in mussels exposed to 10 µg/L of La after 28 days. The acute exposure study by Moreira et al. (2020), revealed an EC_{50} of 6.7 (in 24 h) and 36.1 µg/L (in 48 h) of La exposure in *C. gigas* embryos, a more sensitive stage, reveling that low concentrations of La may be enough to cause effects in bivalves.

In the current study, even though La-exposed organisms under control salinity enhanced GSTs activity and reduced metabolic capacity (ETS) to avoid La accumulation, higher LPO levels were encountered under this exposure, possibly due to a lack of efficient antioxidant defenses activation. Mussels exposed to the REE Gd at the same temperature conditions and experimental time also displayed enhanced LPO levels but not PC (Andrade et al., 2022b). In the current study, although antioxidant and biotransformation capacity decreased at warming conditions, mussels showed lower PC levels and no signs of protein damage, which may be associated with the fact that ETS was also reduced at this elevated temperature. A decrease in PC levels under warming conditions has been formerly described in the same mussel (Andrade et al., 2019b; Freitas et al., 2019b) and also in *R. philippinarum* clams (Almeida et al., 2021). In the present study, mussels treated simultaneously with La and increased temperature showed

enhanced LPO levels in contrast to those subjected to only La or increased temperature independently, which confirms a more deleterious scenario. Likewise, Freitas et al. (2019b) found LPO levels increased while PC levels decreased in mussels subjected to warming and diclofenac. These coincident findings could be explained by a decrease or/and inhibition of antioxidant enzymes by La exposure, resulting in lipid damage, while the metabolic depression caused by both La and warming likely justifies the decrease in PC levels.

Neurotoxicity

Regarding the present findings, AChE was not inhibited in noncontaminated organisms under stressful salinities at 17 °C. However, organisms exposed to La at the lowest salinity showed a significant inhibition of AChE compared to the ones under the other two salinities (30 and 40), thus indicating that a concentration of 10 µg/L under low salinity may not just cause cellular damage but neurotoxicity as well. These results are concomitant to higher concentration of La found in mussels at this condition.

In the current study, no effects at control salinity were observed due to La exposure, warming, or both acting in combination, revealing that under low concentrations of this REE and regardless of the temperature La was not neurotoxic. A similar degree of warming has been shown not to modulate AChE activity in bivalves such as clam *R. philippinarum* and mussel *M. galloprovincialis* (Almeida et al., 2021; Freitas et al., 2019c). Likewise, Figueiredo et al. (2020) demonstrated that in *A. anguilla* neither heat nor a concentration of 1.5 µg/L of La acting alone and in combination led to AChE activity modifications. However, Pinto et al. (2019) showed that in the mussel species *M. galloprovincialis*, La (0.1, 1.0, 10 mg/L) inhibited AChE activity. Most likely, the lack of AChE modulation in our experiment may be due to the low La concentration used. Nevertheless, although a 5 °C temperature change did not appear to affect this enzyme, the continuous release of REEs in shallow estuarine waters may lead to unwanted neurotoxic effects.

Principal Coordinates Analyses

The Principal Coordinates (PCO) analysis as an integrative tool, clearly confirmed the partial information given by the individual biomarker analysis at all the tested treatments at control temperature. In particular, low salinity mimicking an excess of rainfall and/or ice-melting due to climate change, was the most relevant factor in the modulation of biomarker's response, followed by the exposure to La in a second term. Thus, being in agreement with the results previously discussed and further reinforcing the effects on estuarine organisms, such as bivalves. This study suggests that it would be more noxious the co-exposure of rare-earth elements under low salinity conditions in comparison to the effects of each stressor acting individually, with increasing metabolic capacity to fuel up antioxidant and biotransformation defenses, which may compromise other physiological and biochemical mechanisms and, ultimately, organisms' growth and reproduction.

Overall, as shown by the integrative PCO analysis, the temperature was the dominant factor to dictate biochemical changes in mussels at control salinity, with PCO1 explaining almost 60 % of the total variation and clearly discriminating mussels reared at both temperature conditions (17 °C and 22 °C). This separation is supported by the negative correlation found in the antioxidant and biotransformation enzymes since lower values were achieved at a higher temperature. PCO2 explained less than 40 % of the total data variation, distinguishing between La exposed organisms and non-contaminated ones. The most opposed groups corresponded to organisms subjected to La at 22 °C on the positive side and by noncontaminated organisms at 17 °C on the negative side. This division is explained by a positive correlation of the two stressors (La and temperature) combined with LPO and GSTs with higher values reached in La exposed mussels at 22 °C. Whereas on the opposing side of the graph, a negative correlation corresponded to PC and ETS with higher values observed in the control specimens at 17 °C.

Conclusions

Biomarkers revealed that the decreasing of salinity caused an increase in organism responses related to hypotonic stress, evident as oxidative stress, regardless of La presence or absence. However, only mussels exposed to La exhibited increased cellular damage and neurotoxicity. Moreover, these organisms seemed to have their antioxidant defenses compromised compared to noncontaminated counterparts, regardless of salinity. Conversely, the combined effects of La and warming manifested as

metabolic depression, GSTs activation, antioxidant enzymes inhibition, and higher LPO levels. The present study underscores the potential risks of REE La in coastal systems, potentially intensified by variations in salinity and temperature in close proximity to human settlements. It emphasizes the urgency to investigate the impacts of La and other REEs, considering future climate change-related stressors. Their combined influence could jeopardize not only organisms' biochemical processes but also growth and reproduction, bearing potential economic implications.

4.3.2. Gadolinium Exposure

The discussion presented in this section is based on the results from the following publication:

- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2022. Will climate changes enhance the impacts of e-waste in aquatic systems? Chemosphere 288, 132264. https://doi.org/10.1016/j.chemosphere.2021.132264.
- Andrade, M., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Gadolinium accumulation and its biochemical effects in *Mytilus galloprovincialis* mussels under a scenario of global warming. Environ. Sci. Pollut. Res. 30, 116120-116133. https://doi.org/10.1007/s11356-023-30439-2.

Bioconcentration of Gadolinium in Mussels

The present findings demonstrated the capacity of mussels to accumulate Gd in their tissues after 28 days of water-borne exposure to 10 µg/L of Gd. Former studies had already shown that both marine and freshwater mussels were able to accumulate Gd under similar exposure conditions (28 days exposure to 10 µg/L, Hanana et al., 2017a; 28 days exposure to 15 µg/L, Henriques et al., 2019; 21 days exposure to 10 µg/L, Perrat et al., 2017). Still, to our knowledge, there is no information on Gd accumulation under different salinities and increased temperature. Nevertheless, the present study revealed that both salinity and temperature considerably affect Gd accumulation in mussels. Higher Gd concentrations and BCF values were observed in mussels exposed to the lowest salinity, while the opposite was true at the highest salinity (40). This response can be attributed to the decrease of chloride complexation and an increase in free metal ions, the main form of metal uptake in marine invertebrates (Campbell, 1995). Notably, mussels under reduced salinity have shown an increased uptake and concentration of trace elements (Ali and Taylor, 2010; Blackmore and Wang, 2003; Wright, 1995). Additionally, depending on the salinity, organisms may employ other physiological strategies which may affect metal uptake (Wright, 1995). Factors like enhanced metabolism under low salinity conditions, related to increased ETS activity, may play a part in such a response. This phenomenon has been recorded for other contaminants like Pb (Freitas et al., 2019c), As (Moreira et al., 2016), and salicylic acid (Freitas et al., 2020c). As euryhaline osmoconformers, mussels can survive in various osmotic conditions, and when salinity drops, they undergo cell swelling and bursting, necessitating molecular transport and new

protein synthesis. These physiological processes might account for the metabolism enhancement under low salinity conditions (Bradley, 2009).

Increased temperature was also found to enhance Gd accumulation potential and BCF values. Similarly, Mubiana and Blust (2007) observed that with *M. edulis*, there was a positive relationship between the accumulation of non-essential metals (cadmium and Pb) and temperature after 28 days, attributed to enhanced solubility and kinetics at higher temperature. It was also noted that increased temperature enhanced the activity of free metal ions, which are more bioavailable. Moreover, the intensified chemical reactions and diffusion rates further facilitate uptake. However, Gd accumulation behavior under temperature variations is still complex as bioaccumulation can also be influenced by species, tissue and experimental conditions. Unlike our observations, Figueiredo et al. (2022a) demonstrated that in the clam *Spisula solida*, Gd accumulation, following a 7-day exposure, was not influenced by ambient temperature, possibly due to differing exposure durations (7 vs. 28 days). Given the varying outcomes and the looming challenge of global warming, it's crucial to also deeply analyze the intricate interactions between metals like Gd and changing environmental conditions.

