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Daniel Seabra dos Santos

Como diferentes cenários de aumento de temperatura da água do mar podem alterar a resposta do *Mytilus galloprovincialis* ao lítio?

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Marinha Aplicada, realizada sob a orientação científica da Professora Doutora Rosa de Fátima Lopes de Freitas, Professora Auxiliar com Agregação do Departamento de Biologia da Universidade de Aveiro e da Professora Doutora Maria Eduarda da Cunha Pereira, Professora Associada do Departamento de Química da Universidade de Aveiro

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palavras-chave

Aquecimento global; Ondas de calor marinhas; Alterações bioquímicas; *Mytilus galloprovincialis*; Lítio

resumo

Os ecossistemas marinhos têm vindo a ser afetados com o aumento gradual da temperatura devido às alterações climáticas. Cenários de aquecimento e a intensificação de eventos climáticos extremos têm afetado os organismos marinhos, como as ondas de calor marinhas (MHWs), que são períodos de aumento anómalo da temperatura da superfície da água. Para além disso, estes também têm sido ameaçados pela poluição derivado da atividade antropogénica. O lítio (Li) é um poluente emergente que se tornou uma preocupação devido ao seu uso crescente numa variedade de aplicações. Compreender a sua influência nos ambientes marinhos em combinação com os cenários de aquecimento é crucial, pois muito pouco se sabe sobre o seu impacto nos organismos marinhos, especialmente quando se considera também os impactos cada vez mais preocupantes das mudanças climáticas. Posto isto, esta investigação visa avaliar como diferentes cenários de aumento de temperatura podem afetar a resposta de *Mytilus galloprovincialis* aos efeitos de Li. Foram avaliados níveis de bioacumulação nos mexilhões e biomarcadores fisiológicos e bioquímicos após 28 dias de exposição a 250 µg/L de Li em diferentes cenários de temperatura (controlo – 17 ºC; aquecimento - 21 ºC e uma simulação de uma onda de calor marinha – 23 ºC). Os resultados indicam que a concentração de Li nos tecidos aumentou apenas nos mexilhões contaminados quando expostos a 17 e 21 ºC, e que a temperatura não teve qualquer efeito na acumulação de Li. A taxa de respiração dos mexilhões foi maior nos mexilhões contaminados do que nos não contaminados. A taxa metabólica diminuiu em mexilhões expostos a 21 ºC e MHW, enquanto os mexilhões expostos à combinação de Li e MHW aumentaram a sua taxa metabólica. Os mexilhões expostos à MHW e principalmente com Li exibiram danos celulares. Observou-se que o Li não causou neurotoxicidade em *M. galloprovincialis*. Este estudo mostrou que apenas a condição de maior stresse (MHW + Li) induziu efeitos negativos nesta espécie, o que pode afetar negativamente o crescimento e reprodução de toda uma população, bem como a estrutura e funcionamento do ecossistema. No geral, os resultados atuais destacam a importância de estudos futuros em que é necessário combinar diferentes poluentes com cenários de aumento de temperatura, nomeadamente eventos climáticos extremos como MHW.

keywords

Warming; Marine heatwaves; biochemical alterations; *Mytilus galloprovincialis*; Lithium

abstract

Marine ecosystems have been affected by the gradual rise in temperature due to climate change. Warming scenarios and the intensification of extreme climate events, such as marine heatwaves (MHWs), have been negatively affecting marine organisms. In addition, they are also threatened by anthropogenic pollution. Lithium (Li) is an emerging pollutant that has become a major concern due to its increasing use in a variety of applications. Understanding its combination with warming scenarios is crucial, as very little is known about its impact on marine organisms. For this reason, this research aimed to assess how different temperature increase scenarios may affect the response of *Mytilus galloprovincialis* to the effects of Li. Mussel bioaccumulation levels and physiological and biochemical biomarkers were analyzed after 28 days of exposure to 250 µg/L of Li under different temperature scenarios (control – 17 ºC; warming – 21 ºC and a simulation of a marine heatwave - 23 ºC). The results indicate that Li concentration in the tissues increased only in the contaminated mussels when exposed to 17 and 21 ºC, and that temperature had no effect on the accumulation of Li. The respiration rate of mussels was higher in the contaminated mussels than in the non-contaminated ones. The metabolic rate decreased in mussels exposed to 21 ºC and MHW, while mussels exposed to the combination of Li and MHW increased their metabolic rate. The mussels exposed to MHW and Li exhibited cellular damage. It was found that Li was not neurotoxic to *M. galloprovincialis*. This study has shown that only the most stressful condition (MHW + Li) induced negative effects in this species which can negatively affect the growth and reproduction of an entire community as well as the structure and functioning of the ecosystem. In general, the presented results highlight the importance of future studies in which it is necessary to combine the effects of pollutants and climate change, namely extreme weather events such as MHWs.

Contents

Chapter 1 – Introduction

1.1. Marine coastal systems: major stressors ……2

1.1.1. Climate changes: warming and marine heatwaves ……2

1.1.2. Emerging contaminants: lithium in the environment ……4

1.1.3. Responses of bivalves to warming and contaminants ……....6

1.2. *Mytilus galloprovincialis* as bioindicator species ……..…7

1.3. Objectives ……9

Chapter 2 - Materials and Methods

2.1. Sampling area ……..11

2.2. Experimental conditions ……..12

2.3. Lithium quantification in water and mussel´s samples ……14

2.4. Biological responses ……....16

2.4.1. Physiological parameters ……16

2.4.1.1. Mortality ……..16

2.4.1.2. Condition index ……...16

2.4.1.3. Respiration rate ……...17

2.4.2. Biochemical parameters ……...18

2.4.2.1. Metabolic capacity and energy reserves ……..19

2.4.2.2. Oxidative status ……...20

2.4.2.2.1. Antioxidant and biotransformation defenses ……...20

2.4.2.2.2. Cellular damage and redox balance ……....22

2.4.2.3. Neurotoxicity ……..…...23

2.5. Data analyses ……...23

Chapter 3 – Results

3.1. Lithium quantification in water and mussel´s samples …….26

3.2. Biological responses …….……28

3.2.1. Physiological parameters …….28

3.2.1.1. Mortality …….28

3.2.1.2. Condition index ……...……28

3.2.1.3. Respiration rate ……...29

3.2.2. Biochemical parameters ……...30

3.2.2.1. Metabolic capacity and energy reserves ……..30

3.2.2.2. Oxidative status …….…….…….32

3.2.2.2.1. Antioxidant and biotransformation defenses ……...32

3.2.2.2.2. Cellular damage and Redox balance ……35

3.2.2.3. Neurotoxicity …….….37

3.2.3. Multivariate analysis ……37

Chapter 4 - Discussion

4.1. Lithium concentration and bioaccumulation ……..40

4.2. Physiological responses ……..41

4.2.1. Condition index ……...41

4.2.2. Respiration rate ……....42

4.3. Biological responses ……...43

4.3.1. Metabolic capacity and energy reserves ……..43

4.3.2. Oxidative status …….…….…….45

4.3.2.1. Antioxidant and biotransformation capacity ……..46

4.3.2.2. Cellular damage and Redox balance ……..47

4.3.3. Neurotoxicity ……...48

4.4. Contribution to the Sustainable Development goals (2030 Agenda) …….49

Chapter 5 – Conclusions

5. Conclusions ……...51

Chapter 6 – References

6.1. References ……..53

6.2. Webgraphy ……74

Chapter 7 – Appendix

7. Supplementary material ……76

List of figures

Figure 1. *Mytilus galloprovincialis* [1] …….8

Figure 2. Evolution of mussel aquaculture production by weight (million tonnes) in the EU and the rest of the world (1950–2016). Different cores represent the production data of the European Union – blue and red the rest of the world (Avdelas et al., 2021). ……9

Figure 3. *Mytilus galloprovincialis* sampling area [3] …….11

Figure 4. Experimental setup. **A**: Control room 17±1 °C; **B**: Warming room 21 ± 1 °C. …….12

Figure 5. Homogenization process of mussels. The process is composed of inserting the mussel tissue into the mortar (A), then grind and homogenized with liquid nitrogen (B) and finally the tissue is weighed and divided into eppendorf's with 0.5 g for biochemical posterior analysis (C). ……..14

Figure 6. A: Inductively coupled plasma optical emission spectrometry (ICP-OES) used for the quantification of Li in water and mussel's samples; **B**: Representation of the procedure of a typical ICP-OES instrument (in: Khan et al., 2022). …….....15

Figure 7. A: Teflon vessels; **B**: CEM MARS 5 microwave. …….…….16

Figure 8. Organisms (both shells and tissues) after being 48 h in the oven at 60 ºC. …….…….17

Figure 9. Experimental setup of respirometric chambers for the measurement of respiration rate (RR). …….18

Figure 10. Condition Index (CI), in *Mytilus galloprovincialis* exposed to different temperatures (17 °C, 21 °C and Marine heatwave - MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for

non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between non-contaminated and contaminated mussels for each temperature scenario. …….....29

Figure 11. Respiration Rate (RR), in *Mytilus galloprovincialis* exposed to different temperatures (17 °C, 21 °C and Marine heatwave - MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between non-contaminated and contaminated mussels for each temperature scenario. …….....29

Figure 12. A: Electron transport system (ETS) activity; **B:** Protein (PROT) content; **C:** Glycogen (GLY) content, in *Mytilus galloprovincialis* exposed to different temperatures (17 °C, 21 °C and Marine heatwave - MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between non-contaminated and contaminated mussels for each temperature scenario. ……..……31

Figure 13. A: Catalase (CAT) activity; **B:** Glutathione peroxidase (GPx) activity; **C:** Glutathione reductase (GR) activity, in *Mytilus galloprovincialis* exposed to different temperatures (17 °C, 21 °C and Marine heatwave - MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between the non-contaminated and contaminated mussels for each temperature scenario. ……..……...……...……33

Figure 14. A: Carboxylesterase p-nitrophenyl acetate (CbEs-*p*NPA) activity; **B:** Carboxylesterase p-nitrophenyl butyrate (CbEs-*p*NPB) activity; **C:** Glutathione S-transferases (GSTs) activity, in *Mytilus galloprovincialis* exposed to different temperatures (17 °C, 21 °C and Marine heatwave - MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between non-contaminated and contaminated mussels for each temperature scenario. ……..34

Figure 15. A: Lipid peroxidation (LPO) levels; **B:** Protein carbonylation (PC) levels; **C:** Reduced glutathione (GSH) content, in *Mytilus galloprovincialis* exposed to different temperatures (17 °C, 21 °C and Marine heatwave - MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between non-contaminated and contaminated mussels for each temperature scenario.……36

Figure 16. Acetylcholinesterase (AChE) activity, in *Mytilus galloprovincialis* exposed to different temperatures (17 °C, 21 °C and Marine heatwave - MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between non-contaminated and contaminated mussels for each temperature scenario....…….……...37

Figure 17. Centroid's ordination diagram (PCO) based on biochemical markers measured, in *Mytilus galloprovincialis* exposed to different temperatures (17 °C, 21 °C and Marine heatwave - MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data (r > 0.75): Reduced glutathione (GSH); Carboxylesterase *p*-nitrophenyl butyrate (CbEs – *p*NPB); Glycogen (GLY); Condition index (CI); Protein (PROT); Electron transport system (ETS); Lipid peroxidation (LPO); glutathione peroxidase (GPx); Protein carbonylation (PC)..……38

List of tables

Table 1. Lithium (Li) concentrations (µg/L) in artificial seawater samples collected immediately after spiking from each exposure aquarium. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated mussels and uppercase letters for contaminated ones). Values are the mean of 6 values ± standard deviation. MHW: Marine heatwave. …….....26

Table 2. Lithium (Li) concentrations (µg/L) in artificial seawater samples collected immediately after spiking (0 h), 24 h (1 day), 48 h (2 days), 72 h (3 days) and 168 h (7 days) after the beginning of the assay from each blank aquarium. Values are the mean of 2 values ± standard deviation. ……..27

Table 3. Lithium (Li) concentrations (µg/g dry weight) in mussels soft dried tissue and Bioconcentration factor (BCF) (L/Kg) after 28 days of exposure to artificial seawater spiked with 250 µg/L of Li. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated mussels, uppercase letters for contaminated ones) and an asterisk represents significant differences between non-contaminated and contaminated mussels for each temperature scenario. Values are the mean of 3 values ± standard deviation. MHW: Marine heatwave. ...……...28

Table 1 SM. Statistical results (PERMANOVA main test) including *p*-values, pseudo-F and degrees of freedom obtained for concentration of Li in mussel's soft tissue, physiological and biochemical parameters in *Mytilus galloprovincialis*. Significant values ($p < 0.05$) are in bold. …….76

Table 2 SM. Pairwise comparisons (*p*-values (MC) among different temperatures (17 °C, 21 °C and MHW) for each treatment (CTL and Li), and between CTL and Li for each temperature performed in the concentration of Li in mussel's soft tissue and the physiological and biochemical parameters in which the main test was significant. MHW: Marine heatwave. Significant values ($p < 0.05$) are in bold. ……77

Table 3 SM. Statistical results (PERMANOVA test) including *p*-values, pseudo-F and degrees of freedom obtained for concentration of Li in mussel's soft tissue, physiological and biochemical parameters in *Mytilus galloprovincialis*, obtained from an analysis with a two factors design, to test the interaction between Li concentration (factor 1) and temperature (factor 2). Significant values ($p < 0.05$) are in bold. ……78

Chapter 1

Introduction

1. Introduction

1.1. Marine coastal ecosystems: major stressors

Marine coastal ecosystems are among the most ecologically and socio-economic vital systems in the world (Dayton et al., 2005; Harley et al., 2006). Currently, there are more than 600 million people living near the coast, and by 2050, this number will probably reach one million (Boretti & Rosa, 2019; Mackie et al., 2020). The coastal areas are complex and dynamic systems generally defined as an interface between land and sea, being exposed to natural and anthropogenic alterations due to humanity (Andrade et al., 2009), including the areas related to climate changes (Caldeira & Wickett, 2003; Orr et al., 2005). The effects of climate change are seen as major threats to most coastal waters (such as seawater acidification, salinity changes and rising temperatures), reflecting an increase in the vulnerability of coastal ecosystems and living organisms (Ani & Robson, 2021; Freitas et al., 2021). Several studies have shown that seawater temperature is increasing in the oceans in particular marine coastal systems (Fogarty et al., 2008; Levitus et al., 2009). Coastal systems have been also exposed to a wide range of contaminants from human activity, such as "classics" (e.g., organic and inorganic material) (Artifon et al., 2019; Gao et al., 2019), personal care products (PCPs) (Wang et al., 2021) and emerging contaminants (EC) such as pharmaceuticals (Fernández-Rubio et al., 2019), rare earth elements (REEs) (Casse et al., 2019) or nanoparticles (NPs) (Freslon et al., 2014).

