Universidade de Aveiro
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FRANCESCA COPPOLA

IMPACTOS AMBIENTAIS DA ÁGUA DO MAR REMEDIADA UTILIZANDO NANOCOMPÓSITOS E A INFLUÊNCIA DAS ALTERAÇÕES CLIMÁTICAS EM BIVALVES

ENVIRONMENTAL IMPACT OF CONTAMINATED SEAWATER REMEDIATED USING NEW NANOCOMPOSITES AND THE INFLUENCE OF CLIMATE CHANGE IN BIVALVES

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia e Ecologia das Alterações Globais, realizada sob a orientação científica da Doutora Rosa Fátima Freitas, Professora auxiliar com agregação do Departamento de Biologia da Universidade de Aveiro; coorientação científica da Doutora Etelvina Figueira, Professora auxiliar do Departamento de Biologia da Universidade de Aveiro e coorientação científica da Doutora Paula Alexandrina de Aguiar Pereira Marques, Investigadora principal do Departamento de Engenharia Mecânica da Universidade de Aveiro

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o júri

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palavras-chave

Bivalves, Biomarcadores, Histopatologia, Nanomateriais, Remediação, Bioacumulação, Metais (loid) s

resumo

Recentemente, têm sido aplicadas diferentes abordagens para fins de remediação de água, incluindo o uso de nanopartículas (NPs) para remover metais e metaloides da água como alternativas vantajosas aos métodos tradicionais de tratamento de água. Entre essas novas NPs, tem sido dedicado um grande esforço à síntese de nanocompósitos multifuncionais à base de grafeno e de ferrita de manganês tem recebido considerável atenção devido á sua enorme capacidade de remoção de metais (loid) s das águas. No entanto, a investigação dedicada a novos riscos ambientais e específicos relacionados a estes nanomateriais é limitada. Além disso, ainda não foram identificados os impactos induzidos pela combinação de fatores de alterações climáticas (nomeadamente, mudanças de salinidade e aumento de temperatura) e contaminantes, tais como metais (loid) s (por exemplo, mercúrio, arsénio e chumbo), em sistemas aquáticos. Para avaliar os impactos de todos estes fatores, as espécies bentónicas podem ser um bom modelo, pois são afetadas por diversas condições ambientais. Em particular, bivalves como *Mytilus galloprovincialis* (mexilhão) e *Ruditapes philippinarum* (amêijoa) foram identificados por diversos autores como bioindicadores que respondem rapidamente a distúrbios ambientais, possuindo ainda uma ampla distribuição espacial e relevância económica. Assim, a presente tese avaliou a segurança ecotoxicológica da água do mar, previamente contaminada com metais (loid) s, remediada, usando para tal óxido de grafeno funcionalizado com polietilenoimina (GO-PEI) ou / e NPs de ferrita de manganês ($MnFe_2O_4$-NPs), nas espécies *M. galloprovincialis* e *R. philippinarum.* Para tal, foram realizadas análises histopatológicas e bioquímicas, visando obter um maior conhecimento das alterações induzidas em ambas as espécies, por estes materiais, após a remediação. Os resultados obtidos mostraram que os organismos expostos a tratamentos não contaminados (condição controlo) e a água do mar remediada apresentaram padrões biológicos semelhantes, sem diferenças consideráveis expressas em termos de alterações bioquímicas e histopatológicas. Além disso, os resultados presentes revelaram aumentos dos efeitos toxicológicos em bivalves expostos a alterações climáticas, em comparação a organismos expostos a condições controladas de temperatura e salinidade. Estes resultados confirmam a capacidade do GO-PEI e $MnFe_2O_4$-NPs de adsorver metais (loid) s da água sem efeitos tóxicos percetíveis, no entanto, o aumento da temperatura e alterações de salinidade podem afetar as respostas dos bivalves à água do mar remediada. Embora os bivalves expostos às NPs tenham apresentado leves alterações relacionadas com stress oxidativo, dano celular e neurotoxicidade, bem como alterações histopatológicas reduzidas, em comparação ao controlo, os materiais testados aparentam ser uma abordagem promissora e ecologicamente adequada na descontaminação de águas residuais.

keywords

Bivalves, Biomarkers, Histopathology, Nanomaterials, Remediation, Bioaccumulation, Metal(loid)s.

abstract

Recently, different approaches have been applied for water remediation purposes, including the use of nanoparticles (NPs) to remove metals and metalloids from water as advantageous alternatives to traditional water treatment methods. Among these new NPs, the synthesis of multifunctional nanocomposites based on graphene and on manganese-ferrite has received considerable attention due to their huge capacity to remove metal(loid)s from waters. However, research dedicated to new and specific environmental risks related to these nanomaterials is limited. Furthermore, impacts induced by the combination of climatic change factors (namely salinity shifts and increase of temperature) and contaminants such as metal(loid)s (e.g. mercury, arsenic and lead) in aquatic systems, are yet unidentified. To evaluate the impacts of all these factors, benthonic species can be a good model as they are affected by several environmental constraints. Particularly, bivalves as *Mytilus galloprovincialis* (mussels) and *Ruditapes philippinarum* (clams) have been identified by several authors as bioindicators that respond quickly to environmental disturbances, with a wide spatial distribution and economic relevance. Thus, the present thesis evaluated the ecotoxicological safety of remediated seawater previously contaminated with metal(loid)s and remediated by using graphene oxide functionalized with polyethyleneimine (GO-PEI) or/and manganese-ferrite NPs ($MnFe_2O_4$-NPs) on *M. galloprovincialis* and *R. philippinarum* species. For this, histopathological and biochemical alterations were carried out, towards a deeper understanding of the alterations induced in both species by these materials after the remediation. Results obtained showed that organisms exposed to non-contaminated (control condition) and remediated seawater treatments presented similar biological patterns, with no considerable differences expressed in terms of biochemical and histopathological alterations. Moreover, the present findings revealed increased toxicological effects in bivalves under climatic changes in comparison to those under control temperature and salinity. These results confirm the capability of GO-PEI and MnFe2O4-NPs to adsorb metal(loid)s from water with no noticeable toxic effects, although temperature rise and salinity shifts could affect the responses of bivalves to remediated seawater. Although, bivalves exposed to these NPs showed slight oxidative stress, cellular damage and neurotoxicity as well as histopathological alterations in comparison to the control, the materials seem to be a promising eco-friendly approach to decontaminated wastewater.

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CHAPTER 1.

Introduction

1.1 State of the art

The environmental impacts caused by the presence of metals and metalloids in water bodies have been a major concern for a long time (Naimo et al., 1995; Li et al., 2006; Takeda et al., 2014). Elements such as mercury (Hg), arsenic (As), and lead (Pb) have been commonly found in aquatic systems with well-known toxicity effects on inhabiting organisms, including adverse effects on organisms' survival, growth, metabolism, reproduction and redox status, but also on humans through biomagnification (Cravo et al., 2012; Figueira et al., 2011; Regoli and Orlando, 1994; Velez et al., 2015). Nanotechnology has lately introduced new products that are advantageous alternatives to standard water treatment processes in order to limit the discharge of such contaminants into aquatic systems. In fact, nano-based materials may become extremely important to meet increasingly stringent water quality depuration standards, especially for the removal of persistent pollutants at low concentration levels (Adeleye et al., 2016; Tiezheng and Elimelech, 2016). Recently, efforts have been dedicated to the synthesis of multifunctional nanocomposites based on graphene (Henriques et al., 2016) and on manganese-ferrite for application in advanced waste water treatment systems (Smith and Rodrigues, 2015; Tavares et al., 2013). Graphene-based nanomaterials present high adsorption capabilities and can strongly bind chemical contaminants, which often have low or negligible affinity for traditional sorbents. One of these examples was demonstrated by the co-supervising team who produced graphene oxide functionalized with polyethyleneimine (GO-PEI), a very effective material on the sorption of Hg from real waters (Bessa et al., 2020). Also, manganese-ferrite-based nanomaterial present high adsorption capabilities for Pb and As, which often have a weak or negligible affinity for traditional sorbents. As demonstrated by Tavares et al. (2013) $MnFe_2O_4$-NPs presented high absorption capacity to As and Pb from water.

Dedicated research into new and unique environmental risk associated with nanotechnologies is still absent, despite the emergence of these new technological developments and their economic potential. Up to now several studies have described graphene-based materials technical properties and applications, but scarce information is available concerning their impacts in the environment, especially when interacting with metals (Boletim da Propriedade Industrial n.º 101/2016; Klaine et al., 2008; Velez et al., 2016a,b,c; Zhao et al., 2014). Furthermore, no information is known on how predicted climate change could alter these materials properties, toxicity and, consequently, their effects on wildlife organisms. According to recent reports, changes in seawater salinity are predicted to occur in the next 100 years (IPCC, 2013). Warmer temperatures and reduced rainfall increase seawater salinity, while extreme rainy events decrease seawater salinity, which will promote species responses (IPCC, 2013, 2019; Peteiro et al., 2018; Philippart et al., 2011) and changes on contaminants (e.g. metals) behaviour and toxicity (Carregosa et al., 2014; Chapman and Wang 2001; Freitas et al., 2019; Velez et al., 2016b). As a result, determining the effects of predicted salinity shifts in aquatic ecosystems must be a top priority in order to preserve biodiversity, but also investigating the effects of such environmental factors on the toxicity of emerging contaminants, such as newly developed nanomaterials, is critical. Benthic species are a good biological model to evaluate the impacts of environmental changes as they are sensitive to several environmental constrains. Essentially due to their life-history characteristics, as well as their relatively rapid response to pollution, several studies have been using benthic species as

bioindicators for assess anthropogenic and natural stresses (Takeda et al., 2014; Moschino et al., 2012; Velez et al., 2016a,c). Bivalves have been identified by several authors as a group of marine invertebrates that respond quickly to environmental disturbances (Jena et al., 2009; Wang et al., 2012; Morosetti et al., 2020 and more), but few studies addressed the combination of stressors such as climate change related factors and contaminants (Freitas et al., 2016,2019; Pirone et al., 2019; Piscopo et al., 2021). For a better environment protection, the ecological risk assessment of the mentioned stressors must include ecologically relevant endpoints and exposure scenarios (including predicted climate change) to drive accurate safety levels towards biodiversity conservation.

In this context, the current study used benthic marine aquatic species maintained under current and predicted climate change scenarios to investigate the possible toxic effects induced by the use of nanomaterials for seawater remediation, after long-term exposures to avoid risk underestimation, by assessing responses at different biological levels.

1.2 Metal(loid)s

Currently, the increase of pollutants in aquatic environments is closely related with the growth of the world population (Zhang et al., 2015). Several studies have demonstrated that intense urbanization and industrial activities, including mining operations and sludge dumping as well as agricultural production, have greatly contributed to pollution increase in aquatic systems, especially in marine environments (Nardi et al., 2017; Belivermiş et al., 2016; Prokić et al., 2019; Oliveira et al., 2015; Stara et al., 2020; Ventura-Lima et al., 2011). Pollutants frequently end up in lagoons and estuaries, where they tend to concentrate not just in the sediments but also in the species that live there (Capillo et al., 2018; Fattorini et al., 2008; Pagano et al., 2017; Ventura-Lima et al., 2011; Zhang et al., 2015). The present research was devoted to three metal(oid)s, Arsenic (As), Lead (Pb) and Mercury (Hg), considering their wide occurrence in aquatic systems, potential for bioaccumulation and toxicity for aquatic invertebrates and humans. According to the ATSDR 2019 these three elements are in the top three of the Substance Priority List for the most significant risk to human health.

1.2.1 Arsenic

Arsenic (As) was isolated for the first time from a compound by Albertus Magnus (Albert the Great, 1193–1280) in 1250, by heating soap together with arsenic trisulfide (Emsley, 2001). In the environment, it occurs in soil, rocks, sediments and metals ores in the form of oxyhydroxide or sulphide or compounds of various metals (Yan-Chu, 1994). It's use dates back to the 18th century as wood preservative in agriculture against fungi, bacteria and insect (Rahman et al., 2004) as well as pharmaceutical drugs, including arsphenamine and arsenic trioxide (commonly in the treatment of cancer) (Gibaud and Jaouen, 2010). After the World War I, the United States started to use As to build a chemical weapon with organoarsenic (Westing, 1972). Nevertheless, the main use of As is in alloys with lead for the manufacture of vehicles batteries as well as pesticides, herbicides and insecticides (Bissen et al., 2003; Yan-Chu, 1994).

Arsenic concentration in air, sediment and seawater detected around the world ranges between 0.004-20 ng/m^3, 0.005-0.061 µg/g and 0.5-3 µg/L, respectively (Soldi et al, 1996; González et al., 2021; Romano et al., 2013; Neff, 2002). The World Health Organization (WHO, 2010) and the United States Environmental Protection Agency (EPA) have established the As 10 µg/L level for drinking water. However, some countries, including India and Bangladesh, surpass the stipulated limit of 50 µg/L (Ahmed et al., 2004; Mukherjee et al., 2006). Furthermore, the limit of As in waste water is 1000 µg/L under directive 67/548/EEC (2008). Environmental studies identified As in bivalves, with concentrations up to 10.50 mg/Kg (Prato et al., 2019). In mussels *Mytilus galloprovincialis* and clams *Ruditapes philippinarum* concentrations ranged between 0.15-9.05 and 1.8-4.6 mg/Kg (respectively) were detected (Prato et al., 2019; Velez et al., 2015; Spada et al., 2013). However, the maximum levels of As concentration in bivalves for human consumption cannot exceed 86.0 mg/Kg as stabilised by the

EFSA (European Food Safety Authority), USFDA (U.S. Food and Drug Administration) and FSANZ (Food Standards Australia New Zealand) (Costa et al., 2020).

In humans the ingestion of As causes cardiovascular diseases and cancer, as well as influence diabetes incidence and affecting the development of the fetal nervous system (Bhattacharya et al., 2007; Prato et al., 2019). Nevertheless, changes in gene expression, DNA methylation, histone modification, and RNA interference are among the most common impacts caused by As in rat, humans and bivalves (Baccarelli and Bollati, 2009; Rodríguez-Ortega et al., 2003; Venier et al., 2006). Furthermore, chronic exposure to elevated concentration of As leads to oxidative stress and over production of free radical (ROS) with a consequent increase of cellular membrane damage as lipid peroxidation in bivalves (Freitas et al., 2018; Coppola et al., 2018a). The toxicity of As is not only related to the available concentration but it is also connected to its solubility, which is affected by pH. For example, the arsenite (AsO_3^{-3}) is more soluble than arsenate (AsO_3^{-4}) but, at a lower pH, arsenate becomes more mobile and soluble (Bhattacharya et al., 2007). A recent study showed higher metabolic and cytoskeleton proteins alterations in bivalves (oysters *Crassostrea gigas* and *C. angulata*) exposed to a combination of As and low pH than those under a single stress factor (Moreira et al., 2018). Temperature may also affect As toxicity and/or species sensitivity, with studies revealing that under warming conditions As increase the interaction with organisms, namely bivalves, and emphasis their metabolic regulation leading to the increase lipid peroxidation and oxidative stress (Coppola et al., 2018a; Sokolova and Lannig, 2008). Also, low salinity influences the interaction of As with marine organisms, as the case described by Moreira et al (2016) where oysters *C. angulata,* accumulated more As and the consequence increase their metabolic rate involved in physiological osmoregulation.

1.2.2 Lead

In the environment, Lead (Pb) is naturally found in the Earth's crust (Flora, 2009). It is a bluish-white lustrous metal and very soft, highly malleable, ductile, as well a relatively poor conductor of electricity. Also, Pb is very resistant to corrosion, but tarnishes upon exposure to air (Flora, 2009). The use of Pb started between 7000–6500 BCE (Before Common Era) in Asia Minor to produce the metallic Pb beads. At that time Pb had few (if any) applications due to its softness characteristic (Hong et al., 1994). During the Roman Empire Pb was used to manufacture tablets, water pipes, coins and even cooking utensils, as well lethal poisons (Rich, 1994). In medieval times, Pb started to be used for roofing, coffins, cisterns, tanks and gutters, and also for statues and ornaments (Rich, 1994). Only in the middle of the18th century Pb started to be used in agriculture, plumbing and painting and today it is used in car batteries, gasoline and bullets productions (Riva et al., 2012; Rich, 1994; Tolliday, 2014).

Lead concentrations in air, sediment and seawater detected around the world ranges between 0.1-9 ng/m^3, 5-0.10 µg/g, 0.002-0.3 µg/L, respectively (Angel et al., 2016; WHO, 2001,2011). For Pb, the WHO (2010) imposed the limit for drinking water of 10 µg/L, although in December 2013 drinking water limit was moved up to 25 µg/L (WHO, 2018). Moreover, the directive 67/548/EEC (2008) sets, the limit of Pb in wastewater at 1000 µg/L. Environmental studies showed that Pb concentration in bivalves may

reach 0.80 mg/Kg, with values varying between 0.21-2.48 and 0.8-0.33 mg/Kg in *M. galloprovincialis* and *R. philippinarum*, respectively (Prato et al., 2019; Velez et al., 2015; Costa et al., 2020). However, the maximum levels of Pb concentration in bivalves for human consumption cannot exceed to 2.0 mg/Kg as stabilised form EFSA (European Food Safety Authority), USFDA (U.S. Food and Drug Administration) and FSANZ (Food Standards Australia New Zealand) (Costa et al., 2020).

Although, in the human body, 1 % of Pb is stored in the bones after Pb salts absorption and the rest is excreted in the urine and fade within a few weeks of exposure (Emsley, 2011), the ingestion of Pb affects almost every organ and system body, leading to death (ATSDR, 2017). When Pb is consumed, it enters the bloodstream and binds to the sulfhydryl groups found in many enzymes, interfering with their correct action (Perez-Zúñiga et al., 2019). Bouchard et al. (2009) observed that prolonged and constant exposure to a high level of Pb, lead to adverse mental health. Also, studies showed that the ingestion of this metal generate the increase of ROS, like hydroperoxides singlet oxygen ($HO_2^{\bullet}$), and hydrogen peroxide (H_2O_2), and the antioxidant activities decrease in humans and bivalves (Coppola et al., 2020c; Flora, 2009; Flora et al., 2012; Freitas et al., 2019). In molluscs the chronic exposure to concentration of Pb leads to oxidative stress and over production of ROS with a consequent increase of cellular membrane damage as lipid peroxidation in bivalves (Aouini et al., 2018; Meng et al., 2018). For example, Zhang et al. (2010) demonstrated that in the bivalve *Chlamys farreri* exposed to Pb the antioxidant capacity was compromised, resulting in increased levels of lipid peroxidation. The toxicity of Pb it is not only related with the available concentration but is it also connected to its solubility which is affected by climatic changes. Recent studies showed greater metabolic alterations and cellular damages in bivalves (mussels *M. galloprovincialis*) exposed to a combination of Pb with temperatures or with salinity shifts (Freitas et al., 2019; Pirone et al., 2019). Khan et al. (2006) and Bat et al. (2000) demonstrated that rising temperature can increase the sensitivity of aquatic animals (crayfish *Orconectes immunis,* amphipod *Gammarus pulex pulex*) to Pb, which lead to limiting the scope of aerobic metabolism (oxygen extraction, transport and utilization), although reduce the Pb accumulation. Also, studies conducted by Freitas et al. (2019) observed an increase of metabolisms and cellular damage in mussels *M. galloprovincialis* exposed to Pb in combination with low salinity.

1.2.3 Mercury

The use of mercury (Hg) dates back to the time of the Egyptians (1500 BC) but only in 1558 its use grew exponentially due to the invention of the “patio process” to extract silver from ore using Hg (Burkholder and Johnson, 2008). This metal was used in medicine as an ingredient in dental amalgams or as a preservative in vaccines (FDA, 2006). However, it is still used as a principal component in electronic products, thermometers (for measuring high temperatures) and fluorescent lamps (Donnici et al., 2012; Briant et al., 2016; Pereira et al., 2008; Randall and Chattopadhyay, 2013). It exists in several forms, including inorganic vapor (Hg^0) and mercurous (Hg^{2+}) or mercuric (Hg^{+}) salts, as well as organic mercury (methyl, ethyl, phenyl, or similar groups) (Berlin et al., 2007). In the environment Hg is

found as a native metal (rare) or in cinnabar, metacinnabar, sphalerite, corderoite, livingstonite and other minerals, with cinnabar (HgS) being the most common ore as volcanic regions (Rytuba, 2003).

The Hg concentration in air, sediment and seawater detected around the world ranges between 0.1-9.49 ng/m^3, 0.09-1.92 µg/g, 0.1-6 µg/L, respectively (Azad et al., 2019; Portela et al., 2020; Sarasiab et al., 2004; Nguyen et al., 2007; Wan et al., 2009). According to Directive 2013/39/EU (2013), the emission limit in in the field of water (wastewater) is of 50 µg/L and for the drinking water of 0.1 µg/L (WHO, 2018). Moreover, environmental studies showed the range of Hg concentration in bivalves up to 0.072 mg/Kg, namely *M. galloprovincialis* and *R. philippinarum* around 0.03-0.20 and 0.018-0.389 mg/Kg, respectively (Prato et al., 2019; Velez et al., 2015; Costa et al., 2020). However, the maximum levels of Hg concentration in bivalves for human consumption cannot exceed to 1.0 mg/Kg as stabilised form EFSA (European Food Safety Authority), USFDA (U.S. Food and Drug Administration) and FSANZ (Food Standards Australia New Zealand) (Costa et al., 2020).

When Hg is ingested in human bodies, it is extremely toxic and has the ability to interfere with DNA transcription and protein synthesis, including protein synthesis in the developing brain, as well as the destruction of the endoplasmic reticulum and the disappearance of ribosomes (Rojas-Franco et al., 2019). Also, several studies have shown the capacity of Hg to affect biological processes, including biochemical mechanisms involved in organisms' oxidative stress status (Ahmad et al., 2011; Chen et al., 2016; Velez et al., 2015, 2016c; Coppola et al., 2017). In bivalves Chen et al. (2014) observed the increase of oxidative stress and cellular damage as well the mRNA expression alteration in bivalve *Venerupis philippinarum* exposed to Hg concentration. It is known that in sediment and water, inorganic Hg can be converted into toxic organic forms mainly by microbial mediated processes but also by climatic changes such as pH, salinity, and temperature, which is favourable in acidic pH, low salinity, high dissolved organic carbon concentrations as well as high temperatures (Celo et al., 2006). For example, Rodríguez-Romero et al. (2014) showed an increase of various trace elements (including Hg) in the clam R. philippinarum (due to low pH that increased the bioavailability of metals that were bound to sediment.

Recent studies showed increase of metabolic alterations oxidative stress and cellular damages in marine organisms including bivalves exposed to combination of Hg with temperatures (Coppola et al., 2017; 2018b; Denton and Burdon-Jones, 1981; Guinot et al., 2012; Singaram et al., 2013). For example, Morosetti et al. (2020) showed the increase of oxidative stress and cellular damage as well the decrease of metabolic capacity and in bivalve *M. galloprovincialis* exposed to Hg under warming scenario. Also, the salinity shifts combined with Hg may increase the oxidative stress and metabolic capacity as well its accumulation in the bivalves (Chin and Chen, 1993).

1.3 Nanomaterials

The term nanoparticles (NPs) refers to a broad class of materials (natural as combustion products or artificial as products of engineering to perform a specialized function) with a single dimension of less than 100 nanometres, as defined by the International Organization for Standardization and the European Commission (Laurent et al., 2010). Depending on the overall shape these materials can be 0D,1D, 2D or 3D (Tiwari et al., 2012). Size, solubility, chemical composition, shape, aggregation state, crystal structure as well as surface area, energy, charge, morphology and coating are among the physico-chemical features and attributes of each NP (Dreaden et al., 2012). For example, decreasing the size of the NPs leads to an exponential increase in surface area relative to volume, thereby making the NP surface more reactive on itself and to its contiguous milieu (Powers et al., 2007). Based on their physical-chemical characteristics it could be possible enclosed in six large categories: 1) Carbon-based NPs, include fullerenes, carbon nanotubes, graphene and its derivatives, nanodiamonds, and carbon-based quantum dots (Ibrahim, 2013); 2) Metal NPs, purely made of the metal precursors (Dreaden et al., 2012); 3) Ceramics NPs, inorganic non-metallic solids, synthesized via heat and successive cooling (Sigmund et al., 2006); 4) Semiconductor NPs, with properties between metals and nonmetals (Ali et al., 2017); 5) Polymeric NPs, characterized by a particle of solid mass with the capacity to absorb other molecules on top of their surface (Rao and Geckeler, 2011); and 6) Lipid-based NPs, with the focus to designing and synthesis of lipids (Gujrati et al., 2014; Puri et al., 2009).

The widespread usage of nanoparticles (NPs) in industrial, agricultural, and environmental sectors has resulted in a rapid increase in NPs production, which has risen to half a million tons in recent years (www.nanoproject.org; Aithal and Aithal, 2016; Freixa et al., 2018; Morozesk et al., 2018). The biological application of nanoparticles in the diagnosis and treatment of human cancers, for example, is a rapidly evolving area of nanotechnology (Yezhelyev et al., 2006). Also, several NPs (e.g. ZnO, TiO_2, Au, Ag…) have been used to produce the brightest white colour, similar to a pigment in paint (Cho et al., 2013; De Filpo et al., 2013; Su et al., 2014; Wang et al., 2009); to increase the long-term stability in plastics and paper production (Amorim et al., 2018; Capek, 2004; Frattini et al., 2005; Kaegi et al., 2008; Winkler, 2003); to enhance the high hydrophilicity and ability to block UV light in the care products like sunscreen and toothpaste (de la Calle et al., 2019; Lu et al., 2014; Johnson et al., 2011; Sureda et al., 2018; Wahie et al., 2007); and to utilize as antimicrobial activity in food packings or in medicine (Cui et al., 2016; Dani et al., 2008; Shukla et al., 2011; Xu and Han, 2004; Zhu et al., 2018).

Concerns on environmental pollution (especially related with metal(loid)s in the water) have led to the development of different water decontamination approaches, including chemical precipitation (Kevin et al., 2001; Matlock et al., 2001), membrane filtration (Aroua et al., 2007; Muthukrishnan and Guha, 2008; Pugazhenthi et al., 2005), coagulation, flocculation (Heredia and Martín, 2009; Renault et al., 2009), electrochemical methods (Ali et al., 2012; Anjum et al., 2016; Lei et al., 2015). Furthermore, NPs have been also used as nano-sorbent to decontaminate the water (Chen and Mao, 2007; Jackson et al., 2012; Paul et al., 2015; Vilela et al., 2016). For example, carbon nanotubes (CNTs), due to their high

specific surface areas and large pore volumes, have been widely used to remove metal(loid)s from water (Ihsanullah Abbas et al., 2016; Liu et al., 2013; Mauter and Elimelech, 2008; Perreault et al., 2015; Zhao et al., 2011). Tian et al. (2012), detected a remediation of 75% from water contaminated with 10 mg/L of Pb by the use of CNTs (500 mg/L). Also, Mishra and Ramaprabhu (2010) showed a remediation of 58% from water contaminated with 430 mg/L of As using 100 mg/L CNTs. Studies by Moghaddam and Pakizeh (2015) revealed an adsorption of 88% conducted by MnO_2/CNTs 0.02 g in water containing 20 mg/L of Hg. Among innovative NPs, spinel manganese-ferrite ($MnFe_2O_4$) and GO-functionalized with polyethyleneimine (PEI) (GO-PEI), are among the most promising nanomaterials, that showed a high efficiency (around 98%) of decrease in inorganic pollutant content (including metal(loids)s) in freshwater and seawater (Bessa et al, 2020; Henriques et al., 2016; Jang et al., 2016; Mohan and Pittman, 2007; Mishra and Ramaprabhu, 2011; Tavares et al., 2013). Moreover, Meidanchi and Akhavan (2014) reported the magnetic separation application of superparamagnetic $ZnFe_2O_4$/reduced graphene oxide (GO) composites by hydrothermal reaction method to decontaminate the wastewaters. Geng et al. (2012) showed as depositing magnetic Fe_3O_4 nanoparticles on GO sheets can allow an easy separation of dye-adsorbed composites by applying an external magnetic field. Also, Guo et al. (2014) showed the huge adsorption capacity for removing organic pollutants from water by GO-PEI hydrogels. Lei et al. (2012), observed the efficiency of GO foam to absorb heavy metals (e.g. Pb, zinc and cadmium) from the water.

Due to the huge industrial production of carbon nanotubes (CNTs), which has been considered one of the most promising nanomaterials, most recent studies showed, the predicted environmental concentrations (PECs) of CNTs were projected to be around 0.05–5 mg/kg in biosolids (Keller and Lazareva, 2014) and 0.001–1000 µg/L in aquatic environment (Keller and Lazareva, 2014; Zhang et al., 2017). Studies conducted by Handy et al. (2012) and He et al. (2014) at these CNTs concentrations, have shown toxic effects in exposed organisms depending on their physical and chemical characteristics (size, shape, surface area), diffusion capacity, aggregation/ agglomeration properties in suspension, functionalization and interactions with surrounding environment. Available literature already demonstrated the interaction and the toxicity between CNTs and cells (including in marine organisms) which led to increase of oxidative stress and cellular damage as well as cell apoptosis (De Marchi et al., 2018, 2019a; Zhao and Liu, 2012). Nevertheless, CNTs toxicity may also result from the interaction with other contaminants that influence bioaccumulation of each co-exposed contaminant and the induced effects (Sun et al., 2009). Moreover, the effects of CNTs in aquatic organisms depend on their ability to interact and aggregate, which is influenced by water properties such as the pH, temperature and salinity shifts (De Marchi et al., 2018, 2019a,b). De Marchi et al. (2018, 2020) observed the increase of metabolic impairment and oxidative stress in clams *R. decussatus* when exposed to combination of CNTs and salinity shifts even under warming scenario.

Nanomaterials based on graphene, in particular, are in the first class for industrial usage because of their many unique features, including mechanical stiffness, strength, and elasticity, as well as high electrical and thermal conductivity (Ferrari et al., 2015; Novoselov et al., 2012).

Studies conducted by De Marchi et al (2017a) and Khan et al. (2019) showed cellular damage marker as well as increase of oxidative stress when marine organisms where exposed to different concentration (0.10, 1.0, 2.5 and 5 mg/L) and time (72h and 28 days) of GO. Furthermore, studies demonstrated that also the use of magnetic NPs to decontaminate the water are growing, and they could be toxic for the marine organisms as observed by Labiadh et al. (2017) where the induction of oxidative stress and cellular damage in clams *R. decussatus* were inducted after exposure to 100 µg/L of NPs-ZnS:Mn (20%).

GO-PEI and $MnFe_2O_4$-NPs, two of the nanomaterials investigated by our research team, revealed a huge capacity to remove Hg, Pb, and As from seawater. Despite the excellent removal effectiveness, no other research to our knowledge (particularly for coastal aquatic systems) has shown the environmental concerns arising from GO-PEI and $MnFe_2O_4$ NPs or resulting from remediation of saltwater previously contaminated following the application of these materials.

1.4 Climatic change

The significant increase of population and industrialization has been identified as a catalyst for Climate Change (CA), with coastal areas among the most vulnerable marine ecosystems, and it is therefore expected that species found in these habitats are increasingly under threat from these changes. The most important CA-related factors that can affect the biological performance of aquatic organisms include seawater acidification, salinity changes and temperature rise. Recent studies indicate that the increase of temperature and the mean sea level, with the consequent salinization of transitional environments, may be the factors that most affect these ecosystems (Breitberg et al., 2015). The ocean plays key roles, namely in the supply of food, the hydrological cycle, renewable energy, cultural values, trade and transport. For these reasons, approximately 40% of the world's population lives within 100 km of the coast. More than 600 million people currently live-in coastal areas, a number that is expected to exceed 1 billion by 2050. It is also in this region that transitional environments such as estuaries are included. Due to their location and characteristics, these areas are especially vulnerable to CA, particularly with regard to changes induced in the structure and functioning of the ecosystem and loss of biodiversity (Chefaoui et al., 2018; Rilov, 2016, 2019).