Metabolic Capacity and Energy Reserves

In the present study, the higher metabolic capacity at the lowest salinity can be associated to the highest ETS activity regardless of Gd presence. Similar findings were encountered in the same mussel species, with higher ETS activity in those at salinity 28 with respect to those kept at 35 (Freitas et al., 2017b). Moreover, other bivalves such as the clam *R. decussatus* and the oyster *C. angulata*, also showed higher ETS values at lower salinities being associated to hypotonic stress and swell-regulation capacity (Velez et al., 2016b; Moreira et al., 2016). The hypotonic stress has been previously justified in *M. galloprovincialis* by Tomanek et al. (2012) as an increase in the proteins involvement in energy metabolism and ROS scavenging at low salinity, which implies higher energy requirements and, consequently, higher energy reserves expenditure. In the present study, the hypotonic stress further affected Gd exposed mussels at the lowest salinity, as ETS increased significantly compared to the non-contaminated counterparts which may indicate a synergistic effect between Gd and low salinity conditions. Higher ETS activity at the lowest salinity and especially in contaminated mussels may be related with activation of defense mechanisms, namely antioxidant and biotransformation enzymes, a response confirmed in this study and that will be further discussed. On the contrary, at the highest

salinity, mussels tend to reduce their metabolism, especially in those Gd-exposed, which could be related to the fact that under moderate stress conditions (salinity 40 seems less harmful than salinity 20) bivalves reduce their filtration and respiration rate in an attempt to reduce pollutants uptake as common strategies. In fact, in terms of osmoregulation, marine mollusks and echinoderms revealed to be more challenged by osmolality decreases than increases, namely gastropods *Steamonita brasiliensis* (Veiga et al., 2016) and echinoderms *Lytechinus variegatus*, *Echinometra lucunter*, *Arbacia lixula* (Santos et al., 2013) and *Holothuria grisea* (Castellano et al., 2016). Furthermore, it was already demonstrated that bivalves close their valves and reduce their metabolism under unfavorable environments (Anestis et al., 2007; Gosling, 2003; Ortmann, 2003). In the case of toxic metal(oid)s exposures, in the presence of Hg and As, mussels decreased their ETS activity and/or associated filtration capacity in order to prevent or reduce the metal accumulation (Coppola et al., 2017, 2018). Moreover, studies involving the rare-earth Gd and La also demonstrated that mussels, *M. galloprovincialis*, decreased their metabolic activity in a contaminated environment (Henriques et al., 2019; Pinto et al., 2019).

In terms of energy reserves, the consumption of GLY was not affected by salinity or Gd. However, the use of PROT was associated to decreased salinity, thus suggesting that mussels mobilized PROT as a source of energy at lower salinities (accompanied by higher ETS values), and further supporting the hypothesis of hypotonic stress at low salinities. Several authors have described this former relationship between the use of PROT reserves and low salinities in clams *R. philippinarum* (Velez et al., 2016a; Freitas et al., 2016) and in mussels *M. galloprovincialis* (Freitas et al., 2017b). Since hypotonic stress determines increases in ROS production, high energetic demand observed at low salinities will likely initiate the activation of antioxidant defense mechanisms. Regarding the presence of Gd, higher GLY content was revealed in mussels when under the control and high salinity conditions (30 and 40) at 17 °C. This suggests that while hypotonic stress seems to use PROT as energetic source, Gd exposure led to the accumulation of GLY. Similarly, Henriques et al. (2019) demonstrated that the exposure to Gd at control salinities led to an increase in GLY content in mussels as a consequence of an overall metabolic reduction to prevent the accumulation of Gd, as also confirmed in the present study.

In the current study, ETS activity remained consistent at control salinity across treatments despite variations in energy reserves. Specifically, Gd-exposed mussels exhibited elevated GLY content in comparison to the uncontaminated counterpart, irrespective of temperature. This may suggest an adaptative effort to conserve energy expenditure in the presence of Gd. A similar trend was observed in *M. galloprovincialis* exposed to Gd (15-120 µg/L), with increased GLY reserves paralleled with decreased metabolic capacity (Henriques et al., 2019; Trapasso et al., 2021). This strategy was attributed to potential physiological adjustments, including reduced filtration rate and prolonged valve closure, aimed at preventing Gd accumulation. This response was also observed in mussels exposed to the REE La, reinforcing the hypothesis of metabolic maintenance and/or depression under mild stress conditions (Pinto et al., 2019). Still, more research is necessary in bivalves to determine the specific mechanisms adopted to mitigate metal accumulation, such as reductions in filtration and respiration rates. The high PROT reserves exhibited in contaminated mussels at 22 °C suggests an effort to limit protein expenditure (to safeguard functional proteins' vital survival and performance). Simultaneously, an enhanced PROT synthesis may be speculated to possibly repair or replace damaged proteins. The strategy of accumulating energy reserves under stressful conditions has been previously documented in *M. galloprovincialis* at increased temperature and As presence (Coppola et al., 2018), as well as in response to EE2 exposure coupled with elevated temperature (Lopes et al. 2022). Nevertheless, while this adaptative mechanism may compromise immediate metabolic performance, its potential long-term consequences on crucial physiological functions like reproduction and growth warrant further consideration.

Antioxidant and Biotransformation Capacity

At control temperature, higher activity of antioxidant enzymes was observed in organisms at lower salinity, both in contaminated and non-contaminated mussels in general. Similar observations were found by Freitas et al. (2017b) in the same mussel species exposed to different salinity levels (*M. galloprovincialis*) and the activation of defense mechanisms at the lowest salinity was related to a higher metabolic capacity and lower energy reserves. Also in clams *R. philippinarum* and in oysters *C. angulata* increased antioxidant defenses were related to hypotonic stress (Velez et al., 2016b; Moreira et al., 2016). The present findings further revealed that Gd exposure only elevated CAT activity in contaminated mussels at control salinity. In a former study, at the

same control salinity, *M. galloprovincialis* also experienced an increase in antioxidant defenses at different Gd concentrations (15, 30 and 60 µg/L) (Henriques et al., 2019). The lack of the enzymatic activation in the present study in Gd-contaminated organisms at the lowest salinity may be related to the over production of ROS (due to hypotonic stress) that may had caused an override of the antioxidant response capacity, whereas at the highest salinity there is lack of response that would agree with a decreased metabolic performance.

In the present study higher activity of both biotransformation enzymes types was observed at the lowest salinity in non-exposed and Gd exposed organisms. Some authors have already revealed that GSTs activity is affected, although maybe in different ways, by shifts of abiotic factors such as temperature, pH and salinity (Park et al., 2020). For instance, an upregulation of GSTP1 was observed in emerged marine intertidal flatworms at low salinity, and the mechanism that triggered this upregulation was ROS formation under hypoosmotic shock (Rivera-Ingraham et al., 2016). Moreover, these authors theorized this response as a mechanism to overcome future challenges when returning to seawater (*i.e.*, submerged) named “preparation for oxidative stress”, representing a shift to hypersalinity. In this way, in the present study, GSTs activation may not just be related to “ROS formation under hypotonic stress” as mentioned previously but we can also hypothesize that this response may be related to the upregulation of these enzymes to overcome a possible shift to higher salinities. Although there are no studies addressing CbEs responses to salinity shifts, the response observed in the present study could be related to an unspecific metabolic up-regulation embracing several enzymes (ETS, CAT, GST and CbEs), all involved in metabolic defenses. The presence of Gd at control temperature only increased GSTs activity at salinity 30, further evidencing the overriding impact of salinity on mussel’s biotransformation capacity.