1.1.1. Climate changes: warming and marine heatwaves

Coastal ecosystems have been increasingly subject to warming events (Frölicher & Laufkötter, 2018; IPCC, 2021; Ummenhofer & Meehl, 2017), mainly due to the increase in greenhouse gas emissions (e.g., CO_2) as a consequence of population growth and industrialization (Todd et al., 2019). In fact, because of atmospheric temperature rise, oceans and coastal systems have registered a gradual increase in the surface water temperature of 0.88 ºC, between 2001 and 2020 (IPCC, 2021). Recent projections highlighted that the increase in global surface temperature between 2080 and 2100 (IPCC, 2021) will range from 1.0 to 1.8 °C under the scenario of lower greenhouse gases emissions (SPP1 – 1.9), from 2.1

to 3.5 °C in an intermediate scenario (SSP2 – 4.5) and from 3.3 to 5.7 °C considering the worst-case scenario (SSP5 – 8.5). Several studies demonstrated that increasing temperature exceeding that of specie's tolerance range can cause physiological disturbances, affecting their sensory abilities, growth, and reproductive patterns (Costa et al., 2020; Moreira et al., 2018), as well as decreased metabolic rate and respiratory capacity (Jansen et al., 2009; Velez et al., 2017). On the other hand, the increase in temperature enhances the reactive oxygen species (ROS), leading to increase oxidative stress and cellular damage, which was already reported in bivalves exposed to warming conditions, such as mussels (e.g., *Mytilus galloprovincialis*) and clams (e.g., *Ruditapes philippinarum*) (Coppola et al., 2018; Munari et al., 2011; Velez et al., 2017). Furthermore, the increase in temperature can also influence a bioaccumulation of contaminants in organisms (Banni et al., 2014; Coppola et al., 2017; Guinot et al., 2012; Nardi et al., 2017), as well as increasing the sensitivity of organisms to contaminants (Freitas et al., 2019; Pirone et al., 2019), altering their biochemical processes (Banni et al., 2014; Coppola et al., 2018; Nardi et al., 2017; Sokolova & Lanning, 2008).

In addition to the gradual rising temperatures, coastal systems around the world have also been subject to extreme weather events such as Marine Heatwaves (MHWs), commonly defined as prolonged discrete anomalously warm water events that are classified by their duration, intensity, rate of evolution and spatial extent (Hobday et al., 2016, 2018). Recent studies revealed that MHWs are expected to continue in the future, with increasing frequency, duration, and intensity as a result of climate change (Holbrook et al., 2020; Oliver et al., 2019, 2021). The increased frequency of duration of extreme warming events in coastal communities may be a factor responsible for the decline in the abundance and richness of species that inhabit these sites, as well as for the consequent impact on the food chain (Grilo et al., 2011; Holbrook et al., 2019). Several studies conducted after MHW events demonstrated a significant disturbance on the structure and functioning of marine ecosystems and their associated communities, including loss of kelp forests and seagrass beds, coral bleaching, and mass mortality of several marine animals because of toxic algal blooms (Smale et al., 2019; Straub et al., 2019). For example, between 2014-2016, the MHW called "The Blob" provoked a marked decline on the population of kelp off the Pacific coast of Mexico (Arafeh-Dalmau et al., 2019), as well as a massive bloom of toxic diatoms, leading to the death of marine mammals (Cavole et al., 2016; McCabe et al., 2016). Later, in 2016 a well-documented MHW occurred in the northern Great Barrier Reef that disrupted

the symbiotic relationships between corals and their algal symbionts (Hobday et al., 2018). These pronounced effects have led to a rise in number of scientific publications regarding MHWs in an effort to better understand their effects in aquatic environments. For example, Costa et al. (2021) investigated the effects of a simulated MHW on the warm-temperature species *Cymodocea nodosa* under contrasting light regimes. These authors demonstrated that *C. nodosa* was able to adjust its photophysiological processes to successfully handle thermal stress, even under saturating light, drawing a promising perspective for *C. nodosa* resilience under climate change scenarios. Leung and co-authors (2017) investigated the effects of heatwaves on energy balance, body condition and survival of the subtidal gastropod *Thalotia conica*, evidencing a deficit in physiological processes (ingestion, absorption and respiration rate) after 8 weeks of experimental period and a higher mortality at (24 ºC) compared to temperature at (21 ºC), due to the depletion energy reserves to maintain basal balance.

1.1.2. Emerging contaminants: lithium in the environment

Coastal ecosystems are not only threatened by warming and extreme weather events, but also by the continuous discharge of pollutants and waste, which are introduced into the ecosystem due to industrial development and population growth (Jensen & Creinin, 2020; Starling et al., 2019). Estuaries are dynamic sites of the interface between the drainage of the river basin and ocean waters. These ecosystems were seen as areas with high economic potential, leading to high settlement and industrialization around them. As a result, they regularly receive high concentrations of pollutants transported by rivers as well as from the oceans from existing anthropogenic activity in coastal zones (e.g., shipping and wastewater release) (Todd et al., 2019). In the last decades, there has been an increase in the production and application of compounds called emerging contaminants (EC) due to the chemical, agrochemical, pharmaceutical and cosmetic industries (Starling et al., 2019). Emerging contaminants of concern (CECs) are discarded substances in the environment without established regulation and are considered potential threats to ecosystems and the safety of human health (Farré et al., 2008; Martín-Pozo et al., 2019; Rodriguez-Narvaez et al., 2017).

Among existing pollutants, lithium (Li) is a major concern, being considered an emerging contaminant (EC) (Bolan et al., 2021). Lithium is a metal belonging to the group of alkaline, highly reactive, being essentially as a mineral or a stable salt, such as lithium

carbonate (Li_2CO_3), lithium hydroxide (LiOH) and lithium chloride (LiCl) and may also be associated with other compounds such as aluminum silicate (Aral & Vecchio-Sadus, 2008; Bradley et al., 2017; Dessemond et al., 2019; Evans et al., 2013; Viana et al., 2020). Its extraction is done all over the world, mainly: Manona - Zaire; Bikita - Zimbabwe; Greenbushes- Western Australia; La Corne and Lake Bernic — Canada; Kola Peninsula — Russia and Altai Mountains — China (Aral & Vecchio-Sadus, 2008; Moore, 2007). In the environment, this metal comes from geogenic and anthropogenic sources (Aral & Vecchio-Sadus, 2011). For geogenic sources, Li can be released into the environment from geothermal activities, dust and fumes released during volcanic eruptions and hot springs (Bolan et al., 2021). On the other hand, anthropogenic sources of Li are mainly related with extraction, processing of Li minerals and production of batteries for technological purposes (Bernardes et al., 2004; Winslow et al., 2018). In fact, over the years, Li has been gaining importance, since the demand and use of this metal have been growing with the development of technologies and industries due to its numerous applications, namely in green / renewable energy (lithium-ion batteries (LIBs) for electric cars), medicine (pharmaceuticals), nuclear weapons and manufacturing industry (ceramics and glass, lubricants, polymers and metallurgical) (Aral & Vecchio-Sadus, 2011; Bogdanov et al., 2019; Choi et al., 2019; Shahzad et al., 2017). In the last decade, the global production of Li has intensified mainly due to the need for Li batteries, which have a greater energy storage capacity than conventional batteries (790%). This has caused the market to expand by 330 % in the last ten years (Agusdinata et al., 2018). The recent increase in demand for electric cars and renewable energy technologies has also contributed to about 65 % of global Li supply in 2019, an increase of 30 % from 2015 (Tabelin et al., 2021). By 2025, more than 80 % of the total Li market will be used to produce Li batteries (Harper et al., 2019). The increasing number of Li applications, incorrect disposal (e.g., disposal of batteries along with other solid waste from municipal waste) and inefficient recycling strategies result in the release of this element into the environment, already recorded in concentrations up to mg/L (Aral & Vecchio-Sadus, 2008; 2011).

In freshwater ecosystems, such as most non-contaminated rivers in the USA, concentrations of Li of 2.0 µg/L were recorded (Aral & Vecchio-Sadus, 2011; Kszos & Stewart, 2003). In seawater, Li values are higher than freshwater systems, ranging from 170 to 180 µg/L (Aral & Vecchio-Sadus, 2011; Schrauzer, 2002; Zaldívar, 1980). In areas where

there is great pollution by LIBs and industries, Li concentrations reach high values (1.57 mg/L), as is the case in South Korea on the Han River in Seoul (Thibon et al., 2021). In what regards to Li concentrations found in marine organisms, Guérin et al. (2011) revealed the presence of Li in different species from a French fish market. The authors reported that for most seafood the mean value was 0.082 mg/kg, where cockles and sea urchins showed the highest Li values (0.245 and 0.227 mg/kg, respectively).

Although the available information on Li impacts induced on marine organisms is scarce, it is known that this metal has negative effects on marine organisms at high concentrations. Recent studies revealed physiological impacts of LiCl (10 mM) on sea urchins (*Paracentrotus lividus*) with the induction of malformations in the embryos affecting genes with a key role in functional responses, such as stress, detoxification processes, skeletogenesis, development and differentiation (Ruocco et al., 2016). Biochemical effects also observed on mussels (*Mytilus galloprovincialis*) with metabolism depression, lipid peroxidation, loss of redox homeostasis and neurotoxic effects at Li concentration of 750 µg/L (Viana et al., 2020).

1.1.3. Responses of bivalves to warming and contaminants

When assessing the toxicity of pollutants in marine organisms, including those of emerging concern as Li, it is important to consider other environmental variables, such as those related to climate change, such as warming, MHWs but also water acidification and increased salinity. Estuarine species are among the most affected to combinations of various stressors, namely the ones related to temperature shifts and the presence of pollutants. Ectothermic species are vulnerable to temperature changes since an internal temperature increase is associated with warming conditions, which in turn alters organism's biochemical responses and metabolic rates (Brockington & Clarke, 2001; Ganser et al., 2015) and increase sensitivity to pollutants (Coppola et al., 2017, Sokolova, 2004). An increasing number of studies have shown that the combined effect of warming and pollutants has an additive impact on marine species. Since large amounts of energy are expended as a response to heat stress, organisms tend to be more sensitive to contaminants under these circumstances, due to a limited capacity to trigger defense mechanisms (De Marchi et al., 2022). A study by Coppola et al. (2018) demonstrated that *M. galloprovincialis* subjected to

temperature rise and arsenic (As) presented higher oxidative stress than the ones only exposed to As. Also, Nardi et al. (2022) demonstrated that MHWs influenced the response of the mussel *M. galloprovincialis* to the drug Carbamapezine. These authors demonstrated that, when these two factors were combined greater bioaccumulation was observed, while neuro-immune and oxidative impacts were also enhanced. De Marchi et al. (2022) also reported that a simulated MHW intensified the effects of caffeine in bivalve species (*Ruditapes philippinarum* and *Mytilus galloprovincialis*), namely in terms of neurotoxicity and cellular damage.

1.2. *Mytilus galloprovincialis* as bioindicator species

A biological indicator is an organism (or community of organisms) used to identify various pollutants in the environment and diagnose the impacts they may have on a habitat, community, or ecosystem (Markert et al., 2003; Zaghloul et al., 2020). Bioindicators must have essential characteristics including, a wide geographical distribution, a sessile lifestyle or restricted territory, be easily collected, and be well understood in terms of their biochemical and physiological performance (Zaghloul et al., 2020). The Mussel Watch Program is a program for monitoring older estuarine and coastal pollutants carried out in the United States, which initiated the monitoring of metals and organic contaminants (e.g., DDT, PCBs, and PAHs). Since then, mussels have been used globally as bioindicators of pollution in coastal environments (Kimbrough et al., 2008).

M. galloprovincialis (Lamark, 1819) (Figure 1), known as the Mediterranean mussel, belonging to the Mytilidae family, usually occur in intertidal zones up to 40 m deep attached to rocks and piers, within sheltered ports, estuaries, and rocky shores [1]. This species can be found throughout the world, such as in South Africa, Hong Kong, Japan, Korea, Australia, Hawaii, Mexico, California, Washington and the west coast of Canada (Branch & Steffani, 2004), due to human activities, mainly through ballast water and ships hulls. Regarding their reproduction, these organisms are usually dioecious, producing gametes for the first time, at about one year of age. Mussel gametes mature from September to May, when several spawning events occur with a peak in late winter (January - February) (Da Ros et al., 1985). Under adverse conditions, their growth may be slower and sexual maturity may only be

reached at two years of age (Newell, 1989). In terms of growth, it is fast in spring and summer, while in winter the growth rate is lower (Helm & Bourne, 2004).

Figure 1. *Mytilus galloprovincialis [1].*

Currently, this species have been used as study models to evaluate the impacts derived from a wide variety of pollutants, including pharmaceutical and personal hygiene products (Freitas et al., 2021), herbicides (Milan et al., 2018), metals (Coppola et al., 2018; Dimitriadis et al., 2003; Pinto et al., 2019; Pirone et al., 2019) and nanoparticles (Andrade et al., 2019; Leite et al., 2020; Morosetti et al., 2020), as well as, used in biomonitoring programs (Azizi et al., 2018; Benali et al., 2017). Furthermore, this species has high economic importance, as it is part of the world's food chain. Its consumption has been increasing over the years, and a large part of it comes from aquaculture production which has been increasing from 1959 to 2016, reaching 2 million tons (Avdelas et al., 2021; [1]) (Figure 2). Its production is done worldwide, as in some countries (Albania, Bulgaria, Croatia, Egypt, France, Greece, Italy, Morocco, and Portugal) and especially in the coastal waters of Galicia (northwest Spain) (Merdzhanova et al., 2016).

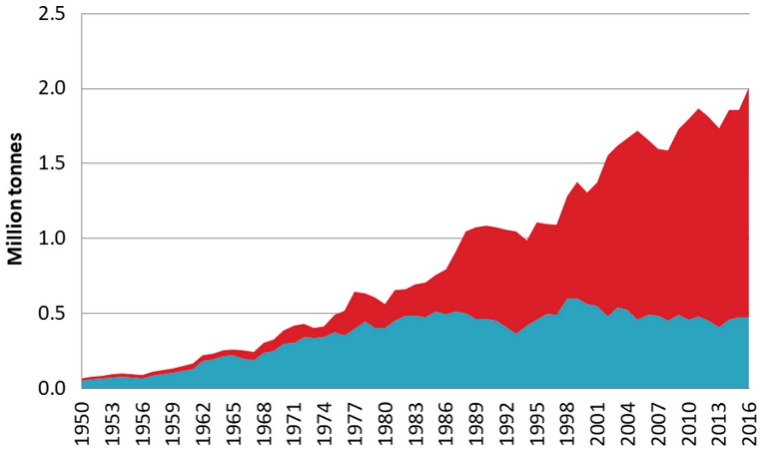

Figure 2. *Evolution of mussel aquaculture production by weight (million tonnes) in the EU and the rest of the world (1950–2016). Different colors represent the production data of the European Union – blue and red the rest of the world (Avdelas et al., 2021).*

1.3. Objectives

Considering the lack of knowledge on the impacts of climate change, namely regarding weather events, on marine species, coupled with the exposure to contaminants from anthropogenic sources, the present study aimed to evaluate how temperature increase scenarios (warming and a marine heatwave) can influence Li toxicity induced in the mussel *Mytilus galloprovincialis*.

The null hypotheses investigated were: there are no significant changes due to the temperature scenarios and the lithium concentration tested, as well as the combination of the two factors. For this, Li concentration in water and mussel's soft tissues, as well as physiological and biochemical responses were evaluated under control temperature, a projected temperature rise, and one MHW scenario, for both non-contaminated and Li-spiked seawater.