Among the main threats to these regions are warming, rising mean sea levels, extreme weather events, increased nutrient and organic matter loads, and acidification. Warming and rising mean sea level have as a direct consequence an increase in seawater intrusion and increased salinity in estuaries, which can be exacerbated by periods of drought and changes in drainage areas due to human activities, with obvious risks to biodiversity and the functioning of these ecosystems (Elliott et al., 2019; Hallett et al., 2018; Marques et al., 2017; Zahid et al., 2018). Among other consequences, changes in salinity in estuaries have been associated with the upstream expansion of brackish and marine benthic and pelagic communities, and a reduction in the diversity and abundance of freshwater fauna (Addino et al., 2019; Hallett et al., 2018; Raimonet and Cloern, 2016; Robins et al., 2016). However, since the distribution of benthic species in estuaries is strongly conditioned by sediment properties (namely grain size), changes in distribution patterns may be strongly conditioned in estuaries, leading to a decrease in species richness (Addino et al., 2019; Hallett et al., 2018; Little et al., 2017).

Another problem associated with these regions is related to the intensive human activities that take place there, responsible for the substantial increase in the amount of organic matter and nutrients in these systems (Maavara et al., 2017). It is known that the interaction of organic matter with heating results in the intensification of bacterial degradation and eutrophication (Maavara et al., 2017; Chen et al., 2018), contributing to the increase in the frequency and extension of hypoxic zones (Breitberg et al., 2015; Gobler and Baumann, 2016). The interaction between heating, increased nutrient load and hypoxia has been shown to be related to increased occurrences of toxic algal blooms (Anderson et al. 2015; Paerl et al. 2016), pathogenic bacteria (Baker-Austin et al., 2017) and mortalities of invertebrates and fish communities (Jeppesen et al., 2018). Hypoxia and acidification conditions can also alter the

sensitivity of organisms to other sources of stress, such as increased temperature and contamination (Mackenzie et al., 2014; Rosas-Navarro et al., 2016; Pörtner et al., 2017).

The increase of temperature and amount of organic matter are also associated with changes in the behaviour and bioavailability of pollutants, changing the toxicity patterns of organic and inorganic substances (Freitas et al., 2016, 2019; Maulvault et al., 2017; Nardi et al., 2017; Pirone et al., 2019). Recent studies also show that the increase in the average temperature in transition systems and the greater exposure to periods of dissection due to prolonged exposure during emergence (especially during extreme drought events) are responsible for physiological changes in intertidal organisms, further enhancing a greater accumulation of pollutants (Andrade et al., 2019). Since the beginning of 20th century, the atmospheric concentration of carbon dioxide (CO_2) has been increasing due to the industrial revolution (IPCC, 2014). Moreover, studies conducted by Pörtner et al. (2014) hypothesized that the concentration in the atmosphere will reach up to ≅1000 ppm until the end of this century unless CO_2 emissions are reduced. Almost 30% of the atmospheric CO_2 is absorbed by the oceans, leading to seawater chemical changes, including a decrease of seawater's pH level (IPCC, 2014).

1.4.1 Increase of temperature

Several studies demonstrated as the seawater temperature is rising in oceans and especially in marine coastal systems (Boyer et al., 2005; Bindoff et al., 2007; Fogarty et al., 2008; Levitus et al.,2009). In particular, a study conducted by Collins et al. (2013) predicted that global ocean warming will increase between 0.5 °C (RCP2.6) and 1.5 °C (RCP8.5) a depth of about 1 km by the end of the century due to the human activity. However, lagoons and estuaries are more susceptible to air temperature's influence because of their restricted heat exchange with open waters (Lloret et al., 2008; Newton et al., 2018; Pan and Wang, 2011) as demonstrated by Jakimavičius et al. (2018), who predicted a temperature rise up to 6 ºC by the year 2100 in the Curonian lagoon. However, during the United Nations Conference on Climate Change, held in Paris, France, in 2015, was assumed to keep temperature rise below 2 °C, preferably 1.5 °C, above pre-industrial levels as a goal regarding global warming (UNFCCC, 2015).
Temperature changes have been shown to be responsible for alterations in the geographic distribution of marine invertebrates, mainly influencing larval development and dispersion, with consequences for the life cycle, establishment and expansion (David and Simon, 2014; Twiname et al. al., 2019). Warming, high frequency and duration of extreme temperatures may also be responsible for changes in the structure and functioning of coastal communities, namely in terms of declining total abundance and species richness, with negative impacts on the structure of the food chain (Anacleto et al., 2014; Grilo et al., 2011; Holbrook et al., 2019; Munari, 2011; Rubio-Portillo et al., 2016). At the organism level, the available information shows that the increase in seawater temperature is responsible for metabolic, physiological and biochemical changes in marine and estuarine invertebrate species with high ecological and/or economic importance, with implications namely in their rates of growth and reproduction (Madeira et al., 2018; Mackenzie et al., 2014; Velez et al., 2017). The increase in

temperature may also be associated with changes in the behaviour and toxicity of pollutants, as well as with a greater sensitivity of organisms to different substances, and there are already studies showing synergistic effects in organisms simultaneously exposed to pollutants and increased temperature (Andrade et al., 2019; Coppola et al., 2018a,b; Freitas et al., 2019; Maulvault et al., 2017; Nardi et al., 2017; Pirone et al., 2019).

1.4.2 Salinity shift

Extreme waters events in the environment could carry out to sea-level rise and are associated with climate change, which is leading to serious consequences on marine biodiversity (IPCC, 2018,2019; Gissi et al., 2020). The importance of salinity shifts is not to be underestimated because salinity regulates the equilibrium, especially in estuaries and coastal areas where it influences water density, circulation and stratification, pH and organic matter solubility (Cai and Wang, 1998; Johnson et al. 2012). A study conducted by Pörtner et al. (2014) has observed as the heavy rainy or long drought periods will increase in frequency and intensity at the end of the century, leading to alterations in seawater characteristics, namely in terms of salinity. Recent studies also revealed that the frequency and intensity of extreme weather events, such as intense rains and prolonged droughts, are increasing, which in transitional environments can act as a disruptive agent (Donnici et al., 2012; Cardoso et al., 2008; Grilo et al., 2011; IPCC, 2018, 2019; IPMA, 2017). These events, associated with rising sea levels, lead to changes in the salinity of coastal systems, with implications for ecosystem processes and services, including changes in the structure and functioning of marine and estuarine communities (Cardoso et al., 2008; Grilo et al., 2011; Muresan et al., 2020; Smyth and Elliott, 2016; Velasco et al., 2019). Salinity changes can also influence the metabolic, physiological and biochemical patterns of several species, also altering the performance of organisms, particularly when exposed to multiple stresses (Carregosa et al., 2014; Freitas et al., 2020, 2019; Moreira et al., 2018; Paital and Chainy, 2010; 2012). Also, the salinity variation caused impact in growth performance, reproduction and immune functions of organisms inhabiting this wildlife (Hauton, 2016). Furthermore, changes in salinity can influence the behavior of pollutants and consequently increase their toxicity (Rodrigues et al., 2014; De Marchi et al., 2020, 2018; Freitas et al., 2020).

1.5 Marine species as bioindicators

Often the costal aquatic systems are the final destination of pollutants where organisms namely bivalves are constantly exposed to, due to their sedentary filter-feeder characteristics (Capillo et al., 2018; Fattorini et al., 2008; Manzo et al., 2017; Pagano et al., 2017; Schiavo et al., 2018). Moreover, these contaminants, associated with climatic change, have already demonstrated harmful effects in inhabiting organisms (La Rosa et al., 2012; Pörtner et al., 2007; Boukadida et al., 2016, among others).
Among the organisms inhabiting coastal systems, bivalves are commonly used in ecotoxicological studies as bioindicator species to assess and monitor substances contamination due to their bioaccumulation capacity, ecological role, wide distribution and high abundance in several aquatic systems as well as for their huge economic value (Ahmad et al., 2011; Coppola et al., 2018a,b; Giani et al., 2012; Kristan et al., 2014; Kucuksezgin et al., 2010; Marques et al., 2018; Livingstone et al., 2000; Velez et al., 2015).

1.5.1 Mytilus galloprovincialis

Mytilus galloprovincialis (Lamarck, 1819) (the Mediterranean mussel) is a specie of bivalve, a marine mollusc in the family Mytilidae (Figure 1). The dimension of this species is greatly influenced by its biotope: intertidal shells often remain small, rarely exceeding 6 cm, while deep-water shells easily measure 9 cm. Maximum 15 cm; common 5-8 cm. Natural and cultivated (*http://www.fao.org/fishery/species*). It is native from the Mediterranean coast but has been introduced to various regions around the world through ship hull fouling and transport of ballast water (Branch and Stefanni 2004). As invasive species, *M. galloprovincialis* is able to compete and replace native mussels, becoming the dominant species due to its characteristics as: faster growth than native mussels, greater

Figure 1- Mussels *Mytilus galloprovincialis*

tolerant to air exposure, and a reproductive output of between 20% and 200% greater than that of indigenous species (Griffiths et al., 2003; Savini et al., 2010; Branch and Stefanni, 2004).

Frequently, this species is selected as a model to studies, due to its strong dispersal potential to typify patterns of genetic structure expected within scenarios of high connectivity as well as their fast reproduction. A study conducted by Da Ros et al. (1985) showed (through the histological analysis of gonads) that gametes of the mussels *M. galloprovincialis* are ripe from September to May, when several spawning events occur with a peak at the end of winter (January - February). The larvae are resistant and have long life (more than one month) (Miller et al., 2018; Lane et al., 1985). Furthermore, this species has economic importance because it is on the base of food chain and largely utilized in aquaculture (where production is based on natural recruitment) (Astorga, 2014). It is cultivated around the world especially in Albania, Bulgaria, Croatia, Egypt, France, Greece, Italy, Morocco, Portugal (Merdzhanova et al., 2016). The consumption of *M. galloprovincialis* in 2018-2019 was balanced around 684.613 tonnes (EU supply balance, 2019).

1.5.2 Ruditapes philippinarum

Ruditapes philippinarum (Adams and Reeve, 1850) is an edible species of saltwater clam in the family Veneridae, the Venus clams (Figure 2). The common names for this species including Manila clam and

Figure 2- Clams *Ruditapes philippinarum*

Japanese clam (Milan et al., 2011; Talley et al., 2015). This spicies is elongate, oval, and sculptured with radiating ribs. It is generally 40 to 57 mm wide, with a maximum width of 79 mm. The shell is quite variable in colour and patterning, being cream-colored to grey with concentric lines or patches. (Hickman et al., 2001). Collected for the first time in 1850 in the Philippines (Goulletquer, 1997), clam *R. philippinarum* is native to sub-tropical and temperate coastal seas as of the western Pacific from the south China Sea (FIGIS, 2004; Qi, 1998). During the 20th century the successful economic of this

species in Asia lead to considerably distribution around the word especially in the Mediterranean Sea (Italy, France, Sardinia, Romania) (Qi, 1998).

The Manila clam reproductive activityis correlated to temperature shifts which is, in turn, related to food supply. Studies from different part of the world highlight that the lower temperature limits the gonads activity, gamete ripening and spawning to be 8, 12 and 14 °C, respectively (Ohba, 1959; Holland and Chew, 1974; Mann, 1979; Xie and Burnell, 1994; Drummond et al., 2006). The first introductions in Europe dates back to 1972–1974 in Arcachon Bay, France by IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer). Then, this species was identified into UK coastal waters in 1983 (Humphreys et al., 2015) and Northern Adriatic Sea in the Venice lagoon (Breber, 2002). Subsequently, was imported in Portugal in 1984 from Ria Formosa (Algarve) probably originated from Spain (Ruano and Sobral, 2000). Although in Europe, these clams represent an invasive species, existing huge demand and spat availability creates an informal and often illegal component of the industry lead to not disturb the native species (Breber, 2002; Pranovi et al., 2006) As a consequence of its economic potential, its faster growing and its higher fecundity than the native species (namely *R. decussatus*), *R. philippinarum* is selected as a model to studies as environmental bioindicator (Breda et al., 2018, Chiesa et al., 2018, Costa et al., 2013; Martín-Díaz et al., 2007; Beninger and Lucas, 1984; Laing,1993). In 2014 Manila clam culture represented 25% of global mollusc production with 31651 tonnes only in Europe (all data from FAO, year 2014).

1.6 Ecotoxicology biomarkers

Normally, biochemical markers (including the ones related with organisms oxidative and neurotoxic status, metabolic capacity and energy reserves content) and histopathological alterations are used to assess changes induced both by pollutants and climate change associated factors in bivalves (Belivermiş et al., 2016; Boukadida et al., 2016; Coppola et al., 2021a; Freitas et al., 2016, 2019; Leite et al., 2020; Moreira et al., 2016, 2018; Morosetti et al., 2020; Nardi et al., 2017; Pirone et al., 2019; Sheehan and McDonagh, 2008).

1.6.1 Biochemical markers

1.6.1.1 Oxidative stress

The anthropogenic factors lead to a stress situation followed by a cascade of biological responses, which can be detect by biomarkers (McCarthy et al., 1991). In 1993 the International Programme on Chemical Safety, led by the World Health Organization (WHO), in coordination with the United Nations and the International Labor Organization, has defined a biomarker as “any substance, structure, or process that can be measured in the body or its products and influence or predict the incidence of outcome or disease” (WHO, 1993). Moreover, in 1998, the National Institutes of Health Biomarkers Definitions Working Group defined a biomarker as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.” (Atkinson et al., 2001). In the last decade, the definition has been broadened to include biological characteristics that can be objectively measured and evaluated as an indicator of normal biological and pathogenic processes (Naylor, 2003). In practice, biomarkers include tools and technologies that can aid in understanding the prediction, cause, diagnosis, progression, regression, or outcome of a treatment (McCarthy et al., 1991, Naylor, 2003). For this, the effects of environmental stress in bivalves are centred on those responses that can potentially lead these organisms to an oxidative stress condition, due to an increase of reactive oxygen species (ROS) generation (McCarthy et al., 1991). Among the ROS, the hydroxyl radical (•OH), the superoxide anion radical ($O_2^{\bullet-}$) and hydrogen peroxide (H_2O_2) are particularly transient species due to their high chemical reactivity and capability to react with DNA, proteins, carbohydrates and lipids in a destructive manner (Regoli and Giuliani, 2014). As a consequence, the biological systems have developed during their evolution adequate enzymatic and nonenzymatic antioxidant mechanisms to regulate their redox homeostasis (Valavanidis et al., 2006). Superoxide dismutase (SOD), catalases (CAT) and glutathione peroxidases (GPx) are the main enzymes involved in the defence against ROS (Regoli and Giuliani, 2014). The SOD activity catalyzes the dismutation of the highly reactive superoxide anion ($O2^{\bullet-}$) to hydrogen peroxide (H2O2) and molecular oxygen (O2) (Abreu and Cabelli, 2010). Then, CAT catalyzes the reduction of H2O2 using another H2O2 molecule (or instead alcohols, phenols, acids or formaldehydes) as an electron donor (Deisseroth and Dounce, 1970). Also (simultaneously with CAT),

the GPx activity catalyzes the reduction of H2O2 or organic hydroperoxides to water or corresponding alcohols using reduced glutathione (GSH) as an electron donor. Glutathione-S transferase (GST) is a family of proteins catalysing the conjugation of GSH with a wide variety of electrophilic substrates. These enzymes also play an essential role in protection against peroxidation, catalyzing the reduction of hydroperoxides by GSH (Prohaska, 1980; Sherratt and Hayes, 2001).

1.6.1.2 Cellular damage

Although the antioxidant enzymes try to cope as the compensatory response of cellular defense systems against oxidative stress, the interactive effects of anthropogenic factors can significantly increase the oxidative damage (in terms of lipid peroxidation) in the organisms (Regoli and Giuliani, 2014). In fact, the increase of ROS attack membrane lipids, following by an autocatalytic oxidation process known as lipid peroxidation (LPO). During this process, lipids are oxidized generating lipid hydroperoxides (LOOH). The increase in lipid hydroperoxidesdisrupts the normal cellular metabolism, triggering adaptive responses and / or causing cell death (Regoli and Giuliani, 2014). Furthemore, oxidative stress can lead to an increase in protein carbonylation (PC) with aggregation, polymerization, unfolding, or conformational change consequences, which may cause the loss of structural or functional activity due to the oxidation of protein (Rodríguez-García et al., 2020).

1.6.1.3 Metabolism and energy reserves

The metabolism plays a key role in organism's survival and function, as well as in stress adaptation and tolerance. The organisms' energy balance can be affected by environmental stress and the additional energy needed to recover and maintain homeostasis that can put strains on the systems involved in energy acquisition, conversion and conservation (Sokolova et al., 2008). For this, carbohydrates, such as glycogen (GLY), are used also as major substances that give the primary energy sources converted into adenosine triphosphate (ATP). The first ATP production occurs in the cytoplasm, then this process takes place in the mitochondria of the cell (Falfushynska et al., 2020). In fact, these energy reserves produce the molecule acetyl coenzyme-A (acetyl-CoA) that enters in the Krebs cycle, also known as TCA (tricarboxylic acid) cycle or the CAC (citric acid cycle) which reduces, nicotinamide adenine dinucleotide and flavin adenine dinucleotide (NAD^+ and FAD^+), respectively to NADH and $FADH_2$ which have the reducing power to generate ATP and also $O_2^{\bullet-}$ in the electron transport system (ETS) located in the inner membrane of the mitochondria (Nolfi-Donegan et al., 2020). Moreover, reduced NADPH and $FADH_2$, represents a universal electron donor, not only to drive biosynthetic pathways which can be used as building blocks for a large number of important processes, including the synthesis of fatty acids. (Falfushynska et al., 2020). Furthermore, the glycogen (GLY) and total protein (PROT) contents are an energy conserved system of antioxidant defence that facilitates the neutralization of oxidizing substances in non-toxic hydroxycarboxylic acids (Lima et al., 2007). This process is carried out by gluthatione reductase (GRed), 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 (Maria and Bebianno, 2011; Regoli and Giuliani, 2014). The GRed enzyme plays an essential role in preventing oxidative stress and maintaining cellular redox homoeostasis. In fact, its activity catalyzes the NADPH-dependent reduction of oxidized glutathione (GSSG) (Regoli and Giuliani, 2014).

1.6.1.4 Neurotoxicity

Among the biomarkers evaluated to date, there is a lot of interest in cholinesterase (ChE) activity as an indicator of the biological effects of exposure to neurotoxic compounds in aquatic organisms. The ChE is a family of enzymes that includes the acetylcholinesterase (AChE), which plays an important role in the neurotransmission system (Karami-Mohajeri and Abdollahi, 2011). The *AChE* is mainly found at neuromuscular junctions and in chemical synapses of the cholinergic type, where its activity serves to terminate synaptic transmission. It represents the most sensitive marker of cholinergic impairment due to its responsibility for the breakdown of acetylcholine released into the synaptic cleft during the transmission of an impulse across a cholinergic synapse (Radić and Taylor, 2001). Focusing on AChE, the inhibitors or anti-cholinesterases inhibit the cholinesterase enzyme from breaking down ACh, increasing both the level and duration of the neurotransmitter action. According to the mode of action, AChE inhibitors can be divided into two groups: irreversible and reversible. Reversible inhibitors, competitive or non-competitive, mostly have therapeutic applications, while toxic effects are associated with irreversible AChE activity modulators (Colovic et al., 2013). The mechanism of the inhibition of AChE, may be responsible for terminating the transmission of the nerve impulse in bivalves especially their gills, the primary uptake route of waterborne pollutants (Almeida and Vasconcelos, 2015). Thus, AChE activity is extensively used as a biomarker of exposure to neurotoxic agents, such as organophosphorus and pesticides, including chlorothalonil in marine organisms (Park et al., 2016).

1.6.2 Histopathological alterations

Nowadays, several studies have been demonstrated the potential tools of histology in bivalves to biomonitoring marine ecosystems and assess health status in aquatic organisms, like bivalves, especially in association to other techniques, (i.e. biomarkers analysis) (Bignell et al., 2008, 2011; Coppola et al., 2020a,b,c; Costa et al., 2013 and more). Furthermore, in United States, Canada, Union Kingdom and Europe the bivalves' histopathology took part in permanent programs as the Clean Seas Environment Monitoring Programme or the Mediterranean (Lyons et al., 2011). Histopathology (of aquatic organisms) incorporates measures of the reproductive and metabolic condition and allows the detection of a range of pathogens that may affect tissue structure and mortality. The digestive gland of molluscs is a main target organ in toxicological studies due to its involvement in metabolic and homeostatic regulation, mechanisms of immune defence regulation, together with processes of detoxification of xenobiotic compounds (Bignell et al., 2011; Cuevas et al., 2015). Moreover, the gills are organs of peculiar interest because they represent the first physical barrier encountered by

pathogens as well as exogenous compounds and are particularly subjected to the accumulation of xenobiotics being involved in the respiration and feeding processes (Pagano et al., 2017; Pirrone et al., 2018). In particular, alterations in tissue morphology, such as atrophy, necrosis (in digestive tubules), cilia lost and enlargement of the central vessel (in gills), haemocyte infiltration and lipofuscin accumulation can be signs of stressful conditions and be related to chronic exposure to a contaminant, both in field and laboratory (Cuevas et al., 2015; Costa et al., 2013; Pinto et al., 2019). For example, haemocyte infiltration, represents an important biomarker of inflammatory response in bivalves, is often observed in specimens exposed to xenobiotics as a defence reaction to cellular damage induced by multiple environmental contaminants (Pagano et al., 2017). Additionally, the presence of lipofuscin like pigments, often related to age, is correlated to lipid peroxidation (Cuevas et al., 2015). In gills the inflammatory response is characterized also by enlargement of the central vessel (Sunila, 1988). The observation of these tissues permits to calculate histopathological indices that integrate different histopathological alterations of the target organs into a single value in order to facilitate the interpretation of the health status of bivalves and to standardize the results (Costa et al., 2013).

CHAPTER 2.

How efficient is graphene-based nanocomposite to absorb Hg from seawater. Assessment of the toxicological impacts induced by remedied water towards marine bivalves.

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Abstract

Advanced investigations on the use of graphene based nanomaterials have highlighted the capacity of these materials for wastewater treatment. Research on this topic revealed the efficiency of the nanocomposite synthetized by graphene oxide functionalized with polyethyleneimine (GO-PEI) to adsorb mercury (Hg) from contaminated seawater. However, information on the environmental risks associated with these approaches are still lacking. The focus of this study was to evaluate the effects of Hg in contaminated seawater and seawater remediated by GO-PEI, using the species *Ruditapes philippinarum*, maintained at two different warming scenarios: control (17 ºC) and increased (22 ºC) temperatures. The results obtained showed that organisms exposed to non-contaminated and remediated seawaters at control temperature presented similar biological patterns, with no considerable differences expressed in terms of biochemical and histopathological alterations. Moreover, the present findings revealed increased toxicological effects in clams under remediated seawater at 22 ºC in comparison to those subjected to the equivalent treatment at 17 ºC. These results confirm the capability of GO-PEI to adsorb Hg from water with no noticeable toxic effects, although temperature could alter the responses of mussels to remediated seawater. These materials seem to be a promise eco-friendly approach to remediate wastewater, with low toxicity evidenced by remediated seawater and high regenerative capacity of this nanomaterial, keeping its high removal performance after successive sorption-desorption cycles.

Keywords

Biomarkers, histopathological index, *Ruditapes philippinarum,* decontamination, mercury, graphene-based nanocomposite.

2.1 Introduction

Coastal marine ecosystems have been influenced by a vast variety of natural and anthropogenic substances such as metal(loid)s (Izagirre et al., 2014; Lamborg et al., 2014; Maulvault et al., 2017; Klaver et al., 2014; Schaller et al., 2011). Increased concentrations of these pollutants it is also associated with world population growth, especially around coastal areas, mainly resulting from industrial and agricultural activities (Fattorini et al., 2008; Marques et al.,2017; Nardi et al., 2018; Randall and Chattopadhyay, 2013; Pereira et al., 2008). Anthropogenic sources of these pollutants include alloy and batteries production, coating, explosive manufacturing, pesticides and phosphate fertilizers (Ayangbenro and Babalola,2017). As a result, several studies already reported high metal(-loid)s concentrations in marine and estuarine systems worldwide, in water, sediments and inhabiting organisms (Bakary et al., 2015; Randall and Chattopadhyay, 2013; Tchounwou et al., 2012). Amongst the most toxic elements in aquatic systems, mercury (Hg) has been recognized as one of the most highly dangerous substances (ATSDR, 2019), a situation that may continue due to its use in electronic products and fluorescent lamps (among others, Donnici et al., 2012; Briant et al., 2016). The booming of nanotechnology and outstanding advances in research concerning graphene-based nanomaterials have provided great promise for wastewater treatment (Mokhtar et al., 2019; Nupearachchi et al., 2017; Stobel et al., 2019; Zhang et al., 2010; Zhou et al., 2015). Recently, several methods have been tested as an attempt to remove metals, including Hg, from contaminated water, such as chemical precipitation (Henke et al., 2001; Matlock et al.,2001), ultra and nano-filtration as well reverse osmosis (Aroua et al., 2007; Muthukrishnan and Guha, 2008; Pugazhenthi et al., 2005) and the use of nanomaterials as sorbents (Ali et al., 2012,2019; Anjum et al., 2016; Babel and Kurniawan, 2003; Huang et al.,2015; Li et al., 2010). Among these techniques, Bessa et al. (2020) studied a low-cost nanocomposite, developed with graphene oxide (GO) combined with polyethyleneimine (PEI) that efficiently removed Hg from water (93% from contaminated seawater with 50 µg/L of Hg). Still, scarce information exists regarding the possible toxicity associated with remediated water obtained from such approaches, especially considering predicted climate change scenarios (Coppola et al., 2019, (2020a,b); Falinski et al., 2020). Besides pollutants, aquatic systems have been also subjected to climate changes, with the intergovernmental panel on climate change (IPCC, 2018) highlighting the increase of global temperature as an imminent climatic problem derived from anthropogenic activities. It was estimated that human activities resulted into a global atmospheric warming close to 1.0 ºC above pre-industrial levels, which may reach 1.5 ºC between 2030 and 2052 (IPCC, 2018; IPCC, 2019). Although at a slower rate, also seawater temperature is rising in oceans and especially in marine coastal systems (Boyer et al.,2005; Bindoff et al., 2007; Fogarty et al., 2008; Levitus et al., 2009). However, differently from open ocean (with a thermal inertia due to its high heat capacity of water and high total volume), shallow water bodies like coastal areas, lagoons and estuaries are more susceptible to air temperature's influence because of their restricted heat exchange with open waters (Lloret et al., 2008; Newton et al., 2018; Pan and Wang, 2011). Jakimavicius et al. (2018) predicted a temperature rise up to 6 ºC by the year 2100 in the Curonian lagoon. Associated with warming of coastal areas several authors have already demonstrated harmful effects in inhabiting organisms (Rosa et al., 2012; Pörtner and Knust, 2007;

Boukadida et al., 2016, among others). As an example, Jiang et al. (2016) highlighted the increase of seawater temperature from 15 to 20 ºC could affect negatively the physiological as well as the biochemical behaviour of the yesso scallop species, *Patinopecten yessoensis*. Based on their sessile nature, filter-feeding habit, high tolerance, and tendency to bioconcentrate pollutants, high distribution and economic importance in Europe as well as in the oriental continent (namely China), *Ruditapes philippinarum* is considered a good sentinel for monitoring marine pollution and climate changes (Bebianno et al., 2004; Freitas et al., 2018; Ji et al., 2006; Jiang et al.,2019; Yang et al., 2013; Velez et al., 2015). The present study aimed to understand the possible impacts derived from Hg remediated seawater using GO-PEI, under control temperature (17 ºC) and predicted warming scenario (22 ºC), in the species *R. philippinarum*, collected from the Red Island, a non-contaminated area in the Yellow Sea (Qingdao, China). Clams were subjected, during 28 days, to five treatments: 1) non-contaminated seawater (CTL); 2) seawater with nanocomposite (graphene oxide combined with polyethyleneimine, GO-PEI); 3) seawater contaminated with mercury (Hg); 4) seawater with mixture of nanocomposite and Hg (GOPEI + Hg) and 5) remediate seawater (RSW). Each treatment was tested under both temperatures. After exposure, clams Hg accumulation levels, histopathological alterations and biochemical responses were evaluated.

2.2 Materials and methods

2.2.1 Laboratory conditions and experimental setup

Clams were collected in the Red Island (Yellow Sea, Qingdao, China), considered as a non-contaminated area (Yang et al., 2013; Jiang et al., 2019), in October 2019. Specimens with similar size were selected: length of 29.2 ± 1.9 mm, width of 21.2 ± 1.5 mm and height of 13.8 ± 1.0 mm. In the laboratory, clams were placed in a 100 L glass aquarium with sand and seawater from the sampling site (salinity 30 ± 1; pH 8.0 ± 0.1; temperature 17.0 ± 1 ºC; dissolved oxygen 7.7 ± 0.2 mg/L) with constant aeration for two weeks. During the first week all organisms were maintained under the same temperature 17 ºC (depuration period). In the second week (acclimation period), half of the clams was kept at 17 ± 1 ºC (control temperature) and another half was subjected to a gradual temperature increase up to 22 ± 1 ºC (warming scenario). During depuration and acclimation periods both groups were fed with a solution of algae (1 g of Algamac protein plus powder per 1.5 L of distilled water) every 3-4 days. The control temperature (17 ºC) was selected based on the mean values recorded along the year in the sampling area (Wang, 2014). The warming scenario (22 ºC) was selected considering predicted increased temperatures in coastal systems (IPCC, 2019; Sun et al.,2011; Zhu et al., 1991). The exposure assay (28 days) was carried out for the two groups (17 and 22 ºC) at the same salinity and pH conditions (salinity 30 and pH 8.0). Organisms at the two temperatures were subjected to five treatments as described in Table 1: control (CTL); GO-PEI; Hg; GO-PEI + Hg; RSW. Per treatment three glass aquaria of 3 L were used, with 12 clams in each replicate. The remediated seawater was obtained after a treatment of 24 h where the seawater was previously contaminated with 50 µg/L of Hg stock solution (Hg 1000 mg/L in 1 mol/L HNO_3, 99.9999% trace metals basis) and remediated with GO-PEI as referred in (Coppola et al. (2020a,b)). The Hg concentration of 50 µg/L used in this study was selected taking into consideration that this is the maximum allowable limit in wastewater discharges from industry, in the European Union (Directive, 2013/39/EU, 2013). The GO-PEI concentration (10 mg/L) was selected according to the capacity of this material to remove Hg (Bessa et al., 2020). During the experiment clams' mortality was checked and one day per week seawater from each aquarium was renewed with the re-establishment of all initial conditions. To feed the animals a commercial Algamac protein plus solution was prepared and 1 mL/L was added to each aquarium three times per week. Before spiking, water samples were collected from all aquaria to assess Hg background levels in seawater medium. To determine the real concentrations of Hg in water medium, every week water samples were collected (from control and contaminated treatments) immediately after water renewal and spiking. At the 28th day, organisms from each aquarium were sacrificed thought the separation of the shell from soft tissue. Three clams per treatment were used for the histological measurements (one from each aquarium); gills and digestive tubules were vivisected and immediately fixed in Davidson's fluid for 24 h at room temperature. The remaining clams were frozen with liquid nitrogen and maintained at 80 ºC. From each clam, soft tissues were manually homogenized (using pestle and mortar) and

separated in five different microtubes with aliquots of 0.3 g fresh weight (FW). Four of them were used for biochemical analyses and the remaining one was lyophilized during 1 week for the Hg quantification.

Table 1. Treatments evaluated (conditions in aquariums).

TREATMENTS	DESCRIPTION
CTL	Hg 0 µg/L + GO-PEI 0 mg/L
GO-PEI	GO-PEI 10 mg/L
Hg	Hg 50 µg L
GO-PEI + Hg	GO-PEI 10 mg/L + Hg 50 µg/L
Remediated seawater (RSW)	Seawater remediated with GO-PEI (10 mg/L) for 24 h after Hg contamination (50 µg/L)

2.2.2 Mercury quantification

The Hg quantification in water samples and clams' tissues were conducted by the Societe Generale de Surveillance - China Standard Technology Development Corporation, SGS - CSTC Standards Technical Services CO., Ltd. (Qingdao, Shandong, China). The concentration of Hg in seawater and organisms were expressed in µg/L and µg/g, respectively, as reported by Coppola et al. (2020b) and Jiang et al. (2019). For each aquarium three replicates were measured. The quantification limits of Hg in seawater and tissue were 5.0 µg/L and 0.005 µg/g, respectively.