In the present study, at 22 °C, Gd-exposed mussels exhibited greater antioxidant capacity compared to their non-contaminated counterpart, in line with the results observed at 17 °C and control salinity between non-contaminated and Gd contaminated mussels. However, at higher temperature (22 °C), both Gd-exposed mussels and non-contaminated ones displayed reduced antioxidant capacity in comparison to those at the lower ambient temperature (17 °C), indicating that elevated temperature alone may compromise antioxidant defenses. This trend is consistent with former findings by Andrade et al. (2022a) and Morosetti et al. (2020), who demonstrated that bivalves subjected to

increased temperature or the combined effect of warming and metal contamination (*e.g.*, La, Hg and CeO_2) displayed diminished defense capacity.

Similarly to the trend observed in antioxidant responses at control salinity, mussels exposed to Gd increased their biotransformation capacity (GSTs and CbEs) in comparison to the non-contaminated counterpart. Notably, at 22 °C, both reported activities were lower than those observed at 17 °C for the non-exposed groups. Once more, Gd exposure induced the detoxification capacity in mussels (Gd-exposed vs. non-contaminated mussels), with temperature being a modulating factor (17 °C vs. 22 °C). Previous studies have already demonstrated that elevated temperature can negatively impact GSTs activity. For instance, Morosetti et al. (2020) indicated a reduction in GSTs activity in *M. galloprovincialis* mussels at increased temperature, either alone or in combination with Hg and CeO_2. Similarly, Lopes et al. (2022) found decreased GSTs activity in mussels exposed to EE2 under increased temperature. Nevertheless, an increase of GSTs activity (which also possesses antioxidant properties) in the presence of Gd can be attributed to heightened oxidative stress derived from a rise in ROS due to metal exposure. In the same mussel species, Henriques et al. (2019) reported enhanced GSTs activity at various concentrations of Gd (ranging from 15 to 120 µg/L). The same observation was described in the digestive gland of the freshwater mussel *D. rostriformis bugensis* after 7 days of exposure to 10 µg/L of Gd (Perrat et al., 2017), further supporting the modulation of GSTs in the presence of Gd. Both studies noted that this increase in GSTs activity reached a peak and subsequently declined, potentially associated to a concentration threshold or a 28-day exposure duration, indicating a link to Gd accumulation. A plausible explanation for this phenomenon lies in the ionic radius of Gd, comparable to that of divalent Ca^{2+}. Consequently, Gd may function as a calcium channel blocker (Sherry et al., 2009) as it can block Ca^{2+}-dependent enzymes such as GSTs, among others (Rogosnitzky and Branch, 2016). This explanation could rationalize the observed enzymatic response during co-exposure to warming and Gd. Notably, the decline in both GSTs and CbEs activities with increased temperature might compromise mussels' defensive mechanisms, thereby amplifying their susceptibility to additional stressors.

Oxidative Damage

While in the present study no cellular damage was evidenced in non-contaminated organisms regardless the salinity, those exposed to Gd displayed increased cellular damage at the highest salinity coincident with a low antioxidant and biotransformation capacity. Similarly, Freitas et al. (2020c) observed that mussels in the presence of the pharmaceutical drug, salicylic acid, experienced cellular damage under the highest salinity (35) accompanied with a low metabolic ETS activity. Thus, the cellular damage observed at the highest salinity in the presence of Gd agrees with a decreased metabolic capacity, which led to a decline on defense mechanisms (observed by CAT, GSTs and CbEs activities). On the other hand, at the lowest salinity, Gd contaminated organisms seemed to be able to activate defense mechanisms (mostly CAT and GSTs) as a consequence of hypotonic stress ROS production, which contributed to prevent or/and reduce LPO and PC levels even in the presence of Gd. Nonetheless, at control temperature Gd exposure was responsible for enhanced LPO levels at all salinities. The study by Henriques et al. (2019) demonstrated that Gd exposure also caused LPO in *M. galloprovincialis* in the range 30–120 µg/L but not at 15 µg/L as the defense mechanisms seemed effective at this lower concentration. In the case of other REEs, such as neodymium (Nd) and dysprosium (Dy), mussels, *M. galloprovincialis*, increased LPO levels at all exposure concentrations from 2.5 to 40 µg/L and was justified by the failure of mussels to activate their defense mechanisms (Freitas et al., 2020a,b). Overall, the different salinities at 17 °C dictated particular responses in Gd-contaminated organisms: i) at the lowest salinity (20), organisms increased their metabolism possibly as a consequence of combined Gd and hypotonic stress factors; ii) at the control salinity, organisms were able to activate CAT and GSTs, and iii) at the highest salinity (40), organisms seemed to reduce their metabolism, as a physiological trait, to cope with the stress caused by increased salinity and Gd. However, since these three former strategies were not able to prevent cellular damage.

In the current study, at a control salinity, Gd was found to cause LPO, whereas not only were the efforts to prevent further Gd accumulation and activate antioxidant and biotransformation enzymes insufficient at the control temperature, but also at 22 °C, the ineffective defense enzymes and metabolic capacity maintenance contributed to the greatest lipid damage. The inefficiency of defense mechanisms leading to elevated LPO levels in *M. galloprovincialis* has been noted under increased temperature and various contaminants, including Hg (Coppola et al., 2017), diclofenac (Freitas et al., 2019b) and rutile titanium dioxide (TiO_2, Leite et al., 2020b). Nonetheless, neither Gd nor temperature induced protein oxidation, suggesting that lipids are more susceptible to ROS excess. While PC can result directly from amino acid side chain oxidation involving metals and hydrogen peroxide, it is also possible that PC arising indirectly from lipid-derived aldehydes is more prevalent (Grimsrud et al., 2008). Additionally, cells have mechanisms such as the induction of heat shock proteins (HSPs) in response to metal-induced and heat stress, playing a protective role in maintaining protein structure integrity (Fabbri et al., 2008). Thus, our observation suggests that lipids are more vulnerable than proteins to Gd exposure and increased temperature. Previous studies have similarly demonstrated that *M. galloprovincialis* experienced LPO while PC levels remain stable when exposed to various contaminants such as anatase TiO_2 (Leite et al., 2020a) and multi-walled carbon nanotubes (MWCNTs, Andrade et al., 2019a), further supporting this hypothesis.

Neurotoxicity

In the present study, at a control temperature, neither salinity, Gd, nor their combination showed signs of neurotoxicity. Additionally, at a control salinity, Gd did not inhibit AChE activity across any temperature tested. Similarly, Henriques et al. (2019) reported unchanged AChE activity under a similar Gd concentration of 15 µg/L in *M. galloprovincialis*. Yet, at higher Gd concentrations (30, 60 and 120 µg/L), AChE inhibition was observed. Nevertheless, in the present study, the rise of temperature alone led to reduced AChE activity, aligning with decreased metabolic performance (as indicated by low antioxidant and detoxifying enzymatic activities) under this condition. This observation is likely connected to the overall metabolic depression due to heat stress.

Integrated Biomarker Response Index and Principal Coordinated Analyses

The application of PCO analysis as an integrative tool in the case of multibiomarker measures and conditions, clearly corroborated the partial results discussed above. The graphical representation at control temperature demonstrated that salinity was the most relevant factor in the modulation of biomarker's response. The fact that similar biochemical responses were given by non-contaminated mussels at salinities 30 and 40, evidence the preferred salinity range for marine mussels, while revealing a clear distinct less favorable pattern at salinity 20. Also, a clear separation between non-contaminated and contaminated mussels was highlighted, which is in line with the biochemical responses given in presence of a realistic Gd concentration, particularly indicated by higher LPO and GLY contents.

In the present study, the IBRvs2 model indicated that, at control salinity, the rise in temperature led to depression of most biomarkers. This outcome aligns with the notion of metabolic depression of the defense enzymes analyzed, likely serving as a strategy to mitigate heat stress and prevent cellular damage. This can be further supported by the PCO analysis, which indicated stronger negative impacts on most of the biomarkers under this increased temperature. Nevertheless, organisms at control salinity exposed to Gd regardless of the temperature, exhibited a lower IBRvs2 score, reflecting a contrasting trend in the deployment of defenses mechanisms. Notably, the Gd-exposure treatment at 17 °C displayed a slightly higher index value, as there was an activation of the defense mechanisms due to Gd presence. In the case of organisms simultaneously exposed to Gd and increased temperature (co-exposure), the activation of defense mechanisms was countered by slight inhibition due to heat stress. In both scenarios, the IBRvs2 index was significantly affected by the elevated LPO values, particularly evident in organisms subjected to increased temperature. This observation supports the notion of Gd's impact, especially in the context of warming. Both IBRvs2 and PCO models revealed that the defense enzymes contribution was more pronounced for Gd-exposed organisms under control conditions than in the context of increased temperature. These finding highlights how warming influences the activation of these enzymes, ultimately leading to heightened cellular damage.