Chapter 2

Materials and Methods

2. Materials and methods

2.1. Sampling area

The specimens of *Mytilus galloprovincialis* were collected in the Ria de Aveiro lagoon (N 40° 38' 40'; W 08° 43' 59') in September 2021, during low tide (Figure 3). The Ria de Aveiro is a coastal lagoon adjacent to the Atlantic Ocean, located on the northwest coast of Portugal 45 km long and 10 km wide (Dias et al., 2000; Dias & Lopes, 2006), connected to the ocean through an artificial channel, and the Antua and Vouga rivers account for about 80 % of the existing freshwater (Dias et al., 1999; Dias et al., 2000). The average depth is about 1 m, except for navigation channels that are about 15 m (Dias et al., 2001; Lopes et al., 2013). The Ria de Aveiro consists of four main channels: S. Jacinto, Mira, Espinheiro and Ílhavo (Dias et al., 2001; Lopes et al., 2013), of which the Channel of S. Jacinto stands out for its largest area of extension with about 29 km, while the Ílhavo channel is the narrowest and smallest, about 15 km long (Dias et al., 2001). The mean temperature of the water of Ria de Aveiro ranges between 14 ºC (minimum in Winter) and 18 ºC (maximum in Summer) [2]. The Ria de Aveiro is one of the most relevant coastal systems in Portugal, with several biotopes of biological importance, such as seagrass meadows and salt marshes (Pardo et al., 2018; Sousa et al., 2017), as well as its higher biodiversity of marine species, it harbours, of which the group of annelids, bivalves and gastropods are the most abundant (Pardo et al., 2018).

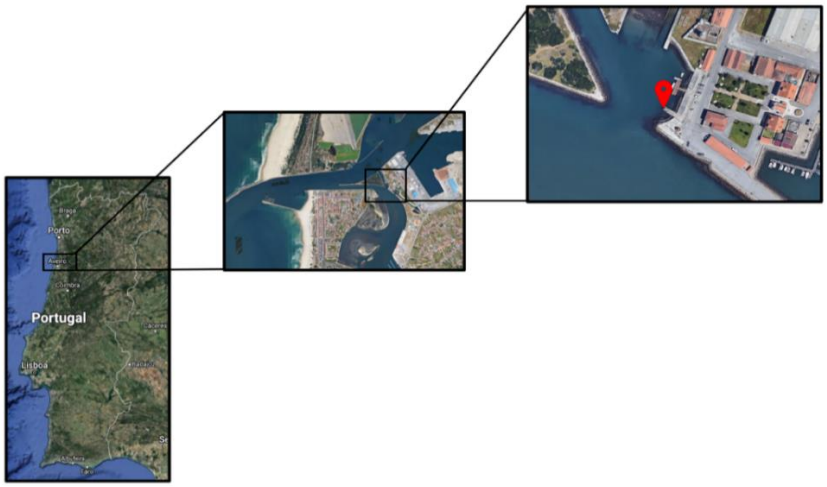

Figure 3. *Mytilus galloprovincialis sampling area [3].*

2.2. Experimental conditions

After sampling, the mussels were transported to the laboratory, where they were placed in tanks for purification and acclimatization for two weeks under laboratory conditions before the experimental test. During this period, the organisms were kept in synthetic seawater (temperature of 17.0 ± 1.0 °C; pH 7.8 ± 0.1; salinity 30 ± 1), prepared with reverse osmosis water with salt (Red Sea Salt ®). The organisms were not fed in the first three days and were then fed with Algamac plus protein three times *per* week (150,000 cells/animal/day). Organisms with average length of 59.1 ± 5.2 mm and 33.9 ± 2.7 mm width were used. Seawater was renewed every day for the first three days and every three days until the end of this period.

During the experimental exposure (twenty-eight days), mussels were exposed to six experimental treatments: CTL 17 °C (no Li spiking and temperature 17 ± 1 °C); Li 17 °C (250 µg/L Li spiking and temperature 17 ± 1 °C); CTL 21 °C (no Li spiking and temperature 21 ± 1 °C); Li 21 °C (250 µg/L Li spiking and temperature 21 ± 1 °C); CTL MHW (no Li spiking and MHW) and Li MHW (250 µg/L Li spiking and marine heatwave with a maximum temperature of 23 °C). In each treatment, three replicates (3 aquaria of 3 L each) with five mussels *per* aquarium were used. The experimental setup performed in this study is illustrated in Figure 4.

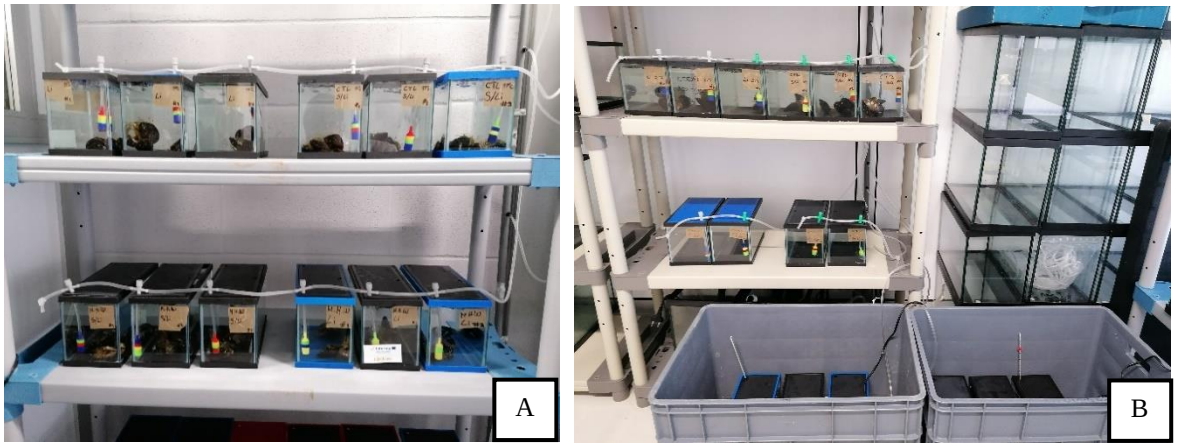

Figure 4. *Experimental setup.* ***A****: Control room 17 ± 1 °C;* ***B****: Warming room 21 ± 1 °C.*

Three temperature scenarios were considered: 17 ºC, control temperature; 21 ºC, resembling a global warming scenario; and a simulated marine heatwave (MHW). Regarding temperature in the MHW scenario, the aquaria were exposed to an initial temperature of 17 ºC for seven days, after which the temperature was gradually increased for five days up to 23 ºC, maintained at this temperature for five days, then the temperature decreased for four days until it reached again 17 ºC, where it was kept for seven days more (total 28 days). This MHW scenario was selected based on studies conducted in the Mediterranean Sea (Garrabou et al., 2022; Rubio-Portillo et al., 2016), which report negative effects on marine biodiversity, and these events are expected to occur in the Ria de Aveiro. The control temperature (17 °C) was set considering the average sea temperature (16 - 19 °C) in September where they were collected [4]; the highest temperature (21 °C) was set to simulate global temperature conditions for 2100 (IPCC, 2021). To maintain temperature levels, the mussels were distributed in two climatic rooms to keep them at two different temperatures: 17 ± 1 ºC (control) and 21 ± 1 °C (which resemble heating conditions); at the time of MHW simulation, the aquaria were placed in the boxes with water and the thermostat was used to regulate temperature up to 23 ºC, represented in Figure 4.

The Li concentration evaluated was selected based on the values obtained from the water samples from the study site (± 280 μg/L), as well as previous studies that tested similar concentrations in bivalves (Aral & Vecchio-Sadus, 2008; Viana et al., 2020). The salinity and pH conditions were the same during the acclimatization and the aquaria were continuously aerated, with a natural photoperiod. Temperature (17 ± 1 or 21 ± 1 °C) and salinity (30 ± 1) were checked daily and adjusted if necessary. Mortality was also checked daily. Throughout the experimental period, the organisms were fed with Algamac Plus (150,000 cells/animals/day) three times a week. Artificial seawater was renewed once a week to restore Li's concentration. Immediately after the spiking, water samples were collected from each aquarium for subsequent quantification of Li, to evaluate Li exposure.

At the end of the exposure period, one of the five organisms *per* aquarium was randomly removed and used to determine the respiration rate and condition index, while the remaining ones were frozen with liquid nitrogen and stored at -80 °C. Subsequently, three frozen mussels from each aquarium (nine *per* condition) were homogenized with a mortar and a pestle under liquid nitrogen (Figure 5). Each homogenized organism was divided into

0.5 g fresh weight (FW) aliquots for biomarkers analyses and the remaining tissue of each mussel was used for Li quantification.

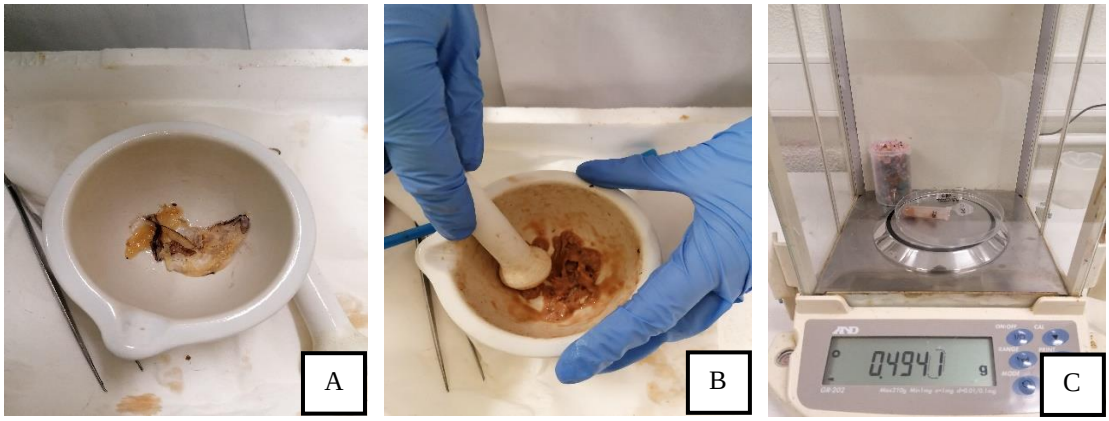

Figure 5. *Homogenization process of mussels. The process is composed of inserting the mussel's tissue into the mortar (A), then grind and homogenized with liquid nitrogen (B) and finally the tissue is weighed and divided into aliquots of 0.5 g for biochemical posterior analysis (C).*

2.3. Lithium quantification in water and mussel's samples

Samples of water were collected in the two first weeks from each aquarium after spiking (three *per* treatment) to assess real Li exposure concentrations. Water was also collected from blanks (aquaria spiked with Li without organisms; two aquaria *per* treatment), immediately after spiking and 24 h, 2 days, 3 days, and 7 days after spiking, to assess the stability of Li under different tested scenarios.

Lithium quantification in water samples was carried out using inductively coupled plasma optical emission spectroscopy (ICP-OES, Jobin Yvon Activa M) (Figure 6A), after dilution with HNO_3 2 % (Viana et al., 2020). The Li quantification limit (LOQ) was 10 µg/L.

Samples are analysed in liquid form in this equipment. The liquid sample is sucked into the nebulizer chamber from a pump, where it is transformed into an aerosol by a nebulization process. Then it is transported to the plasma, where it is vaporised, atomized, as well as excited and/or ionized, thus emitting photons. Through a focusing optical lens, the ICP-OES photons are recollected, thus forming an ICP image with the help of a monochromator that select the wavelength of each element, followed by a photodetector that converted into an electrical signal, which is then detected and transformed into emission

signals. These signals are converted into concentrations of a given element in the samples through a calibration curve (Figure 6B) (Boss & Fredeen, 2004; Khan *et al*., 2022).

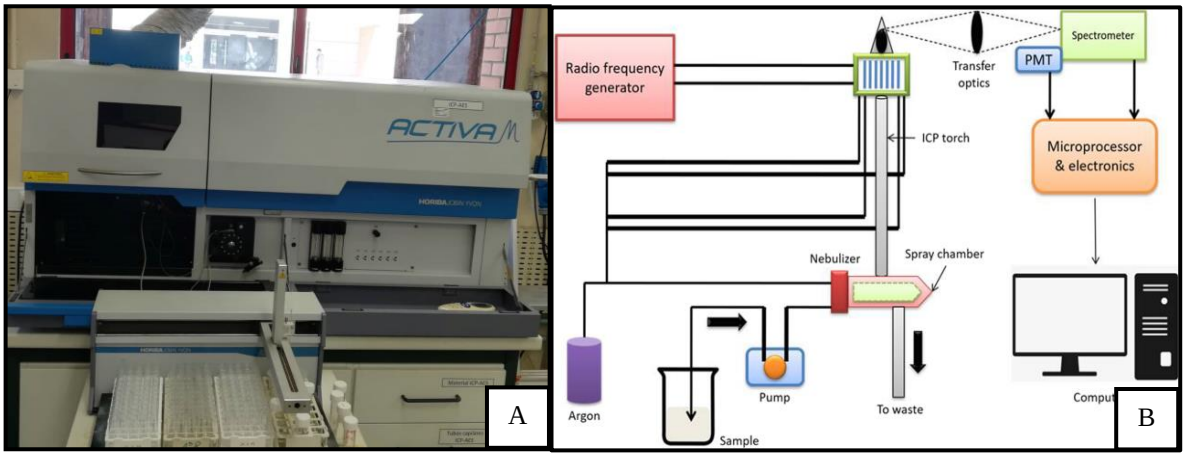

Figure 6. A*: Inductively coupled plasma optical emission spectrometry (ICP-OES) used for the quantification of Li in water and mussel's samples;* ***B****: Representation of the procedure of a typical ICP-OES instrument (in: Khan et al., 2022).*

For the quantification of Li in mussels, the tissues previously homogenized were submitted to microwave-assisted acid digestion using Teflon vessels (Figure 7A), with 200 mg of freeze-dried tissue samples, 1 mL of HNO_3 65 % (v/v), 2 mL of H_2O_2 30 % (v/v) and 1 mL of ultrapure H_2O. Samples were transferred to a CEM MARS 5 microwave (Figure 7B) with temperature ramp up to 175 °C for 15 min, which was then held for another 5 min. After this step, samples were placed in polyethylene vials and ultrapure water was added to a final volume of 25 mL. Digestion and analysis of certified reference materials (NCS ZC 73016), blank samples (containing only the acidic reagent combination), and duplicates provided quality control for Li measurement. The Li quantification in organism tissues was then performed by IPC-OES. The blanks were below the limit of quantification of the ICP-OES for Li (10 µg/L), the percentage of recovery of the reference material was between 80 and 90 % and the coefficient of variation of the duplicate samples ranged from 0.20 to 2.7 %.

The Bioconcentration Factor (BCF) of Li in mussel's tissues, was calculated based on the equation of Arnot & Gobas (2006), which is defined by the ratio between the total concentration of Li in the tissues (Li in the organism from the exposure medium incorporated through its respiratory and dermal surfaces) and the total concentration of the chemical

measured in the water after spiking (BCF = concentration in the organism/concentration in the water after spiking).

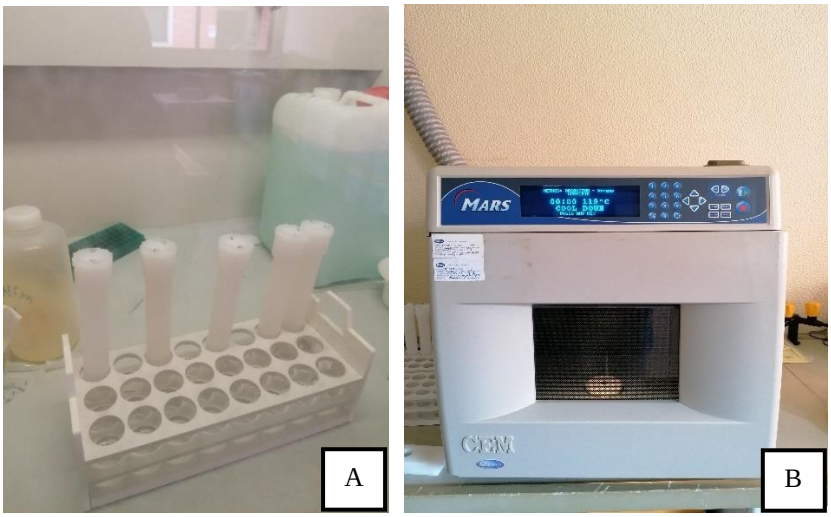

***Figure 7. A**: Teflon vessels; **B**: CEM MARS 5 microwave.*

2.4. Biological responses

2.4.1. Physiological parameters

2.4.1.1. Mortality

During the experimental period (28 days), the organisms were observed three times a week, to check for mortality. Individuals were considered dead when their shells failed to close after an external stimulus.