2.2.3 Quality assurance and quality control

All samples were evaluated in duplicates to obtain parallel results and reduce uncertainties. The average data obtained from tests under the same conditions were used to obtain the final result. All samples including blanks and standard calibration curve were assessed by using the same procedures at the same conditions. The recovery of Hg was 97% in relation to the standard (0,0 -2.0 µg/L) and the relative percentage differences were all within 10%.

2.2.4 Biochemical markers

The electron transport system (ETS) activity is a proxy of the metabolic capacity of an organism and determined as reported by Andrade et al. (2018) and Coppola et al. (2019). It was read in a microplate at 490 nm and expressed in nmol/min/g FW. All the remaining parameters were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) already used in previous studies with the same species or other bivalves (Jiang et al, 2016, 2019), including: i) energy reserves ,namely glycogen (GLY) content (CAS A045-2-2), and protein total(PROT) content (CAS

A043-1-1); ii) enzymatic scavengers as total superoxide dismutase (T-SOD) activity (CAS A001-1-2), catalase(CAT) activity (CAS A007-1-1), and glutathione peroxidase (GSHPX)activity (CAS A005-1-2); iii) cellular damage as malondialdehyde (MDA) levels (CAS A003-1-2); iv) neurotoxicity as true cholineesterase (T-CHE) activity (CAS A024-1-1). All biomarkers were carried out in duplicate and measured using a microplate reader (Multiskan FC, Thermo Fisher Scientific, China).

2.2.5. Histopathological measurements

After 24 h in Davidson fluid, the clams' gills (G) and digestive tubules (D.T.) were placed in ethanol 70%. Afterwards, organisms were sent to the Service bio-Technology Co., Ltd. Laboratory (Wuhan, Hubei, China) for histopathological analyses. Tissue sections were acquired following the method reported by Coppola et al. (2018). The histopathological index (*ih*) was evaluated as described in Leite et al. (2020) where for each slide (6 per tissue) 6 pictures at 50x and 100x magnification were taken for a total of n=36 per organ. The *ih* values were calculated based on biological differences of each surveyed alteration with a value ranging between minimum and maximum severity (1-3) and its degree of dissemination with the score ranging between 0 (feature/alteration not observed in any of the 6 pictures made) and 6 (maximal diffusion where the alteration was detectable in each picture) as reported by Coppola et al. (2020a,b) and Costa et al. (2013).

2.2.6 Statistical analyses

The statistical permutational analysis of variance was conducted for all the obtained results (Hg concentration in clam's tissues, histological alterations and biomarkers) using the software PERMANOVA+add-on in PRIMER v6 (Anderson et al., 2008). Pairwise comparisons were performed and the significant differences were accepted when $p < 0.05$. The null hypotheses tested were: a) for each response (Hg concentration, histological and biochemical markers), no significant differences were detected among treatments (CTL, GO-PEI, Hg, GO-PEI + Hg and RSW) at 17 ºC (uppercase letters in Tables 2 and 3, Figure 1) and 22 ºC (lowercase letters in Tables 2 and 3, Figure 1); b) for each response and for each treatment, no significant differences existed between temperatures (17 and 22 ºC), highlighted with asterisks in Tables 2 and 3, Figure 1. Using the same software (PRIMER v6), Principal Coordinates Analysis (PCoA) was calculated by the Euclidean distance similarity matrix considering Hg concentrations, histopathological and biochemical markers for each treatment and temperature. On top of the PCoA graph, tissues Hg concentration, histopathological and biochemical descriptors were provided as Pearson correlation vectors.

2.3. Results

2.3.1 Mortality

During the experimental exposure, the highest mortality (50%) was recorded in clams submitted to Hg and GO-PEI + Hg at 22 ºC. Moreover, high mortality was also detected in organisms under Hg and GO-PEI + Hg at 17 ºC (47 and 39%, respectively). Mortality was also observed at 22 ºC in non-contaminated (CTL) and GO-PEI exposed clams (both 36%) as well as in clams subjected to remediated seawater (RSW) (33%). The organisms under CTL, GO-PEI and RSW at 17 ºC presented the mortality lower than 19%.

2.3.2 Mercury quantification

The background concentration of Hg in seawater was below the quantification limit (5.0 µg/L). Moreover, the Hg concentration in water samples collected from all aquaria after spiking showed the mean ± standard values close to the nominal concentration (64.1± 8.6 µg/L compared with 50 µg/L). The treatments CTL, GO-PEI and RSW at both temperatures presented a Hg level below the quantification limit (5.0 µg/L). The Hg quantification in clams' tissues from CTL and GO-PEI treatments at 17 ºC showed significantly lower Hg concentrations in comparison to the remaining treatments (Hg, GO-PEI + Hg, RSW), with significantly higher levels in organisms exposed to Hg and GO-PEI + Hg (Table 2). At 22 ºC significantly lower Hg values were observed at CTL and GO-PEI treatments, while significantly higher Hg concentration was found in clams subjected to Hg (Table 2). Between temperatures, significant differences were observed in GO-PEI + Hg and RSW treatments, with higher values at 17 ºC (Table 2).

Table 2. Mercury concentration [Hg] in clams' soft tissues (µg/g) at the end of the experiment. Results are mean ± standard deviation. Significant differences among treatments are represented with different letters: uppercase letters for 17 ºC and lowercase letters for 22 ºC. Differences between both temperatures for each treatment are represented with an asterisk.

[Hg] µg/g	CTL	GO-PEI	Hg	GO-PEI + Hg	RSW
17 ºC	0.021 ± 0.001^{A}	0.024 ± 0.001^{A}	3.2 ± 0.8^{B}	3.9 ± 1.2^{B}	1.8 ± 0.18^{C}
				*	*
22 ºC	0.019 ± 0.002^{a}	0.023 ± 0.003^{a}	3.0 ± 0.04^{b}	1.2 ± 0.08^{c}	0.19 ± 0.009^{d}

Mean values were obtained considering three true replicates per treatment (three different aquaria per treatment; n=3), and from each aquarium one replicate was used.

2.3.3 Biochemical markers

All biochemical results are showed in Table 3.

Table 3. Biochemical markers in *Ruditapes philippinarum* at the end of the experiment: Electron transport system (ETS) activity nmol/min/g FW; glycogen (GLY) content mg/g FW; protein total (PROT) content mg/g FW; total superoxide dismutase (T-SOD) activity U/g FW; catalase (CAT) activity U/g FW; glutathione peroxidase (GSH-PX) activity U/g FW; malondialdehyde (MDA) levels nmol/g FW; neurotoxicity as true choline esterase (T-CHE) activity U/g FW. Results are mean ± standard deviation. Differences among the treatments at 17 and 22 ºC were presented in uppercase letter and lowercase letter, respectively. Significant differences between 17 and 22 ºC are presented with asterisks. The highest values for each biomarker were highlighted in bold, while the lowest values were underlined.

		CTL			**GO-PEI**			**Hg**			**GO-PEI+Hg**			**RSW**		
		17°C		*22°C*	*17°C*		*22°C*	*17°C*		*22°C*	*17°C*		*22°C*	*17°C*		*22°C*
Biochemical markers	*ETS*	27.43±3.54^{A}	*	54.68±7.63^{a}	31.92±3.95^{A}	*	57.21±4.69^{a}	31.25±1.90^{A}		37.09±9.65^{b}	29.16±2.23^{A}	*	**58.89±2.94^{a}**	23.62±1.40^{B}	*	52.20±9.35^{a}
	GLY	8.03±0.35^{A}		7.41±1.13^{a}	11.30±1.63^{B}	*	7.53±0.30^{a}	17.29±0.44^{C}	*	**20.61±2.33^{b}**	14.01±0.62^{D}		17.00±2.26^{b}	8.47±0.57^{A}	*	12.00±0.38^{c}
	PROT	2.66±0.17^{A}	*	3.31±0.73^{a,c}	2.67±0.30^{A}	*	**3.69±0.23^{a}**	2.38±0.37^{A}		2.44±056^{b}	3.64±0.34^{B}	*	1.99±0.85^{b}	3.44±0.67^{B}	*	2.51±0.69^{b,c}
	T-SOD	6.94±0.08^{A}	*	8.82±0.11^{a}	7.06±0.01^{A}	*	8.8±0.1^{a}	7.59±0.54^{A,B}	*	8.88±0.12^{a}	7.89±0.15^{B}	*	**9.97±0.2^{b}**	7.94±0.46^{B}	*	9.89±0.22^{b}
	CAT	7.0±0.28^{A,B}	*	3.57±0.13^{a,c}	**7.34±0.22^{A}**	*	3.6±0.1^{a}	6.54±0.31^{B,C}	*	3.65±0.08^{a}	6.67±0.08^{B,C}	*	3.21±0.24^{b}	5.97±0.32^{C}	*	3.18±0.41^{b,c}
	GSH-PX	2.63±0.73^{A}		3.46±0.85^{a}	2.49±0.48^{A}	*	**5.8±0.8^{b}**	2.07±0.78^{A}	*	5.35±0.84^{b}	1.79±0.94^{A}	*	4.91 ± 1.09^{b}	2.66 ± 0.48^{A}	*	5.24 ± 0.58^{b}
	MDA	124.86±18.86^{A}		129.37±25.44^{a}	234.00±39.71^{B}	*	158.30±56.51^{a}	97.23 ± 9.04^{A}		*145.57±21.23*^{a}	98.44±17.81^{A}	*	295.89±27.42^{b}	115.13±12.38^{A}	*	***348.21±78.53^{b}***
	T-CHE	3.60±0.38^{A}		4.64±0.69^{a,b}	3.74±0.34^{A}		3.0±1.0^{b,c}	6.78±1.14^{B}	*	2.41±0.53^{c}	5.96±0.65^{B}	*	4.58±0.82^{a}	6.49±0.75^{B}		**7.17±1.21^{d}**

Mean values were obtained considering three true replicates per treatment (three different aquaria per treatment; n=3), and from each aquarium three replicates were used.

2.3.3.1 Metabolic capacity

At 17 ºC significantly lower ETS activity was identified in organisms from RSW compared to the remaining treatments. At 22 ºC the lowest values were observed at Hg treatment, with significant differences to the remaining treatments. Between temperatures, significantly higher values were found at 22 ºC except for Hg treatment.

2.3.3.2 Energy reserves

At 17 ºC significantly higher GLY content was detected in clams under GO-PEI, Hg and GO-PEI + Hg treatments in comparison to the remaining ones, with the highest value at Hg treatment and no significant differences between CTL and RSW. At 22 ºC the highest GLY content was found in clams exposed to Hg, followed by clams under GO-PEI + Hg, with significant differences to the remaining treatments. Between temperatures, significantly higher values were found at 17 ºC for GO-PEI treatment, while an opposite response was observed at Hg and RSW treatments. At 17 ºC significantly higher PROT content was obtained in clams subjected to GO-PEI + Hg and RSW treatments in comparison to the remaining ones. At 22 ºC significantly lower PROT values were observed in clams exposed to Hg and GO-PEI + Hg with significant differences to control. Between temperatures, significantly higher values were found at 22 ºC for CTL and GO-PEI treatments, while an opposite response was observed at GO-PEI + Hg and RSW treatments.

2.3.3.3 Enzymatic scavengers

At 17 and 22 ºC significantly higher T-SOD activity was detected at GO-PEI + Hg and RSW treatments in comparison to organisms exposed to CTL and GO-PEI. Between temperatures, significant differences were observed at all treatments, with higher T-SOD activity at 22 ºC. At 17 ºC significantly lower CAT activity was observed in organisms subjected to Hg, GO-PEI + Hg and RSW in comparison to CTL and GO-PEI treatments. The lowest values were found in clams under RSW, and no significant differences between CTL and GO-PEI as well as between Hg and GO-PEI + Hg. At 22 ºC significantly lower values were observed at GO-PEI + Hg and RSW treatments, with no significant differences between CTL, GO-PEI and Hg treatments. Comparing both temperatures, significantly higher CAT activity was recorded at 17 ºC regardless the treatment tested. At 17 ºC GSH-PX activity showed no significant difference among all treatments. At 22 ºC significantly lower values were obtained at CTL treatment compared to the other ones, with no significant differences among GO-PEI, Hg, GO-PEI + Hg and RSW treatments. Between temperatures, significantly higher GSH-PX activity was recorded at 22 ºC regardless the treatment tested, with exception for clams exposed to CTL.

2.3.3.4 Cellular damage

At 17 ºC significantly higher MDA levels were detected in organisms exposed to GO-PEI compared to the remaining treatments. At 22 ºC significantly higher values were observed in clams exposed to GO-PEI + Hg and RSW treatments. Between temperatures, significantly higher values were observed at GO-PEI for 17 ºC, while higher values were detected at 22 ºC in clams subjected to GOPEI + Hg and RSW treatments.

2.3.*3.5* Neurotoxicity

At 17 ºC significantly higher T-CHE activity was observed under Hg, GO-PEI + Hg and RSW compared to other treatments, with no significant differences between CTL and GO-PEI as well as between Hg, GO-PEI + Hg and RSW treatments. At 22 ºC significantly higher activity was found in *R. philippinarum* under RSW in comparison to the remaining treatments, while significantly lower values were found in clams subjected to GO-PEI and Hg compared to the remaining treatments. Between temperatures, significant differences were observed at Hg and GO-PEI + Hg treatments, with higher T-CHE activity at 17 ºC.

2.3.4 Histopathological measurements

2.3.4.1 Digestive tubules

At 17 and 22 ºC the highest digestive tubules histopathological index (*ih*) was observed in organisms exposed to Hg, with significant differences to the rest of treatments (except with RSW at 22 ºC) and no significant differences were detected between CTL and GO-PEI treatments (Figure 1A). Between temperatures, significantly higher values were found at 22 ºC for CTL and RSW treatments (Figure 1A). Figure 2 shows the haemocytes infiltration (arrows), high evidence of lipofuscin aggregates (*) and atrophied (a) in digestive tubules for each treatment at 17 and 22 ºC.

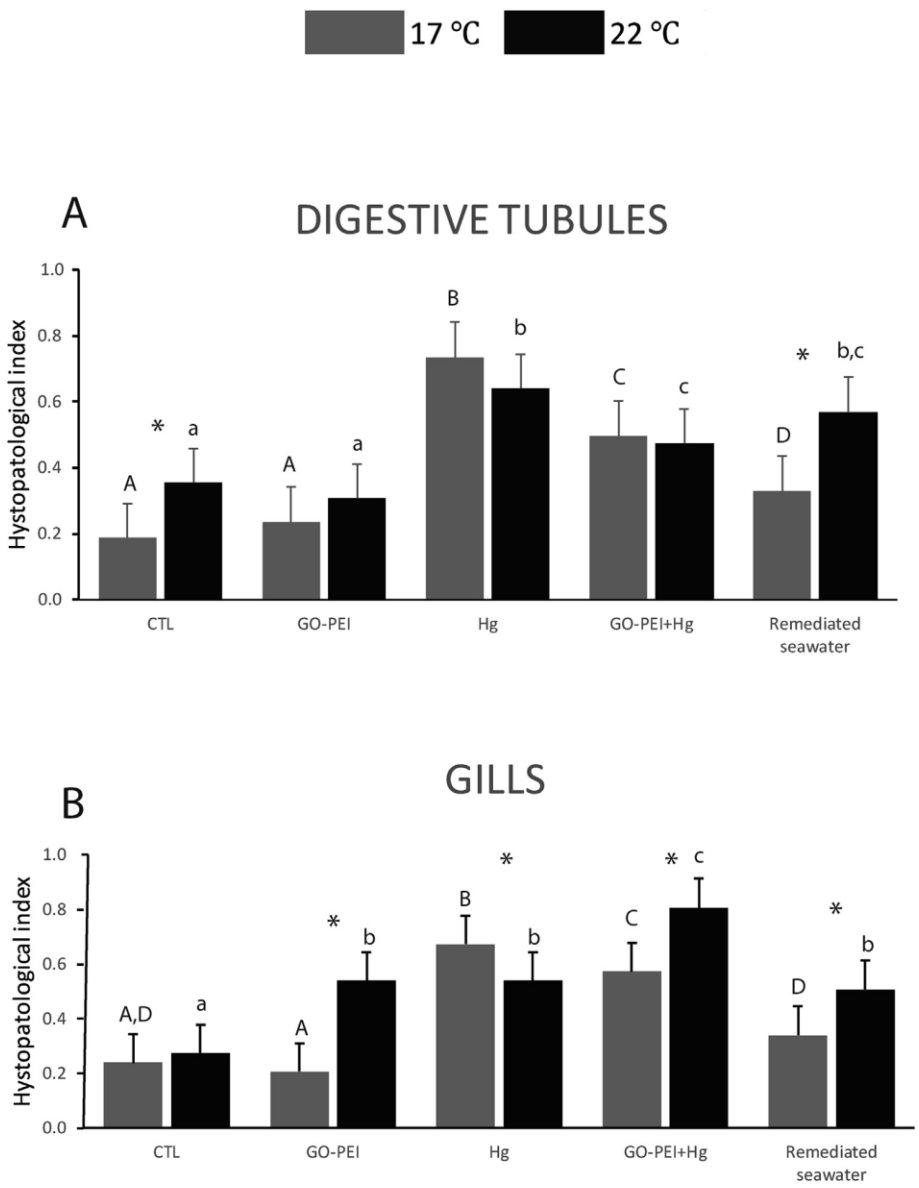

Figure 1. A: Histopathological index in digestive tubule; B: Histopathological index in gills, in *Ruditapes philippinarum* after 28 days-exposure. At each temperature (17 and 22 ºC) the tested treatments were: CTL, GO-PEI, Hg, GO-PEI + Hg and Remediated seawater (RSW). Results are mean + standard deviation. Significant differences among treatments are represented with different letters: uppercase letters for 17 ºC and lowercase letters for 22 ºC. Differences between both temperatures at each treatment are represented with an asterisk.

2.3.4.2 Gills

At 17 ºC significantly higher *ih* values were found in gills of clams subjected to Hg and GO-PEI + Hg as compared to the rest of treatments, with no significant differences between CTL and GOPEI as well as between CTL and remediated seawater treatments (Figure 1B). At 22 ºC the highest *ih* values were observed at GOPEI + Hg treatment, with no significant differences among GO-PEI, Hg and RSW (Figure 1B). Between the temperatures, significantly higher values were detected at 22 ºC for all treatments except for CTL and Hg (Figure 1B). The haemocytes infiltration (arrows), huge enlargement of the central vessel (long arrows), high evidence of lipofuscin aggregates (*) in gills for each treatment under both temperatures were showed in Figure 2.

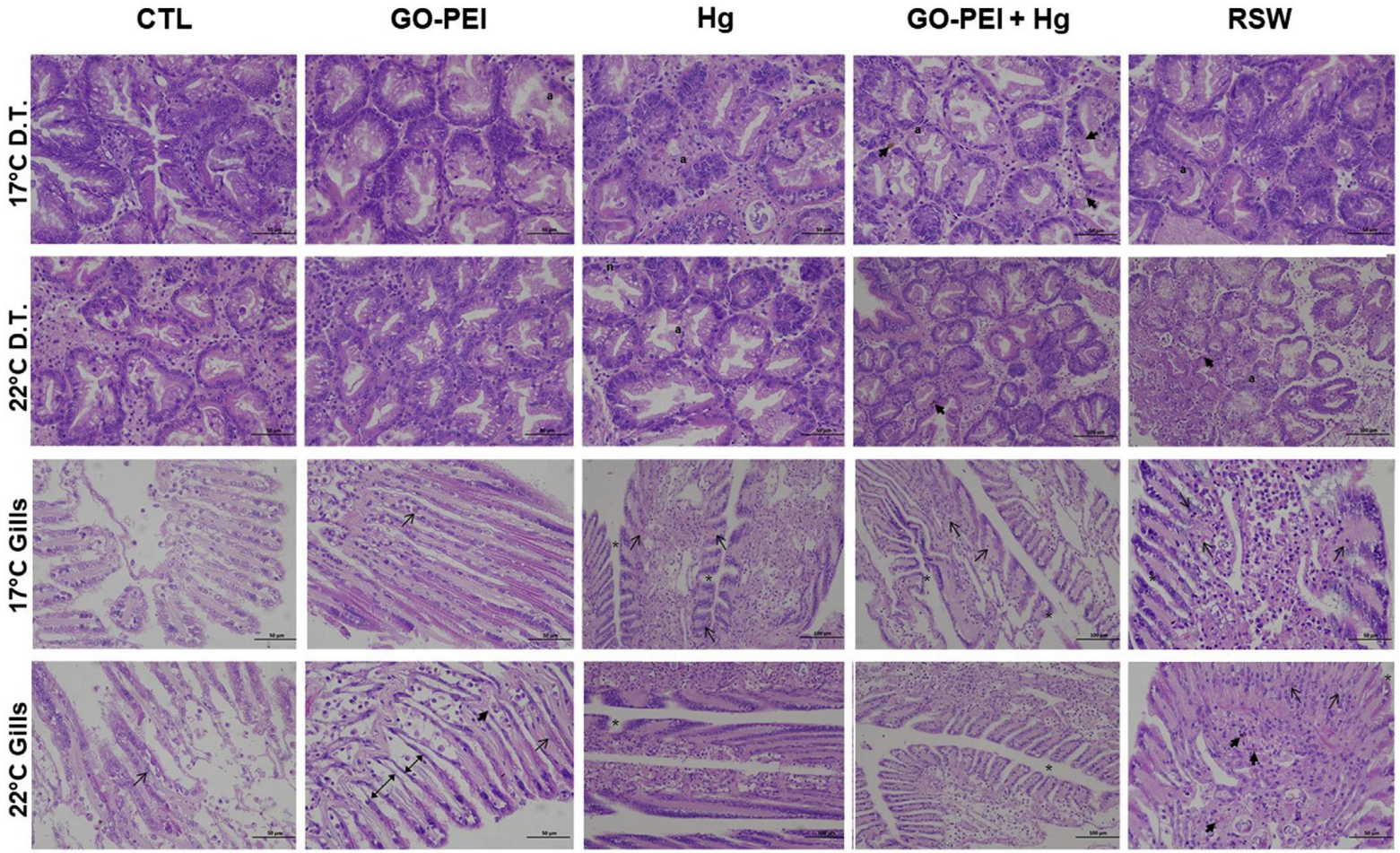

Figure 2. Micrographs of different tissues in *Ruditapes philippinarum* after 28 days-exposure. At each temperature (17 and 22 ºC) the tested treatments were: CTL, GO-PEI, Hg, GOPEI + Hg and Remediated seawater (RSW). Digestive tubule (D.T.): haemocytes infiltration (arrows), atrophied (a) and necrose (n). Gills: haemocytes infiltration (arrows), evident enlargement of the central vessel (long arrows), abundance of lipofuscin aggregates (*). Scale bar=50 and 100 mm.

2.3.5 Multivariate analysis

The Principal Coordinates Analysis (PCoA) calculated for Hg concentration in clams' soft tissue ([Hg]clams), histopathological index in gills (G (*ih*)) and digestive tubules (D.T. (*ih*)) as well as all biochemical markers (ETS, PROT, GLY, T-SOD, GSH-PX, CAT, MDA, TCHE) is shown in Figure 3. The PCoA axis 1 explained 42.1% of the total variation, separating organisms under treatments at 17 ºC in the positive side from others at 22 ºC in the negative side. The PCoA2, with 27.9% of the total variation, separating organisms under CTL, GO-PEI and RSW under both temperatures in the positive side form the remaining treatments in the negative side. PCoA1 positive side was highly correlated with CAT (p > 0.89), while PCoA1 negative side was highly correlated with T-SOD (p > 0.95). PCoA2 negative side was correlated with Hg concentration in tissues, D.T. (*ih*) and GLY (p> 0.71).

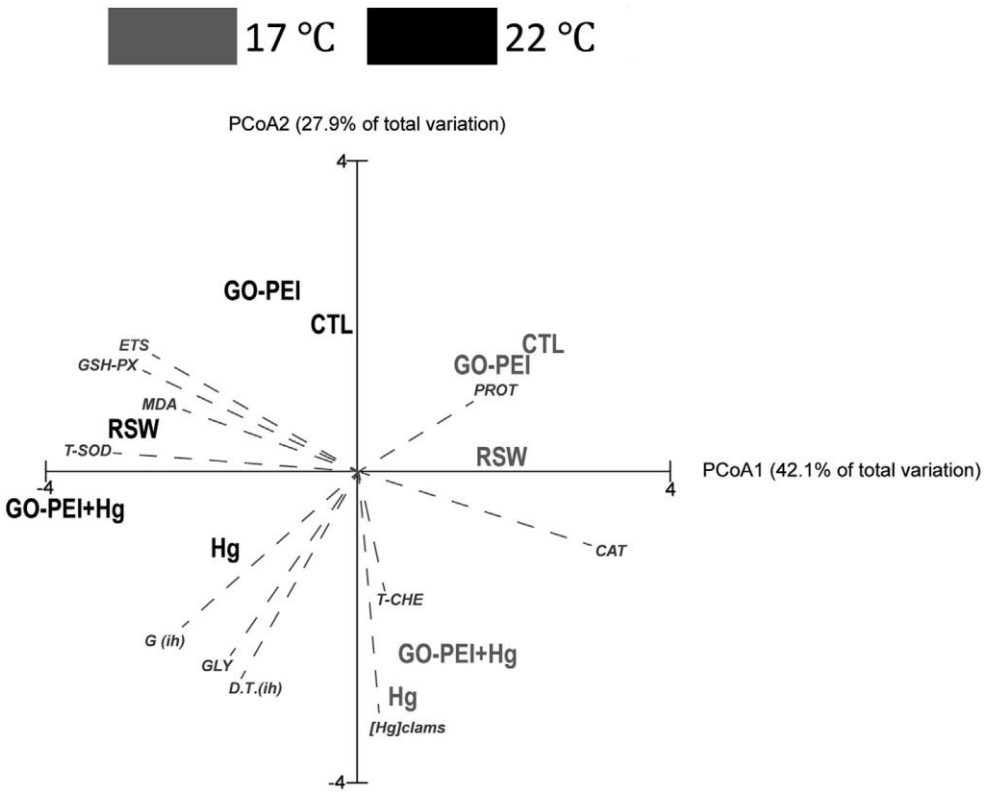

Figure 3. Principal Coordinated Analyses (PCoA) based on Hg quantification, biochemical parameters and histological alteration measured in *Ruditapes philippinarum* after 28 daysexposure. At each temperature (17 and 22 ºC) the tested treatments were: CTL, GO-PEI, Hg, GO-PEI + Hg and Remediated seawater (RSW). Pearson correlation vectors are superimposed as supplementary variables (r > 0.75): ETS, GLY, PROT, MDA, T-SOD, CAT, GSH-PX, T-CHE, [Hg]clams, G (*ih*) and D.T. (*ih*).

2.4 Discussion

Several studies have been assessing the use, efficiency and ecological safety of different synthetic materials for remediation of contaminated waters (namely from metal(loid)s) (Coppola et al.,2019; Mohmood et al., 2016; Nupearachchi et al., 2017; Zhanget al., 2010). Up today, limited information exists on the use of these materials and their potential effects towards organisms (in particular marine species), especially when under predicted climate changes (Andrade et al., 2019; Chen et al., 2016; Morosetti et al., 2020). Also, scarce information is available regarding the possible environmental risks of remediated seawater (Coppola et al., 2019, 2020a,b). Therefore, this study aimed to evaluate the impacts on clams *Ruditapes philippinarum* after chronic exposure to remediated seawater from mercury (Hg), using graphene oxide functionalized with polyethyleneimine (GO-PEI) nanocomposite as adsorbent, under actual and predict temperature increase. In general, the present findings emphasized the efficiency of GO-PEI in removing Hg from seawater, with low Hg accumulation in clams subjected to remediated seawater, especially at 22 ºC. *R. philippinarum* exposed to Hg free treatments (CTL, GO-PEI, RSW) presented similar responses, with clams exposed to Hg treatment presenting greater alterations, regardless the temperature tested. Furthermore, clams exposed to different temperatures showed a different behaviour. The results of this study showed higher mortality in *R. philippinarum* clams maintained at 22 ºC in comparison to clams under control temperature (17 ºC), in particular when exposed to Hg treatment. Previous studies already proved that temperature can influence marine organisms' metabolic capacity and oxidative status (Han et al., 2008; Le Moullac et al., 2007; Velez et al., 2017). Furthermore, it was already demonstrated that warming was responsible for higher sensitivity of organisms to other environmental factors, such as pollutants (Attig et al., 2014; Bat et al., 2000; Coppola et al., 2017, Coppola et al., 2018; Khan et al., 2007; Lanniget al., 2006; Mubiana and Blust, 2007; Sokolova and Lannig, 2008).The present findings further revealed high mortality rate in clams exposed to Hg at control temperature (17 ºC), which is in agreement with other works that demonstrated the high toxicity of Hg in marine organisms (Amachree et al., 2014; Chen et al., 2014; Coppola et al., 2018; Pan and Wang, 2011). Higher mortality rate found in clams exposed to Hg and GO-PEI + Hg at 22 ºC in comparison to organisms exposed to the same treatments but at control temperature were not explained by Hg accumulation in clams' tissues since higher metal concentration was observed in clams under 17 ºC. This response may thus corroborate the hypothesis that warming greatly alter the sensitivity of organisms to pollutants, affecting their biochemical performance and, thus, influencing organisms' general health status. Our findings also revealed that lower Hg levels in organisms at 22 ºC was not associated with strategies to avoid accumulation, namely filtration and respiration depression, since higher metabolic capacity (assessed by ETS activity) was observed in clams under warming conditions. Therefore, it seems that lower Hg accumulation did not result from clams' decreased filtration capacity associated with lower metabolic capacity but may thus result from clams' higher detoxification capacity, which was not evaluated in the present study. Nevertheless, several researches demonstrated that bivalves (clams and mussels) exposed to metals (Hg and Pb) reduce the bioaccumulation by decreasing their metabolic capacity (Freitas et al., 2017b; Casas and Bacher, 2006; Coppola et al., 2018; Velez et al., 2016; Verlecar et al., 2007). Regardless

the accumulation levels, the present findings clearly demonstrated that temperature was the main factor differentiating treatments, with clams' responses clearly distinct between 22 and17 ºC (see Figure 3). Different studies demonstrated that when bivalves are subjected to temperatures exceeding their thermal tolerance they can experience physiological disturbances (Baeket al., 2014; Han et al., 2008; Marigómez et al., 2017; Moreira et al., 2017; Paillard et al., 2004) and biochemical alterations including cellular damage (Maulvault et al., 2018; Matozzo et al., 2013), increase of oxidative stress (Velez et al., 2017; Greco et al., 2011) as well as metabolic alterations associated with respiratory capacities (Tamayo et al., 2013; Pörtner, 2010; Velez et al., 2017). The results here presented showed that among the most noticeable differences is the metabolic activity, with organisms exposed to warming conditions presenting the highest ETS activity in comparison to clams under control temperature. Results reported by Velez et al. (2017) also showed higher ETS activity in *R. philippinarum* exposed to rise of temperature. Coppola et al. (2018) showed that *Mytilus galloprovincialis* subjected to the combination of temperature rise and Hg presented higher accumulation of this metal in their tissues, with a close relationship with an increase on mussels ETS activity. In the present study, higher metabolic capacity observed in clams under 22 ºC was not associated with higher energy reserves expenditure, namely in terms of GLY, especially noticed at Hg, GO-PEI + Hg and RSW treatments. These results may indicate that in the presence of the pollutants, even at lower concentration levels, clams were able to prevent the loss of their energy reserves. Previous studies also demonstrated that in the presence of high temperature bivalves (clam *Macoma balthica* and mussels *M. galloprovincialis*) were also able to preserve their energy reserves content under Cd contamination (Della Torre et al., 2015; Duquesne et al., 2004; Nardi et al., 2017). Nevertheless, the present results revealed that higher metabolism in clams exposed to 22 ºC was associated with a general higher antioxidant capacity. In particular, the results here presented revealed that clams under warming conditions presented, in general, higher enzymatic activity (T-SOD and GSH-PX) than clams exposed to 17 ºC, a defence strategy already demonstrated by Freitas et al. (2017) who observed the increase of enzymatic scavengers' activities (CAT and SOD) in *M. galloprovincialis* under a warming scenario compared to mussels under control temperature. Although higher antioxidant capacity was observed in clams exposed to 22 ºC, higher MDA content was generally observed under warming conditions, evidencing that the defence mechanisms were not sufficient to avoid cellular damage. These results agreed with several works that showed an increase of cellular damage in bivalves under warming scenario and/or metals contamination (Coppola et al., 2018; Freitas et al., 2018; Velez et al., 2016), even after increase in antioxidant enzymes activity (Attig et al., 2014; Nardi et al., 2017; Pirone et al., 2019). As an example, Matozzo et al. (2013) showed that in the clam *Chamelea gallina* and in the mussel *M. galloprovincialis* antioxidant enzymes were activated but still MDA levels increased after one week of exposure to warming scenario (28 ºC). In terms of neurotoxicity, the results here presented showed that increased temperature was responsible for neurotoxic impacts, namely in Hg treatments. Due to the thermo-modulatory function of this enzyme, the thermal stress can be correlated to T-CHE inhibition (Kim et al., 2019), which was already demonstrated by previous researches reporting a significant inhibition of T-CHE activity after exposing the organisms *Pangasianodon hypophthalmus* and *M. galloprovincialis* to metals (zinc (Zn) and Hg) alone and with a simultaneous increase of temperature (Kumar et al., 2020; Morosetti et al., 2020). The