Conclusions

The biochemical parameters assessed revealed diverse responses in mussels across varying salinities and temperatures. Under reduced salinity, mussels faced hypotonic stress, increasing metabolism and enzymatic response. By contrast, at increased salinity, Gd-exposed mussels reduced their metabolism and did not activate their defenses, likely being a physiological trait response to unfavorable conditions but being inefficient in preventing cellular damage. Notably, Gd induced cellular damage at all salinities and temperatures, despite mussels using different strategies to prevent oxidative stress. At control salinity, the cellular damage was accentuated in Gd-exposed organisms at a higher temperature as defensive mechanisms were compromised. As a direct consequence of warming, mussels experienced metabolic depression to avoid heat stress and consequent cellular damage. Intensified rainfall could amplify Gd's bioavailability in coastal ecosystems, posing risks to osmoconformer organisms. Additionally, Gd exposure in a warming context is alarming, emphasizing the need to explore REEs exposures amid climate change stressors, as they might hinder organisms' growth and reproduction with important economic consequences.

4.3.3. Yttrium Exposure

The discussion presented in this section is based on the results from the following publication:

- Andrade, M., Pinto, J., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. Yttrium effects on the Mediterranean mussel under a scenario of salinity shifts and increased temperature. Mar. Environ. Res. https://doi.org/10.1016/j.marenvres.2024.106365.

Bioconcentration of Yttrium in Mussels

In the present study, *M. galloprovincialis* mussels demonstrated to accumulate Y in their tissues, with BCFs reaching up to 31 L/Kg, although salinity and temperature did not influence this accumulation. Similarly, previous studies showed that the same species of mussels, when exposed to Y at a concentration of 10 µg/L, exhibited a BCF of 33 L/Kg (Andrade et al., 2023b) and Nd at the same concentration resulted in a BCF of 43 L/Kg (Freitas et al., 2020b). Nevertheless, knowledge about the bioaccumulation of Y under increased temperature and different salinities in bivalves remains almost unknown. Assessing the impact of temperature and salinity on the bioaccumulation of REEs presents a complex challenge due to the differences in organisms and/or species response, such as behavioral traits and physiological processes, that can influence the way they accumulate pollutants (including REEs) in their tissues. In addition to differences in the analyzed tissue, the chemistry and behavior of the pollutant, and the specific experimental conditions. However, as observed in the present study, previous studies have also shown that *M. galloprovincialis* mussels can effectively maintain the accumulation of REEs regardless of the tested conditions. For instance, mussels maintained their BCF with exposures of 10 µg/L of La (Andrade et al., 2021) and Gd (Andrade et al., 2022b) with the increase of salinity. Exposure to 10 µg/L of La also resulted in the same BCF with increased temperature (Andrade et al., 2022a). These findings suggest that bivalves may employ physiological mechanisms to mitigate the uptake of contaminants under unfavorable conditions. These mechanisms may involve enzymes involved in detoxification, which can modify chemical compounds to make them less harmful and more easily excreted by the organism (dos Santos et al., 2022). This can be achieved by, for instance, through the utilization of GSTs as observed under the lowest salinity and increased temperature treatments, as well as CbEs under the lowest salinity treatment in the present study. Additionally, adaptation traits such as prolonged valve

closure (Anestis et al., 2007; Gosling, 2003) can lead to alterations in their respiration and filtration rates preventing chemical accumulation. Nevertheless, in the present study, the biotransformation activity seemed to be enough as the ETS was maintained or even augmented under increased temperature.

Metabolic Capacity and Energy Reserves

In terms of salinity influence, only the uncontaminated mussels in the present study decreased ETS activity with increased salinity, indicating that organisms may change their metabolism under salinity influence. The decrease of ETS activity with the increase of salinity has been observed previously in *M. galloprovincialis* (Andrade 2021, 2022b; Freitas et al. 2017b), whereas authors suggested that lower salinities influenced organisms due to hypotonic stress and swell-regulation capacity resulting in higher ETS activities than under increased salinities. Tomanek et al. (2012) proposed that hypotonic stress leads to an increase in protein contribution in energy metabolism and ROS scavenging, thus implying a higher energy consumption. This can be observed in the present study, with an overall higher GLY consumption at the lowest salinity, especially in Y-exposed mussels, although ETS and PROT were maintained. These findings suggest a pronounced biological response in contaminated mussels under low salinities, a result supported by the highest IBR score obtained that indicated that this treatment may have been the most impactful. Nevertheless, with the increase of salinity, Y-exposed organisms maintained their ETS activity while depleting GLY, possibly indicating the use of these reserves in defense mechanisms and protective responses and prioritizing mechanisms to mitigate the adverse effects of Y. In Y-exposed mussels under salinity 40 an elevated IBR score was related to the increased GLY usage, suggesting that, while not as impactful as decreased salinity, the osmotic stress resulting from higher salinity also influenced these organisms.

Regarding the temperature influence, uncontaminated mussels decreased their ETS activity and GLY reserves with its increase. Mussels exposed to Y alone (at control salinity and temperature) showed an identical response, indicating that mussels may try to reduce their aerobic respiration under heat stress or Y contamination but still need to use their energy reserves. Andrade et al. (2022a) also observed that uncontaminated mussels used GLY content under increased temperature suggesting its use in an anaerobic pathway linked to physiological and behavioral adaptation such as valve closure to cope with heat stress. The decrease in ETS activity in bivalves is often associated with a reduction in filtration and/or respiration rates, to prevent or limit the accumulation of xenobiotics in bivalves (*e.g.,* Almeida et al. 2015). Additionally, Andrade et al. (2023b) observed that low concentrations of Y (5 and 10 µg/L) caused similar metabolic changes in ETS activity and GLY content in the same mussels' species. Nevertheless, in the present study, the increase in temperature led to increased ETS activity and GLY content in Y-exposed mussels. These results indicate the necessity for organisms to enhance their metabolic activity and possibly increase the synthesis of GLY for additional energy reserves to support defense mechanisms against the harmful effects of heat stress and Y contamination in combination. Similarly, Leite et al. (2020b) observed that *M. galloprovincialis* exposed to rutile TiO_2 under increased temperature exhibited increased ETS activity when exposed to concentrations of 5 and 50 µg/L and increased GLY content at 100 µg/L. These authors suggested that increased temperature augmented TiO_2 toxicity with mussels enhancing their metabolic capacity to activate defense mechanisms at low concentrations and applying a strategy to mitigate the possible effects at a higher concentration by conserving GLY reserves.

Antioxidant and Biotransformation Capacity

In terms of salinity, organisms increased their antioxidant and biotransformation activity with the decrease of salinity, especially in contaminated individuals. This suggested that lower salinity levels not only contribute to increased oxidative stress, potentially due to hypotonic stress, but also highlight a significant influence of Y in inducing this stress. The effect of the decrease of salinity has been previously observed in *M. galloprovincialis*, where an increase of SOD, CAT, GSTs and CbEs activities occurred not only in uncontaminated mussels but also in those exposed to La, and at a lesser extent in organisms exposed to Gd (Andrade et al., 2021; Andrade et al., 2022b). The authors of the mentioned studies proposed that the increase in enzymatic activity acted as

a protective response against the effects of hypotonic stress, which is believed to be associated with an increase in metabolic capacity and the subsequent formation of ROS. In the current study, the presence of Y in the mussels resulted in higher antioxidant activity under all tested salinities as well as higher biotransformation activity under the salinity 20 further emphasizing its impact, especially under lower salinities. Notably, this augmented enzymatic activity strongly contributed to the highest IBR score observed in Y-exposed mussels at the lowest salinity, providing further support for these findings.