2.4.1.2. Condition Index

The condition index (CI) was determined using one individual *per* aquarium, three *per* treatment, the same used for the respiration rate determination. The organisms (shells and tissues) were dried in an oven at 60 °C for 48 h and then weighed (Figure 8). The values were calculated based on the following equation:

$$CI\ (\%) = \frac{dry\ tissue\ weight}{dry\ shell\ weight}\ x\ 100$$

where CI (%) corresponded to the percentage of the ratio between the dry weight of soft tissues (g) and the dry weight (DW) of the shell × 100 (Matozzo et al., 2012).

Figure 8. *Organisms (shells and tissues) dried after 48 h in the oven at 60 ºC.*

2.4.1.3. Respiration Rate

The respiration rate (RR) was measured at the end of the experimental period (28 days), in one individual from each aquarium. Each chamber had an oxygen sensor inserted into the wall with silicon mass, where they were filled with artificial seawater used during the experimental period. Each individual was placed in a respirometric chamber randomly organized under dark and fully oxygenated concentrations, where it acclimated for 30 min to avoid the influence of manipulation on RR (Figure 9). After this period, chambers were filled until they make up their full capacity to prevent the formation of air bubbles. During 2 h, using a multichannel fiber optic oxygen meter (Multi channel Oxygen Meter, PreSens GmbH), the RR was recorded as a function of the decrease in O_2 concentration (mg/L), measured every 15 min. Measurements were performed using a simple static respirometry. RR was expressed in mg O_2 consumed *per* g DW and correction to O_2 variation in blanks (chambers with no organisms) was employed (Andrade et al., 2019).

Figure 9. *Experimental setup of respirometric chambers for the measurement of respiration rate (RR).*

2.4.2. Biochemical parameters

Biochemical alterations were assessed by measuring thirteen biochemical markers related to: i) organism's metabolic capacity and energy reserves content (electron transport system (ETS) activity; energy reserves: total protein (PROT) and glycogen (GLY) content); ii) antioxidant and biotransformation capacity (activity of the enzymes catalase (CAT), glutathione peroxide (GPx), glutathione reductase (GR); carboxylesterase *p*-nitrophenyl acetate (CbEs-*p*NPA), carboxylesterase *p*-nitrophenyl butyrate (CbEs-*p*NPB), glutathione S-transferases (GSTs); iii) cellular damage and redox balance (lipid peroxidation (LPO) levels, protein carbonylation (PC) levels, reduced glutathione (GSH) content); iv) and neurotoxicity (acetylcholinesterase (AChE) activity).

For each parameter, 0.5 g fresh weight (FW) of soft tissue *per* organism were used (three individuals *per* aquarium and nine organisms *per* treatment) (Figure 4) previously extracted with specific buffers for each biomarker in the proportion 1:2 (w/v): 50 mmol/L potassium phosphate (pH 7), 1 mmol/L EDTA, 1 % (v/v) Triton X-100, 1 mmol/L DTT (phosphate buffer) to determine PROT, GLY, CAT, GPx, GR, CbEs-*p*NPA, CbEs-*p*NPB, GSTs, AChE; 20 % (w/v) trichloroacetic acid (TCA) to proceed LPO; 0.1 mol/L Tris-HCl (pH 8.5) with 15 % (w/v) PVP, 153 μmol/L magnesium sulfate ($MgSO_4$) and 0.2 % (v/v) Triton X-100 (Tris buffer) to quantify ETS activity; and potassium phosphate 0.1 mol/L EDTA 5 mmol/L buffer (pH 7.5) with 0.1 % (v/v) Triton X-100 and 0.6 % (w/v)

sulfosalicylic acid (KPE buffer) to measure GSH content (Coppola et al., 2017; Pinto et al., 2019). Samples were homogenized using a TissueLyzer II (Qiagen) at a frequency of 20 1/s for 90 s and centrifuged at 4 ºC at different intensities and times depending on the buffer and fraction to analyze: samples extracted with the phosphate buffer and TCA were centrifuged at 10,000 *g* for 20 min; samples extracted with the Tris buffer were centrifuged at 3,000 *g* for 20 min; while samples with the KPE buffer were centrifuged at 10,000 *g* for 10 min. Finally, the supernatants were removed and stored at -80 °C or used immediately. The samples were duplicated in each parameter to provide data quality control, and their absorbance was measured using a microplates reader (Biotek Synergy HT).

2.4.2.1. Metabolic capacity and energy reserves

The activity of the ETS corresponds to a measure of the metabolic state of the organisms, allowing estimating the consumption of energy at the mitochondrial level (Andrade et al., 2018; De Coen & Janssen, 1997). The evaluation of the activity was quantified using the King & Packard method (1975) with modifications by De Coen & Janssen (1997). The absorbance was measured at 490 nm for 10 min at intervals of 25 s. The extinction coefficient $\varepsilon = 15.9$ $(mmol/L)^{-1}$ cm^{-1} was used to measure the amount of formazan produced. Tetrazolium salt 2-(4-iodophenyl)-3-(4-nitro-phenyl)-5-phenyl-2*H*-tetrazolium chloride (INT) was used to quantify ETS activity. This salt is reduced by dehydrogenase enzymes present in the ETS of aerobic organisms. These enzymes convert INT (artificial electron acceptor to register the electron transmission rate) into insoluble formazan (Hatzinger et al., 2003). The amount of formazan produced is used as a measure of the total respiratory or ETS activity. The results were expressed in nmol *per* min *per* g of FW.

The sulfuric acid method was used to determine the GLY concentration (Dubois et al., 1951). This method was developed for small analyses of sugars and related substances. Phenol is used at a concentration of 5 % (v/v), since it physically damages the cell wall, altering its permeability without cell death. Phenol when found in the presence of sulfuric acid can be used for quantitative colorimetric microdetermination of sugars and their methyl derivatives, oligosaccharides and polysaccharides. Quantification is allowed by the sulfuric acid because it can break down any mono-poly-oligo-dysacarid (sugars). The amount of colour produced in a constant concentration of phenol is proportional to the amount of sugar present. A calibration curve was created using glucose patterns (0 – 5 mg/mL). After an

incubation period of 30 min at room temperature, absorbance was measured at 492 nm, and the results were represented in mg *per* g of FW.

The protein content was quantified from the spectrophometric Biuret method described by Robinson & Hogden (1940). To produce a calibration curve, bovine serum albumin standards (0 – 40 mg/mL) were used. Copper Sulfate Anhydrous (cooper II) was used, which in the presence of sodium potassium tartrate, can form a complex with peptide bonds of amino acids causing the solution to change color from blue to purple. According to the Beer's Law, the absorption read is directly proportional to the amount of protein in the sample. Absorbance was measured at 540 nm and the results were reported in mg *per* g of FW.

2.4.2.2. Oxidative status

2.4.2.2.1. Antioxidant and biotransformation defenses

Organisms (including mussels) naturally produce reactive oxygen species (ROS): atomic oxygen (O_2), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (HO), during aerobic metabolic pathways under normal conditions (Regoli & Giuliani, 2014). However, when in excess, they can react in the plasma membrane, destroying it, causing negative effects on cells, such as lipid peroxidation in the cell membrane, protein oxidation, DNA damage and enzyme inactivation (Regoli & Giuliani, 2014; Regoli & Winston, 1999). To avoid the impacts caused, organisms have developed mechanisms to remove ROS and protect against oxidative stress, namely antioxidant enzymes (SOD, CAT, GPx and GR).

The activity of CAT was measured using the method developed by Johansson & Borg (1988). The method consists in the reaction of the enzyme with methanol in the presence of a concentration based on potassium hydroxide (H_2O_2). The formaldehyde produced is measured photometrically with 4-amino-3-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald). Potassium periodate is used as an oxidizing agent that catalyses a reaction between formaldehyde and Purpald, turning it into potassium hydroxide. To develop a calibration curve formaldehyde standard (0 – 150 μmol/L) were employed. The absorbance was measured at 540 nm and the activity was measured in U *per* g of FW, with U indicating the quantity of enzyme that produce the synthesis of 1.0 nmol formaldehyde *per* min.

The activity of GPx was evaluated using Paglia & Valentine (1967). GPx catalyzes the reduction of cumene hydroperoxide (an organic peroxide), oxidizing a reduced glutathione (GSH) to form disulphide glutathione (GSSG). In turn, this is reduced by glutathione reductase (GR), in which NADPH forms $NADP^+$ and recycles GSH as donor to catalyse H_2O_2 reduction in H_2O. GPx activity is measured by the reduction of NADPH to $NADP^+$, through enzymatic activity. Absorbance was measured at 340 nm at intervals of 15 s for 5 min. The enzymatic activity was calculated using the extinction coefficient $\varepsilon = 6.22$ $(mmol/L)^{-1}$ cm^{-1}. The results were represented as U *per* g of FW, with U represents the number of enzymes which catalyse the conversion of 1.0 nmol NADPH *per* min.

Glutathione reductase (GR) is an enzyme present in biological tissue, which catalyzes the reduction of GSSG in GSH (1 mol the GSSG in 2 mol GSH) using NADPH. Its activity was measured according to the Carlberg & Mannervik method (1985). The GR activity was monitored by the oxidation of NADPH in $NADP^+$, and the reduction of the oxidation of NADPH is proportional to the activity of the GR in the sample (the greater the amount of NADPH, the greater the GR activity). The activity was calculated based on the extinction coefficient $\varepsilon = 6.22$ $(mmol/L)^{-1}$ cm^{-1} and the absorbance was measured at 340 nm at intervals of 15 s for 5 min. The activity was measured in U *per* g of FW, with U representing the amount of enzymes that catalyze the conversion of 1.0 nmol NADPH oxidized *per* min.

The activity of CbEs was quantified following the Hosokawa & Satoh method (2001) with adaptations made by Solé et al. (2018). Carboxylesterases (CbEs) are type B esterases that can be measured with different substrates (naphthyl derived and nitrophenyl), indicative of different isoforms (Sanchez-Hernandez & Wheelock, 2009). In this case, it was used *p*-nitrophenyl acetates (*p*NPA) and butyl *p*-nitropheny (*p*NPB). The hydrolysis rate of *p*NPA and *p*NPB is determined by a continuous spectrophotometric enzymatic assay. Absorbance was measured at 405 nm for 5 min with 15 intervals between readings and the extinction coefficient $\varepsilon = 18$ $(mmol/L)^{-1}$ cm^{-1} was used to determine the activity. The results were expressed in nmol *per* min *per* g of FW.

GSTs are phase II detoxification enzymes, located mainly in the cytosol. The enzymes GSTs are best known for transferring reduced glutathione (GSH) to hydrophobic organic compounds, making the conjugates more soluble, thus facilitating their excretion, as well as functioning as transporters by binding to toxins. The activity of GSTs was measured according to Habig et al. (1974), with alterations by Carregosa et al. (2014). GSTs catalyse

the reaction of the substrate 1-Chloro-2,4-dinitrobenzen (CDNB) conjugation with the thiol group of GSH, forming a thioether. The extinction coefficient $\varepsilon = 9.6\ (mmol/L)^{-1}\ cm^{-1}$ was used to calculate the amount of thioether produced. At 340 nm, absorbance was measured spectrophotometrically at 15 intervals to 5 min. The enzymatic activity was measured in U *per* g of FW, with U equalling the amount of enzyme needed to produce 1 nmol of dinitrophenyl thioether *per* min. The rate of increase is directly proportional to the activity of the GSTs in the sample.

2.4.2.2.2. Cellular damage and redox balance

Under oxidative stress conditions, when defense mechanisms are not able to eliminate the excess of ROS produced, cellular damage can occur. In particular, ROS will interact with polyunsaturated fatty acids of the membrane, causing lipid peroxidation (LPO) and can also oxidize cellular proteins (protein carbonylation, PC) (Regoli & Giuliani, 2014).

The occurrence of LPO was determined according to Buege et al. (1978). LPO can be estimated by the quantification of thiobarbituric acid reactive substances (TBARS), which form in the reaction between LPO by-products (such as malondialdehyde – MDA) with 2-thiobarbituric acid (TBA). MDA is a highly reactive compound resulting from the final product of lipid peroxidation and its produced quantity was determined with the extinction coefficient $\varepsilon = 156\ (mmol/L)^{-1}\ cm^{-1}$, which was measured at 532 nm. The results were given in MDA nmol *per* g of FW.

Protein carbonylation (PC) levels were measured from the alkaline DNPH method, published by Mesquita et al. (2014). Determination of carbonyl groups is a way to measure protein oxidation, since oxidation of amino acid residues into proteins leads to the formation of protein carbonyls. 2,4-Dinitrophenylhydrazine (DNPH) reacts with carbonyl groups leading to protein-DNP hydrazone formation (yellow, orange or red precipitation). Sodium hydroxide (NaOH) is used to neutralize the derivatization of DNPH, allowing to read hydrazone conjugated with proteins. The extinction coefficient $\varepsilon = 22.308\ (mmol/L)^{-1}\ cm^{-1}$ was used to determine PC levels, which were measured at 450 nm. The results were represented as the number of nmol of protein carbonyl groups produced by g of FW.

Reduced glutathione (GSH) is a tripod (γ-glutamilcisteinil glycine) more abundant in the cytosol, having the ability to directly neutralize several reactive species through their

oxidation process with GSSG. In addition, it is a cofactor of several glutathione-dependent enzymes, and its intracellular concentration is an indicator of oxidative stress (Regoli & Giuliani, 2014). The presence of GSH was determined according to the method of Rahman et al. (2007). To do this, (DTNB) was used, which is a 5,5′-dithio-bis sulfhydryl reagent (2-nitrobenzoic acid) to oxidize GSH, forming the yellow derivative and 5′-thio-2-nitrobenzoic acid (TNB). A quantification of GSH content was made based on GSH standards (0 – 90 μmol/L). The absorbance was measured at 412 nm and the results expressed in nmol *per* g of FW.

2.4.2.3. Neurotoxicity

Acetylcholinesterase (AchE) activity is used as a biomarker of exposure to neurotoxic compounds in marine organisms. This enzyme is important for the functioning of the neuro-muscular system and is responsible for the degradation of neural acetylcholine from transmitters to choline in cholinergic synapses and neuromuscular junctions (Karami-Mohajeri & Abdollahi, 2011; Matozzo et al., 2005). For the determination of its activity (AChE), the substrate acetylthiocholine iodide (ATChI, 5 mmol/L) was used, as described by Ellman et al. (1961), with modifications by Mennillo et al. (2017). The activity of the enzyme is measured depending on the increase in the yellow color produced from thiocholine when it reacts with the dianion of 5-thio-2-nitrobenzoic acid, TNB. Using the extinction coefficient $\mathcal{E}$ = 13.6 $(mmol/L)^{-1}$ cm^{-1}, the enzyme activity was measured at 412 nm at intervals of 25 s for 5 min and expressed in nmol *per* min *per* g of FW.