obtained results are also in agreement with a study by Costa et al. (2020) which showed increased neurotoxicity in two species of clams (*R. decussadus* and *philippinarum*) in response to increased temperature. In terms of histopathological alterations, differences between temperatures were also observed in clams under warming scenario and at control treatment temperature, with histopathological impairments including atrophied, haemocytes infiltration and necrose in digestive glands evident at 22 ºC. The study conducted by Leite et al. (2020) also showed that temperature rise caused histopathological alterations in gills as increase of haemocytes infiltration, enlargement of the central vessel and abundant lipofuscin aggregates in *M. galloprovincialis* mussels. Regardless of the temperature tested, the present findings also demonstrated that clams' responses were closely associated with the presence of Hg (Hg and GO-PEI + Hg treatments), with greater alterations in clams exposed to Hg alone than in the presence of GO-PEI. In particular, the results here presented evidence the low toxicity of remediated seawater, with biological responses observed in clams exposed to this treatment similar to the ones observed in clams exposed to CTL and GO-PEI acting alone, regardless the tested temperature (see PCoA graph). Under higher stressful conditions, namely the presence of Hg (Hg and GO-PEI + Hg treatments), the results obtained showed that Hg contaminated clams were not able to present significantly higher antioxidant capacity in comparison to CTL, remediated seawater or GO-PEI exposed clams. Such results may explain high cellular damages in clams exposed to Hg (especially GO-PEI + Hg) at 22 ºC, while lower LPO levels in clams subjected to Hg at 17 ºC may result from lower ETS activity at this temperature, a mechanism that generates reactive oxygen species. A recent study conducted by Coppola et al. (2019) observed lower oxidative stress and cellular damage in mussels *M. galloprovincialis* when exposed to seawater with low arsenic (As) concentration due to the decontamination of the water by manganese-ferrite ($MnFe_2O_4$) nanoparticles. However, recent literature has demonstrated an increase of non- and enzymatic defences (e.g. lipid peroxidation; superoxidase dismutase; catalase) in clams *R. philippinarum* exposed to different pollutants, mainly metal(loid)s (in specific 100 µg/L of As, 200 µg/L of cadmium (Cd), 1000 µg/L of lead (Pb) and 50 µg/L of Hg) and nanomaterials (Freitas et al., 2018; Marques et al., 2017; Velez et al., 2015). Jiang et al. (2019) showed a rise of antioxidant activity and cellular damage in the same species exposed at low Hg concentration (10 µg/L). The present study further demonstrated that Hg strongly induced the increase of the T-CHE enzyme at 17 ºC. Acetylcholinesterase degrades acetylcholine, a neural transmitter, in choline in cholinergic synapses and neuromuscular junctions (Matozzo et al., 2005). For this reason, the activity of acetylcholinesterase has been used as a biomarker of neurotoxicity. Among metals, Hg is known as a neurotoxic substance, namely to bivalves, by interrupting the nervous transmission. In fact, previous studies demonstrated the inhibition of acetylcholinesterase in bivalves, including in mussels and in clams, can occur due to the presence of metals (Attig et al., 2010; Cajaraville et al., 2000; Chalkiadaki et al., 2014; Matozzo et al., 2005). Nevertheless, since the inhibition of acetylcholinesterase is followed by accumulation of acetylcholine, the elevation of this compound can also indicate neurotoxicity. This was previously demonstrated by Liu et al. (2011) exposing the clams *R. philippinarum* to Hg. Such findings may explain the results obtained in the present study. Regarding histopathological impacts, our finding clearly demonstrated greater alterations in clams in the presence of Hg, especially when acting alone, with increase of haemocytes infiltration atrophied and necrose in

digestive tubule tissue. These results are in according with previous studies conducted by Leite et al. (2020) and Cuevas et al. (2015), which showed atrophy indigestive tubules, following a reduction in the thickness of epithelia followed by the expansion of lumen in mussels *M. galloprovincialis* exposed to metals (copper (Cu), Hg, Pb, Zn and titanium (Ti)). Other works also demonstrated that the presence of metals lead to histopathological alterations (haemocytes infiltration and necrose) in gills and digestive tubules of bivalves exposed to Pb (Hariharan et al., 2014) and Cu (Sabatini et al., 2011). Overall, the present findings demonstrated low impact of remediated seawater towards *R. philippinarum* clams. Such findings are related with the capacity of GO which allows the removal of Hg, highlighting the potential use of this nanomaterial to obtain water quality intended for human consume (Abraham et al., 2017; Kovtun et al., 2019; Tung et al., 2017). Bessa et al. (2020) had already demonstrated the efficiency of GO-PEI (10 mg/L) to remove Hg (50 µg/L) from different water type. While evidencing the capacity to remove Hg from seawater, the present study also demonstrated the low toxicity of the nanomaterial used, with sub-cellular alterations observed in clams exposed to CTL, GO-PEI and remediated seawater being similar, and differing from clams exposed to GO-PEI + Hg and especially Hg. Recent studies conducted with bivalves (oysters, *Crassostrea virginica*; mussels, *M. galloprovincialis*; clams, *R. philippinarum*) exposed to different concentrations of GO (from 1to 25 mg/L) evaluated possible toxic effects of this nanomaterial (Britto et al., 2020, 2021; Meng et al., 2020; Katsumiti et al., 2017; Khan et al., 2019a,b), showing limited toxic effects of the nanomaterial towards the exposed bivalves. Similarly, the present findings evidence that GO-PEI could be implemented as environment friendly sorbent in industries effluents for water purification before being discharged in aquatic systems. Bessa et al. (2020) further demonstrated the ability of GO-PEI to be regenerated, keeping its high removal performance after successive sorption desorption cycles allowing its reuse, therefore reducing the environmental impact of its utilization. Although previous studies with graphene nanomaterials always evidenced low toxicity, further studies may be relevant to evaluate the potential use of GO-PEI to remove other pollutants from seawater and the toxicity of resulting remediated water. Recovery of pollutants, although not addressed in this study, should be advised towards a circular and sustainable economy. Commonly, residuals contaminated with Hg are explored by specific industries, that successfully recover Hg from different waste sources (including GO-PEI nanomaterials). However, the recovery of elements from remediated water is still scarcely explored.

2.5 Conclusion

In general, less biochemical physiological and histopathological alterations were detected in *R. philippinarum* exposed to remediated seawater in comparison to clams subjected to Hg and/or GO-PEI treatments, at control temperature. Furthermore, higher alterations were observed at 22 ºC compared to control temperature. This study emphasizes the capability of GO-PEI nanocomposite as anew technology to remove the metal Hg from seawater with low toxic effects in *R. philippinarum* species, although temperature may increase the sensitivity of clams.

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CHAPTER 3.

The role of temperature on the impacts of remediated water towards marine organisms.

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Abstract

Marine organisms are frequently exposed to pollutants, including trace metals, derived from natural and anthropogenic activities. In order to prevent environmental pollution, different approaches have been applied to remove pollutants from wastewater and avoid their discharge into aquatic systems. However, organisms in their natural aquatic environments are also exposed to physico-chemical changes derived from climate change-related factors, including temperature increase. According to recent studies, warming has a negative impact on marine wildlife, with known effects on organisms physiological and biochemical performance. Recently, a material based on graphene oxide (GO) functionalized with polyethyleneimine (PEI) proved to be effective in the remediation of mercury (Hg) contaminated water. Nevertheless, no information is available on the toxic impacts of such remediated water towards aquatic systems, neither under actual nor predicted temperature conditions. For this, the present study assessed the toxicity of seawater, previously contaminated with Hg and remediated by GO-PEI, using the clam species *Ruditapes philippinarum* exposed to actual and a predicted temperature conditions. The results obtained demonstrated that seawater contaminated with Hg and/or Hg+GO-PEI induced higher toxicity in clams exposed to 17 and 22 °C compared to organisms exposed to remediated seawater at the same temperatures. Moreover, similar histological and biochemical results were observed between organisms exposed to control and remediated seawater, independently of the temperatures (17 and 21 °C), highlighting the potential use of GO-PEI to remediate Hg from seawater without significant toxicity issues to the selected marine species.

Keywords

biomarkers; toxicity; *Ruditapes philippinarum*; GO-PEI; seawater remediation; mercury; bioaccumulation.

3.1 Introduction

Studies conducted in the last decade have demonstrated that the increase of greenhouse effect gases, such as carbon dioxide, is intrinsically related with global warming (Dahlke et al., 2012; IPCC, 2007, 2018; Manciocco et al., 2014; Sokolova and Lannig, 2008). Global warming is not only responsible for atmospheric temperature rise but also for the increase in mean water temperature in aquatic systems. According to the Intergovernmental Panel on Climate Change (IPCC) (IPCC, 2018), global warming is likely to reach 1.5 ˚C between 2030 and 2052 if it continues to increase at the current rate. Temperature increase may be of greater magnitude in estuaries and coastal lagoons due to their physical-chemical characteristics, including low water exchange (Newton et al., 2018; Earp et al., 2018; Pan et al., 2011). Associated with temperature increase in aquatic systems, it is predicted that inhabiting organisms may be subjected to deleterious effects as already shown by different authors (Boukadida et al., 2016; Pörtner et al 2007; Rosa et al., 2012). Studies with bivalves already showed that temperatures exceeding an organism's thermal tolerance range can cause physiological perturbations with consequences on growth and reproduction of mussels (Bodin et al., 2004; Hiebenthal et al., 2012; MacKenzie et al., 2014; Petes et al., 2008), as well as decrease of metabolic rate and respiratory capacity in clams and mussels (Jansen et al., 2009; Pörtner et al., 2010; Velez et al., 2017). Warming conditions can also enhance reactive oxygen species (ROS) production in the cells, leading to oxidative stress in different marine species, including bivalves (Freitas et al., 2020; Coppola et al., 2017,2018a; Kefaloyianni et al., 2005; Verlecar et al., 2007). Particularly, biochemical alterations have been observed in different clam's species in response to temperature rise, including increased antioxidant capacity (Velez et al., 2017; Greco et al., 2010) and cellular damage (Maulvault et al., 2018; Matozzo et al., 2013). Recent studies further demonstrated that change in temperature negatively impacted bivalve's embryo-larval development (Moreira et al., 2018; Van Colen et al., 2018). In the aquatic environment organisms may not only be subject to climate changes but are also exposed to pollutants, such as metals, derived from natural and anthropogenic activities, associated with world population growth (Tankoua et al., 2012; Giani et al., 2012; Pereira et al., 2008; Randall et al., 2013; Suriya etal., 2012). Coastal ecosystems have been particularly affected by metals (e.g., lead (Pb), mercury (Hg), cadmium (Cd) and others) with well-known toxic effects towards aquatic organisms (Coppola et al., 2018a,b; Al Naggar et al., 2018; Bielen et al., 2016; Freitas et al., 2016; Velez et al., 2016). Studies conducted with top list hazardous elements (ATSDR, 2015) as Hg, Cd and arsenic (As) already showed their capacity to interfere on bivalve's biochemical performance (Coppola et al., 2018a,b; Freitas et al., 2018; Nardi et al., 2017; Samuel et al., 2005; Zhang et al., 2010; Velez et al., 2015). In particular, studies assessing the effects of Hg in bivalves showed that this metal induced histological, physiological and biochemical impairments in oysters (*Saccostrea cucullata*, *Crassostrea gigas*) (Pan et al., 2011; Gagnaire et al., 2004), clams (*Anodonta anatina*, *Corbicula fluminea*, *Ruditapes decussatus* and *R. philippinarum*) (Pan et al., 2011; Velez et al., 2016; Oliveira et al., 2017), mussels (*Perna viridis*, *Septifer virgatus*, *Mytilus galloprovincialis*, and *M. edulis*) (Bodin et al., 2004; MacKenzie et al., 2014; Attig et al., 2014; Mubiana et al., 2007) and cockles (*Cerastoderma edule*) (Freitas et al., 2012). Although environmental threats

caused by Hg are well-known, the concentration of this metal has increased in the environment due to its use as main component in electronic products, thermometers(for measuring high temperatures) and fluorescent lamps (Randall et al., 2013; Donnici et al., 2012; Briant et al., 2016). In the aquatic environment Hg has been identified in coastal and bay waters in concentrations ranging from 0.10 ng/L (Chesapeake Bay, MD, USA) to 1200 ng/L (Marano and Grado lagoons, Venice, Italy), reaching 2700 ng/L in Bohai Sea coast (China); whereas in open seawater Hg concentrations range from 0.08 ng/L (Pacific Ocean) to 0.20 ng/L (Atlantic Ocean) (Faganeli et al., 2012; Pereira et al., 2019). As an attempt to remove metal (oid)s, in particular Hg, from water and avoid their discharge into aquatic systems, different approaches have been applied such as chemical precipitation (Matlock et al., 2001; Henke et al., 2001), ultrafiltration (Aroua et al., 2007; Muthukrishnan et al., 2005), reverse osmosis (Pugazhenthi, et al., 2005), nanofiltration (Aroua et al., 2007; Muthukrishnan et al., 2005; Pugazhenthi, et al., 2005), and sorption on nanomaterials (Ali et al., 2011; Anjum et al., 2019; Huang et al., 2015; Li et al., 2010). The main concerns regarding these methodologies are the factthat they are low cost but inefficient, or efficient but expensive (Babel et al., 2003; Gehrke et al., 2015; Mohan et al., 2007). In order to overcome these issues, Henriques et al. (2016) synthesized and characterized new nanostructured materials (NSMs), based on graphene oxide (GO) that proved to be effective to remove Hg from water. The remarkable breakthroughs in research on graphene-based materials (GBM) have revealed its great potential for environmental remediation. GO can be produced by oxidation of graphite in laboratory (Gonçalves et al., 2009), composed on a substrate or porous material and used as a membrane. Different GBM have been developed for water desalination, sorption processes, degradation of organic contaminants and the removal of potential toxic elements from polluted waters (Cohen-Tanugi et al., 2012; Fang et al., 2017; Sahraei et al., 2017; Bessa et al., 2020). Recently our group developed a material based on GO functionalized with polyethyleneimine (PEI) that proved to be effective in the remediation of Hg 50 µg/L contaminated seawater, with 81% of removal efficiency after just 6 h (Bessa et al., 2020). Nevertheless, up to now, no information exists on the toxicity of the remediated seawater, i.e., no information is available on possible effects in aquatic organisms exposed to water after the remediation treatment. Although recent literature has demonstrated the impacts of temperature in bivalves physiological and biochemical performance, the cooccurrence of temperature increase and pollutants is not yet well understood. The simultaneous occurrence of temperature rise and the presence of pollutants may result in organisms increased sensitivity to each of the stressors but may also alter pollutants' toxicity, leading to additive or antagonist effects as reported in several studies (Coppola et al., 2017; Moreira et al., 2018; Van Colen et al., 2018; Attig et al., 2007; Banni et al., 2014a,b; Izagirre et al., 2014). According to Coppola et al. (2019b), oxidative stress was enhanced in *M. galloprovincialis* exposed to Hg under warming conditions. For the afore mentioned, the present study aimed to assess the possible toxicity of seawater, previously contaminated with Hg and remediated by GO-PEI, using the clam species *R. philippinarum* under different temperature scenarios, to assess the effects of temperature rise on the impacts induced by remediated water. Previous studies already demonstrated that this clam species is a good bioindicator, being commonly used in field and laboratory studies to evaluate the effects derived from the exposure to different pollutants, including metals (Velez et al., 2016; Zhang et al., 2010; Marques et al., 2018), drugs (Almedia et al., 2015; Costa et al., 2019; Cruz et

al., 2016), or nanoparticles (Coppola et al., 2019; De Marchi et al., 2017). *R. philippinarum* specimens were exposed for 28 days, at different treatments, including clean seawater (control-CTL); remediate seawater; and seawater containing Hg (50 µg/L), GO-PEI (10 mg/L) or the mixture of both. Each treatment was conducted under control (17 ˚C) and increased (22 ˚C) temperatures. At the end, Hg concentrations in clam's soft tissues, histopathological alterations, as well as biochemical responses related to clams' metabolic, cellular damage and oxidative stress status were measured.

3.2 Materials and methods

3.2.1 Laboratory Conditions and Experimental Setup

The species *Ruditapes philippinarum* were collected in the Mira channel (Ria de Aveiro lagoon, Portugal), with a mean total weight of 12.1 ± 2.6 g, mean length of 3.53 ± 0.29 cm and a mean width of 4.53 ± 0.42 cm. In the laboratory clams were placed under acclimation (one week), with water conditions similar to the sampling site. After this initial period, clams were divided in two groups: one exposed at 17 ± 1 °C and another at 22 ± 1 °C (with a gradual temperature increase), for the acclimation to test conditions during an extra week. Throughout these two weeks all organisms were maintained in artificial seawater (salinity 30 ± 1) at pH 8.0 ± 0.1 and constant aeration. Seawater was renewed every 2–3 days, after which animals were fed with Algamac protein plus. Environmental conditions measured during clam's field sampling (temperature 17 °C, pH 8.0, salinity 30) were considered as control levels that were also in agreement with the mean values observed during the year in the sampling area (IPMA, 2019). The highest tested temperature (22 °C) was selected considering predicted global warming conditions (IPCC, 2018). After this period, organisms were maintained during 28 days in two groups under test temperatures, salinity and pH conditions, with organisms divided in five different treatments as described in Table 1. Per treatment three aquaria were used with six individuals in each aquarium (5 L glass aquaria).

Table 1. Experimental treatments. GO-PEI: Graphene oxide functionalized with polythyleneimine; Hg: mercury.

TREATMENTS	DESCRIPTION
CTL	Artificial seawater (Hg 0.0 µg/L + GO-PEI 0.0 mg/L)
GO-PEI	Artificial seawater with GO-PEI 10 mg/L
Hg + GO-PEI	Artificial seawater with Hg 50 µg/L and GO-PEI 10 mg/L
Hg	Artificial seawater with Hg 50 µg/L
REMEDIATED SEAWATER	Artificial seawater previously contaminated with Hg (50 µg/L), and remediated by GO-PEI (10 mg/L) during 24 h

The concentration of mercury (Hg) used in the present study, 50 µg/L, was selected taking into consideration that this is the maximum allowable limit in wastewater discharges from industry (Directive 2013/39/EU). A concentration of 10 mg/L of graphene oxide (GO) functionalized with polyethyleneimine (GO-PEI) was selected according to the capacity of this nanostructured material (NSMs) to remove Hg from seawater (preliminary assays). The remediated seawater was prepared by the contamination of clean seawater with Hg using a defined volume of a stock solution (1000 mg/L of Hg, Sigma Aldrich) followed by remediation with GO-PEI (10 mg/L) during 24 h, after which the material was separated from the seawater by filtration. Throughout the experimental period, water conditions were checked daily as

well as clams' mortality. Animals were fed with Algamac protein three times per week. During this period, seawater from each aquarium was renewed weekly and treatments reestablished, including temperatures, salinity and concentrations of Hg and GO-PEI. Seawater samples from each aquarium were collected immediately after weekly water exchange for Hg quantification, to compare real concentrations with nominal ones. At the end of the exposure clams were meticulously opened to separate the shell from soft tissue. One clam (soft tissue) per aquarium (three per treatment) was fixed in Bouin's fluid for 24 h atroom temperature for the histological evaluation. For biochemical analyses and Hg quantification sixorganisms per treatment (two per aquarium) were frozen in liquid nitrogen and manually homogenized with a mortar and a pestle. Each organism' soft tissue was divided into aliquots of 0.3 g fresh weight(FW) and stored at −80 °C.

3.2.2 Synthesis and Characterization of Graphene Oxide Functionalized with Polyethyleneimine

Graphene oxide water dispersion (0.4 wt % concentration from Graphenea) was directly mixed with ethyleneimine polymer (PEI) solution at 50% (*w*/*v*) in water, with molecular weight (M.W.) ~750,000 and a ratio GO/polymer of 24% *v*/*v*. The pH of both solutions, GO and polymer, was adjusted to 2 before mixing, using 0.1 mol/L NaOH or HCl solutions. After mixing, the solution was rapidly shaken for 10 s to form a hydrogel. The hydrogel was frozen at −80 °C obtaining three-dimensional (3D) porous structures. The lyophilized samples were then washed in MilliQ water for 12 h to remove acidic residues. Finally, samples were freeze-dried again resulting in a foam-like macrostructure (Figure 1).

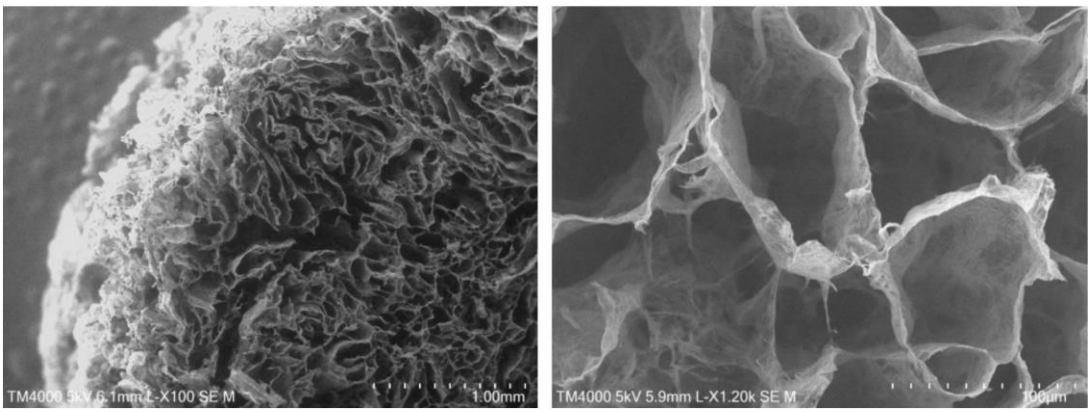

Figure 1. SEM (scanning electron microscopy) images of GO-PEI macrostructure obtained after lyophilization evidencing the porous nature of this materials.

3.2.3 Mercury Quantification

The quantification of Hg in seawater aliquots was performed following Henriques et al. (2019) methods using cold vapor atomic fluorescence spectroscopy (CV-AFS). The concentration of Hg in the organism's tissues was quantified by thermal decomposition atomic absorption spectrometry with gold amalgamation, as described in Costley et al. (2000).

3.2.4 Biochemical Markers

The selected biomarkers included: (i) metabolic capacity (electron transport system, ETS); (ii) antioxidant enzymes activity (superoxide dismutase, SOD; glutathione peroxidase, GPx; glutathione reductase, GRed); (iii) extent of cellular damage levels (lipid peroxidation, LPO; protein carbonylation, PC); (iv) redox balance (ratio between reduced (GSH) and oxidized (GSSG) glutathione content).The biochemical parameters were determined as described in Coppola et al. (2019). All biochemical parameters were performed in duplicate and measurements made on a microplate reader (BioTek Synergy HT).

3.2.5 Histopathological Measurements

After the experimental period, the clams fixed in Bouin's fluid for histopathological analyses were placed in ethanol 70% which was changed daily to wash out the fixative left over. Afterwards, organisms were gradually dehydrated from ethanol 70% to absolute alcohol in graded alcohols, cleared in xylene, embedded in paraffin (56–58 °C), and serial sections (7 µm thick) were obtained using a microtome as described in Pinto et al. (2019). Histopathological alterations in gills and digestive tubules were identified as described previously (Leite et al., 2020; Coppola et al., 2020).

3.2.6 Integrated Biomarker Response

The integrated biomarker response (IBR) index was calculated according to Beliaeff and Burgeot (2020) and detailed in Coppola et al. (2019). Biomarkers were arranged in the following order: ETS, SOD, GPx,GRed, LPO, PC, and GSH/GSSG. Values were discussed in terms of a general response given by the final IBR value, where higher values correspond to higher clams' response.

3.2.7 Statistical Analyses

Mercury concentration in seawater and the clam's soft tissues, biochemical markers and histopathological indices, obtained for each tested treatment, were submitted to a statistical hypothesis testing using permutational analysis of variance (2008)]. The null hypotheses tested were: (i) for each response (Hg concentration in seawater and clams, biomarkers and histopathological indices), no significant differences were observed among treatments (CTL, GO-PEI, Hg+GO-PEI, Hg and remediated seawater) at 17 °C (uppercase letters in Table 2) and 22 °C (lowercase letters in Table 2); (ii) for each response and for each treatment no significant differences existed between temperatures (17 and 22 °C), represented in Table 2 by an asterisk. The matrix expressing histopathological and biochemical markers as well as Hg concentrations per treatment under both temperatures were normalized and the Euclidean distance calculated among centroids was visualized in principal coordinates ordination (PCO) analysis. In the PCO graph, the variables presenting a correlation higher than 75% with treatments spatial distribution were represented as superimposed vectors.

3.3 Results

3.3.1 Mortality

At the end of the experimental period the highest mortality (44%) was recorded in clams submitted to Hg at both temperatures (17 and 22 ˚C) and in organisms exposed to GO-PEI at 17 ˚C and Hg+GO-PEI at 22 ˚C (44%). Lower mortality was observed in GO-PEI at 22 ˚C and Hg+GO-PEI treatments at 17 ˚C (11% and 33%, respectively). The organisms exposed to remediated seawater at both temperatures presented the same mortality (33%). A mortality rate of 22% was recorded in CTL treatment at 17 and 22 ˚C. Due to high mortality rates observed in all tested conditions, including control, the results achieved must be considered with precaution.

3.3.2 Mercury Concentration in Seawater and Clams

Mercury concentration in seawater samples collected weekly in aquaria (immediately after water renewal and spiking) from Hg+GO-PEI and Hg treatments at 17 and 22 ˚C were very close to the nominal concentration (50 µg/L), while Hg levels in CTL and GO-PEI treatments at both temperatures were below the limit of quantification (<LOQ) (Table 2). Under 17 ˚C, significantly lower Hg concentrations were observed in seawater samples collected from remediated seawater comparing with those contaminated by Hg+GO-PEI and Hg treatments. Similar results were observed under 22 ˚C. Between temperatures no significant differences were found regardless the treatment (Table 2). At 17 ˚C, significantly lower Hg concentration was found in clams exposed to remediated seawater compared with Hg values measured in clams subjected to Hg+GO-PEI and Hg treatments. Under 22 ˚C, clams showed significantly lower Hg concentrations when exposed to remediated seawater and to Hg+GO-PEI in comparison to organisms under Hg treatment. Between temperatures, significant differences were found in all treatments except for GO-PEI, with clams exposed to 17 ˚C presenting significantly lower Hg concentrations in specimens under CTL and Hg treatments while significantly higher values were observed at 17 ˚C in clams exposed to Hg+GO-PEI and remediated seawater.

3.3.3 Biochemical Markers

All results obtained from biochemical markers were expressed as mean ± standard deviation and values are shown in Table 2.

3.3.3.1 Metabolic Capacity

At 17 ˚C significantly lower ETS activity was detected in organisms exposed to Hg+GO-PEI and remediated seawater compared to the remaining treatments. At 22 ˚C, significant differences were observed among treatments with exception to clams exposed to Hg+GO-PEI and CTL. Organisms exposed to Hg (22 ˚C) and remediated seawater (17 ˚C) showed the highest and the lowest ETS activity, respectively. Between temperatures, significant differences were observed at CTL and GO-PEI treatments, with higher metabolic capacity under 17 ˚C compared to 22 ˚C.

3.3.3.2 Antioxidant Enzymes Activity

At 17 and 22 ˚C, significantly higher SOD activity was observed in clams exposed to Hg in comparison to organisms under the remaining treatments. Between temperatures, significant differences were observed at CTL and Hg treatments, with higher SOD activity at 17 ˚C compared to 22 ˚C. At 17 ˚C, significant differences in GPx activity were observed between CTL, GO-PEI and Hg+GO-PEI, with the highest values in *R. philippinarum* under Hg+GO-PEI treatment. No significant differences were observed between clams exposed to CTL and remediated seawater, as well as among GO-PEI, Hg and remediated seawater. At 22 ˚C, significantly lower antioxidant defence was observed at CTL compared to GO-PEI and Hg treatments. Between temperatures, significantly higher GPx activity was only recorded in clams exposed to Hg+GO-PEI at 17 ˚C compared to treatment at 22 ˚C. At 17 ˚C, significant differences in terms of GRed activity were observed between GO-PEI, Hg and remediated seawater showing the highest values in organisms under GO-PEI treatment. Specimens exposed to remediated seawater did not present significant differences between CTL and Hg+GO-PEI.In addition, Hg+GO-PEI condition did not evidence difference with Hg exposed clams. At 22 ˚C,significant differences were observed among all treatments except between clams exposed to Hg and remediated seawater. Under this temperature (22 ˚C), organisms exposed to GO-PEI and Hg treatments showed the highest and the lowest GRed activity, respectively. Between temperatures, significantlylower enzymatic activity was measured in clams under CTL at 17 ˚C compared to 22 ˚C, while bivalves exposed to Hg treatments presented significantly higher enzymatic activity at 17 ˚C.

Table 2. (i) Hg quantification: water samples ([Hg] W) µg/L collected immediately after the weekly water renewal for each treatment; clams ([Hg] C) mg/Kg collected at the end of the experiment. Levels not detectable (below the limit of quantification, <LOQ). (ii) Biochemical markers in *Ruditapes philippinarum* collected 28 days after the beginning of the experiment: electron transport system (ETS) activity nmol/min/g fresh weight (FW); superoxide dismutase activity (SOD) U/g FW; glutathione peroxidase (GPx) activity U/g FW; glutathione reductase (GRed) activity U/g FW; lipid peroxidation levels (LPO) nmol MDA/g FW; protein carbonyl levels (PC) nmol/g FW; ratio between reduced and oxidized glutathione (GSH/GSSG). (iii) Histopathological markers collected 28 days after the beginning of the experiment: Gills (Ih G) ih; digestive tubules (Ih DT) ih. Results are mean + standard deviation. Statistical differences among the treatments at 17 and 22 ºC (The meaning of the letters is in the legend. Uppercase letter are used to identify statistical differences among treatments at 17 ºC. While lowercase letter are used to identify statistical differences among treatments at 22 ºC were presented with different uppercase letters and lowercase letters, respectively. Significant differences between treatments 17 ºC vs 22 ºC (*) are presented with asterisks. The highest values for each biomarker were highlighted in bold, while the lowest values were underlined.