Regarding the influence of temperature, uncontaminated mussels in the present study exhibited only a decrease in GSTs activity at increased temperature, which could be attributed to a decrease in metabolic capacity as indicated by lower ETS activity. The decrease of GSTs in *M. galloprovincialis* due to heat stress has been observed in previous studies (*e.g.*, Leite et al., 2020b; Morosetti et al., 2020), where increased temperature caused lower metabolic capacity in mussels as well. In the present study, contaminated mussels' response to Y was intensified under increased temperature presenting higher GSTs activity, suggesting that the presence of both increased temperature and Y may stimulate the enhancement of biotransformation enzymes, agreeing with the increased metabolic capacity (higher ETS activity) observed. Similarly, Andrade et al. (2022a) observed that *M. galloprovincialis* mussels exposed to La activated their GSTs enzymes, indicating a response to the presence of this element, and the combination with increased temperature resulted in a greater detoxification capacity. In fact, exposure to Y at each temperature tested in the current study resulted in elevated antioxidant and biotransformation activity as well, indicating an impact of Y on the defense mechanisms similar to the observations made under different salinities. Likewise, Freitas et al. (2019b) observed that the same mussel species activated their antioxidant defenses when exposed to triclosan and diclofenac, regardless of the tested temperature, with the authors emphasizing the organisms' ability to employ these mechanisms to respond to ROS, even when their metabolic capacity was reduced.

Oxidative Damage

In terms of salinity influence, only the uncontaminated mussels showed decreased lipid damage with decreased salinity or increased protein damage with increased salinity. The absence of cellular damage at salinity 20 may be attributed to the significant activation of antioxidant and biotransformation enzymes seen in response to hypotonic

stress, enabling mussels to better counteract oxidative stress and prevent cellular damage. Similarly, Freitas et al. (2019b) observed that uncontaminated mussels decreased their LPO levels when exposed to salinity 25 compared to 30, whereas antioxidant enzyme activities such as SOD and CAT increased. Nevertheless, Y-exposure under salinities 20 and 30 resulted in increased LPO levels, indicating that Y exerts greater toxicity in organisms under these salinities, despite the increased activation of defense mechanisms. Andrade et al. (2022b) observed that the same mussel species exposed to Gd were able to avoid and even reduce LPO and PC levels with decreasing salinity, due to the activation of defense mechanisms. However, higher LPO levels were still found in contaminated mussels compared to uncontaminated ones under each tested salinity.

Regarding the temperature influence, uncontaminated mussels decreased their LPO levels with increasing temperature, likely associated with lower ETS activity as it is known to generate ROS as a byproduct of its normal function. Likewise, Morosetti et al. (2020) found that the increase in temperature caused lower levels of LPO and PC in *M. galloprovincialis* mussels, being justified as well by the decrease of ETS observed and sequent lower ROS generation. Temperature influence in the current study, reduced PC levels of Y-exposed mussels as well, however as the metabolic capacity increased (higher ETS activity), this decrease could be linked with the employment of GSTs, as well as other unmeasured defense mechanisms, that were useful in avoiding cellular damages. Nevertheless, under each tested temperature, Y-exposed mussels exhibited higher levels of LPO compared to their uncontaminated counterpart. These findings suggest that despite the activation of defense mechanisms, mussels were unable to completely counteract the harmful effects of Y contamination by themselves. The inefficiency of defense mechanisms has been observed previously in *M. galloprovincialis* exposed to different contaminants, such as Gd (Henriques et al., 2019; Trapasso et al., 2021), Hg (Coppola et al., 2017), diclofenac (Freitas et al., 2019b) and TiO_2 (Leite et al., 2020b).

Neurotoxicity

In terms of salinity influence, mussels in the present study decreased their AChE activity as salinity levels increased, particularly in the case of contaminated organisms. This result suggests that salinity plays a role in influencing AChE activity, and the presence of Y may intensify this effect. In a study conducted by Pfeifer et al. (2005) on

Mytilus sp., it was observed that increasing salinity resulted in a significant decrease in AChE activity, being proposed that this reduction in AChE activity could be attributed to the alterations in the extra- and intracellular inorganic ion concentrations that occur during osmotic adaptation processes. Additionally, Andrade et al. (2021) reported that while no significant differences were observed in AChE activity of uncontaminated mussels, a decrease in AChE activity was observed in mussels exposed to La with an increase in salinity. Nevertheless, at lower and higher salinities in the current study, the Y-exposure resulted in an increase in AChE activity in mussels, except for the organisms under the control condition. This indicates that Y exposure may have a stimulatory effect on AChE activity in mussels under stressful salinities, potentially due to an alteration in cholinergic neurotransmission or other physiological responses.

Regarding the temperature influence, only contaminated mussels in the present study showed an increase in AChE with an increase in temperature. Furthermore, when comparing contaminated mussels to uncontaminated ones, Y exposure under increased temperature seemed to increase AChE activity as well. These findings suggest that the combination of Y contamination and increased temperature causes an enhancement in AChE activity in mussels. In fact, previous studies have reported that an increase in temperature can potentially influence the increase of AChE activity in bivalves (Costa et al., 2020a,b; Pfeifer et al. 2005), and the increase of this esterase activity has also been related to cellular apoptosis under stressful conditions (Zhang et al., 2002).

Conclusions

Overall, the present study found that hypotonic and heat stress in *M. galloprovincialis* mussels were intensified in the presence of Y. Particularly, effects of osmotic adaptation to decreased salinity such as increases in metabolism and defense enzyme activities. With increased temperature, contaminated mussels also enhanced metabolic capacity, possibly activating defense mechanisms such as GSTs. These responses helped the contaminated organisms to mitigate the cellular damage caused by Y accumulation. Nevertheless, under increased temperature and lower salinity, Y-exposure resulted in higher oxidative stress and defense mechanisms activity, although inefficient to avoid cellular damages, indicating enhanced toxic effects of Y under these conditions. Notably, the combination of Y-exposure with lower salinity exhibited the most pronounced impact, indicating that low salinity represents the worst treatment. Thus, the

present study highlights the potential impact of low environmentally relevant Y concentrations on coastal organisms experiencing salinity fluctuations and predicted temperature increases. This study emphasizes the importance of investigating emerging contaminants like REEs in various climate change scenarios.

4.3.4. Terbium Exposure

The discussion presented in this section is based on the results from the following publication:

- Andrade, M., Pinto, J., Soares, A.M.V.M., Solé, M., Pereira, E., Freitas, R., 2023. How predicted climate changes will modulate the impacts induced by terbium in bivalves? Chemosphere. https://doi.org/10.1016/j.chemosphere.2024.141168.

Bioconcentration of Terbium in Mussels

In the present study, mussels bioaccumulated Tb when it was present in the water, as demonstrated by BCFs higher than 29 L/Kg, independently of the temperature and salinity tested. Furthermore, this was reinforced by Tb-exposed mussels that accumulated Tb dozens of times higher (0.26-0.42 µg/g DW) than non-contaminated mussels (up to 0.009 µg/g DW), aligning with natural levels observed in *M. galloprovincialis* along the Portuguese coast (0.002-0.009 µg/g DW in fall and 0.002-0.013 µg/g DW in spring) (Figueiredo et al., 2022b). Recently, Andrade et al., 2023a observed that the same mussel species exposed to a different range of Tb concentration (5, 10, 20 and 40 µg/L) displayed similar BCFs (between 24 and 33 L/Kg), with coincident tissue values to the ones found in the present work (0.274 ± 0.026 µg/L DW) under the same exposure conditions. Previous studies have also demonstrated that this species can bioaccumulate other REEs under similar salinities and temperatures reaching similar BCFs. For instance, a BCF of 33 L/Kg in Y-exposed mussels and of 43 L/Kg in Nd-exposed mussels were observed after the same period (28 days) and under 17 °C and salinity 30 as control physical conditions (Andrade et al. 2023b, Freitas et al. 2020b). Across different salinity levels (20, 30 and 40), mussels exposed to La showed greater BCFs ranging from 46 to 155 L/Kg (Andrade et al., 2021), while Gd exposure resulted in lower BCFs between 11 and 16 L/Kg (Andrade et al., 2022b). For these two REEs, La and Gd, significant higher BCFs were achieved at lower salinity of 20. Under different temperatures (17 °C and 22 °C), La-exposed mussels exhibited values of 75 L/Kg and 77 L/Kg (Andrade et al., 2022a). Overall, these findings suggest that accumulation was independent of the water salinity and temperature levels. This regulation may involve various physiological and adaptive mechanisms such as the modulation of their filtration and respiration rates (Bal et al., 2021; Mayer-Pinto et al., 2020), as well as prolonged closure of their valves (Anestis et al., 2007; Gosling, 2003), in response to environmental stressors. Mussels can also employ detoxification mechanisms such as the use of biotransformation enzymes, which

facilitate the transformation and excretion of different substances (dos Santos et al., 2022). This may explain the different BCFs observed under controlled conditions between different mentioned REEs, whereas La (Andrade et al., 2021, 2022a) showed higher BCF, followed from Nd (Freitas et al. 2020b) and Y (Andrade et al. 2023b), and with Gd (Andrade et al. 2022b) having the lowest one.