2.5. Data analyses

The physiological parameters (RR and CI), biochemical markers (ETS, PROT, GLY, CAT, GR, GSTs, CbEs, LPO, PC, GSH and AChE) as well as Li concentrations in mussel's tissues and water samples were submitted to non-parametric statistical hypothesis testing using Permutational Analysis of Variance (PERMANOVA + add-on in Primer v6) with two factors design: Li concentration (without Li and with Li) as factor 1 and temperature condition (exposed to the control temperature, warming scenario – 21 ºC and MHW scenario) as factor 2 (Table 3 - SM). The Euclidean distance similarity matrix was then

analysed following the unrestricted permutation of the raw data (9999 permutations) and the sums of squares were calculated: Type III (partial). Significant differences were defined as values lower than 0.05 ($p < 0.05$) and when significant differences were found in the main test (Table 1 - SM), paired comparisons were performed (Table 2 - SM). The null hypotheses tested were: i) regarding Li concentrations measured in water from the exposure aquaria, there were no significant differences among tested temperature scenarios (17 ºC, 21 ºC, MHW); significant differences among temperature scenarios are represented in Table 1 with different letters (lowercase letters for non-contaminated treatments; uppercase letters for contaminated treatments); ii) regarding Li concentrations in mussel tissues, there were no significant differences among tested temperature scenarios (17 ºC, 21 ºC, MHW); significant differences among temperature scenarios are represented in Table 3 with lowercase letters for uncontaminated treatments and uppercase letters for contaminated treatments; iii) regarding Li concentration in mussel tissues, there were no significant differences between contaminated and non-contaminated mussels for each temperature scenario; significant differences between non-contaminated and contaminated mussels were represented in Table 3 with an asterisk; iv) for each biomarker and a give Li concentration (control or Li-spiked), there were no significant differences among tested temperature scenarios (17 ºC, 21 ºC, MHW); significant differences among temperature scenarios are shown in figures with different letters (lowercase letters for non-contaminated mussels and uppercase letters for contaminated mussels); v) for each biomarker at each temperature scenario, there were no significant differences between non-contaminated and contaminated mussels; significant differences between non-contaminated and contaminated mussels were represented in figures with an asterisk.

To perform the Principal Coordinates (PCO), the original data were divided by factor with the different treatments and transformed by applying a normalization. Then, the Euclidean distance similarity matrix was used to calculate the distance between centroids matrix based on each treatment. The Pearson's correlation vectors for physiological and biochemical descriptors with correlation higher than 75 % were placed on top of the PCO graph.

Chapter 3

Results

The following results were used in the papers already published:

Santos, D., Leite, C., Pinto, J., Soares, A. M. V. M., Pereira, E., & Freitas, R. (2022). How will different scenarios of rising seawater temperature alter the response of marine species to lithium? *Science of The Total Environment, 856*, 158728. https://doi.org/10.1016/J.SCITOTENV.2022.158728

3. Results

3.1. Lithium concentration in water and mussel's tissues

The concentrations of Li measured in non-contaminated aquaria varied between 243 and 260 µg/L, with significant differences between 17 ºC and temperature stressful scenarios (21 ºC and MHW). In the aquaria subjected to Li contamination, values varied between 530 and 545 µg/L, with no significant differences among treatments (Table 1).

Table 1. *Lithium (Li) concentrations (µg/L) in water samples collected immediately after spiking from each exposure aquarium. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated mussels and uppercase letters for contaminated ones). Values are the mean of 6 values ± standard deviation. MHW: marine heatwave.*

Treatments	Temperature (°C)	Li concentration
Non-contaminated	17	243 ± 10^{a}
	21	256 ± 2^{b}
	MHW	260 ± 1^{b}
Contaminated	17	530 ± 4^{A}
	21	535 ± 12^{A}
	MHW	545 ± 1^{A}

Regarding Li concentrations in blank samples, the results obtained demonstrated the stability of this element along seven days of exposure (between weekly water renewals), with a loss of around 2 % at 17 and 21 ℃ (Table 2).

Table 2. *Lithium (Li) concentrations (µg/L) in water samples collected immediately after spiking (0 h), 24 h (1 day), 48 h (2 days), 72 h (3 days) and 168 h (7 days) after the beginning of the assay from each blank aquarium. Values are the mean 2 values ± standard deviation.*

	Sampling period				
Temperature (º C)	**0 h**	**24 h**	**Day 2**	**Day 3**	**Day 7**
17	552 ± 3	534 ± 23	527 ± 10	524 ± 11	541 ± 10
21	548 ± 28	529 ± 6	593 ± 9	551 ± 3	535 ± 10

Lithium in mussel's soft tissues, revealed that non-contaminated mussels presented concentrations of 1.2 ± 0.3 µg/g dry weight (DW) (17 ºC), 1.0 ± 0.1 µg/g DW (21 ºC) and 1.6 ± 0.5 µg/g DW (MHW). In contaminated mussels, concentrations of Li were 2.7 ± 0.9 µg/g DW (17 ºC), 2.1 ± 0.2 DW µg/g (21 ºC) and 3.1 ± 0.8 DW µg/g (MHW) (Table 3). Between contaminated and non-contaminated mussels, significant differences were found in temperature scenarios of 17 ºC (p = 0.0498) and 21 ºC (p = 0.0019) (Table 2 SM). No significant interaction was observed between Li and temperature (p = 0.7809) (Table 3 SM).

The values of Bioconcentration Factor (BCF) (Table 3) obtained for each exposure condition were 9.7 ± 3.3 L/kg (17 ºC), 7.6 ± 0.8 L/kg (21 ºC) and 10.3 ± 2.7 L/kg (MHW). For contaminated mussels, no significant differences were observed between temperature scenarios (p = 0.4406) (Table 1 SM).

Table 3. *Lithium (Li) concentrations (µg/g dry weight) in mussels soft dried tissue and Bioconcentration Factor (BCF, L/Kg) after 28 days of exposure to artificial seawater spiked with 250 µg/L of Li. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated mussels, uppercase letters for contaminated ones) and an asterisk represents significant differences between non-contaminated and contaminated mussels for each temperature scenario. Values are the mean of 3 values ± standard deviation. MHW: Marine heatwave.*

Treatments	Temperature (°C)	Li concentration	BCF
Non-contaminated	17	$1.2 \pm 0.3^{a*}$	-
	21	$1.0 \pm 0.1^{a*}$	-
	MHW	1.6 ± 0.5^{a}	-
Contaminated	17	$2.7 \pm 0.9^{A*}$	9.7 ± 3.3^{A}
	21	$2.1 \pm 0.2^{A*}$	7.6 ± 0.8^{A}
	MHW	3.1 ± 0.8^{A}	10.3 ± 2.7^{A}

3.2. Biological responses

3.2.1. Physiologic parameters

3.2.1.1. Mortality

There was no mortality during 28 days exposure period, and the subsequent consequences are considered sublethal.

3.2.1.2. Condition index

The condition index (CI) showed no significant variations among temperature scenarios regardless the absence or presence of Li, although mussels subjected to the MHW showed lower values. No significant differences were observed between contaminated and non-contaminated mussels at each temperature scenario (Figure 10; Table 1 SM). No significant interaction was observed between Li and temperature ($p = 0.8654$; Table 3 SM).

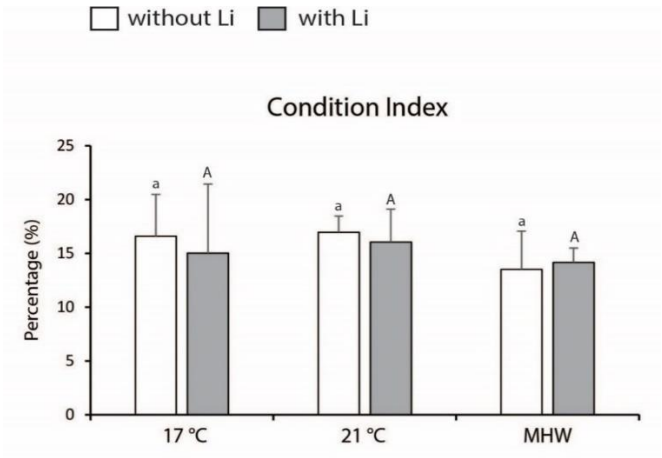

Figure 10. *Condition Index (CI), in Mytilus galloprovincialis exposed to different temperatures (17 °C, 21 °C and Marine heatwave-MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences (p < 0.05) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between the non-contaminated and contaminated mussels for each temperature scenario.*

3.2.1.3. Respiration rate

The respiration rate (RR) was higher in organisms exposed to Li, regardless the temperature scenario but significant differences to non-contaminated mussels were only observed at 21 ºC. Regardless the absence or presence of Li, no significant variations were observed among temperature scenarios (Figure 11; Table 1 SM). No significant interaction was observed between Li and temperature (p = 0.9359; Table 3 SM).

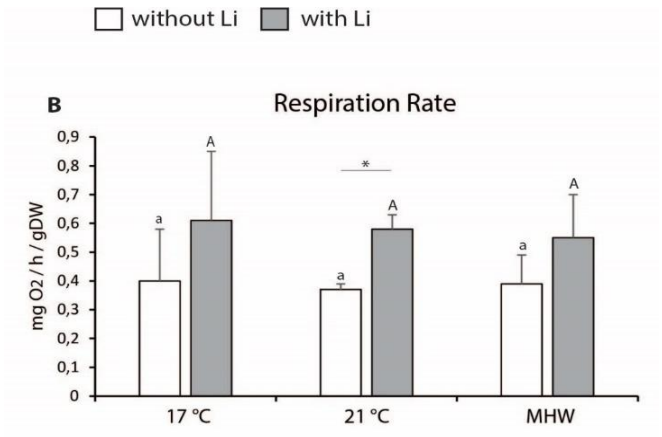

Figure 11. *Respiration Rate (RR), in Mytilus galloprovincialis exposed to different temperatures (17 °C, 21 °C and Marine heatwave-MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences (p < 0.05) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between the non-contaminated and contaminated mussels for each temperature scenario.*

3.2.2. Biochemical parameters

3.2.2.1. Metabolic capacity and energy reserves

The electron transport system (ETS) activity in mussels not exposed to Li showed significantly lower values when maintained at 21 ºC or under the MHW scenario in comparison to mussels exposed to control temperature (17 ºC). Contaminated mussels showed significantly higher ETS (increased metabolism) when under the MHW scenario in comparison to mussels maintained at 21 ºC, but no differences were observed between control and increased temperature scenarios. Significant differences between non-contaminated and contaminated mussels were observed only under the MHW treatment, with the non-contaminated individuals presenting significantly lower ETS values than the contaminated ones (Figure 12A; Tables 1 and 2 SM). A significant interaction was observed between Li and temperature ($p = 0.0011$; Table 3 SM).

In terms of protein (PROT) and glycogen (GLY) concentrations, no significant differences were observed among temperature scenarios, regardless of the absence or presence of Li. Also, no significant differences were found between contaminated and non-contaminated mussels for each temperature scenario (Figures 12B and C; Table 1 SM). No significant interaction was observed between both factors (Li and temperature), with $p = 0.2774$ for PROT and $p = 0.3816$ for GLY (Table 3 SM).

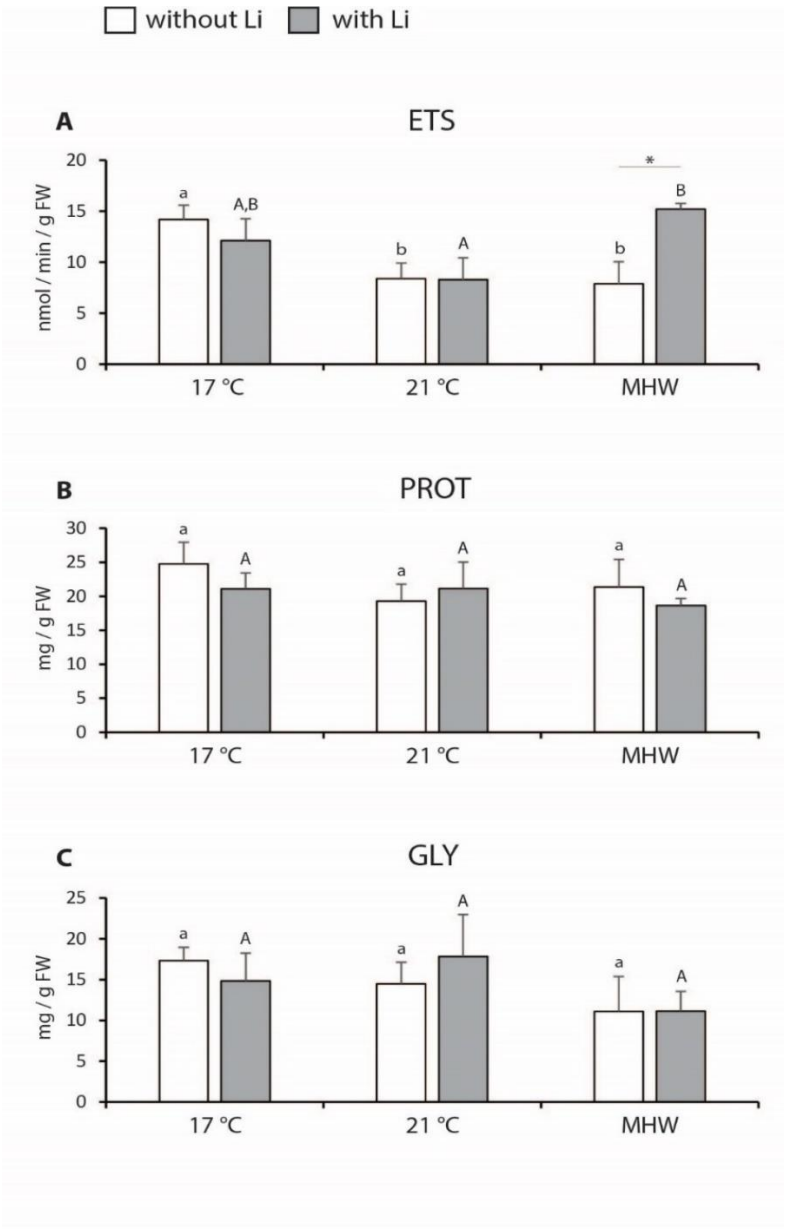

Figure 12. A*: Electron transport system (ETS) activity;* ***B****: Protein (PROT) content;* ***C****: Glycogen (GLY) content, in Mytilus galloprovincialis exposed to different temperatures (17 °C, 21 °C and Marine heatwave-MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences (p < 0.05) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between the non-contaminated and contaminated mussels for each temperature scenario.*

3.2.2.2. Oxidative status

3.2.2.2.1. Antioxidant and biotransformation defenses

The activity of catalase (CAT) showed no significant differences among temperature scenarios, regardless of the absence or presence of Li. Also, no significant differences were found between contaminated and non-contaminated mussels for each temperature scenario (Figure 13A; Table 1 SM). No significant interaction was observed between both factors (Li and temperature), with $p = 0.4188$; Table 3 SM).

The activity of glutathione peroxidase (GPx) showed no significant differences between the control temperature (17 ºC) and the MHW scenario in both non-contaminated and contaminated organisms. On the other hand, when exposed to 21 ºC, mussels showed significantly lower GPx values in relation to 17 ºC (non-contaminated $p = 0.0039$; contaminated $p = 0.0368$) and MHW (non-contaminated $p = 0.0042$; contaminated $p = 0.0014$) groups. Between contaminated and non-contaminated mussels no significant differences were found, regardless the temperature scenario (Figure 13B; Tables 1 and 2 SM). No significant interaction was observed between both factors (Li and temperature), with $p = 0.3256$ (Table 3 SM).