		CTL			**GO-PEI**			**Hg+GO-PEI**			**Hg**			**Remediated seawater**		
		17°C		*22°C*	*17°C*		*22°C*	*17°C*		*22°C*	*17°C*		*22°C*	*17°C*		*22°C*
Hg quantification	*[Hg]W*	< LOQ		< LOQ	< LOQ		< LOQ	50.0 ± 3.90^{A}		49.6 ± 3.26^{a}	50.4 ± 2.95^{A}		49.4 ± 5.09^{a}	11.5 ± 3.71^{B}		11.5 ± 3.71^{b}
	[Hg]C	0.18 ± 0.02^{A}	*	0.29 ± 0.01^{a}	0.14 ± 0.03^{B}		0.16 ± 0.0056^{b}	7.3 ± 0.63^{C}	*	3.6 ± 0.29^{c}	9.1 ± 1.9^{C}	*	12 ± 2.5^{d}	4.7 ± 0.34^{D}	*	2.9 ± 0.98^{c}
Biochemical markers	*ETS*	31.7 ± 4.86^{A}	*	25.5 ± 5.44^{a}	35.5 ± 5.99^{A}	*	15.5 ± 3.42^{b}	13.2 ± 0.76^{B}		20.2 ± 7.09^{a,b}	30.1 ± 4.65^{A}		**39.0 ± 2.82^{c}**	11.1 ± 0.60^{C}		11.1 ± 1.34^{d}
	SOD	0.43 ± 0.030^{A}	*	0.24 ± 0.040^{a}	0.46 ± 0.050^{A}		0.34 ± 0.03^{a}	0.41 ± 0.05^{A}		0.33 ± 0.02^{a}	**0.77 ± 0.07^{B}**	*	0.54 ± 0.13^{b}	0.28 ± 0.03^{A}		0.29 ± 0.01^{a}
	GPx	0.03 ±0.005^{A}		0.03 ± 0.004^{a}	0.04 ± 0.004^{B}		**0.05 ± 0.01^{b}**	0.06 ± 0.008^{C}	*	0.04 ± 0.004^{a,b}	0.04 ± 0.009^{B,C}		0.04 ± 0.006^{b,c}	0.04 ± 0.01^{A,B}		0.04 ± 0.006^{a,b}
	GRed	0.030 ± 0.0040^{A,D}	*	0.060 ± 0.010^{a}	0.14 ± 0.020^{B}		**0.12±0.010^{b}**	0.060 ± 0.010^{C,D}		0.070 ± 0.0070^{c}	0.060 ± 0.010^{C}	*	0.030 ± 0.0070^{d}	0.035 ± 0.014^{D}		0.041 ± 0.013^{d}
	LPO	15.4±0.75^{A}		14.9 ± 1.09^{a,d}	16.2 ± 0.64^{A}	*	13.5 ± 0.48^{a}	20.6 ± 0.58^{B,C}		22.6 ± 3.67^{b}	22.0 ± 0.25^{B}	*	**28.2 ± 0.384^{c}**	17.3 ± 3.049^{A,C}	*	14.9 ± 0.645^{d}
	PC	0.90 ± 0.13^{A}		0.89 ± 0.06^{a}	0.99 ± 0.19^{A}		0.97 ± 0.11^{a}	0.95 ± 0.08^{A}		0.88 ± 0.04^{a}	**1.03 ± 0.13^{A}**		*0.87 ± 0.06^{a}*	0.89 ± 0.007^{A}		0.95 ± 0.12^{a}
	GSH/GSSG	0.49±0.05^{A}	*	**0.75 ± 0.04^{a}**	0.13 ± 0.02^{B}	*	0.23 ± 0.04^{b}	0.13 ± 0.01^{B}	*	0.21 ± 0.03^{b}	0.12 ± 0.008^{B}	*	0.22 ± 0.04^{b}	0.11 ± 0.02^{B}	*	0.23 ± 0.04^{b}
Histopathological index	*Ih G*	0.05 ± 0.02^{A}	*	0.08 ± 0.03^{a}	0.15 ± 0.02^{B}		0.13 ± 0.05^{b}	0.17 ± 0.08^{B}		0.18 ± 0.07^{c}	0.27 ± 0.05^{C}	*	**0.33 ± 0.05^{d}**	0.12 ± 0.06^{B}		0.16 ± 0.06^{c,b}
	Ih DT	0.23 ± 0.09^{A}		0.16 ± 0.07^{a,b}	**0.38 ± 0.001^{B}**	*	0.21 ± 0.19^{a}	0.23 ± 0.07^{A}	*	0.09 ± 0.001^{b}	0.31 ± 0.001^{C}	*	0.37 ± 0.05^{c}	0.21 ± 0.11^{A}		0.19± 0.13^{a}
IBR				2.81	2.01		4.16	3.44		2.39	3.38		**4.27**	2.20		2.09

3.3.3.3 Cellular Damage

Under 17 ˚C, significantly higher LPO levels were observed only in organisms exposed to Hg compared to the remaining treatments with exception to Hg+GO-PEI. Clams under CTL conditions showed significantly lower cellular damage when compared with those at Hg+GO-PEI and Hg treatments. At 22 ˚C, significant differences were observed among all treatments with exception to CTL, GO-PEI and remediated seawater. Between temperatures, significantly higher cellular damage was shown at GO-PEI and remediated seawater treatments at 17 ˚C compared to 22 ˚C, while significantlylower LPO levels were found in organisms exposed to Hg at 17 ˚C. No significant PC levels at 17 ˚C and/or 22 ˚C differences were observed among treatments. No significant differences were observed between temperatures regardless of the treatment tested.

3.3.3.4 Redox Balance

At 17 ˚C, significantly higher GSH/GSSG ratio were observed in *R. philippinarum* exposed to CTL when compared to all the other treatments. Similar results were obtained in organisms under 22 ˚C. Between temperatures, significant differences were observed among all treatments, with higherGSH/GSSG ratio under 22 ˚C compared to 17 ˚C.

3.3.4. Histopathological Measurements

All results obtained from histopathological measurements were expressed as mean ± standard deviation for seawater and clams (Table 2).

3.3.4.1 Gills

Figure 2 shows the haemocytes infiltration (arrows), evident enlargement of the central vessel (long arrows), abundance of lipofuscin aggregates (*) in gills for each treatment at 17 ˚C and 22 ˚C.At 17 ˚C significant gills histopathological (I_h) differences were presented between all treatments with the exception among GO-PEI, Hg+GO-PEI and remediated seawater. Moreover, organisms exposed to CTL and Hg showed the lowest and the highest histopathological alterations, respectively. At 22 ˚C significant I_h differences were identified among all treatments in comparison to CTL. No significant differences were observed among clams exposed to remediated seawater compared to GO-PEI and Hg+GO-PEI. Organisms exposed to CTL and Hg showed the lowest and the highest gill alterations, respectively. Between the temperatures, significant differences were identified at CTL and Hg,with higher histopathological alterations under 22 ˚C compared to 17 ˚C.

3.3.4.2 Digestive Tubules

The haemocytes infiltration (arrows), abundance of lipofuscin aggregates (*) and atrophied (at) in digestive tubules of each treatment at both temperatures are shown in Figure 2. At 17 ˚C significant differences were shown among the treatments with the exception between CTL, Hg+GO-PEI and remediated seawater. At this temperature (17 ˚C), organisms exposed to remediated seawater showed the lowest I_h values, while the highest values were found at GO-PEI treatment. Organisms under 22 ˚C showed no significant I_h differences among CTL, GO-PEI, Hg+GO-PEI and remediated seawater. Organisms exposed to Hg showed the highest I_h values. Between the temperatures, significantly higher I_h values were found at 17 ˚C for GO-PEI and Hg+GO-PEI treatments, while in clams exposed to Hg higher values were obtained at 22 ˚C.

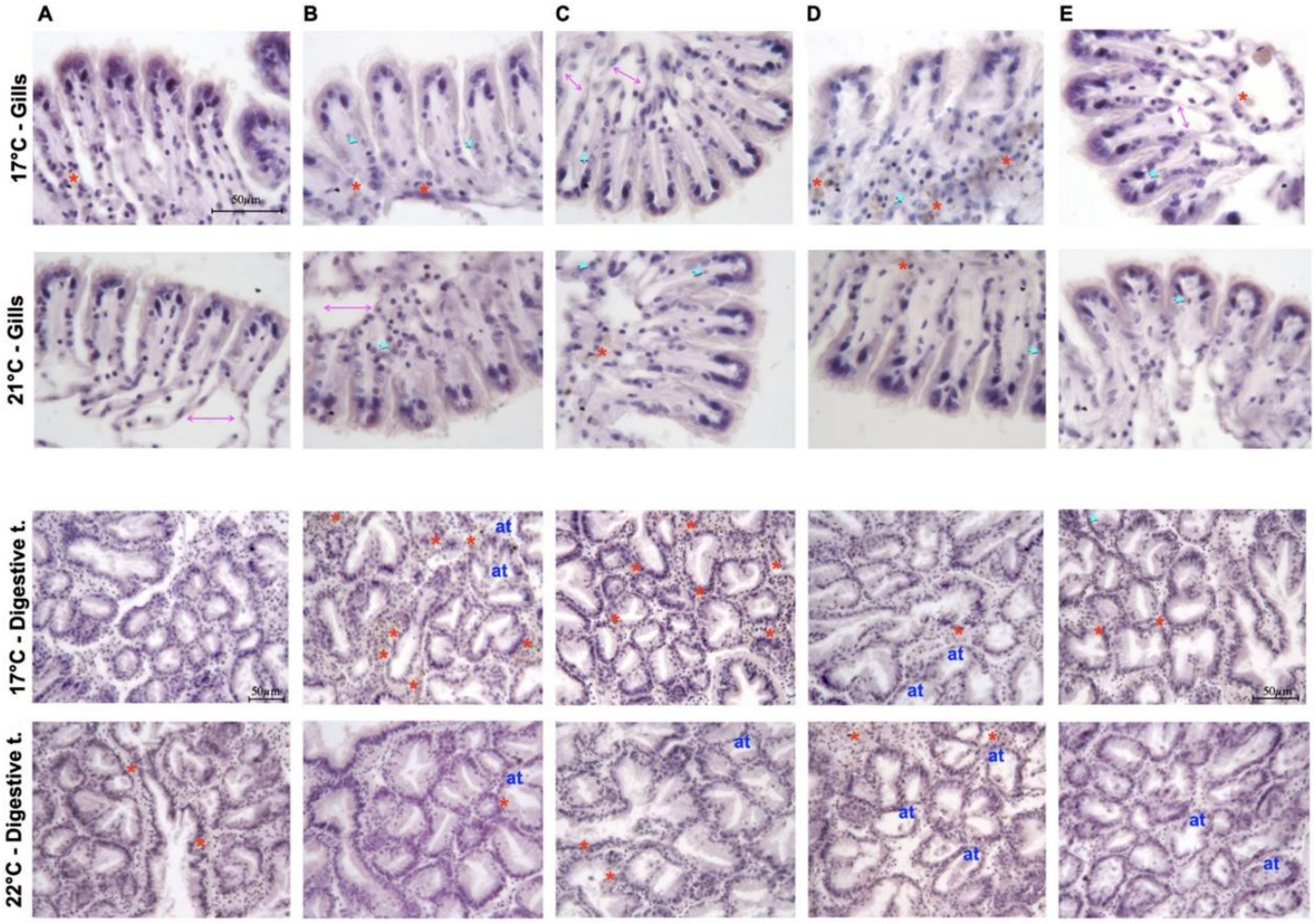

Figure 2. Micrographs of different tissues in *Ruditapes philippinarum* exposed to different treatments stained with haematoxylin. (i) Gills: haemocytes infiltration (light blue arrows), evident enlargement of the central vessel (pink long arrows), abundance of lipofuscin aggregates (red asterisks); (ii) digestive tubules: haemocytes infiltration (light blue arrows), abundance of lipofuscin aggregates (red asterisks) atrophied digestive tubules (blue *at*). Scale bar = 50 µm.

3.3.5 Integrated Biomarker Response (IBR)

The highest IBR value (4.27) was found for the clams exposed to Hg at 22 °C, which indicates higher impacts in Hg contaminated organisms under warming conditions. By contrast, lower IBR values were observed in organisms exposed to GO-PEI at 17 °C (2.01) and remediated seawater(2.20 and 2.09 at 17 and 22 °C, respectively). The results obtained for organisms exposed to the remaining treatments were showed in Table 2.

3.3.6 Multivariate Analysis

The principal coordinates ordination analysis (PCO) obtained for Hg in clams and water,biochemical and histopathological alterations is shown in Figure 3, with the PCO axis 1 explaining 45.3% of the total variation and PCO axis 2 21.6%. PCO1 separated organisms exposed to CTL (17 and22 °C), remediated seawater (17 and 22 °C) and GO-PEI (22 °C) in the positive side from the remaining treatments in the negative side. PCO2 separated organisms exposed to GO-PEI (17 °C) and Hg (17 °C) in the negative side from the remaining treatments in the positive side. Remediated seawater (17 and22 °C) as well as CTL (17 and 22 °C) clams were associated with GSH/GSSG values; clams exposedto GO-PEI (17 °C) were close related with the highest GRed and PC values; Hg contaminated clams at 17 °C were associated to the highest values of SOD; while Hg-contaminated clams at 22 °C were associated with the highest values of LPO and Hg concentrations in water and tissues.

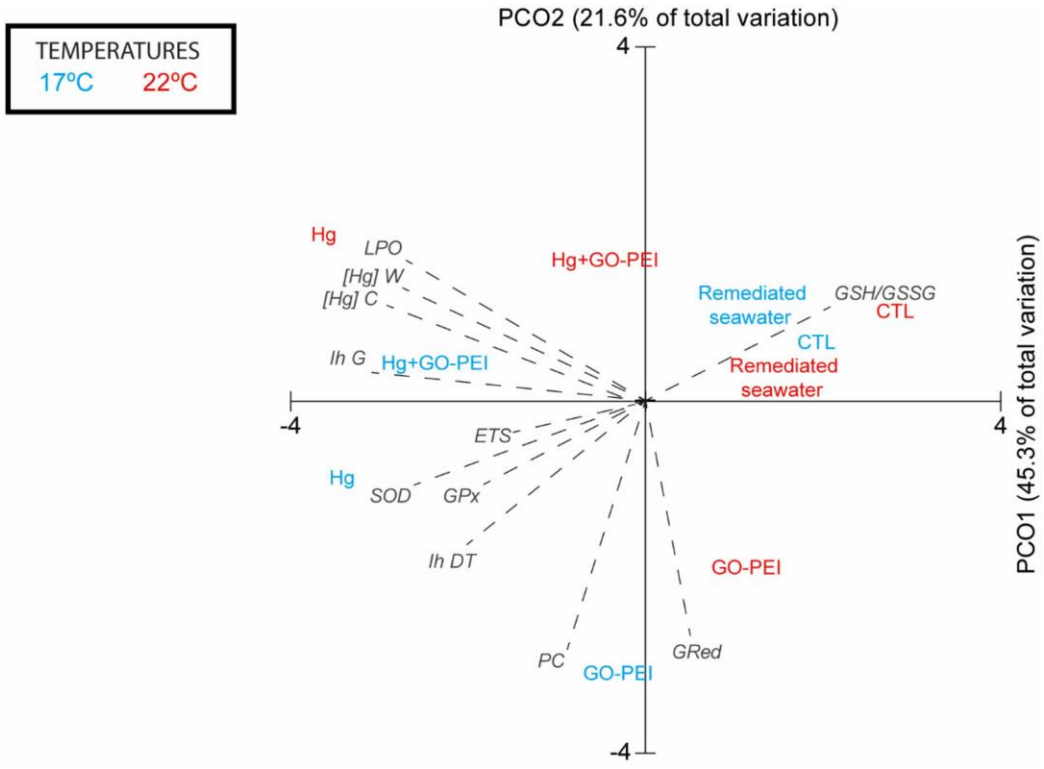

Figure 3. Principal coordinated ordination (PCO) analyses based on biochemical parameters, measured in *Ruditapes philippinarum* exposed to different conditions (CTL, GO-PEI, Hg+GO-PEI, Hg and Remediated seawater). Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data (r > 0.75): ETS, PC, SOD, LPO, GPx, GRed, GSH/GSSG, [Hg] C and [Hg] W,*Ih* DT and *Ih* G.

3.4 Discussion

In the present study the increase of seawater temperature influenced the accumulation of Hg, with higher concentration measured in *Ruditapes philippinarum* exposed to warming conditions (22 °C)compared to organisms at 17 °C. Higher Hg concentration in clams exposed to 22 °C may be related to increased metabolic capacity (measured by the electron transport system activity) observed at 22 °C. The present findings further revealed that clams under Hg+GO-PEI and remediated seawater treatments presented higher Hg concentration at control temperature (17 °C) than in warming conditions (22 °C), and in this case no differences were observed in terms of clam's metabolic capacity at both temperature regimes. These results can indicate that in the presence of GO-PEI (Hg+GO-PEI and remediated seawater treatments) Hg is not as easily accumulated at 22 °C as it is at 17 °C, a result that will need further investigation. Similarly, Leite et al. (2020) demonstrated that *Mytilus galloprovincialis* presented the highest accumulation of rutile at 18 °C compared to 22 °C. The authors explained that at higher temperature the lowest accumulation could be explained by higher precipitation of larger aggregates limiting the availability and accumulation of the contaminated particles. Nevertheless, higher accumulation of metals in bivalves under temperature rise in comparison to control temperature was previously observed in mussels (*M. galloprovincialis*) exposed to As (Coppola et al., 2018a), which was also associated with increased metabolic activity in these species. Nevertheless, Sanni et al. (2008) demonstrated that temperature (12, 20 and 28 °C) did not influence the accumulation of Cd in the oyster *Crassostrea virginica*; and Izagirre et al. (2014) showed similar results for the species *M. galloprovincialis* exposed to Cd at 18 and 26 °C. Therefore, the present and previous studies indicate that bioconcentration may not only depend on the exposure concentration levels, exposure time and temperature conditions but also theelement and its behavior. The present findings clearly demonstrated that biochemical responses and histopathological alterations were close related to stress induced by Hg bioaccumulation, with a lower influence of thetemperature on a clam's performance. These findings are evidenced by the PCO analysis, where clams exposed to control (CTL) and remediated seawater at both temperatures were grouped together in terms of biochemical and histopathological responses; clams exposed to GO-PEI at 17 and 22 °C were graphically close indicating similar biochemical performance of organisms under these conditions; while clams exposed to Hg and Hg+GO-PEI were apart from all the other treatments, indicating similar effects induced by the presence of Hg. These findings suggest that: (i) regardless of the temperature, clams exposed to remediated seawater were exposed to low stress conditions due to lower Hg exposure concentration and accumulation, with biochemical and histopathological responses similar to CTL organisms; (ii) clams exposed to Hg at both temperatures and Hg+GO-PEI at 17 °C presented the highest Hg concentrations in their tissues and showed similar biochemical performance and histopathological alterations. Similar biochemical and histopathological alterations induced in clams exposed to remediated seawater and CTL conditions at both temperatures revealed a low effect of temperature and indicate the low toxicity of remediated seawater as a consequence of low Hg concentration in this water. Higher GSH/GSSG values observed in clams under control conditions (17 and 22 °C) evidence the maintenance of the redox balance under these conditions, regardless of the temperature of exposure.It is well known that under non-stressful conditions organisms tend to

have higher reduced glutathione (GSH) in comparison to oxidized glutathione (GSSG) content, with higher GSH/GSSG values at non-stressful conditions (Coppola et al., 2018a,b; Marques et al., 2018; De Marchi et al., 2017). The results obtained indicate that the concentrations of Hgin remediated water were not high enough to induce significant alterations in clams compared to organisms in control conditions. Previous studies exposing bivalves to similar Hg concentrations also demonstrated limited biochemical impacts (Velez et al., 2015,2016). Furthermore, similar findings were already revealed by Coppola et al. (2019) when exposing bivalves to seawater previously contaminated by As and remediated by manganese-ferrite ($MnFe_2O_4$) nanoparticles. Clams exposed to GO-PEI, both under control and increased temperatures, evidenced limited biochemical and histopathological alterations, although higher than alterations observed in organisms under remediated seawater and CTL treatments but lower than clams exposed to Hg and Hg+GO-PEI treatments. The present results also demonstrated no interactive effects between the presence of GO-PEI and temperature rise, with no clear separation on a clam's responses exposed to GO-PEI at control and increased temperature. Previous studies investigating the impacts of similar nanoparticles evidenced limited biochemical alterations in bivalves (Coppola et al., 2019; Freitas et al., 2012; De Marchi et al., 2017). In the presence of Hg, both with or without GO-PEI, clams evidenced higher Hg concentrationsresulting in greater biochemical and histopathological alterations. This is due to the activation of antioxidant mechanisms in clams exposed to these conditions, which were inefficient to avoid cellular damage, especially under warming conditions. Considering that clams exposed to Hg and Hg+GO-PEI under warming conditions presented higher cellular damage than at 17 °C, and since Hg concentrations were lower in clams exposed to Hg+GO-PEI at 22 °C than at 17 °C, these results indicate that temperature rise will enhance the impacts caused by Hg and/or the sensitivity of clams towards this metal. Data on the IBR index also corroborate these results, with the highest values in clams exposed to Hg at 22 °C. Studies conducted by Coppola et al. (2018b) also showed the increase of oxidative stress in *M. galloprovincialis* when exposed to a combination of Hg and warming scenario. In the same species, Coppola et al. (2020) further demonstrated that the combination between GO-PEI and Hg caused lower oxidative stress and cellular damage than organisms only exposed to treatmentswith Hg.

3.5 Conclusions

Overall, the present study clearly showed that remedied seawater induces less biochemical andhistopathological alterations than Hg and GO-PEI treatments. Furthermore, the temperature rise seemed to enhance the impacts cause by Hg (both acting alone or combined with GO-PEI), which can negatively impact the clam's population growth and reproduction in future warming conditions and in the presence of Hg.

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CHAPTER 4.

Oxidative stress, metabolic and histopathological alterations in mussels exposed to remediated seawater by GO-PEI after contamination with mercury.

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Abstract

The modern technology brought new engineering materials (e.g. nanostructured materials) with advantageous characteristics such as a high capacity to decontaminate water from pollutants (for example metal(loid)s). Among those innovative materials the synthesis of nanostructured materials (NSMs) based on graphene as graphene oxide (GO) functionalized with polyethyleneimine (GO-PEI) had a great success due to their metal removal capacity from water. However, research dedicated to environmental risks related to the application of these materials is still non-existent. To evaluate the impacts of such potential stressors, benthic species can be a good model as they are affected by several environmental constraints. Particularly, the mussel *Mytilus galloprovincialis* has been identified by several authors as a bioindicator that responds quickly to environmental disturbances, with a wide spatial distribution and economic relevance. Thus, the present study aimed to evaluate the impacts caused in *M. galloprovincialis* by seawater previously contaminated by Hg and decontaminated using GO-PEI. For this, histopathological and biochemical alterations were examined. This study demonstrated that mussels exposed to the contaminant (Hg), the decontaminant (GO-PEI) and the combination of both (Hg + GOPEI) presented an increment of histopathological, oxidative stress and metabolic alterations if compared to organisms under remediated seawater and control conditions. The present findings highlight the possibility to remediate seawater with nanoparticles for environmental safety purposes.

Keywords

Toxicity, nanostructured materials, *Mytilus galloprovincialis,* metals, bioaccumulation, remediated seawater.

4.1 Introduction

The increase of pollutants in aquatic environments is closely related to the world population growth along with industrial and agricultural activities (Yi et al., 2011; Morais et al., 2012; Zhang et al., 2015). The rapid development and expansion of industry and other anthropogenic activities, such as mining, fuel and energy production, use of pesticides and fertilizers, metallurgy and high tech industries lead to the discharge of effluents containing potentially toxic elements (PTEs) into the aquatic environment, which puts aquatic ecosystems quality at risk (Chowdhury et al., 2016; Buffet et al., 2014; Ventura-Lima et al., 2009, 2011). Among the most common pollutants, mercury (Hg) stands out as frequent environmentally hazard (ATSDR, 2015) in the field of water policy, whose emissions should be progressively phased out by 2021 (Directive 2013/39/EU, 2013). Nevertheless, this metal is still the main component in electronic products, thermometers (for measuring high temperatures) and fluorescent lamps (Donnici et al., 2012; Briant et al., 2016; Pereira et al., 2008; Randall and Chattopadhyay, 2013). As a response to these hazards, throughout the last years a growing interest in the removal of PTEs from effluents has been observed, in particular Hg due to its harmful character to the environment and human health (Xiong et al., 2014; Zhang et al., 2014). Different methodologies to the decontamination of water have been developed, including chemical precipitation, among others, 2,4,6-trimercapto-1,3,5-triazine, trisodium salt nonahydrate (TMT) ($Na_3S_3C_3N_3 \cdot 9H_2O$) to precipitate metals (Hg, Zn, Pb, Cd) (Matlock et al., 2001; Henke et al., 2001); membrane filtration as ultrafiltration, reverse osmosis, nanofiltration (Aroua et al., 2007; Muthukrishnan and Guha, 2008; Pugazhenthi et al., 2005); coagulation and flocculation, as the use of chitosan, alum or ferric salts (Heredia and Martín, 2009; Renault et al., 2009); electrochemical methods and nanomaterials (Ali et al., 2012; Anjum et al., 2016; Huang et al., 2015; Li et al., 2010). Most of these methodologies are low cost but inefficient, or efficient but very costly (Babel and Kurniawan, 2003; Gehrke et al., 2015; Mohan and Pittman, 2007). For these reasons, it is important to develop alternative methods and new materials, such as nanostructured materials (NSMs), which have proved good effectiveness to remove metal(loid)s from water (Chen and Mao, 2007; Jackson et al., 2012; Paul et al., 2015; Vilela et al., 2016). Since the discovery of graphene in 2004, the 2-dimensional hexagonal network of carbon atoms graphitic material has received worldwide attention and research (Adeleye et al., 2015, 2016). Presently, graphene-based materials (GBM) are extensively investigated in electronics, biological engineering, being widely used in a vast range of applications including filtration systems, lightweight/strong composite materials, photovoltaic and energy storage, due to its excellent electrical conductivity, high mechanical strength and thermal conductivity, high impermeability to gases and optical transparency (Henriques et al., 2016; Yang et al., 2018). Due to its multifunctionality, GBM started to be investigated as an alternative to conventional water treatment methods, resulting very promising (Ali et al., 2018; Nupearachchi et al., 2017; Xiong et al., 2014; Zhang et al., 2014). For example, graphene oxide (GO) showed that oxygen functional groups play a key role in the absorption of metals, especially multivalent metal ions (Bian et al., 2015; Feng et al., 2011; Vilela et al., 2016). More recently, studies highlighted the performance of GO-functionalized with polyethyleneimine (PEI), GO-PEI, in freshwater, with removals of 35% for As, 96% for Hg, 99% for Cd, 96% for Pb, and 92% for Cr and 98% for Ni, using only 50 mg/L of GO-PEI

(all contaminants at equal initial concentration of 2.5 µmol/L) (Henriques et al., 2016; Rute, 2017). This material proved also to be very effective to remove Hg ions from seawater, with 81% of removal after just 6 h (results under publication). Despite the high removal efficiency, it is imperative to assess environmental toxicity risks derived from the application of these materials, especially for coastal aquatic systems, as this information is inexistent. In this context, benthic species are good biological models as they accumulate and reflect the impacts of different substances (Attig et al., 2014; Coppola et al., 2017, 2018; Freitas et al., 2018; Nardi et al., 2017; Velez et al., 2015; Hu et al., 2015; Banni et al., 2014). Studies conducted on oysters (*Saccostrea cucullata, Crassostrea gigas* (Gagnaire et al., 2004; Pan and Wang, 2011)), clams (*Anodonta anatina, Corbicula fluminea, Ruditapes decussatus, Ruditapes philippinarum* (Oliveira et al., 2017; Pan and Wang, 2011; Velez et al., 2016b)), mussels (*Perna viridis, Septifer virgatus, Mytilus galloprovincialis, Mytilus edulis* (Attig et al., 2014; Mubiana and Blust, 2007; Pan and Wang, 2011)) and cockles (*Cerastoderma edule* (Freitas et al., 2012)) showed the capacity of PTEs to impair organism's redox status, increasing the activity of antioxidant enzymes, and to decrease organisms metabolic capacity, namely when exposed to Hg (Raftopoulou and Dimitriadis, 2011; Azizi et al., 2018; Coppola et al., 2017, 2018; Mubiana and Blust, 2007; Nardi et al., 2017). The present study evaluated the toxicity of seawater, previously contaminated with Hg and remediated by GO-PEI, using the mussel *M. galloprovincialis.* This species is one of the most popular environmental bioindicators, presenting a wide spatial distribution and economic relevance (Fattorini et al., 2008; Kristan et al., 2014; Velez et al., 2016a, 2016b, 2016c; Coppola et al., 2017; Richir and Gobert, 2014; Mejdoub et al., 2017). It is a sedentary filter-feeder organism and possess a large capacity to accumulate pollutants (Coppola et al., 2018; Livingstone et al., 2000; Selvin et al., 2000). The experimental setup consisted of the exposure of mussels, during 28 days, to different treatments: clean seawater (control); remediated seawater; and seawater containing Hg (50 µg/L), GO-PEI (10 mg/L) or the mixture of both. At the end of the exposure period, Hg concentrations in mussel's soft tissues, histopathological alterations, as well as biochemical responses related to mussels' metabolic, oxidative stress and neurotoxic status were evaluated.

4.2 Materials and methods

4.2.1 Experimental treatments

The species *Mytilus galloprovincialis* was collected in the Ria de Aveiro, Portugal (40°38′51.1”N 8°44′05.5”W), with a mean body weight of 21.3 ± 6.61 g, mean length of 6.18 ± 0.46 cm and a mean width of 3.52 ± 0.27 cm. Organisms were transported from the field to the laboratory using plastic containers filled with seawater from the sampling site. A depuration and acclimation period of two weeks was performed placing mussels in 100 L tanks filled with artificial seawater (salinity 30 ± 1) (Tropic Marin® SEA SALT from Tropic Marine Center) at the temperature of 17.0 ± 1.0 °C and pH 8.0 ± 0.10. Seawater was renewed daily during the first three days. Then water was renewed every two-three days and animals were fed with Algamac protein plus (150.000 cells/animal). Temperature 17 °C, pH 8.0 and salinity 30 were selected considering values measured during mussel's collection and mean values observed during the year in the sampling area (IPMA, 2017). After this period, organisms were maintained under the same temperature, salinity and pH conditions but exposed to five different treatments as described in Table 1.

Table 1. *Mytilus galloprovincialis* exposed for 28 days in following experiment treatments.

TREATMENTS		DESCRIPTION
A:	**CTL**	Clean seawater (Hg 0.0 µg/L + GO-PEI 0.0 mg/L)
B:	**GO-PEI**	Clean seawater fortified with GO-PEI 10 mg/L
C:	**Hg + GO-PEI**	Clean seawater fortified with Hg 50 µg/L and GO-PEI10 mg/L
D:	**Hg**	Clean seawater fortified with Hg 50 µg/L
E:	**Remediated seawater**	Seawater previously contaminated with Hg (50 µg/L), and remediated by GO-PEI (10 mg/L) during 24 h.

Per treatment three aquaria were used with six individuals each. The concentration of mercury (Hg) used in the present study, 50 µg/L, was selected taking into consideration that this is the maximum allowable limit in wastewater discharges from industry (Directive 2013/39/EU, 2013). The graphene as graphene oxide (GO) functionalized with polyethyleneimine (GO-PEI) 10 mg/L was selected according to the capacity of this nanostructured material (NSMs) to remove Hg (preliminary assays). The remediated seawater was prepared by spiking clean seawater with Hg (1000 mg/L of Hg, Sigma Aldrich) first, and then remediating it using GO-PEI (10 mg/L) for 24 h, after which the material was separated from the seawater by filtration (remediated seawater). During the experimental period of 28 days, temperature and salinity were daily checked as well as mortality. Animals were fed with Algamac protein plus (150.000 cells/animal) three times per week. During the experiment, seawater from each aquarium was renewed weekly and treatments reestablished, including temperature, salinity and concentrations of Hg and GO-PEI. Seawater samples from each aquarium were collected immediately after weekly water exchange for Hg quantification aiming to compare real concentrations with nominal ones. At the

end of the exposure period (28 days), two organisms per treatment were fixed in Bouin's fluid for the histopathological evaluation and the remaining ones were frozen in liquid nitrogen. For biochemical and Hg concentration analyses mussel's soft tissues were carefully separated from shells and manually homogenized with a mortar and a pestle under liquid nitrogen. Each organism was divided into aliquots of 0.5 g fresh weight (FW) and stored at −80 °C.

4.2.2 Synthesis and characterization of graphene oxide functionalized with polyethyleneimine

Graphene oxide (GO) water dispersion (0.4 *wt* % concentration from Graphenea) was directly mixed with ethyleneimine polymer (PEI) solution 50 % (*w*/*v*) in water with M.W. ~750,000 (Sigma Aldrich) with a ratio of 24% *v*/*v* (GO/polymer). The pH of both solutions, GO and polymer, was adjusted to 2 before mixing, using 0.1 mol/L NaOH or HCl solutions. After mixing, the solution was rapidly shaken for 10 s to form a hydrogel. The hydrogel was freeze-dried (Telstar LyoQuest HT-40, Beijer Electronics Products AB, Malmoe, Sweden) at −80 °C obtaining three dimensional (3D) porous structures. The lyophilized samples were then washed in MilliQ water for 12 h to remove acidic residues. Finally, samples were freeze-dried again resulting in a foam (Figure 1).