Metabolic Capacity and Energy Reserves

In the present study, salinity had little influence in uncontaminated mussels as it did not cause any changes in ETS activity but, energy reserves (GLY and PROT) were spent at the lowest salinity, suggesting their use for essential processes such as osmoregulation and cellular function maintenance, which could be a metabolic response independent of ETS activity. Several studies have previously observed in *M. galloprovincialis* an increase consumption of GLY (Freitas et al., 2017b) and PROT (Andrade et al., 2021, 2022b) at low salinity attributed to hypotonic stress and the regulation of cellular swelling capacity under this condition. It has been theorized that, as a response to hypotonic stress, mussels rely on protein usage as energy source and to scavenge ROS (Tomanek et al., 2012). This was the case for Tb-exposed organisms at the lowest salinity were a preference for PROT rather than GLY as fuel contrasted with unexposed mussels that used both energy sources. Andrade et al. (2021, 2022b) observed that La- and Gd-exposed mussels also mobilized PROT at low salinity. By contrast, at the highest salinity, Tb-exposed mussels increased their ETS activity along with presenting higher GLY and PROT tissue content. While together with an enhancement of their metabolic capacity, the increase of PROT may be indicative of enhanced enzyme synthesis, the increase in GLY content may indicate a strategy of energy preservation. Likewise, an increase in GLY and PROT content at higher salinity has been observed in clams *R. philippinarum* exposed to carbamazepine (Almeida et al., 2022) and As (Freitas et al., 2016), as well as in *M. galloprovincialis* mussels exposed to Pb (Freitas et al., 2019c).

An increase in water temperature implied an increase in ETS activity of both mussel groups, using GLY rather than PROT as the main energy reserves. Such findings indicate that mussels increase their metabolic capacity in response to heat stress and this is accompanied by a usage of GLY as fuel more evident than in the case of osmotic stress. Similarly, Freitas et al. (2019c) observed increases in ETS activity and PROT

content in *M. galloprovincialis* as a result of heat stress and Pb. Additionally, Andrade et al. (2019a) revealed that both unexposed and MWCNTs-exposed mussels of the same species, exhibited higher ETS activity and utilization of GLY reserves with increasing temperature. The authors attributed these changes to the mussels' enhanced respiratory chain associated with increased respiration rate in response to heat stress, leading to higher energy costs to maintain their oxidative balance. Notably, in the current study, Tb-exposed and unexposed mussels under the highest temperature regime maintained the same metabolic capacity and energy reserves usage. This suggests that Tb-exposed mussels may employ another metabolic strategy to mitigate Tb accumulation rather than further increasing metabolism under heat stress, potentially through the modulation of their filtration/respiration rates and prolonged valve closure, as discussed previously. Alternatively, it's possible that mussels, whether contaminated or not, reached their metabolic steady state at 22 °C, and further increase in ETS activity may not have been feasible unless there was a process of former thermal tolerance as described by Georgoulis et al. (2021) in *M. galloprovincialis*.

Antioxidant and Biotransformation Capacity

Salinity modulation of antioxidant defenses was only evidenced in Tb-contaminated mussels. At decreased salinity, most of antioxidant enzymes were activated, and GSTs activity also tended to increase (especially when compared to the hyper-saline media of 40), indicating enhanced enzymatic defenses under hypotonic stress. Andrade et al. (2021) described in the same bivalve species exposed to La, an increase in defenses such as antioxidant enzymes and GSTs (but also CbEs) with decreasing salinity. This response was attributed to ROS formation under hypotonic stress and the upregulation of all these enzymes as a defense mechanism against salinity shifts. Nevertheless, in the current study, Tb exposure alone inhibited the activity of all the antioxidant enzymes in mussels at the control salinity, an effect that seemed to be masked under the influence of other stressful factors such as salinity shifts. The consequence of decreased antioxidant defenses could lead to cellular damages. A similar inhibition in antioxidant enzymes in *M. galloprovincialis* under control salinities was reported to salicylic acid (Freitas et al., 2020c), a mixture of carbon nanotubes (CNTs) and triclosan (De Marchi et al., 2020a), and La (Andrade et al., 2021) exposures. These former studies also highlighted the role of salinity shifts in modulating this response, masking the singular effects of each contaminant. Nevertheless, in the present study,

mussels activated GR and GSTs defenses at high salinity conditions in an efficient way. Freitas et al. (2019c) also observed an increase in GSTs activity under increased salinities in the same mussel species when exposed to Pb, despite the lack of activation or even inhibition of antioxidant enzymes, demonstrating the organisms' ability to activate these conjugation enzymes under such conditions.

Under the increased temperature scenario of the present study, mussels showed a decrease in GR activity, regardless of the presence of Tb. These findings suggest that, when combined, Tb and temperature can inhibit GR activity, with the latter possibly associated with increased ROS formation associated to higher ETS activity at this condition. De Marchi et al. (2020b) also observed lower GR activity levels in uncontaminated and CNTs-exposed *M. galloprovincialis* at higher temperatures. Nevertheless, Tb-exposed organisms under the high temperature regime compared to their uncontaminated counterpart or Tb-exposed mussels under control temperature, were still able to activate other defenses such as CAT, GSTs and GPx but not SOD to mitigate any ROS produced. Morosetti et al. (2020) reported increased GPx activity in the same mussel species exposed to Hg and a mixture of CeO_2 with Hg as temperature increased, a trend which was not observed in uncontaminated mussels. Nonetheless, considering the role of GPx (reduction of lipid hydroperoxides and H_2O_2 by oxidizing reduced glutathione, GSH, to oxidized glutathione, GSSG), GSTs (detoxification by conjugating GSH with xenobiotic substances) and GR (reduction of GSSG back to GSH), prolonged inhibition of GR could impact the activity of both GSH-dependent: GPx and GSTs, resulting in a decreased effectiveness of these defenses.

Oxidative Damage

In terms of salinity modulation, mussels experienced an enhanced oxidative stress status with increased salinity, as mussels at this condition presented higher LPO levels accompanied by a lower GSH:GSSG ratio. Particularly, in those Tb-exposed it was associated with decreased GSTs activity and increased ROS production due to higher ETS activity. Similarly, Freitas et al. (2016) found that increased salinity pressure caused oxidative stress in clams *R. philippinarum*, resulting in higher LPO levels and a lower GSH:GSSG ratio. Nevertheless, in the current study, uncontaminated mussels also increased PC levels with both changes in salinity (20 and 40) as there was a lack of enzymatic response. This result was more evident at salinity of 40, where PC levels were

also higher than in Tb-exposed mussels, probably due to the efficient activation of GSTs in the contaminated group. Also in mussels, Freitas et al. (2019c) noted a similar pattern as uncontaminated mussels increased their PC levels at extreme osmotic values, and that at the highest salinity PC damage was less evident in those exposed to Pb also attributed to their activation of GSTs.

An increase in temperature caused a decrease in PC levels, more evident in uncontaminated mussels, suggesting that despite antioxidant and biotransformation enzymes were not enhanced, other defense mechanisms may have been activated. For instance, in response to elevated temperature, cells may activate heat shock proteins, which serve as intrinsic mechanisms to protect the functionality of proteins and preserve cellular integrity, thus shielding against damage (Fabbri et al., 2008). Nevertheless, Tb-exposed mussels in the present work further reduced their PC levels while increasing their LPO ones with the increase in temperature. Such findings indicate that mussels can mitigate protein damages possibly through increased PROT synthesis (seen through increased PROT reserves), but the elevated ROS formation associated with higher ETS activity and Tb exposure leads to lipid damage despite their efforts to enhance part of their enzymatic activities. Likewise, Freitas et al. (2019b) reported that with increasing temperature both uncontaminated and diclofenac (DIC)-exposed mussels showed decreased PC levels, with contaminated organisms increasing LPO levels as the activation of antioxidant enzymes and GSTs was insufficient to neutralize the ROS excess. In terms of Tb exposure by itself, contaminated mussels in the current study compared to uncontaminated ones exhibited increased cellular damage under the highest temperature, suggesting an enhanced toxicity of Tb under heat stress. These findings may have been related to organisms' attempt to mitigate Tb accumulation by not increasing their metabolism and allocating resources primarily to specific enzymes, but being insufficient in preventing cellular damage. Previous studies have observed the ineffectiveness of defense mechanisms in organisms exposed to various contaminants, including MWCNTS (Andrade et al., 2019a), Pb (Freitas et al., 2019c), and DIC (Freitas et al., 2019b), under heat stress.