The activity of glutathione reductase (GR) showed no significant differences among temperature scenarios, regardless of the absence or presence of Li. Also, no significant differences were found between contaminated and non-contaminated mussels for each temperature scenario (Figure 13C; Table 1 SM). The interaction between both factors revealed no significant effects on GR activity ($p = 0.2352$; Table 3 SM).

Carboxylesterase *p*-nitrophenyl acetate (CbEs-*p*NPA) activity revealed no significant differences among temperature scenarios, regardless of the absence or presence of Li. Also, no significant differences were found between contaminated and non-contaminated mussels for each temperature scenario (Figure 14A; Table 1 SM). The interaction between Li and temperature was not significant ($p = 0.3091$; Table 3 SM).

Carboxylesterase *p*-nitrophenyl butyrate (CbEs-*p*NPB) activity showed no significant differences among temperature scenarios for non-contaminated mussels. Li exposed mussels showed significantly lower CbEs-*p*NPB activity when subjected to the MHW scenario in comparison to mussels exposed to 21 ºC. No significant differences were found between contaminated and non-contaminated mussels for each temperature scenario

(Figure 14B; Table 1 and 2 SM). The interaction between Li and temperature was not statistically significant (p = 0.1575; Table 3 SM).

The glutathione S-transferases (GSTs) activity showed no significant differences among temperature scenarios, regardless of the absence or presence of Li. Also, no significant differences were observed between contaminated and non-contaminated mussels for each temperature scenario (Figure 14C; Table 1 SM). No significant interaction was observed between Li and temperature (p = 0.3436; Table 3 SM).

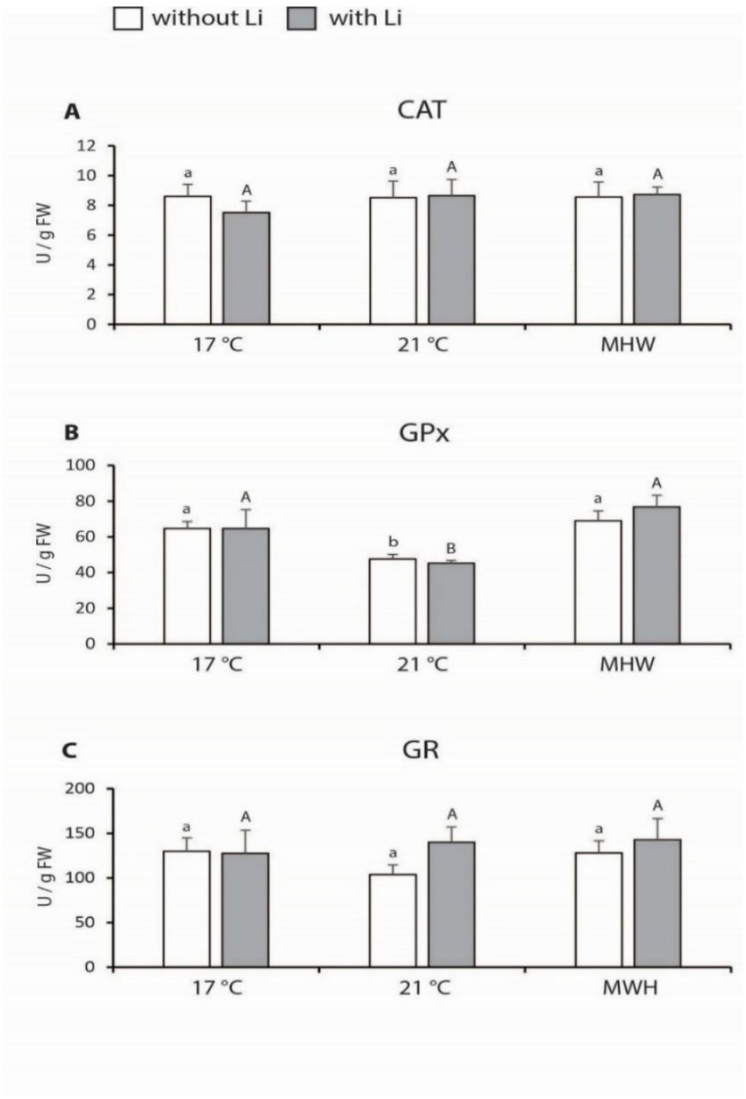

Figure 13. A*: Catalase (CAT) activity;* ***B****: Glutathione peroxidase (GPx) activity;* ***C****: Glutathione reductase (GR) activity, in Mytilus galloprovincialis exposed to different temperatures (17 °C, 21 °C and Marine heatwave-MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences (p < 0.05) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between the non-contaminated and contaminated mussels for each temperature scenario.*

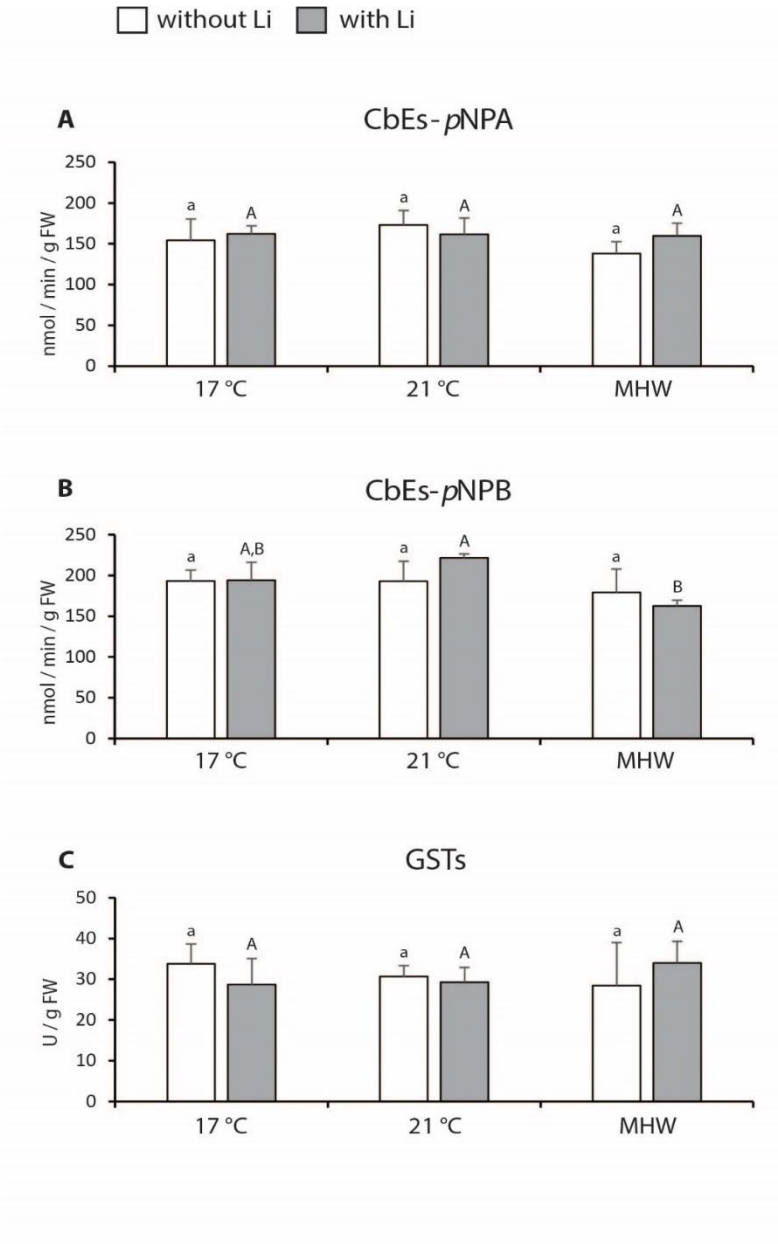

Figure 14. A*: Carboxylesterase p-nitrophenyl acetate (CbEs-pNPA) activity;* ***B****: Carboxylesterase p-nitrophenyl butyrate (CbEs-pNPB) activity;* ***C****: Glutathione S-transferases (GSTs) activity, in Mytilus galloprovincialis exposed to different temperatures (17 °C, 21 °C and Marine heatwave-MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between* the *non-contaminated and contaminated* mussels *for each temperature scenario.*

3.2.2.2.2. Cellular damage and redox balance

The levels of lipid peroxidation (LPO) in non-contaminated mussels did not present significant differences between temperature scenarios when compared to control (21 ºC and MHW); there were only significant differences between those of 21 ºC with MHW. On the other hand, in the presence of Li mussels subjected to the MHW presented significantly higher LPO levels than mussels maintained at 17 or 21 ºC. Significant differences between contaminated and non-contaminated mussels were only observed under the MHW scenario, with higher values in the presence of Li (Figure 15A; Table 1 and 2 SM). The interaction between both factors (Li and temperature) was not significant (p = 0.0626; Table 3 SM).

In terms of protein carbonylation (PC) levels and reduced glutathione (GSH) content, both for Li-exposed or non-exposed mussels, no significant differences were observed among temperature scenarios; between contaminated and non-contaminated mussels, no significant differences were observed at each temperature scenario (Figures 15B and 15C; Table 1 SM). No significant interaction was found between both factors (p = 0.1374 for PC and p = 0.2609 for GSH) (Table 3 SM).

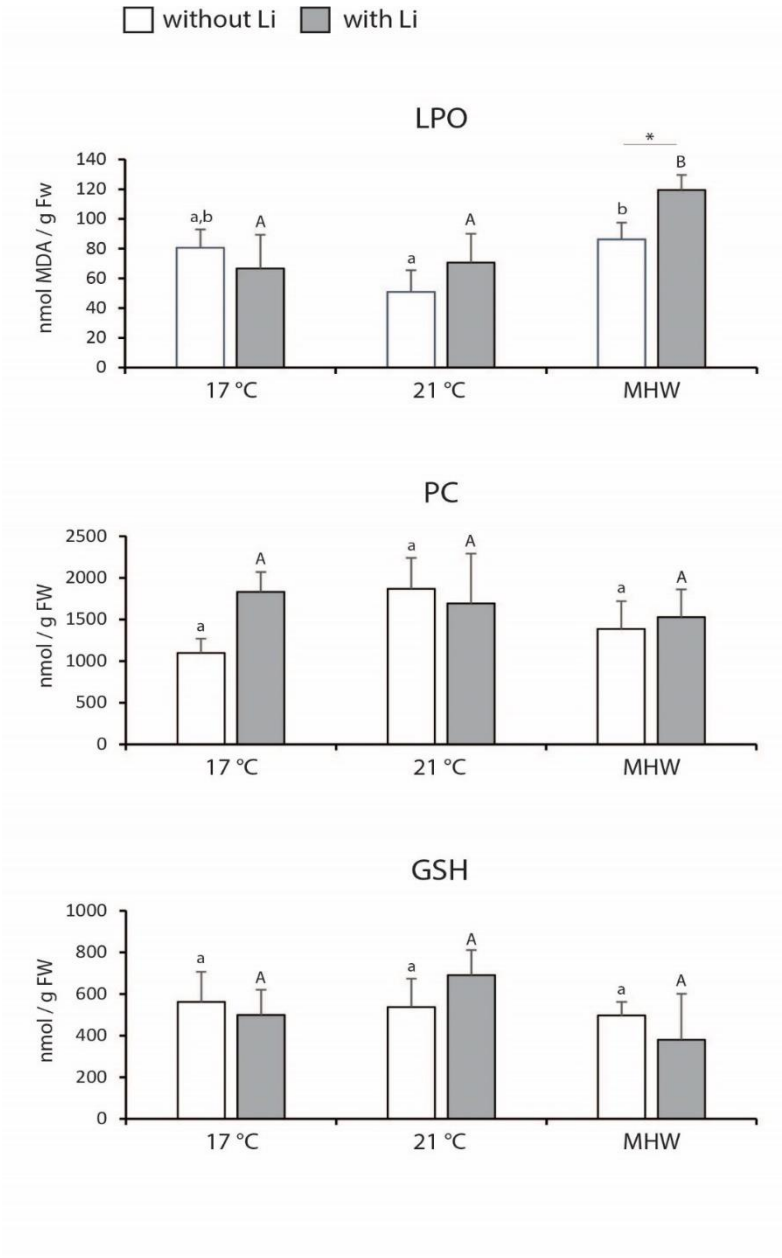

Figure 15. A*: Lipid peroxidation (LPO) levels;* ***B****: Protein carbonylation (PC) levels;* ***C****: Reduced glutathione (GSH) content, in Mytilus galloprovincialis exposed to different temperatures (17 °C, 21 °C and Marine heatwave-MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences (p < 0.05) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between the non-contaminated and contaminated mussels for each temperature scenario.*

3.2.2.3. Neurotoxicity

The acetylcholinesterase (AChE) activity showed no significant differences among temperature scenarios, regardless of the absence or presence of Li. Also, no significant differences were observed between contaminated and non-contaminated mussels for each temperature scenario (Figure 16; Table 1 SM). No significant interaction was observed between Li and temperature (p = 0.2544; Table 3 SM).

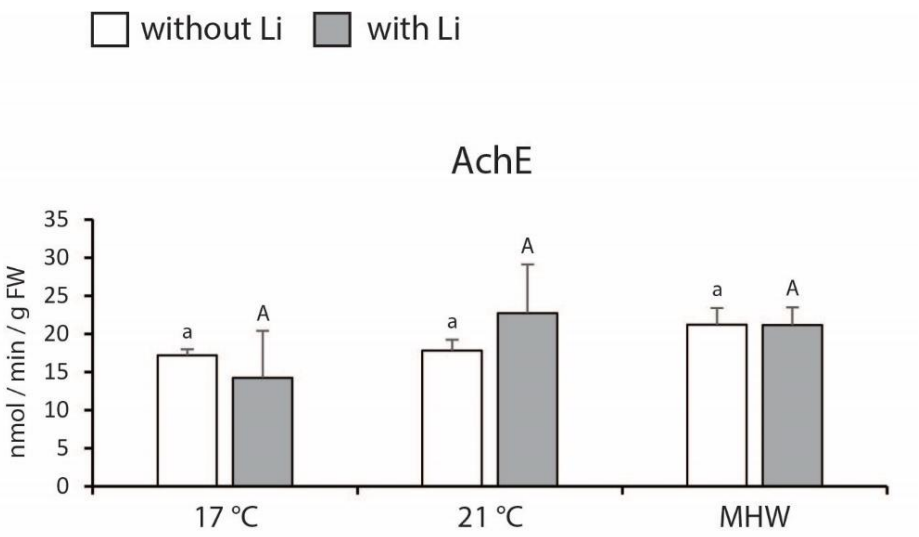

Figure 16. *Acetylcholinesterase (AChE) activity, in Mytilus galloprovincialis exposed to different temperatures (17 °C, 21 °C and Marine heatwave-MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Results are means with standard deviations. Different letters represent significant differences ($p < 0.05$) among tested treatments (lowercase letters for non-contaminated, uppercase letters for contaminated ones) and asterisks represent differences between the non-contaminated and contaminated mussels for each temperature scenario.*

3.2.3. Multivariate analysis

The results of the PCO analysis are shown in Figure 17. The PCO1 accounted for 46.4 % of the variation, with non-contaminated and contaminated organisms at 21 ºC in the positive side while mussels exposed to Li under the MHW were on the negative side of PCO1. The PCO2 axis explained 16.9 % of the overall variation, separating the non-contaminated individuals under control temperature and those contaminated at 21 ºC on the

positive side, from the remaining treatments on the negative side. The variables GLY, GSH, CbEs-*p*NB and CI showed the best correlation with the positive side of PCO1 (0.68627; 0.83869; 0.87741 and 0.69503, respectively), while ETS, LPO and GPx were the parameters best correlated with PCO1 negative side (0.70206; 0.92292 and 0.96287, respectively). On the other hand, the variable PROT (0.8393) showed the best correlation with positive side of PCO2 and the PC (0.72079) for negative side.