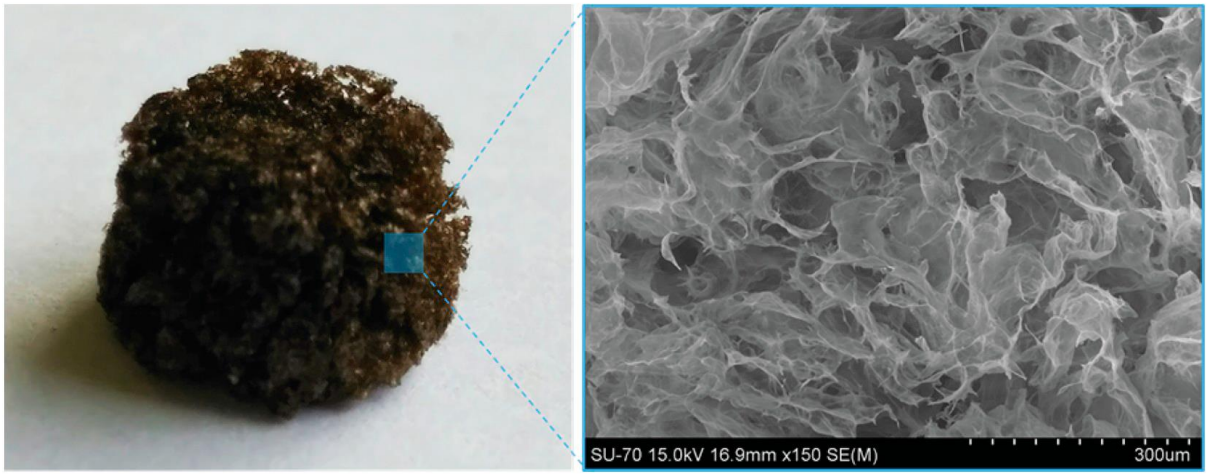

Figure 1. Photograph and SEM (*Scanning Electron Microscopy*) images at magnification of GO-PEI macrostructures after lyophilization.

4.2.3 Mercury quantification

The quantification of Hg in seawater aliquots was performed by cold vapor atomic fluorescence spectroscopy (CV-AFS), using a PSA 10.025 Millennium Merlin Hg analyser and SnCl2 (2% *m/v* in HCl 10 % *v/v*) as a reducing agent (Henriques et al., 2019). The calibration curve ($r^2 \geqslant 0.999$) was plotted and checked daily, using five standard solutions (0.0 – 0.5 µg/L). Detection and quantification limits obtained through blank measurements (n = 15) were 0.007 µg/L and 0.021 µg/L, respectively. Three replicate measurements were carried out for each sample (acceptable relative standard deviation between replicates: <5 %). The concentration of Hg in organism's tissues was quantified by thermal decomposition atomic absorption spectrometry with gold amalgamation (LECO model AMA-254), as described in Costley et al. (2000). Analytical quality control was performed by using Certified Reference Material, TORT 2 (*Lobster hepatopancreas*; 0.27 ± 0.06 mg/Kg (total Hg)), which was analysed every day prior to the beginning of the analysis and repeated at the end of the day. All percentages of recovery were within the accepted range of 99 – 113 %. Blanks, with the empty vessel, were analysed between samples to assure that no Hg was remained between analysis.

4.2.4 Biochemical markers

The selected biomarkers included: i) metabolic capacity (electron transport system activity, ETS); ii) energy-related parameters (glycogen content, GLY; total protein content, PROT); iii) phase I antioxidant enzymes activity (namely, enzymes superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx; glutathione reductase, GRed); iv) phase II antioxidant isoenzymes activity (glutathione-S-transferases, GSTs); v) extent of cellular damage (lipid peroxidation levels, LPO; protein carbonylation levels, PC); vi) redox balance (assessed by the ratio between reduced (GSH) and oxidized (GSSG) glutathione); and vii) neurotoxicity (acetylcholinesterase activity, AChE). The biochemical parameters were determined as described in Carregosa et al. (2014) and Coppola et al. (2019). All biochemical parameters were performed in duplicate and measurements made on a microplate reader (Biotek). All extractions for each biomarker were performed with specific buffers: i) samples used for ETS quantification were extracted in homogenizing buffer (0.1 mol/L Tris-HCl, pH 8.5, 15 % (*w/v*) PVP, 153 mmol/L magnesium sulfate ($MgSO_4$) and 0.2 % (*v*/v) Triton X-100); ii) for LPO determination samples were extracted with 20 % (*v/v*) trichloroacetic acid (TCA); iii) for GSH/GSSG quantification extraction was done using KPE buffer made with 0.1 % Triton X-100 and 0.6 % sulfosalisylic acid in KPE (0.1 mol/L potassium phosphate buffer, 5 mmol/L EDTA, pH = 7.5); iv) for the quantification of the remaining biomarkers (GLY, PROT, SOD, CAT, GPx, GRed; PC, GSTs, AChE) extraction was done using potassium phosphate buffer (50 mmol/L potassium dihydrogen phosphate; 50 mmol/L potassium phosphate dibasic; 1 mmol/L ethylenediamine tetra acetic acid disodium salt dihydrate (EDTA). These samples were disrupted using the TissueLyser II, which has the capacity of simultaneously disrupts multiple biological samples through high-speed shaking in plastic tubes with

stainless steel, tungsten carbide for 60 s at 4 °C. After this procedure samples were centrifuged for 25 min (or 10 min for GSH/GSSG) at 10000 *g* (or 3000 *g* for ETS) and 4 °C, and supernatants collected and stored at −80 °C or immediately used.

4.2.4.1 Metabolic capacity

The ETS activity was measured based on King and Packard (1975) and the modifications performed by De Coen and Janssen (1997). Absorbance was measured during 10 min at 490 nm with intervals of 25 s and the extinction coefficient (Ɛ) of 15,900 $(mol/L)^{-1}$ cm^{-1} was used to calculate the amount of formazan formed. Results were expressed in nmol per min per g FW.

4.2.4.2 Energy related parameters

For GLY quantification the sulphuric acid method was used, as described in Dubois et al. (1956). Glucose standards were used (0–10 mg/mL) to obtain a calibration curve. Absorbance was measured at 492 nm. The GLY content was expressed in in mg per g FW. The PROT content was determined following the Biuret method (Robinson and Hogden, 1940). Bovine serum albumin (BSA) was used as standards (0 – 40 mg/mL) to obtain a calibration curve. Absorbance was measured at 540 nm. The PROT content was expressed in mg per g FW.

4.2.4.3 Phase I antioxidant enzymes activity

The activity of SOD was quantified by following the method of Beauchamp and Fridovich (1971). The standard curve was obtained using SOD standards (0.25 – 60 U/mL). Absorbance was measured at 560 nm after 20 min of incubation at room temperature. The activity was expressed in U per g FW, where one unit (U) represents the quantity of the enzyme that catalyses the conversion of 1 µmol of substrate per min. The activity of CAT was quantified according to Johansson and Borg (1988). The standard curve was obtained using formaldehyde standards (0 – 150 µmol/L). Absorbance was measured at 540 nm. The enzymatic activity was expressed in U per g of FW, where U represents the amount of enzyme that caused the formation of 1.0 nmol formaldehyde per min at 25 °C. The activity of GPx was quantified following Paglia and Valentine (1967). Absorbance was measured at 340 nm in 10 s intervals during 5 min and the enzymatic activity was determined using the extinction coefficient Ɛ = 6.22 $(mmol/L)^{-1}$ cm^{-1}. The activity was expressed in U per g FW, where U represents the number of enzymes that caused the formation of 1.0 µmol NADPH oxidized per min. The activity of GRed was determined according to Carlberg and Mannervik (1985). Absorbance was measured at 340 nm and the enzymatic activity was determined using ε = 6.22 $(mmol/L)^{-1}$ cm^{-1}. The activity was expressed in U per g FW, where U represents the enzymes amount that caused the formation of 1.0 µmol NADPH oxidized per min.

4.2.4.4 Phase II antioxidant isoenzymes activity

The activity of GSTs was quantified following Habig et al. (1974). Absorbance was measured at 340 nm and the enzymatic activity was determined using ε = 9.60 $(mmol/L)^{-1}$ cm^{-1}. The activity was expressed in U per g FW, where U is defined as the amount of enzyme that catalysis the formation of 1 µmol of dinitrophenyl thioether per min.

4.2.4.5 Cellular damage

Levels of LPO were determined following the method described by Ohkawa et al. (1979). LPO levels were measured through the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation. Absorbance was measured at 535 nm and the extinction coefficient ε = 156 $(mmol/L)^{-1}$ cm^{-1} was used to calculate LPO levels, expressed in nmol of MDA formed per g of FW. Levels of PC were obtained following Mesquita et al. (2014) protocol. Absorbance was measured at 450 nm and the extinction coefficient ε = 22,308 $(mol/L)^{-1}$ cm^{-1} was used to calculated PC levels, expressed in nmol per g of FW.

4.2.4.6 Redox balance

GSH and GSSG were used as standards (0 – 60 µmol/L) and absorbance was measured at 412 nm (Rahman et al., 2007). The calculation of the ratio between oxidized and reduced glutathione was done in account the number of thiol equivalents (GSH/2 $*$ GSSG).

4.2.4.7 Neurotoxicity

Acetylthiocholine iodide (ATChI 5 mmol/L) substrates were used for the determination of AChE activity following the method of Ellman et al. (1961). Enzyme activities were recorded continuously for 5 min at 412 nm. The activity was expressed in nmol per min per g FW.

4.2.5 Histopathological markers

After the experimental period, mussels used for histopathological analyses were fixed in Bouin's fluid for 24 h at room temperature. Then, each specimen was placed in ethanol 70 % which was changed daily to wash out the fixative. Afterwards, organisms were gradually dehydrated from ethanol 70 % to absolute alcohol in graded alcohols, cleared in xylene and embedded in paraffin (56 – 58 °C). Serial sections (10 µm thick) obtained at microtome were mounted on albumin-coated slides and

stained with hematoxylin and toluidine blue as described in Pinto et al. (2019). At the end of this process, all specimens were analysed to identify histopathological alterations in gonads (female and males), gills and digestive tubules. For each individual and treatment, the histopathological index (I_h) in gills and digestive tubules was obtained based on Costa et al. (2013).

4.2.6 Integrated biomarker response

To integrate results obtained from biochemical markers and to try to understand the general mussel's biochemical response, the integrated biomarker response (IBR) was used, which was calculated according to Beliaeff and Burgeot (2002). Thus, results obtained from all studied biomarkers were used in order to evaluate the general response of *M. galloprovincialis* to different treatments. IBR index was calculated as described by Pinto et al. (2019). Biomarkers were arranged in the following order: SOD, CAT, LPO, GLY, GSTs, GPx, AChE, GRed, PROT, PC, ETS and GSH/GSSG. Values were discussed in terms of a general response given by the final IBR value, where higher values correspond to higher mussels' response.

4.2.7 Statistical analyses

Mercury concentration in seawater samples and mussel's soft tissues, biochemical markers and gills and digestive tubules histopathological indices, obtained for each tested treatment, were submitted to a statistical hypothesis testing using permutational analysis of variance, employing PERMANOVA+add-on in PRIMER v6 (Anderson et al., 2008). The null hypotheses tested were: i) for Hg concentration in seawater and mussels, no significant differences exist among treatments; ii) for each biochemical marker, no significant differences exist among treatments; iii) for histopathological results, no significant differences exist among treatments. Significant differences, between each pair of treatments, were assigned for a p-value < 0.05. In Tables 2 and 3 significant differences among treatments are represented with different lowercase letters. In figures (biochemical and histopathological markers) significant differences among treatments are represented with different uppercase letters (p-values are identified in Table 4).

4.3 Results

4.3.1 Mortality

At the end of the experimental period high mortality (44 %) was recorded in mussels submitted to Hg 50 µg/L (treatment D), while low mortality (11 %) was observed in treatments B (GO-PEI) and C (GO-PEI and Hg). No mortality was recorded in control (A) and remediated seawater (E) treatments during the entire experiment.

4.3.2. Mercury concentration in mussels and seawater

Mercury concentrations in seawater (Table 2) from treatment D was very close to the nominal concentration (50 µg/L). For treatment C the concentration of Hg was lower (35 ± 3.8 µg/L, while in treatments A and B were below the limit of quantification (Table 2). The remediated seawater (E) showed concentrations of 12 ± 1.9 µg/L, significantly lower than those recorded in Hg contaminated treatments (C and D, Table 2).

Table 2. Mercury concentration (µg/L) measured in water samples collected immediately after the weekly water renewal for each condition (A, B, C, D and E). Results correspond to the mean value and standard deviation of the four weeks. Different lowercase letters represent differences among the treatments. *n* = 12.

Hg water concentration (µg/L)	
A	≤ 0.01[a]
B	≤ 0.01[a]
C	35 ± 3.8[b]
D	49 ± 3.7[c]
E	12 ± 1.9[d]

Mercury concentrations in whole mussels´ soft tissue (Table 3) showed significant differences among treatments. In particular, organisms kept during 28 days exposed to GO-PEI presented significantly lower Hg concentrations of 0.081 ± 0.009 mg/Kg dry weight (DW) in comparison to the remaining treatments (A, C, D and E) (Table 3). Significantly higher concentration of Hg (42 ± 11 mg/Kg, DW) was detected in mussels exposed to treatment D in comparison to the other ones (A, B, C and E) (Table 3). Moreover, organisms exposed to remediated seawater (treatment E) showed significantly lower Hg concentration of 3.4 ± 0.4 mg/Kg (DW) than those exposed to Hg contaminated seawater (C and D, Table 3).

Table 3. Hg concentration in mussels soft tissues (mg/Kg), 28 days after the beginning of the experiment. Concentrations were measured in organisms from different conditions: (A, B, C, D and E). Different lowercase letters represent differences among the treatments. n = 9.

Hg mussels concentration (mg/Kg)	
A	0.17 ± 0.027^{a}
B	0.081 ± 0.0087^{b}
C	27 ± 4.7^{c}
D	42 ± 11^{d}
E	3.4 ± 0.41^{e}

4.3.3 Biochemical markers

4.3.3.1. Metabolic capacity

No significant differences were found between B and D treatments (Figure 2A, Table 4). Significantly lower values were found in these treatments in comparison to organisms under control (A) and remediated seawater (E) (Figure 2A, Table 4). No significant differences on ETS activity were observed among treatments A, C and E (Figure 2A, Table 4).

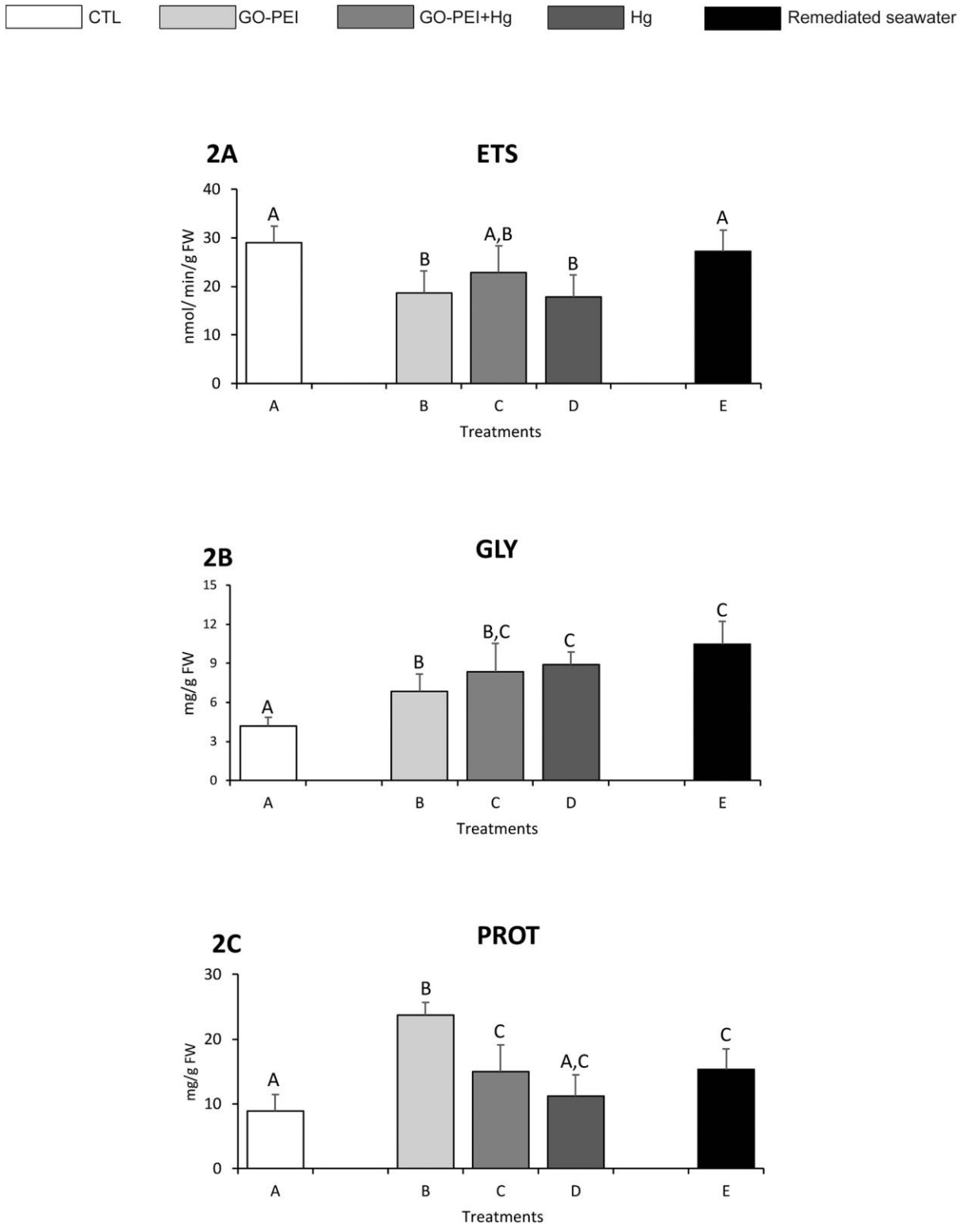

Figure 2. A: Electron transport system activity (ETS); B: Glycogen content (GLY); C: Total protein content (PROT) in *Mytilus galloprovincialis* exposed to different treatments (A, B, C, D and E) at the end of the experiment. Results are mean (*n*=9) + standard deviation. Significant differences among the treatments are presented with uppercase letters.

Table 4. *p-values, F- values, DFn, and DFd* (Degrees of freedom numerator and denominator, respectively) obtained by pair-wise comparisons between treatments (A, B, C, D and E) for each biomarker: Electron transport system (ETS); Glycogen (GLY); Total protein (PROT); Superoxide dismutase (SOD); Catalase (CAT); Glutathione peroxidase (GPx); Glutathione reductase (GRed); Glutathione-S-transferases (GSTs); Lipid peroxidation (LPO); protein carbonyl (PC); ratio between reduced and oxidized glutathione (GSH/GSSG); Acetylcholinesterase (AChE) and histopathological index: Gills; Digestive Tubules; Significant differences ($p \leq 0.05$) are highlighted in bold.

ρ < 0.05	**ETS**	**GLY**	**PROT**	**SOD**	**CAT**	**GPx**	**GRed**	**GSTs**	**LPO**	**PC**	**GSH/ GSSG**	**AChE**	**Gills**	**Digestive Tbulue**
A vs B	0.0005	0.0003	0.0001	0.0031	00.0068	0.0001	00.0002	00.0051	0.6755	00.0001	0.0001	0.0001	0.2376	0.0001
A vs C	0.0767	0.0001	0.0020	0.0025	0.0001	0.0001	0.0001	0.0004	0.0002	0.0001	0.0001	0.0001	0.2795	0.0148
A vs D	0.0006	0.0001	0.1670	0.0021	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0001	0.0008	0.0001
A vs E	0.4620	0.0001	0.0020	0.5634	0.1772	0.0001	0.0920	0.0451	0.0002	0.0001	0.0001	0.0001	0.0089	0.0447
B vs C	0.267	0.1255	0.0031	0.6695	0.0004	0.0591	0.1476	0.0115	0.0001	0.7717	0.0001	0.0142	0.8239	0.0252
B vs D	0.7690	0.0094	0.0005	0.8035	0.0001	0.0262	0.8466	0.0001	0.0001	0.5395	0.0004	0.0001	0.0020	0.0266
B vs E	0.0100	0.0028	0.0059	0.0070	0.4333	0.0562	0.0240	0.7822	0.0001	0.0001	0.0001	0.1206	0.1721	0.0350
C vs D	0.2126	0.4900	0.0873	0.8063	0.0027	0.7566	0.2952	0.0097	0.0005	0.6223	0.0070	0.0001	0.0015	0.0019
C vs E	0.2609	0.0750	0.8074	0.0027	0.0003	0.0002	0.0008	0.0464	0.0405	0.0001	0.0011	0.4220	0.1219	0.8237
D vs E	0.0078	0.1176	0.0722	0.0033	0.0001	0.0002	0.0389	0.0001	0.0001	0.0001	0.0001	0.0001	0.0698	0.0016
***F*-value**	*5.053*	*15.423*	*13.672*	*8.151*	*45.950*	*43.947*	*9.683*	*20.672*	*63.450*	*107.850*	*294.58*	*120.72*	*7.829*	*17.497*
F (DFn, DFd)			4, 40										4, 25	

4.3.3.2. Energy related parameters

Significantly lower GLY content was observed in organisms exposed to control (A) in comparison to organisms under the remaining treatments (Figure 2B, Table 4). Organisms exposed to remediated seawater (E) as well as organisms exposed to Hg (treatment D) showed significantly higher GLY content than organisms under control (A) and GO-PEI (B) (Figure 2B, Table 4). No significant difference was detected in organisms exposed to Hg + GO-PEI in comparison to the other ones with exception to the control (Figure 2B, Table 4). Significantly higher PROT content was observed in organisms exposed to GO-PEI contaminated seawater (B) in comparison to the remaining treatments (Figure 2C, Table 4). No significant differences in terms of PROT content were detected in organisms exposed to Hg and A, C and E treatments. Moreover, no significant differences were found between C and E treatments (Figure 2C, Table 4). On the other hand, the PROT content in organisms exposed to control (A) was significantly lower than the values observed in mussels exposed to B, C and E treatments (Figure 2C, Table 4).

4.3.3.3. Antioxidant and biotransformation enzymes activity

Significantly lower SOD activity was observed in organisms exposed to control (A) and organisms exposed to remediated seawater (E) in comparison to organisms exposed to contaminated treatments (B, C and D) (Figure 3A, Table 4). No significant differences on SOD activity were observed among B, C and D treatments (Figure 3A, Table 4). Significantly higher CAT activity was observed in organisms exposed to treatments D and C, with the highest values were in organisms exposed to Hg (treatment D) (Figure 3B, Table 4). No significant differences in terms of CAT activity were observed in organisms exposed to remediated seawater in comparison to organisms exposed to A and B treatments (Figure 3B, Table 4). Moreover, significantly lower antioxidant enzymes activity was observed in organisms exposed to control in comparison to B, C and D treatments (Figure 3B, Table 4). Significantly lower GPx activity was observed in organisms exposed to control (A) in comparison to the remaining treatments (Figure 3C, Table 4). No significant difference was observed in mussels exposed to Hg + GO-PEI and B treatment as well as between Hg + GO-PEI and D treatment. Mussels exposed to remediated seawater presented antioxidant activity similar to organisms exposed to GO-PEI (treatment B) (Figure 3C, Table 4). Significantly lower GRed activity was observed in organisms exposed to control (A) and to remediated seawater (E) in comparison to contaminated treatments (B, C and D) (Figure 3D, Table 4). No significant differences in terms of GRed activity were observed among treatments B, C and D (Figure 3D, Table 4). Significantly lower GSTs activity was observed in organisms exposed to control (A) in comparison to organisms exposed to contaminated treatments (B, C and D) (Figure 4, Table 4). Significantly higher GSTs activity was observed in organisms exposed to Hg (C and D) with the highest values in organisms exposed to Hg alone (treatment D) in comparison to the remaining treatments (Figure 4, Table 4). Mussels exposed under remediated seawater showed no significant differences in comparison to organisms at control and GO-PEI treatments (Figure 4, Table 4).

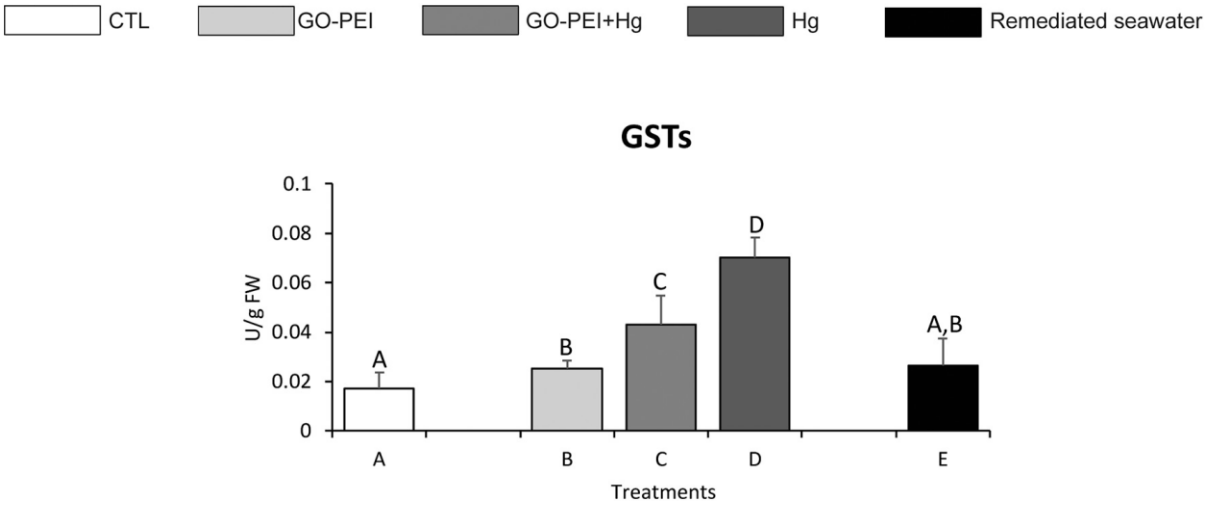

Figure 4. Glutathione-S-transferases activity (GSTs), in *Mytilus galloprovincialis* exposed to different treatments (A, B, C, D and E) at the end of the experiment. Results are mean (n = 9) + standard deviation. Significant differences among the treatments are presented with uppercase letters.

4.3.3.4. Cellular damage

Significantly lower LPO levels were observed in organisms exposed to control (A) and to GO-PEI (B) in comparison to remaining treatments (Figure 5A, Table 4). Significantly higher LPO levels were observed in organisms exposed to Hg treatments (C and D), with the highest values in organisms exposed to Hg alone (treatment D) (Figure 5A, Table 4). Mussels exposed to remediated seawater presented higher LPO than values recorded at control and GO-PEI treatments but significantly lower than values observed in organisms exposed to C and D treatments (Figure 5A, Table 4). Significantly lower PC content was observed in organisms exposed to control (treatment A) in comparison to organisms exposed to the remaining treatments (Figure 5B, Table 4). No significant differences in terms of PC values were observed in organisms exposed to contaminated seawater (B, C and D) (Figure 5B, Table 4). Mussels exposed to remediated seawater presented an intermediate PC value between control and contaminated treatments (Figure 5B, Table 4).

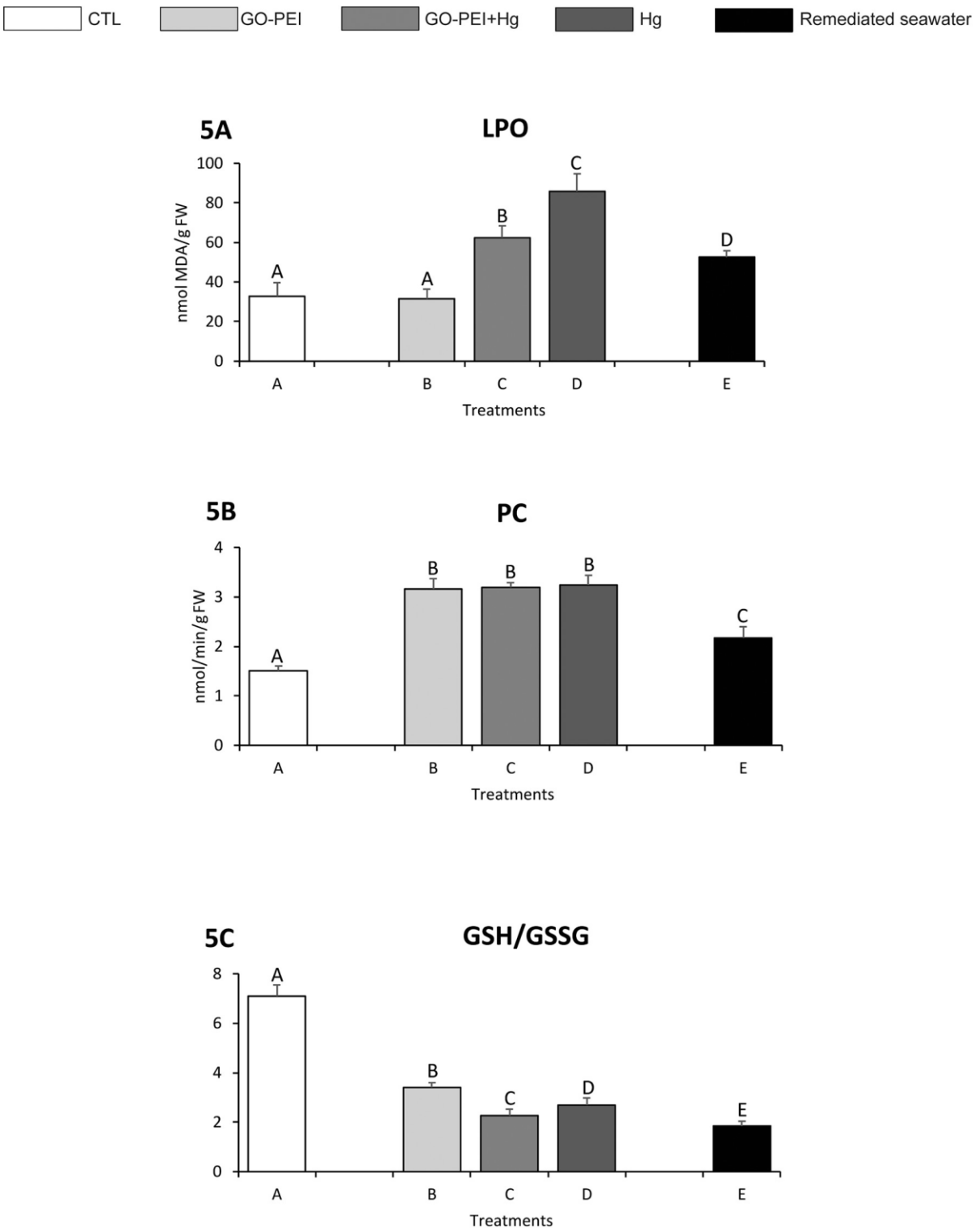

Figure 5. A: Lipid peroxidation levels (LPO); B: Protein carbonyl levels (PC); C: Ratio between reduced and oxidized glutathione (GSH/GSSG); in *Mytilus galloprovincialis* exposed to different treatments (A, B, C, D and E) at the end of the experiment. Results are mean (n = 9) + standard deviation. Significant differences among the treatments are presented with uppercase letters.

4.3.3.5. Redox balance

Significantly higher GSH/GSSG values were observed in organisms exposed to control (treatment A) treatment in comparison to contaminated (B, C and D) and remediated (E) seawater, with the lowest values in organisms exposed to remediated seawater (Figure 5C, Table 4).

4.3.3.6. Neurotoxicity

Significantly higher AChE activity was observed in organisms exposed to control in comparison to remaining treatments, with the lowest values in organisms contaminated with Hg (D) (Figure 6, Table 4). Mussels exposed to remediated seawater presented an intermediate AChE activity, with no differences to treatments B and C (Figure 6, Table 4).