Neurotoxicity

In terms of salinity shifts, both mussel groups increased AChE activity with increased salinity, suggesting that salinity can influence this activity in mussels regardless

of Tb presence. However, Tb-exposure alone led to AChE inhibition at control salinity, potentially indicating that the inhibitory effect of Tb may have been masked under extreme salinities, possibly as a consequence of osmotic stress. Previous studies have demonstrated AChE inhibition in *M. galloprovincialis* caused by rare-earth elements such as Gd (Henriques et al., 2019) and La (Pinto et al., 2019) under optimal salinity conditions. Salinity has been regarded as a confounding factor in assessing AChE activity in contaminated bivalves (Pfeifer et al., 2005; Tankoua et al., 2011), with conflicting results across species. For instance, AChE activity increased with increasing salinity in clams *Scrobicularia plana* (Tankoua et al., 2011) and mussels *Unio ravoisieri* (Lassoued et al., 2023), while it decreased in a more saline media in marine *Mytilus* sp. mussels (Pfeifer et al., 2005). These alterations have been attributed to osmotic stress affecting acetylcholine levels and changes in extra- and intracellular inorganic ion concentrations during osmotic adaptation, although the underlying mechanisms still remain fully understood.

Regarding the increase in temperature, both mussel groups exhibited an increase in AChE activity with the increase of temperature, implying a general increase in metabolism (as seen for ETS). Previous studies with bivalves (Costa et al., 2020a,b; Pfeifer et al., 2005) have demonstrated that temperature can enhance AChE activity, which may be linked to cellular apoptosis in response to extreme stressful conditions (Zhang et al., 2002). Nonetheless, Tb exposure by itself in the current study seemed to exert effects solely under normal conditions, resulting in AChE inhibition. These findings suggest that temperature, as seen by salinity, could potentially act as a confounding factor in the assessment of AChE inhibition as a consequence of Tb contamination.

Conclusions

Overall, the present study revealed that osmotic and heat stress led to significant biochemical alterations particularly in Tb-exposed *M. galloprovincialis* mussels. Increased salinity resulted in oxidative stress, cellular damage, and increased AChE activity in the Tb-exposed group although metabolic capacity associated to antioxidant defenses and possible cell repair mechanisms also increased. By contrast, decreased salinity also affected Tb-exposed mussels but differently by effectively activating defense mechanisms to avoid cellular damage from hypotonic stress. In turn, increased temperature equally affected both uncontaminated and Tb-exposed mussels, leading to GR inhibition and increased AChE activity despite efforts to increase metabolic capacity and reduce protein

damage. In the case of Tb-contaminated mussels, several antioxidant enzymes were activated but they were not enough to avoid lipid damage. Tb alone caused antioxidant enzymes inhibition and neurotoxicity which may had led to reduced enzymatic efficiency with the concomitant increase of salinity and temperature, although these two physical variables may act as a co-founding factors. These findings emphasize the importance of studying emerging contaminants such as Tb in the context of climate change scenarios, where low concentrations of these elements may become more pronounced on marine organisms under a changing scenario.

4.3.5. Final Considerations

In the present section (4.3.), the comprehensive assessment of the impacts of REEs, namely La, Gd, Y, and Tb, on *M. galloprovincialis* mussels under different salinities and increase of temperature was performed. Both changes of salinity and increase of temperature demonstrated to cause osmotic and heat stress in the organisms. Furthermore, each of the elements exhibited distinct effects: La compromised antioxidant defenses and caused cellular damage, Gd and Y activated enzymatic defenses inefficient at preventing cellular damage, and Tb inhibited antioxidant defenses while causing neurotoxicity.

Interestingly, each REE presented distinct profiles of interaction with the mussels under varying conditions, highlighting the complexity and specificity of these interactions. For instance, with decreased salinity, both uncontaminated and contaminated mussels exhibited hypotonic stress, with La and Y exposures in particular intensifying the biochemical effects. Conversely, at increased salinity, Gd exposure reduced even more the metabolism and defense activation, resulting in cellular damage. Additionally, the effects of Tb where masked under osmotic stress, whereas under decreased salinity mussels activated their enzymatic defenses to avoid cellular damages but increased salinity there was increased oxidative stress, acetylcholinesterase, metabolism, and antioxidant activities, albeit insufficient to prevent cellular damage. Furthermore, the presence of all REEs increased cellular damage under elevated temperatures, with La and Gd having some defense mechanisms inhibited and Y and Tb activating others.

The effects of these REEs, particularly when coupled with fluctuating salinities and increased temperature, are not only of ecological concern but also bear potential socio-economic ramifications, given the importance of *M. galloprovincialis* in coastal ecosystems. Recognizing the intricate web of interactions and the potential cascade of

impacts, this section reinforces the critical need for a multidimensional approach when evaluating emerging contaminants in marine ecosystems. As we move forward in an era characterized by shifting climate scenarios, understanding the synergistic effects of REEs and environmental stressors will be paramount for ensuring the health and sustainability of marine habitats.

CHAPTER 5. CONCLUDING REMARKS AND FUTURE PERSPECTIVES

5.1. Concluding Remarks

In today's world, where technology changes rapidly, there's a new problem that society is facing: e-wastes, due to discard of electronic and electric equipment, now recognized as one of the fastest growing waste streams globally. Each year, millions of tons of e-waste are generated, and this number has surged by 9.2 million metric tons since 2014, with projections pointing towards a staggering 74.7 million tons by 2030 (Forti et al., 2020). Unfortunately, a substantial portion of this waste is not subjected to recycling; instead, it ends up in landfills or is shipped to less affluent regions, predominantly in Asia and Africa. This practice has led to illegal and unsafe recycling methods, resulting in environmental harm and raising ethical and health concerns due to the release of hazardous substances.

Also, there's another critical aspect often missed in discussions about e-waste: rare-earth elements (REEs). These elements are present in many electronic devices, from smartphones to medical equipment. These elements, while essential, tend to be neglected regarding their environmental impact. The significance of this oversight becomes clearer when considering the fast-growing demand for REEs, expected to grow by 4.2 % annually from 2023 to 2030, driven primarily by advances in sustainable technologies like electric vehicles and wind turbines (GlobalData, 2023; Goodenough et al., 2017). As a result, the increasing use of REEs is closely connected to the escalating e-waste crisis.

A considerable amount of these materials finds its way into the coastal environments, entering either directly or indirectly through runoff into rivers and streams, presenting a serious threat to marine ecosystems. In these environments, marine organisms, already under stress due to various human activities, now face an added burden from these novel contaminants. Detecting and quantifying these pollutants is arduous due to their low environmental concentrations, so far. However, as the tide of e-waste continues to surge, so is the accumulation of REEs within marine species, ultimately affecting final consumers.