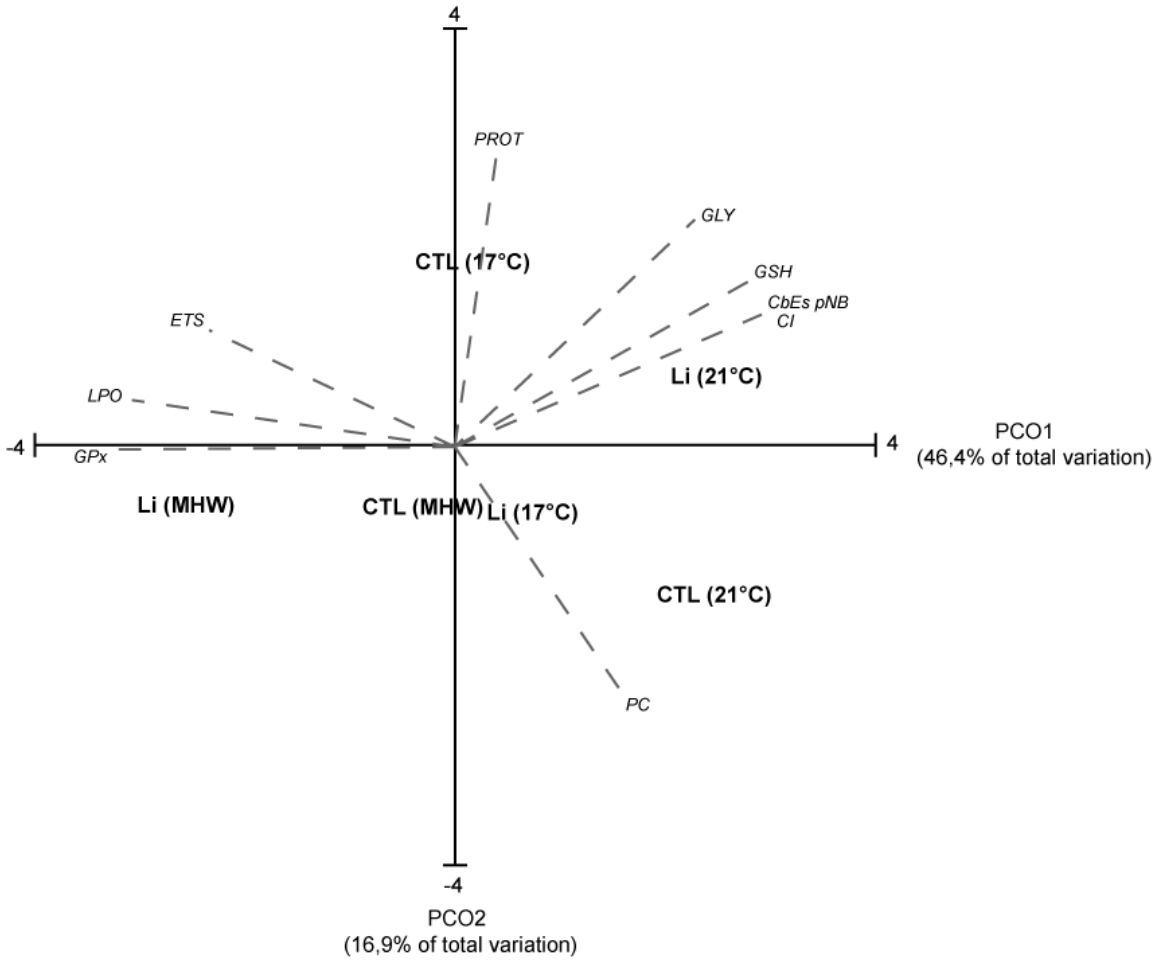

Figure 17. *Centroid's ordination diagram (PCO) based on biochemical markers measured, in Mytilus galloprovincialis exposed to different temperatures (17 °C, 21 °C and Marine heatwave-MHW) in the absence and presence of lithium (250 µg/L) for 28 days. Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data (r > 0.75): Reduced glutathione (GSH); Carboxylesterase p-nitrophenyl butyrate (CbEs – pNPB); Glycogen (GLY); Condition index (CI); Protein (PROT); Electron transport system (ETS); Lipid peroxidation (LPO); glutathione peroxidase (GPx); Protein carbonylation (PC).*

Chapter 4

Discussion

4. Discussion

The present study aimed to investigate the physiological (condition index and respiration rate) and biochemical performance of the species *M. galloprovincialis*, when exposed to Li, and to understand how warming and MHW conditions influence the toxicity of Li in organisms.

4.1. Lithium concentration and bioaccumulation

The results showed that Li concentration in tissues was significantly higher (approximately 2 times more) in contaminated organisms, at a temperature of 17 ºC and 21 ºC, in comparison with control organisms. A similar pattern was observed in the study by Henriques et al. (2019) where it showed that mussels were able to accumulate gadolinium (Gd) in tissues during 28 days at 17 ºC in comparison to non-contaminated mussels. Shen et al. (2022) also demonstrated that the same species had the ability to accumulate copper (Cu), zinc (Zn), and lead (Pb) during 30 days at 20 ºC. These results are in line with the data obtained on the respiration rate, since, although the values were not significantly different, there was a higher respiration rate in contaminated mussels compared to the control mussels, and according to Relexans et al. (1988) this behaviour represents a physiological response to contamination. On the other hand, comparing temperatures scenarios, no significant differences were observed for both the Li concentration in tissues and BCF, which indicates that the temperature did not influence the bioaccumulation of Li in *M. galloprovincialis*. Similar to this study, Pirone et al. (2019) showed that the concentration of lead (Pb) in mussel's tissue did not presented differences when comparing different temperatures, and Andrade et al. (2022) showed that between different temperature scenarios no changes of BCF were found when mussels were exposed to lanthanum (La). Lan et al. (2020) demonstrated that higher temperatures led to an increase in bioaccumulation factor (BAF) in oysters for Cu, Zn, mercury (Hg), and cadmium (Cd) but no change was observed for arsenic (As) and Pb when comparing temperature scenarios. Nevertheless, other studies, such as the study by Coppola et al. (2017) found that the mussels under higher temperatures presented a higher accumulation of As after 14 and 28 days. In the present study, Li accumulation was not higher in mussels exposed to higher temperatures, probably due to a

common mechanism in bivalves, which is the valves closure (Gosling et al., 2003), which causes a decrease in filtration.

4.2. Physiological responses

4.2.1. Condition index

The increase in temperature and the presence of contaminants in the marine environment can change the physiological condition of aquatic organisms, including bivalves (Andrade et al., 2019; Azpeitia et al., 2016; Matozzo et al., 2012). The condition index (CI) is one of the physiological parameters used to indicate the general health status of organisms (Andral et al., 2004). In view of the results obtained, both temperature changes and the presence of Li seemed to not represent significant threat to mussel's CI, which may indicate that tested conditions were not sufficiently stressful to alter the health status of organisms or on the other hand (namely short exposure period), indicate an adaptive behaviour of the individual to resist stress conditions common to an intertidal environment, as shown by Romero-Freire et al. (2020). The authors observed that the same species (*M. galloprovincialis*) collected at different sites showed different physiological responses when exposed to a higher temperature, while the Galician mussels decreased the CI with increasing temperature, the Tunisian mussels maintained the CI with increasing temperature. Similarly, Andrade et al. (2019) showed no significant changes in CI in the same species when exposed to increased temperature and carbon nanotubes, when acting alone or in combination. Contrary to what this study demonstrated, Nilin et al. (2012) observed that cockles with higher concentrations of Hg had lower CI, which may indicate that Hg induces more negative effects than Li. Mackenzie et al. (2014) showed that mussels exposed to a higher temperature during six months have a lower CI, demonstrating that the impacts on CI at different temperatures may depend on the exposure duration.

4.2.2. Respiration rate

The respiration rate (RR) is another physiological parameter used to evaluate the influence of diverse stress conditions, including ocean warming, acidification and anthropogenic pollution (Freitas et al., 2017; Gestoso et al., 2016; Wang et al., 2015). The results obtained in the present study demonstrated that temperature alone did not influence the RR of mussels, but the presence of Li exerted an impact, showing a tendency towards higher respiration rate of the different temperature scenarios, especially noticed at 21 ºC, with higher RR in Li contaminated mussels compared to the non-contaminated. The increase of the RR was already demonstrated as a physiological response of adaptation to the exposure of contaminants (De Marchi et al., 2017; Rivera-Ingraham et al., 2021), associated to the increase of metabolism and activation of antioxidant defenses to avoid cellular damage. On the other hand, Gestoso et al. (2016), showed that in the same species with the increase in temperature (21 ºC), there was no increase in RR compared to 16 ºC, the increase of RR were only noticed at the same temperature when combined with pH reduction (7.65). This increase can be justified by the stress caused by the reduction of pH, thus increasing the metabolic rate, to maintain the integrity of soft tissues. Nevertheless, the maintenance of mussel's RR at different temperatures revealed the capacity of the organisms to deal with temperature changes, which might be associated with their capacity to reduce filtration rate at the tested temperature scenarios (21 ºC and MHW). Although the filtration capacity was not measured in the present study, published literature already demonstrated that a reduction in RR can be associated with a decrease in bivalve's filtration rate (Tang & Riisgård, 2018). The present findings further indicate that mussels may present higher capacity to adapt to temperature shifts than to the presence of Li and/or that Li represents a greater threat to mussels than temperature rise. Closely related with bivalve's RR is their metabolism (Guppy & Withers, 1999; Kim et al., 2001), with published literature demonstrating that the metabolic decrease was associated with bivalve's valves closure (Gosling et al., 2003; Tang & Riisgård, 2018). Previous studies also demonstrated that the capacity of organisms to change their metabolism is fundamental for their survival as well as for stress adaptation and tolerance (Freitas et al., 2021; Sokolova et al., 2012).

4.3. Biological responses

4.3.1. Metabolic capacity and energy reserves

Metabolism can be a vital role in the survival and function of the body, but also in the adaptation and tolerance to stress (Sokolova et al., 2012), through the supply of adenosine triphosphate (ATP) from biological oxidations that require acceptors of nicotinamide adenine dinucleotide (NAD^+) and adenine flavin dinucleotide (FAD). As reduced coenzymes, NADH and $FADH_2$ are converted back into NAD^+ and $FADH_2$ when their electrodes are transferred, through the electron transport system (ETS) (Haunerland et al., 2003). The electron transport system is a series of four protein complexes that harbour redox reactions, leading to the establishment of an electrochemical gradient through the internal mitochondrial membrane providing energy that leads to the creation of ATP through oxidative phosphorylation of adenosine diphosphate (ADP) [5]. The regulation of energy expenditure and the amount of available energy is essential for the fitness of the body (reproduction, development, and growth). However, environmental stress can affect energy balance, since the body will expend additional energy to recover and maintain homeostasis, which can impair the systems involved in the acquisition, conversion, and conservation of energy (Sokolova et al., 2008, 2012). In the present study, non-contaminated mussels decreased their metabolic capacity at stressful temperature scenarios (21 ºC and MHW) and the same behavior was observed at 21 ºC for Li exposed mussels. However, in the presence of Li under MHW scenario mussels tended to increase their metabolism in relation to control temperature and especially to 21 ºC. Considering previous studies, the present results can indicate that under lower stress conditions (absence or presence of Li at 17 or 21 ºC) mussels were able to reduce their metabolism to avoid injuries. Reduced metabolism in comparison to control values was observed in bivalves under low-stress conditions, namely subjected to warming conditions as demonstrated in the study in the mussel *M. galloprovincialis* at 21 ºC, when compared to the control temperature (17 ºC) (Coppola et al. 2017). The same results were observed in the same species with low concentrations of La (0.1 mg/L) in the study by Pinto et al. (2019). In addition, Nunes et al. (2017), also observed in the species *Ruditapes philippinarum* a metabolic reduction with a concentration of 0.25 µg/L of paracetamol. The present study further demonstrated that in the presence of Li and under a MHW scenario the metabolism shut down was no longer a valid response and mussels

tended to increase their metabolic capacity. This response might indicate greater stress levels and the need of bivalves to activate their defense mechanisms, associated to enhanced ETS activity. In fact, as demonstrated by the PCO graph, mussels exposed to Li and maintained under MHW scenario were close related to ETS activity and antioxidant capacity. Similarly, previous studies demonstrated that when the stress level increased bivalves defense strategy involves an increase in the metabolic capacity. For example, in the study by De Marchi et al. (2022), it was observed that *M. galloprovincialis* under conditions of multiple stresses (Caffeine + MHW), metabolic activity was higher in relation to individual or no stress conditions.

Carbohydrates (such as glycogen (GLY), proteins (PROT) and lipids (LIP)) are the major source of energy to fuel up metabolism (Haunerland et al., 2003). Bivalves have GLY as the main polysaccharide in their organism, being the first energy reserve to be used, thus playing an important role in energy metabolism (Kaushik et al., 2022; Patrick et al., 2006), while PROT has as main functions of growth, maintenance and repair of organs, tissues and cells of organisms. Glycogen is made up of several glucose residue units, which is the main neutral sugar present in bivalve's tissues. It has been reported that, when organisms are under stress, they can increase their energy expenditure to fuel defense mechanisms (Bielen et al., 2016). Glycogen is also used as a crucial substance that contributes to the primary energy sources that are converted into ATP. The first stage of ATP synthesis takes place in the cytoplasm, hence this process takes place in the cell's mitochondria (Falfushynska et al., 2020). Furthermore, the GLY and PROT contents are an energy conserved system of antioxidant defense that facilitates the neutralization of oxidizing substances in non-toxic hydroxycarboxylic acids (Lima et al., 2007). This process is carried out by glutathione reductase (GR), which is used by glyoxalase enzymes to neutralize highly toxic α-ketoaldehydes (α-KA) that may be formed in cellular oxidative processes; glyoxalase I (Glx-I) utilizes GSH as coenzyme to form an intermediate thiolester and S-D-lactoylglutathione (S-D-LGSH), while glyoxalase II (Glx-II) converts the thiolester to a d-hydroxyacid (DHA) with regeneration of GSH (Regoli & Giuliani, 2014).

Glycogen and PROT levels are indicators of an organism's energy status and reserve capacity (Ansaldo et al., 2006; Vasseur & Cossu-Leguille, 2003). In terms of PROT and GLY content, the present results showed that mussels were able to avoid the expenditure of their energy reserves, among temperature scenarios, regardless the absence or presence of

Li. This result was also observed in the study by Viana et al. (2020), when the mussels *M. galloprovincialis* were exposed to Li at a concentration of 250 and 750 µg/L. In the present study the fact that the energy reserves remained similar regardless the temperature scenario, can indicate that although a decrease in the mussel's metabolism was observed (except under MHW), this response was not accompanied by an accumulation of energies reserves, demonstrating that mussels were still using their reserves to fuel up defense mechanisms. Similarly, the study by Pinto et al. (2019) showed that the same species exposed to a concentration of La (10 mg/L) decreased metabolic capacity avoiding energy consumption when compared to control. The present findings also revealed that although ETS activity increased in contaminated mussels subjected to the MHW scenario, mussels maintained their energy reserves similar to control, which could indicate that mussels were making an effort to accompany their energy reserves expenditure with energy reserves production. Previous studies by Almeida et al. (2021) also demonstrated that GLY and PROT contents were maintained similar to control levels although bivalve's metabolism increased in the presence of the drug cetirizine. Although higher ETS activity was observed when mussels were submitted to the MHW scenario in the presence of Li, the limited effects observed could result from the fact that organisms were exposed for 7 days to 17 ºC after the temperature rise, which may have acted as a recovery period. Minuti et al. (2021) demonstrated that after a MHW the sea urchin *Heliocidaris erythrogramma* increased food consumption to replenish the energy deficit. This could explain the fact that mussels, in the present study, did not reduce their energy reserves after a 28 days exposure period, with the maintenance of CI in comparison to control organisms.