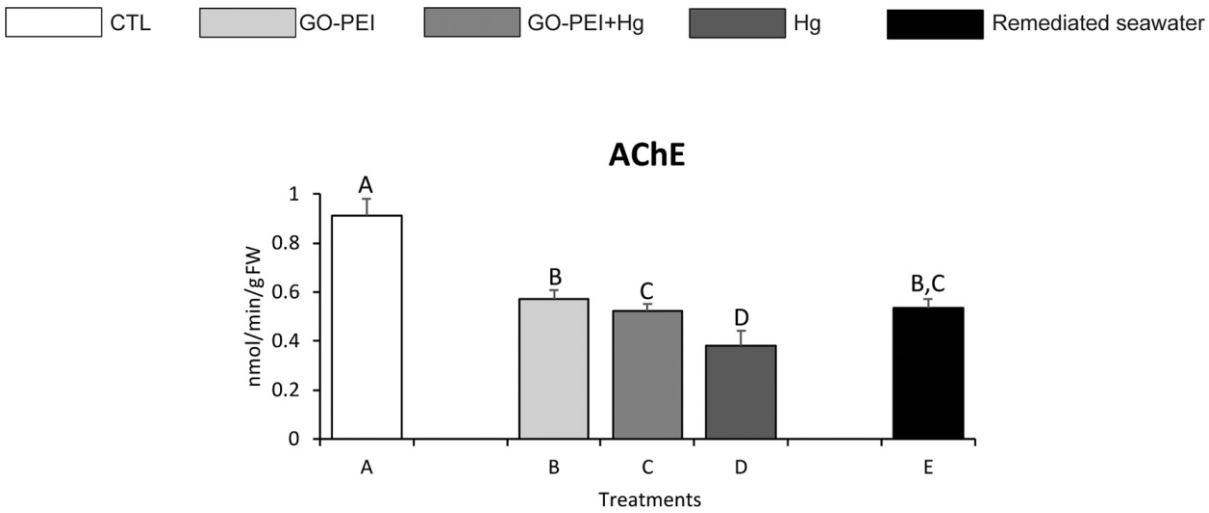

Figure 6. Acetylcholinesterase activity (AChE), in *Mytilus galloprovincialis* exposed to different treatments (A, B, C, D and E) at the end of the experiment. Results are mean (n = 9) + standard deviation. Significant differences among the treatments are presented with uppercase letters.

4.3.4. Histopathological markers

4.3.4.1. Gonads

Organisms exposed to control (A) and seawater with GO-PEI (B) showed abundant vitellogenic oocytes in gonads. Mussels exposed to Hg + GO-PEI and Hg (treatments C and D) showed a reduction in the number of the oocytes and the presence of necrotic forms (Figure 7). In females, no oocytes were detected in remediated seawater (E) (Figure 7). In males, no differences between control and treated mussels were observed (data not shown). No enough morphological effects were detectable to calculate the I_h in gonads.

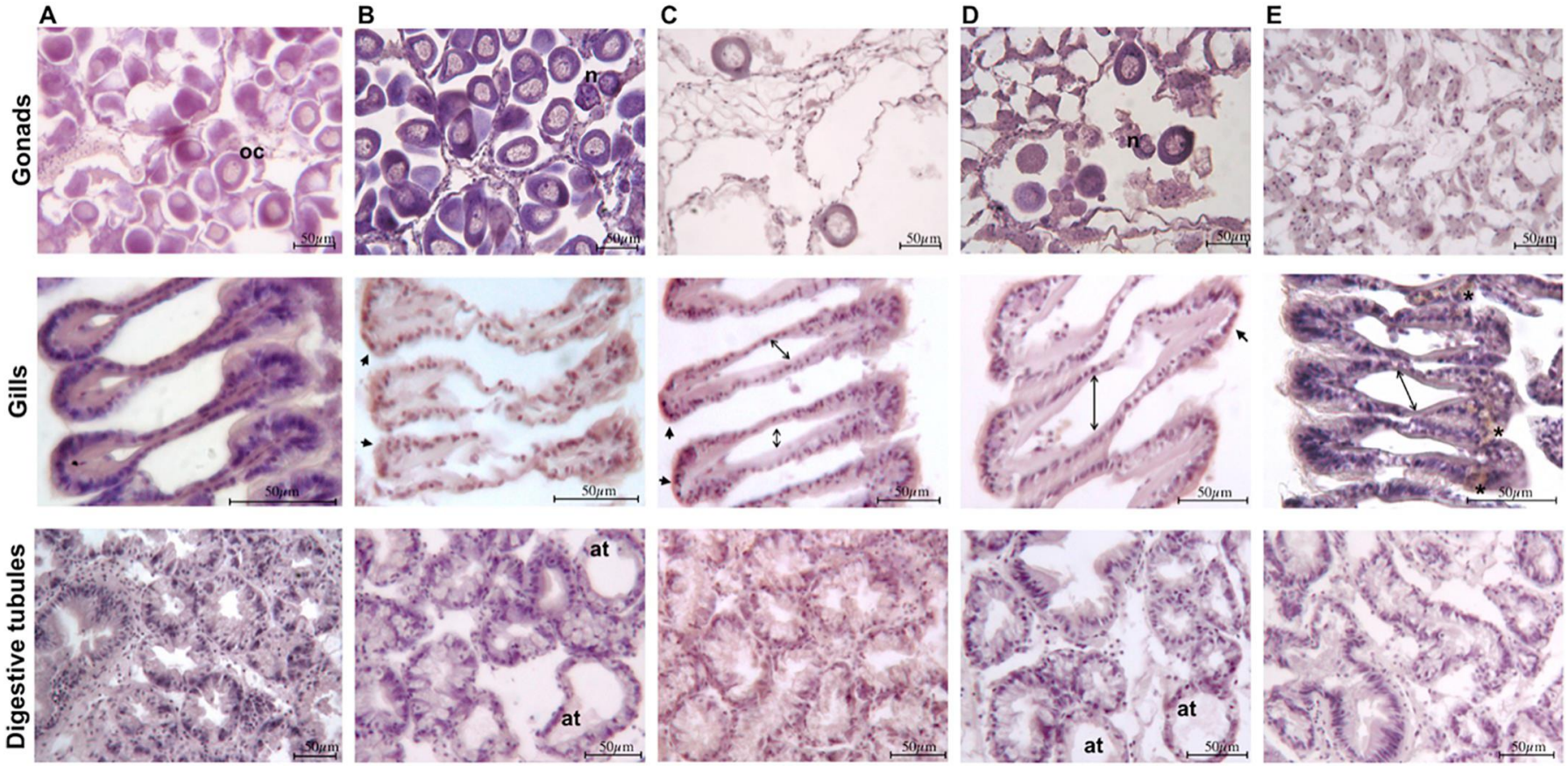

Figure 7. Micrographs of different tissues in *Mytilus galloprovincialis* exposed to different treatments stained with hematoxylin i) Ovaries: A and B exhibited normal ovarian follicles containing maturing oocytes (oc); Follicles after gametes release in mussels exposed to C, D and E. Necrotic (n) oocytes in B ovary. ii) Gills: A mussel gills with normal structure with frontal and lateral cilia (arrows); evident enlargement of the central vessel in C, Hg and E mussel (arrows); loss of cilia in B, C and D mussel (arrowheads); abundance of lipofuscin aggregates (*) in E mussels. iii) Digestive tubules: A, C and E digestive tubules showing a normal structure; atrophied digestive tubules (at) showing large lumen and thin epithelium in mussel exposed B and E. Scale bar = 50 μm

4.3.4.2. Gills

Gills of mussels kept in control condition (A) displayed epithelial cells with a regular distribution of lateral frontal cilia and no morphological abnormalities. The central vessel resulted tight. A very low number of lipofuscin aggregates and infiltrated haemocytes were observed (Figure 7). Exposure to Hg (D) leads to several histopathological alterations including cilia loss, haemocytes infiltration and enlargement of the central vessel (Figure 7). Mussels exposed to remediated seawater (E) showed less histopathological alterations but still enlargement of the gill vessel and lipofuscin aggregates were observed (Figure 7). For gills, significantly lower I_h was obtained for organisms exposed to treatment A in comparison to treatments D and E (Figure 8A, Table 4). No significant differences were observed between I_h of mussels exposed to A, B and C treatments (Figure 8A, Table 4). Significantly higher I_h values were found in organisms exposed to Hg alone (treatment D) in comparison with organisms exposed to GO-PEI and combination of both (treatments B and C, respectively) (Figure 8A, Table 4). No significant differences in I_h were observed among B, C and E treatments (Figure 8A, Table 4).

4.3.4.3. Digestive tubules

Mussels' digestive tubules under control treatment (A) (Figure 7) result constituted by a single layer of cells surrounding a narrow or occluded tubular lumen. Atrophy increment was observed in organisms exposed to GO-PEI and Hg (treatments B and D, respectively), but especially in the treatment D (Figure 7). Lipofuscin aggregates were detected in organisms exposed to Hg+GO-PEI (C), while under remediated seawater (E) digestive tubules showed an increase in haemocyte infiltration. In all analysed organisms' necrosis was never detected. Significantly lower I_h was obtained in organisms exposed to treatment A in comparison to the remaining treatments (Figure 8B, Table 4). Comparing I_h values significantly higher values were observed in organisms exposed to Hg (treatment D) in comparison to the other ones (Figure 8B, Table 4). No significant differences were found between mussels exposed to C and E (Figure 8B, Table 4).

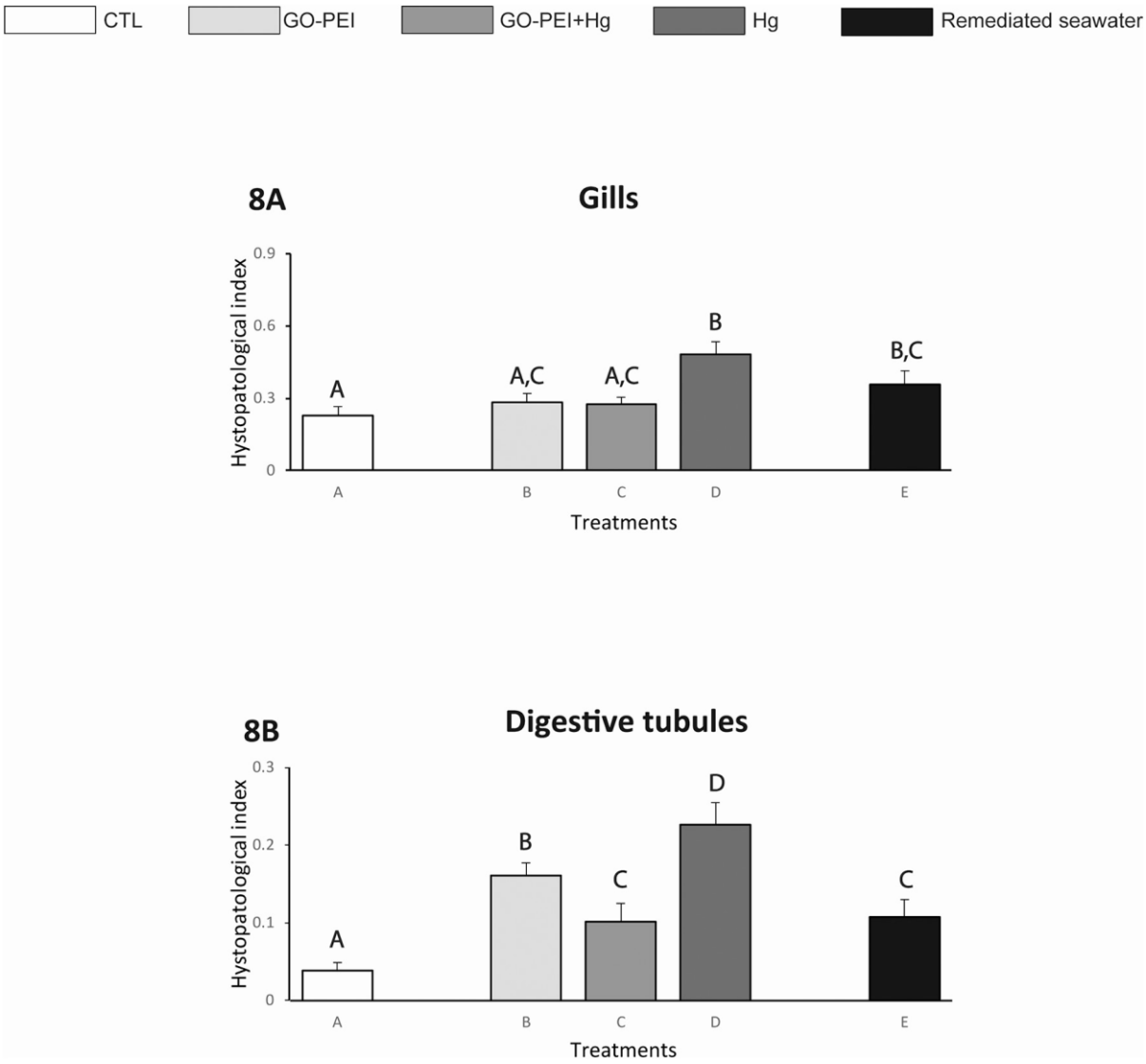

Figure 8. A: Histopathological index in gills; B: Histopathological index in digestive tubules in *Mytilus galloprovincialis* exposed to different treatments (A, B, C, D and E) at the end of the experiment. Results are mean (*n* = 3) + standard deviation. Significant differences between treatments are represented with uppercase letters.

4.3.5. Integrated biomarker response (IBR)

The highest IBR value (8.60) was found for the mussels exposed to Hg, while the lowest value (0.10) was observed in the remediated seawater (treatment E). Moreover, the results obtained for organisms exposed to treatments B and C showed IBR score of 2.01 and 4.08, respectively.

4.4 Discussion

In the last few years, a series of investigations involving the use of different synthetic materials (e.g. nanoparticles (NPs)) for water remediation (namely from metal(loid)s), have been conducted (Nupearachchi et al., 2017; Zhang et al., 2010; Coppola et al., 2019; Gehrke et al., 2015; Mohmood et al., 2016; Davidescu et al., 2015). However, the combination between nanoparticles (NPs) and metal (loid)s (for example Hg and As) can generate an interactive toxic influence in the environment, especially in aquatic systems (Coppola et al., 2019; Moore, 2006; Fabrega et al., 2011). Previous studies based on the NPs such as zinc oxide (ZnO_2-NPs), titanium dioxide (TiO_2-NPs), gold nanoparticles (AuNPs) and carbon nanotubes (CNTs) were focused only on NPs toxicity to marine organisms as mussels (*Mytilus edulis* and *M. galloprovincialis*), oysters (*Crassostrea gigas*) and clams (*Ruditapes philippinarum*) (Sun et al., 2016; Volland et al., 2015; Freitas et al., 2018; De Marchi et al., 2017a; Pan et al., 2012; Canesi et al., 2015; Trevisan et al., 2014). These studies reported alterations on organism's metabolic capacity and energy reserve contents as well as increase of oxidative stress and cellular damage when the organisms were in contact with NPs and metal(loid)s. However, scarce information is available on the use of NPs to remove contaminants from water and the possible toxic impacts of this remediated water towards marine organisms. Recently, Coppola et al. (2019) observed lower cellular damage and oxidative stress but higher metabolism in *M. galloprovincialis* after 28 days of exposure to remediated seawater (previously contaminated with As (1000 µg/L) and decontaminated by $MnFe_2O_4$, NPs (50 mg/L)) in comparison to contaminated seawater. Still, to the best of our knowledge, no studies evaluated the potential toxic effects, in marine organisms (e.g. bivalve), resulting from seawater decontaminated from metal(loid)s using graphene oxide functionalized with polyethyleneimine (GO-PEI). The present study demonstrated that when under Hg contaminated seawater mussels *M*. *galloprovincialis* presented higher concentration of mercury (Hg) in their tissues in comparison to organisms exposed to remediated seawater. These results are in line with previous studies that showed the capacity of bivalves (e.g. oysters, clams and mussels) to accumulate metal(loid)s (Hg, lead (Pb) and As) when exposed to high concentrations of these contaminants (Coppola et al., 2019, 2018; Pirone et al., 2019; Freitas et al., 2019; Moreira et al., 2017). Previous studies also demonstrated that the accumulation of metal (loid)s by bivalves may lead to changes on their metabolic capacity (assessed by measuring the activity of ETS) and the occurrence of cellular damages, including peroxidation of the lipid membrane and carbonylation of proteins. Furthermore, as already demonstrated from the literature, under elevated concentrations of metal(oid)s bivalves are also able to activate their antioxidant defences in order to eliminate reactive oxygen species (ROS), and avoid or limit cell damages. The present study showed that mussels exposed to Hg (50 µg/L, corresponding to the maximum permissible concentration of Hg in wastewater discharges (treatment D)), had a decrease on their metabolic capacity in comparison with the control (treatment A). This decrease in mussel's metabolism was also observed in previous studies that assessed bivalve's metabolism after metal(loid)s exposure (Coppola et al., 2017, 2018; Bielen et al., 2016; Freitas et al., 2017). A similar pattern was observed in mussels exposed to GO-PEI (treatment B). A decrease on organism's metabolic capacity was already observed in other bivalve species (mussels*, Mytiuls*

coruscus, and clams, *Ruditapes philippinarum*) exposed to NPs of carbon and ZnO_2 (De Marchi et al., 2017a; Huang et al., 2015). This fact may indicate that mussels exposed to potentially toxic substances can prevent their impacts by reducing their metabolic capacity through the closure of their valves as identified by Gosling (2003). When analysing mussels exposed to the combination of Hg and GO-PEI, results revealed an ETS capacity similar to control and remediated seawater. These findings contrast with a study conduct by Freitas et al. (2018) that showed a decrease of *R. philippinarum* metabolic capacity exposed to As and carbon nanotubes treatment. Probably the binding of Hg by GO-PEI led to a decrease in water toxicity, either because a) the GO-PEI + Hg complex remained in solution but is less toxic to organisms than free Hg, or b) the GO-PEI + Hg complex settled, being unavailable for uptake by the mussels. Moreover, a similar metabolic capacity was observed when comparing mussels exposed to remediated seawater (treatment E) with control, which may result from lower Hg concentration observed in mussel's tissues under this condition. Similarly, Coppola et al. (2019) demonstrated that the ETS activity was significantly higher in organisms exposed to decontaminated seawater in comparison to organisms exposed to As contaminated seawater, with values obtained in mussels under decontaminated treatments closer to control than to contaminated ones. As a response to metabolic decrease, the results obtained revealed higher PROT and especially GLY content in mussels exposed to Hg (treatment D) when compared to the control, demonstrating the capacity of organisms to save their energy reserves under reduced metabolic activity. In accordance to these findings, other studies have also showed lower metabolic activity associated with an increase of energy reserves content in marine organisms exposed to metal(loid)s, namely in *M. galloprovincialis* exposed to Ti (titanium), Hg and As (Coppola et al., 2017, 2018; Freitas et al., 2017; Monteiro et al., 2019). A similar pattern was observed when mussels were exposed to GO-PEI (treatment B) which is also in accordance with previous studies by De Marchi et al. (2017a, 2017b) that showed an increase of GLY and PROT content associated with lower ETS when the polychaete *Diopatra neapolitana* was exposed to graphene oxide (GO). Moreover, the increase of energy reserves was observed in mussels exposed to combination of GO-PEI and Hg (treatment C) in comparison to control. In accordance to these results, Freitas et al. (2018) highlighted the increase of energy reserves in clams *R. philippinarum* exposed to combination of As and functionalized multi-walled carbon nanotubes (f-MWCNTs). The present findings further revealed an increase of energy reserve content in organisms exposed to remediated seawater (treatment E) compared to control organisms. These findings may indicate that mussels under this treatment were able to avoid the expenditure of energy reserves, although their metabolic capacity was similar to control values. Although mussels tend to decrease their metabolic capacity when exposed to any of the contamination treatments (B, C and D), an increase of antioxidant enzymes activities was observed in mussels exposed to these treatments, demonstrating the capacity of bivalves to activate their antioxidant defences to fight against the excess of ROS produced by the presence of Hg, GO-PEI or both contaminants acting in combination. In particular, mussels exposed to Hg (treatment D) increased the activity of antioxidant enzymes in comparison to the control and remediated seawater (treatment E). A similar pattern was observed when mussels exposed to GO-PEI (treatment B). Also, mussels exposed to the combination of Hg and GO-PEI showed an increase of SOD, CAT, GRed, GPx activities in comparison to organisms exposed to control and remediated seawater (E). These results

are in line with analogues studies based on marine organisms (mussels, *M. galloprovincialis* and clams, *R. philippinarum*) exposed to metal(loid)s (Pb, Hg and As) (Pirone et al., 2019; Velez et al., 2016ab; Coppola et al., 2017, 2018), NPs (De Marchi et al., 2017ab; Xia et al., 2017; Cid et al., 2015; Gomes et al., 2012), or the combination of NPs and metal(oid)s (Coppola et al., 2019; Freitas et al., 2018). Moreover, similar antioxidant activity was observed comparing mussels exposed to control and remediated seawater (treatment E) which indicate that remediated seawater induced lower toxicity than seawater with Hg, GO-PEI or GOPEI with Hg (treatments D, B and C, respectively) and low Hg accumulated in mussels resulted in a similar defence activity. Similarly, a study conducted by Coppola et al. (2019) demonstrated that mussels exposed to control and As remediated seawater presented similar antioxidant enzymes activity while mussels exposed to contaminated treatments showed activation of their antioxidant defences. Regarding the antioxidant phase II defence mechanisms, GSTs enzymes are known for their ability to catalyse the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates (Regoli and Giuliani, 2014). In the present study, comparing to control, GSTs clearly increased their activity on mussels exposed to Hg (treatment D), the combination of both contaminants (treatment C) and at a lower extent in mussels exposed to GO-PEI acting alone. These results are in line with previous studies that revealed that GSTs are activated by the presence of contaminants (including As, Pb and Hg) in bivalves including clams and mussels (Freitas et al., 2016, 2017, Velez et al., 2016abc, Coppola et al., 2017, 2018; Jaishankar et al., 2014). Previous studies also showed an increase in bivalves' biotransformation activity related to combination of NPs and metal(loid)s exposure compared to the control (Freitas et al., 2018; Coppola et al., 2019). Lower GSTs activity in mussels exposed to treatment C and especially treatment B may indicate that GSTs are not involved in the biotransformation of NPs of carbon, namely in the biotransformation of GO-PEI into less toxic excreted substances. Similarly, De Marchi et al. (2017) showed that clams exposed to f-MWCNTs presented lower GSTs activity compared to non-contaminated clams. In this study, organisms at control treatment had a similar GSTs activity of mussels exposed to remediated seawater. Lower GSTs activity in mussels exposed to remediated seawater may indicate that the concentration of Hg in this treatment was not enough to activate these enzymes, which agrees with the recent work by Coppola et al. (2019) that reported similar GSTs activity in mussels exposed to As seawater decontaminated and control organisms. Although antioxidant and biotransformation enzymes were activated in contaminated mussels (treatments C and D), these defence mechanisms were not enough to prevent cellular damages in mussels exposed to these treatments (identified by higher LPO and PC levels). The increase of antioxidant and biotransformation defences may prevent cellular damages, avoiding the occurrence of LPO and PC (Li et al., 2008; Yang et al., 2008; Chang et al., 2011; Coppola et al., 2017, 2018; Freitas et al., 2019). In the present study Hg, GO-PEI, and the combination of both showed cellular damages and loss of redox balance (shown by lower GSH/GSSH ratio), demonstrating the inefficient capacity of antioxidant and biotransformation enzymes to eliminate the excess of ROS and avoid LPO and PC, as well as the maintenance of redox homeostasis. Similar results were observed by Coppola et al. (2018) in mussels exposed to Hg concentration, by De Marchi et al. (2017a) in *D. neapolitana* under the presence of high GO concentration reduced the GHS/GSSG ratio and increased the LPO value, and by Freitas et al. (2018) that showed the induction of cellular damages on mussels

exposed to f-MWCNTs and As. Furthermore, although at a limited extent, mussels exposed to remediated seawater showed higher LPO and PC levels than control organisms and lower GSH/GSSG values. These findings may indicate that although less Hg was accumulated, lower metabolic changes were observed, and limited activation of antioxidant and biotransformation enzymes was observed in mussels exposed to this treatment that still presented oxidative stress and cellular damages. Opposite results were found by Coppola et al. (2019) that reported a decrease of LPO and PC values followed by increase of GSH/GSSG ratio in mussels under seawater decontaminated from As by $MnFe_2O_4$ NPs in comparison to non-remediated conditions. Such findings may indicate higher toxicity of Hg remediated seawater in comparison to As remediated seawater. In marine bivalves the neurotoxic impacts of different pollutants were evidenced by the decrease on AChE activity due to its high affinity for many neurotoxic compounds such as metal(loid)s and NPs (Wang et al., 2009; Maisano et al., 2017; Coppola et al., 2019; Freitas et al., 2018). The present results evidenced that mussels exposed to contaminated and remediated seawater showed neurotoxicity effects, with lower AChE activity under these treatments in comparison to control organisms. In accordance, several other studies revealed similar impacts with inhibition of AChE activity when organisms are under the presence of metal(loid)s (Coppola et al., 2017, 2018; Freitas et al., 2017; Pirone et al., 2019), f-MWCNTs (De Marchi et al., 2018) or the combinations of f-MWCNTs and As (Freitas et al., 2018). Also Coppola et al. (2019) showed similar results in mussels *M. galloprovincialis* exposed to seawater previously contaminated with As and remediated by $MnFe_2O_4$ NPs. The results obtained with IBR index showed higher values in mussels exposed to Hg contaminated seawater. These results are in agreement with several studies which showed higher IBR values in bivalves exposed to pollutants, including Hg (Bigot et al., 2011; Pinto et al., 2019; Yuan et al., 2017; Marigomez et al., 2013; Serafim et al., 2012). On the other hand, the lowest IBR index was observed in *M. galloprovincialis* exposed to GO-PEI. This result disagreed with previously similar study by Coppola et al., 2019 which showed an increase of IBR index when mussels were exposed to NPs. Also, Xia et al. (2017) detected the highest oxidative stress and histopathological alteration in marine scallop *Chlamys farreri* under higher concentration of TiO_2 NPs correlated to increase of IBR values. Still, to our knowledge, no studies evaluated the potential toxic effects, in marine organisms (e.g. bivalve), resulting from seawater decontaminated from metal(loid)s using GO-PEI or the combination of Hg and GO-PEI. In what regards to histopathological alterations, the present study also demonstrated the toxic action of Hg in mussels' tissues. In particular, obtained results showed that female gonads exposed to Hg had semi-empty follicles with the few damaged oocytes, while loss of cilia, haemocytes infiltration and enlargement of the central vessel were observed in gills. Similar histopathological alterations were observed by other authors when exposing mussels to contaminants (Amachree et al., 2014; Cuevas et al., 2015; Sonawane, 2015; Sunila, 1988), including the study by Maisano et al. (2017) that showed several histopathological changes on mussels' gills collected from a heavily Hg polluted area. The lack of cilia may lead to difficulties in filtering food and breathing problems, compromising the survival of the animals (Pagano et al., 2016). Haemocyte infiltration is often observed in organisms exposed to xenobiotics and it is a sign of inflammation. Also, the digestive tubules of mussels exposed to Hg resulted seriously damaged showing an increase of atrophy with a reduction in thickness of epithelia accompanied by the enlargement of the tubules lumen

and haemocytes infiltration. The digestive tubules of molluscs is the main organ for detoxification of xenobiotic compounds, and it has therefore been extensively used for toxicity assessment (Moore and Allen, 2002, Livingstone et al., 1992). Therefore, severe alterations in this organ could seriously affect survival of the organisms. The present study further showed that while in female gonads GO-PEI induces necrosis and apoptotic events in mature oocytes, in gills the nanostructured materials did not have any significant effect. The I_h for gills resulted quite similar to control, suggesting no uptake of the GO-PEI at gills level. At digestive tubules level, instead, exposure to GO-PEI led an increase of histopathological damages resulting in a higher I_h not only than control, but also than C (GO-PEI + Hg) and E (remediated seawater) treatments while lower than treatment D (Hg exposure). The main alterations encountered on digestive tubules were the presence of cellular atrophy with changes in the morphology of the epithelial cells, that became thinner. Although these results have no comparison in the literature due to the lack of studies performed with GO-PEI, a study on zebrafish exposed to GO for 14 days revealed several cellular alterations of the liver tissue (Chen et al., 2016). Furthermore, another study, using GO, showed numerous degenerative changes in gut and testis cells of the cricket *Acheta domesticus* (Dziewięcka et al., 2017). Up today no studies are available on histopathological alterations induced by the combination of Hg and GO-PEI. However, in this work the gills tissue did not appear to be affected. In fact, the gills' I_h results were similar to treatments A and C. Also, for the digestive tubules the histopathological impact of the Hg combined to GO-PEI resulted lower than Hg and GO-PEI by them self, even though the I_h calculated was slightly higher than control. These findings suggest that the combination of Hg and GO-PEI may prevent damages induced by Hg and hypothetically other metals too. Regarding the results of organisms exposed to remediated seawater, the gills showed higher I_h at treatment E in comparison to control but lower values in comparison to the treatments B and D. The most common histopathological alteration observed in gills on treatment E was the presence of lipofuscin aggregates. Lipofuscin results from the oxidation of membrane lipids and proteins by free radicals. The presence of lipofuscin aggregates may indicate oxidative stress or defective detoxification of free radicals in the affected cells (Hohn and Grune, 2013).

4.5. Conclusion

The present study clearly shows that mussels exposed to contaminated treatments presented higher impacts than the ones under remediated seawater, with greater activation of antioxidant and biotransformation mechanisms, cellular damage, loss of redox balance and decrease of metabolic capacity in mussels exposed to Hg, GO-PEI and the combination of both contaminants. The present study further demonstrated that mussels exposed to contaminated seawater presented higher histopathological alterations than mussels under remediated seawater and control treatments. The results obtained with IBR index also corroborate the results obtained with higher values obtained for Hg exposed mussels, followed by mussels exposed to Hg + GO-PEI and mussels exposed to GO-PEI with the lowest values. Overall, these results are innovative since, up to our knowledge, no published information is available on the toxic effects induced in mussels when exposed to Hg contaminated seawater remedied by GO-PEI, which also revealed the low toxicity.

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CHAPTER 5.

The influence of temperature increase on the toxicity of mercury remediated seawater using the nanomaterial graphene oxide on the mussel *Mytilus galloprovincialis*

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Abstract

Mercury (Hg) has been increasing in waters, sediments, soils and air, originated by natural events and anthropogenic activities. In aquatic environments, especially marine systems (estuaries and lagoons), Hg is easily bioavailable and accumulated by aquatic wildlife, namely bivalves due to their lifestyle characteristics (sedentary and filter-feeding behavior). Therefore, in the last years, different approaches have been developed with the objective to remove metal(loid)s from the water, including the employment of nanomaterials. However, coastal systems and marine organisms are not exclusively challenged by pollutants, but also by climate changes such as progressive temperature increment. Therefore, the present study aimed to i) evaluate the toxicity of remediated seawater, previously contaminated by Hg (50 mg/L) and decontaminated by the use of graphene based nanomaterials (graphene oxide (GO) functionalized with polyethyleneimine, 10 mg/L), towards the mussel *Mytilus galloprovincialis*; ii) assess the influence of temperature on the toxicity of decontaminated seawater. For this, alterations observed in mussels' metabolic capacity, oxidative and neurotoxic status, as well as histopathological injuries in gills and digestive tubules were measured. This study demonstrated that mussels exposed to Hg contaminated seawater presented higher impacts than organisms under remediated seawater. When comparing the impacts at 21 °C (present study) and 17 °C (previously published data), organisms exposed to remediated seawater at higher temperature presented higher injuries than organisms at 17 °C. These results indicate that predicted warming conditions may negatively affect effective remediation processes, with the increasing of temperature being responsible for changes on organisms' sensitivity to pollutants or increasing pollutants toxicity.