The present work contributes to confirm, for the first time, that actual low environmental concentrations of REEs like yttrium (Y) and terbium (Tb) have an impact in valuable estuarine and intertidal organisms such as the mussel *Mytilus galloprovincialis*. These mussels are considered excellent sentinels since they are widespread, resilient, and play essential roles in marine ecosystems and aquaculture. The study has revealed the accumulation of these elements within mussels and their subsequent biological cellular effects. In particular, mussels exhibited shifts in metabolic capacity and energy

usage in response to these REEs exposure in the range of concentrations tested (0 to 40 µg/L), raising concerns for aquatic ecosystems, especially given the potential long-term effects of low anthropogenic concentrations on growth, reproduction, and defense mechanisms. Nevertheless, while both Y and Tb induced oxidative stress in the mussels, the nature and extent of their impacts varied. Yttrium primarily caused minor physiological changes at a concentration of 5 µg/L, with these effects decreasing as the concentration increased (10, 20 and 40 µg/L). In contrast, Tb exhibited a more complex response: an initial increase in defenses responsiveness from 5 to 10 µg/L, a return to baseline levels at 20 µg/L, and a mild increase again at 40 µg/L. Remarkably, although Lanthanum (La) and Gadolinium (Gd) were observed to induce several biochemical changes in another bivalve species at similar low concentrations, more pronounced alterations were reported at intermediate concentrations by other researchers, potentially indicating more significant adverse effects compared to Y and Tb. These differences in toxicity among these elements likely stem from their unique chemical properties and how they interact with cellular components and processes. In this aspect the present observations might suggest a trend where lighter REEs demonstrate greater toxicity compared to their heavier counterparts. This can be concerned given that lighter REEs are more abundantly found in the environment, potentially posing a higher risk of ecological and biological impact.

This novel chemical challenge becomes even more complex when we anticipate climate change scenarios as an additional cause of stress. The rise in global temperatures, attributed to human activities like fossil fuel combustion, is expected to lead to an increase of 1.0 to 5.7 °C by the end of this century, depending on different greenhouse gases emission projections (IPCC, 2021). Climate change further aggravates the situation by altering ocean salinity and other factors through the melting of polar ice, increasing precipitation and evaporation, and more frequent extreme weather events. Such environmental changes will likely alter the behavior of REEs in relation to marine organisms by affecting the physical and chemical properties of these elements. Consequently, REEs might become more readily available or reactive in the marine environment, potentially leading to increased accumulation on or interaction with marine life. Moreover, climate-induced changes might inadvertently cause marine organisms to absorb more of these elements.

The present work demonstrated how climate change factors, particularly changes in salinity and temperature, exacerbate the impact of a low but relevant concentration (10 µg/L) of La, Gd, Y, and Tb on mussels. The magnitude of these REEs effects in the physiology of bivalves greatly depended on these environmental conditions. For instance,

the hypotonic stress from reduced salinity seemed to intensify the La, Gd, and Y effects, leading to increased enzymatic responses and cellular damage. In another hand, Tb-exposed mussels demonstrated a more resilient response, activating defense mechanisms enough to avoid cellular damage. In the scenario of increased salinity, both La- and Y-exposed mussels adapted to hypersalinity by utilizing protein reserves and slightly elevating enzyme activities, managing osmotic and metabolic changes. However, both Gd and Tb showed compromised metabolic and defense activities, with Gd potentially leading to a metabolic shutdown and Tb exhibiting susceptibility through significant oxidative stress and cellular damage. In terms of increased temperature its effects were not so linear with mussels exposed to both La and Gd showing compromised defense mechanisms and Y mussels activating most of these. Terbium in particular had some defense enzymes compromised although being able to activate some of the evaluated antioxidants. Among these elements, only the mussels exposed to La did not exhibit cellular damage under conditions of warm stress. Overall, while all elements induced stress in all tested conditions, organisms exposed to La and Y seemed to be the most affected under lower salinity and the ones exposed to Gd and Tb under increased salinity and increased temperature. This study highlights that while by themselves lighter REEs may inherently appear more toxic, the influence of climate change factors can significantly modify the predicted toxicity profiles of these elements, revealing that environmental conditions play a crucial role in dictating the severity and nature of their impact. Additionally, among all the tested elements, the decrease in salinity intensified the harmful effects of REEs more drastically than an increase in salinity or in temperature. This suggests that areas experiencing freshwater influx, such as estuaries, may face a higher risk of REE-induced toxicity.

This emerging challenge highlights the urgency for sound research to scientifically inform policymakers and take the right decisions. A comprehensive understanding of the concentration levels and impact of REEs in marine organisms under a changing environment is essential for developing effective strategies to safeguard marine ecosystems and maintain the services they provide. Such a task requires collaborative efforts from scientists, policymakers, and environmental organizations, working together to turn scientific insights into practical policies.

5.1.1. Thesis Relevance and Elements of Innovation

The relevance of the present thesis lies in its investigation of the ecological implications of REEs, key components of e-waste, on marine ecosystems. The study gains in-depth knowledge by examining elements like La, Gd, Tb, and Y, each used in various industrial and technological applications, in both water and marine biota, addressing a crucial gap in the current environmental research by offering a comprehensive view of the ecological implications they may cause. Specifically, it provides insights into how these contaminants, often overlooked in marine pollution studies, affect key species like the model mussel *M. galloprovincialis*.

The present thesis makes a significant advancement in understanding the ecological repercussions of REEs in dynamic environments such as estuaries. The comprehensive approach of quantifying these elements both in water and in biota, coupled with detailed analysis of their biochemical impacts on mussels, often not considered together in the literature, distinguishes the present research. It also stands out by examining these elements in environmentally relevant concentrations, a departure from conventional studies that typically focus on high and non-relevant concentrations. Notably, it also explores the complex interactions between REEs and marine organisms under the conditions of a changing climate, factors that when considering together have been scarcely addressed in existing literature. The extended study over prolonged experimental periods allows also for a nuanced understanding of the dynamic and long-term effects of these elements.

The findings of the present work closely align with key objectives of international environmental protection strategies, including the United Nations' 2030 Agenda (UN General Assembly, 2015). They resonate with Sustainable Development Goals 13 (Climate Action) and 14 (Life Below Water). In particular, by exploring the complex interactions between climate change factors and the accumulation and toxicity of REEs in marine organisms, the present research directly supports the goals of SDG 13, providing empirical data crucial for understanding and mitigating the impacts of climate change on marine ecosystems, thus aiding in the development of informed climate policies and resilience strategies. In relation to SDG 14, the study's in-depth analysis of the effects of e-waste contaminants on marine life offers valuable insights for conserving ocean health and biodiversity. The findings emphasize the need for improved waste management and pollution control measures to sustainably use and protect our oceans, seas, and marine resources, aligning closely with the SDG aim to prevent and significantly reduce marine pollution of all kinds.

Furthermore, within the European Union's Horizon Europe 2021-2027 framework, the findings also fit into Pillar 2, contributing to Cluster 5 by addressing the clean energy transition and environmental impact of e-waste. The research identifies potential risks associated with e-waste, emphasizing the need for its careful management, which is a crucial component in achieving a cleaner, more resilient environment. Additionally, in Cluster 6, the research aligns with the goals of Circular Bio-based Europe and the Climate Neutral, Sustainable and Productive Blue Economy. Here, the study enhanced the understanding of marine ecosystem health and underscores the need to safeguard biodiversity, especially in the context of global change and increasing technological reliance.

Overall, this thesis advances our knowledge on the concentrations and effects of REEs in marine organisms under a changing environment setting a new precedent for future ecotoxicological research. It provides pivotal data and insights for policymakers and stakeholders tasked with protecting marine ecosystems and fostering sustainable development in the face of global environmental challenges.

5.2. Future Perspectives

Future research can offer an array of opportunities, beginning with a deeper exploration of the rare-earth elements' interactions, which may yield additive, synergistic, or antagonistic outcomes. Building upon this, there's immense potential in extending this investigation to encompass a wider array of marine organisms. By specifically focusing and simulating their unique environmental habitats, such as the intertidal and subtidal zones, we can also gain a more comprehensive perspective of the marine ecosystem. Concurrently, the molecular mechanisms that underpin the observed biochemical responses warrant a closer look. Advanced techniques like transcriptomics and proteomics can be instrumental in this regard. However, to enrich the current understanding, there's a pressing need to introduce biomarkers not just at the sub-cellular level, but also at the physiological, organismic, and ecosystem levels. This multi-level approach can provide a richer insight, especially when considering the application of mesocosms. Another imperative is a detailed assessment of REE distribution in marine ecosystems. Identifying regions particularly vulnerable to shifts in salinity or temperature could spotlight potential high-risk areas. As knowledge is accumulated on the harmful effects of REEs, developing mitigation strategies—both technological and biological—becomes crucial. On a parallel track, crafting policy recommendations that target regions

widespread with e-waste dumping practices its of big importance. In the larger picture, an integrated approach that synergistically addresses both climate change and e-waste contamination is pivotal for the sustainable future conservation of marine ecosystems.

CHAPTER 6. REFERENCES

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