4.3.2. Oxidative status

Reactive oxygen species (ROS) are generated during cellular pathways in organisms (Halliwell & Gutteridge 2007), but when organisms are under stress conditions the production of ROS increase and in order to neutralize/eliminate than the organisms may activate defense mechanisms (activation of antioxidant and biotransformation enzymes) (Regoli & Giuliani, 2014; Sturve et al., 2008; Townsend & Tew, 2003). Nevertheless, when defense mechanisms are not sufficient to protect the cells from ROS cellular damage can occur (Regoli & Giuliani, 2014).

4.3.2.1. Antioxidant and biotransformation capacity

Under stress conditions, organisms activate their defense mechanisms, namely antioxidant enzymes: catalase (CAT); glutathione reductase (GR); glutathione peroxidase (GPx), to eliminate the excess of ROS produced and protect cells against oxidative damage (Regoli & Giuliani, 2014). Catalase is a hemoprotein, mostly present in peroxisomes but can be found in cytosol, with a principal function of divide hydrogen peroxide (H_2O_2), that is a ROS, into water (H_2O) and oxygen (O_2) (Fahimi & Cajaraville 1995; Halliwell & Gutteridge 2007). Glutathione peroxidase is the principal peroxidase for the hydroperoxides detoxification (Orbea et al., 2000). This enzyme is in the cytosol, nucleus, and lysosomes (Orbea et al., 2000) and its purpose is to catalyze the reduction of H_2O_2 to H_2O using reduced glutathione (GSH) as an electron donor (Regoli & Giuliani, 2014). Glutathione reductase is an important enzyme (flavoprotein) for maintaining the intracellular redox and the GSH/GSSG ratio because reconverts oxidized glutathione (GSSG) to GSH, using NADPH as a cofactor (Carlberg & Mannervik, 1985; Regoli & Giuliani, 2014). Defense mechanisms also include biotransformation enzymes, such a group of phase II detoxification enzymes, known as glutathione S-transferases (GSTs) enzymes. This group of enzymes is involved in cellular detoxification processes producing products that make compounds more soluble for their excretion in the cell (Habig et al., 1974; Regoli & Giuliani, 2014; Townsend & Tew, 2003) Some GST enzymes also reduce lipid hydroperoxides in alcohol, using the oxidation of GSH to GSSG (Regoli & Giuliani, 2014; Sturve et al., 2008). This group of enzymes can be found in the cytosol, mitochondria and microsomes (Raza, 2011). Carboxylesterase (CBEs) are biotransformation enzymes of the phase I involved in detoxification processes, by catalyzing the hydrolysis of several compounds creating a product known as a metabolite that is more water-soluble than the original one, contributing to the contaminant's excretion. These enzymes are present in in the cytoplasm and endoplasmic reticulum (Hosokawa, 2008; Laizure et al., 2013; Ribalta et al., 2015). According to the results obtained, there was no activation of antioxidant enzymes (CAT, GPx and GR) and biotransformation (GSTs and CBEs) by mussels, regardless of the tested treatment (except for GPx in contaminated mussels under warming scenario – 21ºC). Overall, this response can be explained by the fact that the reduction of metabolism at increased temperature (21 ºC) was the strategy used to reduce the stress induced by temperature and the presence of Li was not sufficient to activate these enzymes. Still, the tendency showed by mussels to increase GPx activity when exposed

to Li and maintained under the MHW scenario may be associated with increased metabolism. Other studies, such as the one conducted by Morosetti et al. (2020), showed that *M. galloprovincialis* when exposed for 28 days to cerium oxide nanoparticles (CeO_2) at a temperature of 22 ºC, showed no activation of enzymes (CAT, GPx, GR and GSTs) in contaminated mussels under warming conditions comparing to control ones.

4.3.2.2. Cellular damage and redox balance

When defense mechanisms are insufficient to protect cells, ROS initiates an oxidative process that causes cell damage and lipids are oxidized in a process known as lipid peroxidation (LPO) (Regoli & Giuliani, 2014). LPO is a chain of reactions where free radical binds to a hydrogen atom (H), forming a lipid radical (L) that will react with oxygen (O_2) producing a peroxidized lipid radical (LOO). This, in turn, gives rise to a peroxidized lipid (LOOH) and a lipid radical by removing 1H from another lipid, causing a reaction again. Lipid peroxidation produces molecules and degradation products, such as aldehydes, acetone and malondialdehydes (MDA) which are formed from the breakdown of unsaturated fats and used as a lipid peroxidation index. LPO is terminated when L or LOO is destroyed by a fat-soluble antioxidant (Hampel et al., 2016). In addition, excess ROS can promote protein oxidation, called protein carbonylation (PC), leading to aggregation, polymerization, unfolding or conforming alterations that can confer a loss of structural or functional activity (Suzuki et al., 2010). In turn, it can be directly induced by the action of oxidative stress, which involves the direct action of ROS or metal-catalyzed oxidation of amino acids side chains, particularly proline, arginine, lysine, and threonine or indirectly by reactions of secondary by-products (Rodríguez-García et al., 2020), which imply a carbonylation of lysine, cysteine, and histidine, which may be caused by their reaction with reactive carbonyl groups produced during the oxidation of carbohydrates (e.g., glyoxal (GO), methylglyoxal (MGO)) and lipids (e.g., HNE, MDA or acrolein (ACR)).

The results obtained in the present study demonstrated that only when subjected to a MHW, and especially in the presence of Li, mussels showed higher LPO levels than compared to the remaining tested treatments, although no PC was observed. Similar results were already obtained with the same species in previous multi-stressor studies, such as Andrade et al. (2019) where the presence of functionalized multi-walled CNTs (f-

MWCNTs) and a temperature rise to 21 ºC induced an increase in LPO while showing negligible effects to PC relative to control.

Increased LPO levels in Li exposed mussels under MHW was accompanied by a slight decrease in reduced glutathione (GSH) content, indicating a possible loss of redox balance in mussels experiencing the combined effect of MHW and Li. Reduced glutathione is an antioxidant compound that acts on the cytoplasm as a direct neutralizer of ROS through its oxidation to oxidized glutathione (GSSG). In turn, it also acts as a cofactor of other antioxidant enzymes such as GPx (Regoli & Giuliani 2014). In view of the results, it seems that there was not enough oxidative stress for GSH to turn into GSSG to eliminate ROS, as is also proven by the non-alteration of GPx and GR enzymes in the same treatment. Results of a previous study showed no changes in GSH in *M. galloprovincialis*, when contaminated with different concentrations (5, 50 e 100 µg/L) of titanium (Ti) (Monteiro et al., 2019). Nevertheless, a significant increase on LPO levels and ETS activity in Li exposed mussels maintained under the MHW scenario support the hypothesis that this was the most stressful condition tested.

4.3.3. Neurotoxicity

Acetylcholinesterase (AChE) belongs to the cholinesterase family (ChE), which has been used as a biomarker for the detection of possible neurotoxic impacts caused by different compounds existing in the marine environment (Bocquené & Galgani, 1991). It is found at cholinergic synapses and neuromuscular junctions in both vertebrates and invertebrates (Boison, 2007). This enzyme is important to the neurotransmission because is involved in the hydrolysis of the neurotransmitter acetylcholine into choline and acetylcholine acid at neuromuscular junctions (Karami-Mohajeri & Abdollahi, 2011; Massoulié et al., 2008). Acetylcholine is a neurotransmitter used in efferent systems and also in some central circuits (Woolf, 1991). This in turn is synthesized by the enzyme choline acetyltransferase in the cytoplasm of cholinergic neurons and degraded in the synaptic cleft by the enzyme AChE. Inhibitors or anti-cholinesterase inhibit the enzyme cholinesterase from breaking down an acetylcholinase, causing an increase in the duration of the neurotransmitter action. Regarding the mode of action, AChE inhibitors can be classified as toxic effects of AChE activity associated with adverse or reversible effects, having a fundamental role in therapeutic applications (Colovic et al., 2013). This biomarker provides ample neurobiotic information

for xenobiotic assessments that interfere with cholinesterase function (Lionetto et al., 2003). The results obtained in the present study revealed that no neurotoxicity was induced in the presence of Li, regardless of the temperature scenario tested. However, Viana et al. (2020) demonstrated that at the control temperature (18 °C), Li caused neurotoxicity at higher concentrations, highlighting the low toxicity of the tested concentration.

4.4. Contribution to the Sustainable Development goals (2030 Agenda)

The "2030 Agenda for Sustainable Development" of the United Nations aims towards a sustainable and resilient development path. It is designed around five pillars (5Ps), which are people, planet, prosperity, peace, and partnership (Khajuria et al., 2022). These pillars are designed to involve the three elements of sustainability, that are economic, environmental, and social elements (Khajuria et al., 2009). The knowledge gained from this research, concerning the influence of temperature increase and MHW on the effects of Li on the mussel species *M. galloprovincialis*, will contribute to the fulfilment of some of the sustainable developments (SDGs) of the 2030 Agenda [6], in particular by providing data on Li concentration in ecosystems on marine species, the effects on marine organisms and consequently on humans, as well as the effects of increased temperature and as MHW on marine species. These data will contribute to: (i) establish appropriate regulatory guidelines on Li limit concentration in water in order to reduce contamination and consequently human intake (SDGs 3); (ii) develop strategies to improve wastewater treatment plants, methods of recycling and reusing the equipment's containing Li in order to decreased the release of Li into the environment, as well as the creation of cleaner and environmental alternative technologies to Li (SDGs 6, 9 and 12); iv) to understand how the increase in climate change and the effects of Li affect marine life, as well as its influence when acting simultaneously, in order to protect marine species (SDGs 13 and 14).

Chapter 5

Conclusions

5. Conclusions

Temperature increase scenarios have intensified in the last decade, as well as the increasing use of Li and the consequent increase in its concentration in the marine environment. The study assessed the influence of two temperature increase scenarios (warming and MHW) on the toxicological effects of Li on the bivalve *M. galloprovincialis*, providing data on the possible risk that Li and climate change have to the marine organism.

The results showed that only extreme temperature increase events (MHW) changed mussel's response to Li, causing oxidative stress and increased metabolism. The fact that there are no negative effects on no-contaminated and contaminated individuals at 21 ºC, can be explained by their adaptation to the gradual increase in temperature that mussels have been acquiring in aquatic system due to the action of the tides. The temperature factor did not influence the accumulation of Li or the respiration rate. Overall, the biochemical responses of the mussel *M. galloprovincialis* highlighted that the most stressful condition was observed when mussels were subjected to an MHW in the presence of Li.

Currently, there is still little knowledge about the impacts of Lithium on marine species when combined with other environmental factors derived from climate change (acidification, increased salinity and extreme weather events). The present results obtained underline the importance, in future studies, evaluating the effects of Li with other environmental factors, as well as different contaminants existing in the environment, with extreme meteorological events (MHW).

This study allows concluding that the increase in temperature, especially marine heatwaves, influence the toxicological effects of Li on the mussel *M. galloprovincialis* and can predict that in future heating scenarios may have ecological consequences, socio-economic impacts and possibly result in the loss of the biodiversity.

Chapter 6

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6.1. References

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6.2. Webgraphy

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Chapter 7

Appendix

7. Supplementary material

Table 1 SM - Statistical results (PERMANOVA main test) including *p*-values, pseudo-F and degrees of freedom obtained for concentration of Li in mussel's soft tissue, physiological and biochemical parameters in *Mytilus galloprovincialis*. Significant values ($p < 0.05$) are in bold.

	Degrees of freedom (df)	**Pseudo-F value**	***p*-value (MC)**
Li concentration in mussels	5	6,4395	**0,0045**
CI	5	0.4151	0.8338
RR	5	1.6589	0.2139
BCF	2	0.9549	0.4406
ETS	5	10.4070	**0.0008**
PROT	5	1.5095	0.2547
GLY	5	2.0963	0.1364
CAT	5	0.7422	0.6057
GPx	5	13.2330	**0.0002**
GR	5	1.6607	0.2217
CbEs-*p*NPA	5	1.2415	0.3563
CbEs-*p*NPB	5	3.1871	**0.0464**
LPO	5	6.5376	**0.0033**
PC	5	1.9231	0.1659
GSH	5	1.5223	0.2574
AChE	5	1.9891	0.1475

Table 2 SM - Pairwise comparisons (*p*-values (MC)) among different temperatures (17 °C, 21 °C and MHW) for each treatment (CTL and Li), and between CTL and Li for each temperature performed in the concentration of Li in mussel's soft tissue and the physiological and biochemical parameters in which the main test was significant. MHW: Marine heatwave. Significant values ($p < 0.05$) are in bold.

Comparisons	Li concentration in tissue	ETS	GPx	CbEs-*p*NPB	LPO
CTL 17 °C *vs* CTL 21 °C	0.4635	**0.0080**	**0.0039**	0.9813	0.0557
CTL 17 °C *vs* CTL MHW	0.2425	**0.0123**	0.3346	0.4839	0.5829
CTL 21 °C *vs* CTL MHW	0.1110	0.7506	**0.0042**	0.5599	**0.0273**
Li 17 °C *vs* Li 21 °C	0.3331	0.0926	**0.0368**	0.0984	0.8310
Li 17 °C *vs* Li MHW	0.6477	0.0698	0.1630	0.0749	**0.0200**
Li 21 °C *vs* Li MHW	0.1228	**0.0058**	**0.0014**	**0.0005**	**0.0197**
CTL 17 °C *vs* Li 17 °C	**0.0498**	0.2335	0.9968	0.9554	0.4105
CTL 21 °C *vs* Li 21 °C	**0.0019**	0.9404	0.2554	0.1178	0.2345
CTL MHW *vs* Li MHW	0.0634	**0.0043**	0.1854	0.3764	**0.0183**

Table 3 SM - Statistical results (PERMANOVA test) including p-values, pseudo-F and degrees of freedom obtained for concentration of Li in mussel's soft tissue, physiological and biochemical parameters in *Mytilus galloprovincialis*, obtained from an analysis with a two factors design, to test the interaction between Li concentration (factor 1) and temperature (factor 2). Significant values ($p < 0.05$) are in bold.

	Degrees of freedom (df)	**Pseudo-F value**	***p*-value (MC)**
Li concentration in mussels	2	0.2511	0.7809
CI	2	0.1424	0.8654
RR	2	0.0686	0.9350
ETS	2	12.0490	**0.0011**
PROT	2	1.4197	0.2774
GLY	2	1.0612	0.3816
CAT	2	0.9261	0.4188
GPx	2	1.2179	0.3256
GR	2	1.6291	0.2352
CbEs-*p*NPA	2	1.2757	0.3091
CbEs-*p*NPB	2	2.1900	0.1575
GSTs	2	1.1629	0.3436
LPO	2	3.5363	0.0626
PC	2	2.3701	0.1374
GSH	2	1.5135	0.2609
AChE	2	1.5522	0.2544