Keywords

metals; warming; bivalves; oxidative stress; nanomaterial; histopathology

5.1 Introduction

Among hazardous chemical elements, mercury (Hg) is on the top ten list of toxic contaminants in the world (Coppola et al., 2020a; ATSDR, 2019). Mercury is ubiquitous in waters, sediments, soils and air, and can be originated by natural events, like erosion and volcanic eruptions (Guilherme et al., 2008; Spada et al., 2012; Vaselli et al., 2015). However, the presence of this element in the environment, especially in the aquatic systems, results mostly from anthropogenic activities such as the burning of fossil fuels, gold mining and, more recently, improper disposal of electronic products (Cooper et al., 2011; Davydova, 2005; Donnici et al., 2012; Pereira et al., 2008; Randall et al., 2012). In aquatic environments, especially marine coastal systems (estuaries and lagoons), most of the metal(loid)s, including Hg, have the capacity to be associated with sediments and to be present in the water column and biota tissues (Spada et al., 2012; de Souza Machado et al., 2016; Portela et al., 2020; Sunderland et al., 2009). Although in open seawater Hg concentration ranges from 0.5 to 3.0 ng/L (Faganeli et al., 2012), Hg was detected in concentrations up to 27 µg/L in coastal waters (Gworek et al., 2016). Moreover, Sunderland et al. (2009) highlighted that at the current emission rate, Hg concentrations in the North Pacific Ocean will rise by 50 % in 2050 compared to levels recorded in 1995. This situation may happen in other areas around the world since Hg is still commonly used, including new technological applications (Davydova, 2005). The bioavailability of Hg in aquatic environments enhances concerns, namely regarding marine organisms as bivalves due to their lifestyle characteristics, including their sedentary and filter-feeding behavior, that facilitates contaminants accumulation and, consequently, may generate toxicity (Angelidis and Catsiki, 2002; Casas and Bacher, 2006; Galvão et al., 2009; Marques et al., 2018). Thus, due to their characteristics, bivalves are among the best bioindicator species of environmental pollution (Morrison and Brown, 2003; Moschino et al., 2012; Hamza-Chaffai, 2014; Fernández et al., 2010). Previous studies showed that, besides their capacity to accumulate Hg (ranging from 0.015 to more than 40 µg/g), exposure to Hg can induce oxidative stress and neurotoxicity, cellular damage and histopathological alterations in bivalves (Coppola et al., 2020a; Cuevas et al., 2015; Chen et al., 2014; Coppola et al., 2018; Freitas et al., 2017; Sonawane, 2015).

5.1.1 Strategies to remediate the contaminated waters

In the last decade different methodologies such as ultrafiltration, reverse osmosis and electrochemical methods have been developed to remediate polluted waters before being discharged into coastal systems (Coppola et al., 2020a; Ali et al., 2012; Anjum et al., 2016; Huang et al., 2016; Li et al., 2010; Coppola et al., 2020b,c). Nevertheless, these methodologies are frequently low cost but inefficient, or efficient but expensive (Babel and Kurniawan, 2003; Dabrowski et al., 2004; Gehrke et al., 2015; Mohan and Pittman, 2007). To overcome these limitations, alternative methods and materials, especially based on nanostructured materials, have been synthetized and tested (Chen et al., 2007; Jackson et al., 2012; Paul et al., 2015; Vilela et al., 2016) Among several materials, graphitic carbon atoms have demonstrated an excellent electrical conductivity, high mechanical strength and thermal conductivity, high impermeability to gases and optical transparency (Henriques et al., 2016; Yang et al.,

2018). Due to its characteristics, graphene has been used in a vast diversity of applications (Ali et al., 2018; Nupearachchi et al.,2017; Zhang et al., 2014). As an example, Bessa et al. (2020) synthesized and characterized a new nanomaterial based on graphene oxide (GO) and functionalized with polyethyleneimine (GO-PEI) that proved to be effective (easy to prepare and low cost) for removing Hg from seawater in 24 hours. This excellent performance was attributed to the synergistic effect resultant from the interactions between GO and PEI, giving a high content of N-rich groups and negative zeta potential over a wide pH range (from 2 to 12). Based on this study, Coppola et al. (2020a) demonstrated that under control temperature (17 ˚C) seawater contaminated with Hg (50 µg/L) and remediated using GO-PEI did not present toxic effects in mussels *Mytilus galloprovincialis* after chronic exposure. These authors concluded that GO-PEI was able to reduce significantly the concentration of Hg in seawater, being safe to wildlife if discharged into aquatic systems. However, Sanchez et al. (2012) reported that GO is able to interact with biomolecules causing the generation of ROS (reactive oxygen species) in target cells as a potential mechanism for toxicity. In fact, although high hydrophobic surface area, GO may lead to significant interactions with membrane lipids causing direct physical toxicity or adsorption of biological molecules. In what regards to PEI toxic effects towards aquatic invertebrates Petersen et al. (2011) revealed that PEI coatings increased nanotubes toxicity in *Daphnia magna*.

5.1.2 Impacts of temperature in marine organisms

Coastal ecosystems and marine biological resources are not exclusively at risk due to pollution, but also to natural pressures, including daily and seasonal temperature changes, with predicted scenarios indicating an increase of seawater temperature up to 2 °C until the end of the century (Fogarty et al., 2008; IPCC, 2018). Associated with the temperature rise in aquatic systems, different authors have shown deleterious effects in inhabiting wildlife, including bivalves. In particular, studies revealed that exposure to warming conditions lead to perturbations on bivalves' physiological performance, including reduced aerobic scope and the energy available for fitness-related functions, impacts on shell growth and mortality (Berthelin et al., 2000; Hiebenthal et al., 2012; Mackenzie et al., 2014; Mubiana and Blust, 2007; Sokolova et al., 2012). Also, changes on bivalves' metabolic capacity, oxidative status and neurotoxicity were revealed in bivalves exposed to temperature rise (Freitas et al., 2018; Moreira et al., 2018; Maulvault et al., 2018; Matozzo et al., 2013; Kefaloyianni et al., 2005; Verlecar et al., 2007; Velez et al., 2017). In addition, the interaction between the increase of temperature and contaminants can affect bivalve's sensitivity, changing their vulnerability towards each stressor, but may also change pollutants bioavailability and toxicity (Coppola et al., 2018; Moreira et al., 2018; Attig et al., 2014; Ivanina et al., 2009; Freitas et al., 2019; Lannig et al., 2006; Sokolova and Lannig, 2008). For example, Pirone et al. (2019) showed that *M. galloprovincialis* presented higher oxidative stress and cellular damage when exposed to the combination of lead (Pb) and warming conditions in comparison to those mussels under each single stressor.

Considering the lack of information regarding the impacts that remediated seawater may induce to aquatic wildlife, the present study aimed to: i) evaluate the toxic effects induced in the mussel *M. galloprovincialis* by remediated seawater (previously contaminated with Hg and remediated by GO-PEI); ii) assess the influence of temperature on the toxicity of remediated seawater.

5.2 Materials and methods

5.2.1 Experiment setup

Adult mussels of the species *Mytilus galloprovincialis* were collected in the Ria de Aveiro lagoon (Portugal) during the low tide, at the end of August 2019. More than one hundred mussels were collected, which were transported to the laboratory using plastic containers filled with seawater from the sampling site. Specimens presented a mean body weight of 13.1 ± 2.1 g, mean length of 5.7 ± 0.68 cm and a mean width of 3.0 ± 0.42 cm. In the laboratory, mussels were maintained for one week at constant temperature, pH and salinity (17 °C, pH 8.10 and 30, respectively) (depuration). The artificial seawater (salinity 30±1, prepared with Tropic Marin® SEA SALT dissolved in osmose water) was renewed every 2 days during this week. Afterwards, organisms were divided in two groups and placed in two different climatic rooms: one exposed at 17 ± 1 °C (identified as control; CTL) and other at 21 ± 1 °C for the acclimatization, during an extra week. Temperature of 21 °C was selected to resemble predicted warming conditions considering projections by IPCC (IPCC, 2018, 2019). After acclimation, bivalves were exposed for 28 days at different treatments, including: CTL, clean seawater (at 17 and 21 °C; CTL 17 and CTL 21, respectively); Hg, seawater containing Hg (50 g/L, 21 °C); GO-PEI, graphene oxide (GO) functionalized with polyethyleneimine, (10 mg/L, 21 °C); GO-PEI+Hg, GO-PEI and Hg (at 21 °C); RSW, seawater after remediation (at 21 °C) (Table 1). For each condition three aquaria (3L) were used, with five individuals per aquarium. During acclimation and exposure periods animals were fed (with Algamac protein plus (150.000 cells/animal/day) every other day and maintained in artificial seawater at pH 8.1, photoperiod 12 h light and 12 h dark, and constant aeration.

Table 1. Experimental treatments: CTL: control; GO-PEI: graphene oxide (GO) and functionalized with polyethyleneimine; Hg: mercury; RSW: remediated seawater.

CONDITIONS	DESCRIPTION
CTL 17	Hg 0.0 µg/L + GO-PEI 0.0 mg/L at 17°C
CTL 21	Hg 0.0 µg/L + GO-PEI 0.0 mg/L at 21°C
GO-PEI	GO-PEI 10 mg/L + Hg 0.0 µg/L at 21°C
GO-PEI+Hg	GO-PEI 10 mg/L + Hg 50 µg/L at 21°C
Hg	Hg 50 µg/L + GO-PEI 0.0 mg/L at 21°C
RSW	Remediated seawater afore contaminated with Hg (50 µg/L) and decontaminated by GO-PEI (10 mg/L) for 24h at 21°C

The selected Hg concentration (50 µg/L) used to resemble contaminated water (treatment: Hg) was based on the permitted concentration of this metal in wastewater (Directive 2013/39/EU, 2013) and previous studies testing the capacity of GO-PEI to remove Hg from contaminated water (Bessa et al., 2020). Considering that in the aquatic ecosystems most of the Hg is found in the inorganic form (Hg)

(Celo et al., 2006), in the present study the inorganic form $Hg(NO_3)_2$ (Sigma Aldrich) was used. A certified standard solution of Hg was used (1000 ± 2 mg L^{-1} of Hg(II) in HNO_3 0.5 mol L^{-1}, from Merck). The amount of GO-PEI (10 mg/L) used for water remediation was selected according to previous studies where it was demonstrated the capacity of this material to remove Hg from seawater (Bessa et al., 2020). The remediated seawater (treatment: RSW) was prepared as described by Coppola et al. (2020a). Throughout an experimental period of 28 days, temperatures, salinity and pH were daily checked as well as mussels' mortality. During the 28-days of exposure, the seawater was renewed weekly and conditions reestablished, including temperature, salinity and concentrations of the metal and nanomaterial. Furthermore, to compare real Hg exposure concentrations with Hg nominal concentrations, seawater samples from each aquarium were collected immediately after spiking following weekly seawater renewals. After the experimental period (28 days), mussels' soft tissues were used to analyze and evaluate the Hg concentrations, histopathological alterations, oxidative stress and neurotoxicity. The results obtained were discussed comparing biological responses observed at 17 °C (Coppola et al, 2020a) and 21 °C (present study). For the histological measurement one mussel from each aquarium (three per treatment) was meticulously opened and the soft tissue was separated from shell and fixed in Bouin's fluid for 24 hours at room temperature to analyze gills and digestive glands as described by Leite et al. (2019). Three organisms from each aquarium (9 per treatment) were frozen in liquid nitrogen for Hg quantifications and biochemical assays. The whole soft tissues from each mussel were homogenized under liquid nitrogen, and divided into five aliquots of 0.5 g fresh weight (FW). From each individual, four aliquots were used for biochemical analyses (each one for a specific buffer and respective biomarkers) and one for Hg quantification.

5.2.2 Graphene oxide with ethyleneimine polymer

The GO-PEI material was synthetized under laboratory conditions, mixing graphene oxide (GO) in water solution (0.4 wt % concentration from Graphenea) with ethyleneimine polymer (PEI) solution 50 % (w/v) in water with M.W. 750000 (Sigma Aldrich) with a ratio of 24 % v/v (GO/polymer) with pH 2 as described by Coppola et al. (2020a) and Bessa et al. (2020). High Mw (750 k) of highly branched PEI and GO nanosheets (1:3 ratio) were used to produce a hydrogel in aqueous acidic medium. Although the synthesis methodology was quite reproductible, each batch of material was analyzed via Attenuated Total Reflectance Fourier Transform Infrared (ATR-FTIR) in a Bruker Tensor 27 FT-IR spectrometer (Bruker Corporation, Massachusetts, USA). The spectra were recorded between 400 and 4000 cm^{-1}, with a resolution of 4 cm^{-1} and 256 scans. The microstructure was evaluated using a scanning electron microscope (FEGSEM HITACHI S4100), to prove the synthesis reproducibility. The capacity of GO-PEI to remove Hg was tested each week during the experiment (for a total of 4 weeks). The water samples from the RSW condition were collected each week after the remediation treatment and Hg was quantified to validate the remediation process.

5.2.3 Mercury quantification

The Hg in seawater was analyzed as described by Henriques et al. (2016) and Coppola et al. (2020a) using cold vapor atomic fluorescence spectroscopy (CV-AFS), in a PSA 10.025 Millennium Merlin Hg analyzer and the results were expressed in µg/L. The concentration of Hg in organisms' soft tissues was quantificated by thermal decomposition atomic absorption spectrometry with gold amalgamation (LECO model AMA-254) following Costley et al. (2000) and Coppola et al. (2020a) and the results were expressed in µg/g.

5.2.4 Biological responses: metabolic capacity, oxidative stress and neurotoxicity biomarkers

The biochemical markers were performed following the methods described in the supplementary material, including: i) metabolic capacity - electron transport system activity (ETS) expressed in nmol/min/g FW and determined as reported and modified by De Coen and Janssen (1997) and King and Packard (1975); ii) antioxidant enzymes activity - superoxide dismutase (SOD) and catalase (CAT) expressed in U/g FW and determined following Beauchamp and Fridovich (1971) and Johansson and Borg (1988), respectively; iii) antioxidant isoenzymes activity - glutathione-S-transferases (GSTs) expressed in U/g FW and determined as described by Habig et al. (1976); iv) extent of cellular damage - lipid peroxidation levels (LPO) and protein carbonylation levels (PC) expressed in nmol MDA/g FW and nmol/g FW and determined according to Ohkawa et al. (1969) and Mesquita et al. (2014), respectively; v) redox balance - ratio between reduced (GSH) and oxidized (GSSG) glutathione, determined by Rahman et al. (2007); vi) neurotoxicity - acetylcholinesterase activity (AChE) expressed in nmol/min/g FW and determined following Ellman et al. (1961).

5.2.5 Biological responses: histopathological measurements

The fixed tissues for the histological alterations were processed as described previously (Coppola et al., 2020a; Pinto et al, 2019). The digestive glands and gills were carefully dissected from mussels. After gradually dehydrated from ethanol 70 % to absolute alcohol in graded alcohols and cleared in xylene, each piece was embedded in paraffin and cut with a microtome (7 µm thick for each slide) to evaluate the histological alterations. The evaluation of the histopathological index *(ih*) was done following Leite et al. (2019): six slides (with three sections each) were processed for gills and digestive glands. For each slide, six pictures at 40x magnification were taken (n = 36 pictures per mussel's tissue); for each picture it was noted the presence/absence of the considered histological damage (for gills: haemocytes infiltration, evident enlargement of the central vessel, abundance of lipofuscin aggregates; and for digestive glands: haemocytes infiltration, atrophied, necrosis) giving a score (a) from 0 (none)

to 6 (diffuse). The alteration level (w) was given for each damage from 1 (minimum severity) to 3 (maximum severity) based on Costa et al. (2013).

5.2.6 Integrated biomarker response

The integrated biomarker response (IBR) was calculated to understand the general mussels' health status, using biomarkers results (ETS, SOD, CAT, GSTs, LPO, PC, GSH/GSSG and AChE), following Beliaeff and Burgeot (2002) method.

5.2.7 Statistical analysis

All results obtained (Hg concentrations in seawater and mussel's soft tissues, biochemical markers and histopathological index) were submitted for the statistical analysis using PERMANOVA (Permutational multivariate analysis of variance) add-on package for PRIMER v6 software (2008). Pearson correlation was used to perform pairwise comparison, with 9999 permutations. Significance (*p*-value) was calculated using a Monte Carlo test. Significant differences between each pair of treatments were assigned for a *p*-value < 0.05. The null hypothesis (H0) tested the existences of no significant differences among treatments: i) for Hg concentration in seawater and mussels; ii) for each biochemical marker; iii) for histopathological alterations. Significant differences among CTL 17, CTL 21, GO-PEI, GO-PEI+Hg, Hg and RSW treatments are represented in figures with different letters.

5.2.8 Multivariate analysis

The matrix gathering the histopathological index, biochemical markers as well as Hg concentrations was used to calculate the Euclidean distance similarity matrix, which was simplified through the calculation of the distance among centroids (i.e. the mean position of all the points representing a given treatment). The resulting matrix was submitted to ordination analysis (Principal Coordinates, PCO). In the PCO graph, the variables presenting a correlation higher than 75 % were represented a super imposed vector.

5.3 Results

At the end of the exposure period (28 days) 100 % survival was observed.

5.3.1 Mercury quantification

The nominal Hg concentration and the capacity of GO-PEI to remediate the seawater were checked after each weekly renewal. Hg concentrations measured in water from GO-PEI+Hg and Hg treatments showed similar values to the nominal concentrations (52.1 ± 2.2 and 52.4 ± 2.8 µg/L, respectively). Furthermore, Hg concentration in seawater samples from RSW treatment confirmed the 83 % capacity of GO-PEI to remove Hg (Hg concentrations in RSW: 9.6 ± 1.5 µg/L). The concentration of Hg in the seawater samples from CTL 17 and CTL 21 treatments (clean seawater at 17 and 21 °C) as well as in samples from GO-PEI treatment were below the limit of quantification (Table 2).

Table 2. Mercury concentration in: i) seawater samples (µg/L) collected immediately after the weekly water renewal for each treatment (results correspond to the mean ± standard deviation of four weeks; 3 samples per treatment and per week); ii) mussels' soft tissues (µg/g) collected 28 days after the beginning of the experiment (results correspond to the mean ± standard deviation; 3 mussels per aquarium, 9 mussels per treatment). Different uppercase letters represent differences among the treatments. LOQ (limit of quantification) for PSA 10.025 Millennium Merlin was of ≤ 0.01 µg/L.

Conditions	Hg water concentration (µg/L)	Mussel's Hg concentration (µg/g)
CTL 17	< LOQ	0.17 ± 0.027^{A}
CTL 21	< LOQ	0.08 ± 0.03^{B}
GO-PEI	< LOQ	0.09 ± 0.01^{B}
GO-PEI+Hg	52 ± 2.3^{A}	13.09 ± 4.502^{C}
Hg	52 ± 2.8^{A}	16.19 ± 1.052^{C}
RSW	9.6 ± 1.5^{B}	6.09 ± 1.73^{D}

In the soft tissue of *M. galloprovincialis* the lowest Hg concentrations were measured in RSW compared with those values obtained in mussels exposed to GO-PEI+Hg and Hg conditions (Table 2). The concentration of Hg in mussels' tissues showed no significant differences between GO-PEI+Hg and Hg conditions (Table 2). Moreover, mussels under CTL 17, CTL 21 and GO-PEI conditions presented Hg concentrations below 1 µg/g.

5.3.2 Biological assays: metabolic capacity, oxidative stress and neurotoxicity biomarkers.

The ETS activity showed significantly higher values in mussels exposed to RSW compared with mussels exposed to the remaining treatments (Figure 1, Table 3). Significantly higher SOD activity was observed in mussels exposed to GO-PEI compared with the remaining treatments. No significant differences were observed between mussels exposed to CTL 21 and Hg as well as among mussels exposed to CTL 17, GO-PEI+Hg and RSW (Figure 2A, Table 3).

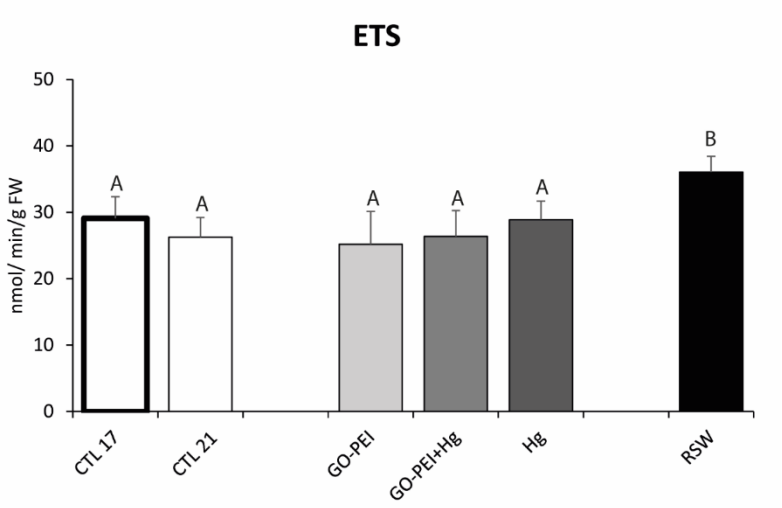

Figure 1. Electron transport system activity (ETS), in *Mytilus galloprovincialis* exposed to different treatments and temperatures (CTL 17, exposure at 17 °C; CTL 21, GO-PEI, GO-PEI+Hg, Hg and RSW, exposure at 21 °C) at the end of the experiment (28 days). Results are mean ± standard deviation (n = 9). Significant differences among the conditions are presented with uppercase letters.

The activity of CAT was significantly higher in mussels exposed to 21 °C compared with mussels under 17 °C. Significant differences were also observed between mussels exposed to RSW and exposed to Hg, with higher values in remediated water (Figure 2B, Table 3). Significantly higher GSTs activity was observed in mussels exposed to GO-PEI compared with the remaining treatments. No significant differences were observed among mussels exposed to CTL 21, Hg and RSW as well as

among mussels exposed to GO-PEI+Hg, Hg and RSW (Figure 2C, Table 3).

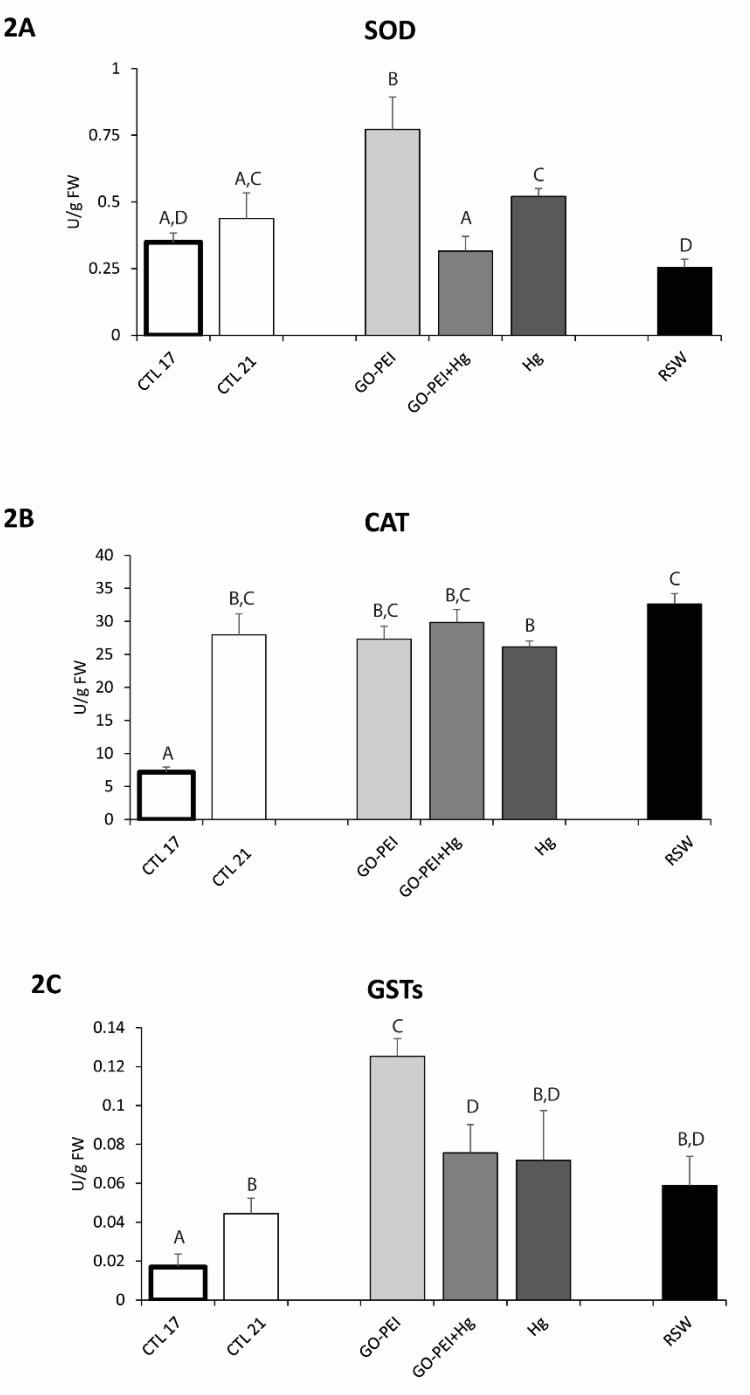

Figure 2. A: Superoxide dismutase activity (SOD); B: Catalase activity (CAT); C: Glutathione S-transferases activity (GSTs), in *Mytilus galloprovincialis* exposed to different treatments and temperatures (CTL 17, exposure at 17 °C; CTL 21, GO-PEI, GO-PEI+Hg, Hg and RSW, exposure at 21 °C) at the end of the experiment (28 days). Results are mean ± standard deviation (n = 9). Significant differences among the conditions are presented with uppercase letters.

Table 3. *p*-values, F-value, and F (DFn, DFd) (Degrees of freedom numerator and denominator) obtained by pair-wise comparisons between treatments (CTL17, CTL21, GO-PEI, GO-PEI+Hg, Hg and RSW) for each biomarker: Electron transport system activity (ETS); Superoxide dismutase activity (SOD); Catalase activity (CAT); Glutathione-S-transferases activity (GSTs); Lipid peroxidation levels (LPO); protein carbonyl levels (PC); ratio between reduced and oxidized glutathione (GSH/GSSG); Acetylcholinesterase activity (AChE) and histopathological index: Gills; Digestive Tubules; Significant differences ($p < 0.05$) are highlighted in bold.

	ETS	**SOD**	**CAT**	**GSTs**	**LPO**	**PC**	**GSH/GSSG**	**AChE**	**Gills**	**Digestive Tubules**
CTL 17 vs CTL 21	0.1948	0.3809	0.3809	**0.0003**	0.0889	**0.0001**	**0.0001**	**0.0001**	**0.0321**	**0.0001**
CTL 17 vs GO-PEI	0.0654	**0.0093**	0.0093	**0.0001**	**0.0026**	**0.0001**	**0.0001**	**0.0014**	**0.0001**	**0.0001**
CTL 17 vs GO-PEI+Hg	0.1849	0.6506	0.6506	**0.0001**	**0.0482**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
CTL 17 vs Hg	0.7773	**0.0379**	0.0379	**0.0032**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
CTL 17 vs RSW	**0.0005**	0.2197	0.2197	**0.0002**	**0.0171**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
CTL 21 vs GO-PEI	0.639	**0.0363**	0.0363	**0.0002**	**0.0024**	**0.0001**	0.4829	0.4065	**0.0001**	0.9999
CTL 21 vs GO-PEI+Hg	0.9579	0.1049	0.1049	**0.0063**	**0.0272**	**0.0002**	0.8785	0.5988	**0.0001**	0.9999
CTL 21 vs Hg	0.402	0.2774	0.2774	0.1064	**0.0001**	**0.0001**	**0.0042**	0.5498	**0.0001**	**0.0001**
CTL 21 vs RSW	**0.0001**	**0.022**	0.022	0.1012	**0.0011**	0.8038	0.7682	0.1474	**0.0001**	0.9999
GO-PEI vs GO-PEI+Hg	0.5827	**0.0023**	0.0023	**0.0065**	**0.0081**	0.9759	0.4029	0.1906	0.999	0.906
GO-PEI vs Hg	0.2006	**0.0492**	0.0692	**0.0181**	**0.0186**	**0.0324**	0.1087	0.1784	**0.0001**	**0.0001**
GO-PEI vs RSW	**0.0001**	**0.0014**	0.0014	**0.0001**	**0.0116**	**0.0011**	0.8561	0.951	0.2278	0.9101
GO-PEI+Hg vs Hg	0.4002	**0.0001**	0.0001	0.8328	0.123	**0.0436**	**0.0045**	0.8413	**0.0001**	**0.0001**
GO-PEI+Hg vs RSW	**0.0004**	**0.0478**	0.0478	0.1776	0.7441	**0.0023**	0.7046	**0.0001**	0.227	0.9116
Hg vs RSW	**0.0004**	**0.0001**	0.0001	0.4525	0.094	**0.0001**	0.2458	**0.0009**	**0.0001**	**0.0001**
F-value	*6.8*	*7.7088*	*22.168*	*13.823*	*10.034*	*71.835*	*56.966*	*13.846*	*47.304*	*39.377*
F (DFn, DFd)	*5,48*								*5,12*	

Significantly higher LPO levels were observed in mussels exposed to GO-PEI compared with the remaining treatments. No significant differences were observed among mussels exposed to GO-PEI+Hg, Hg and RSW (Figure 3A, Table 3).

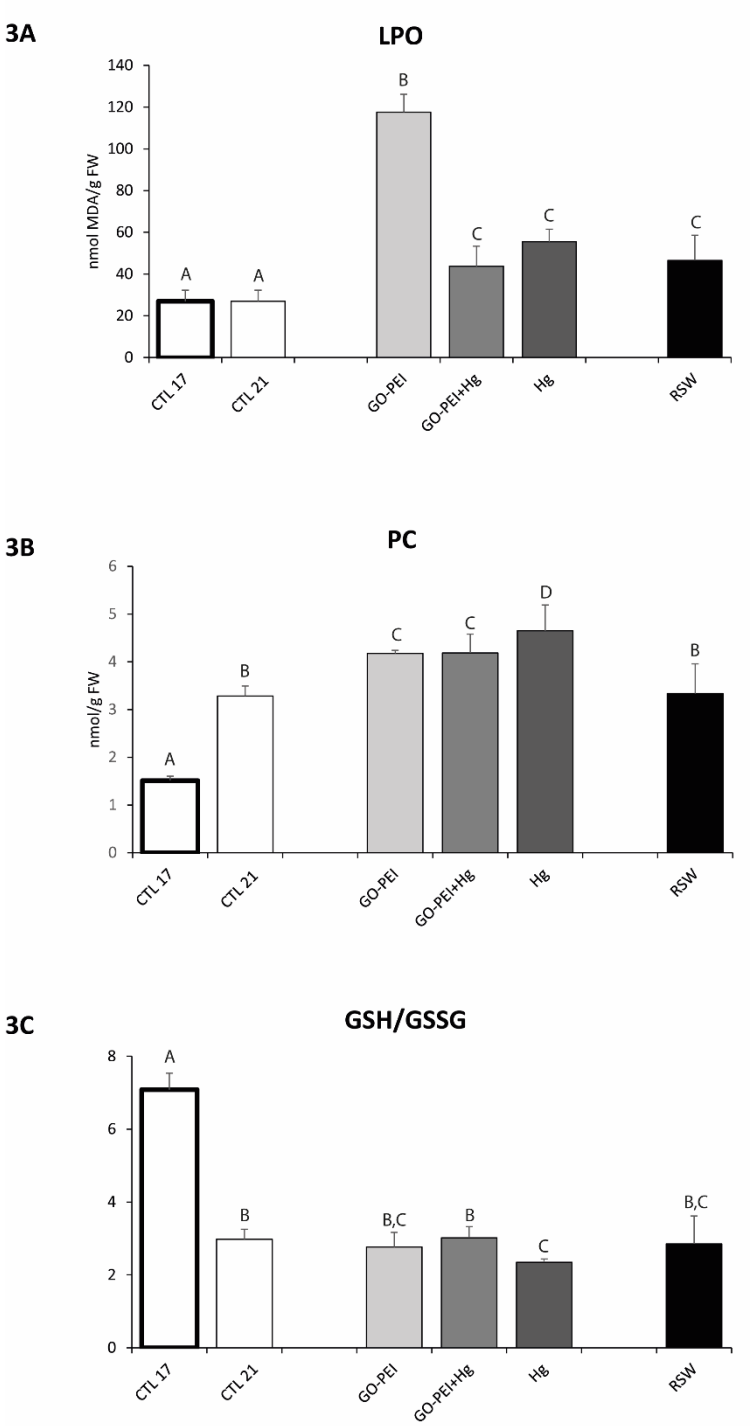

Figure 3. A: Lipid peroxidation levels (LPO); B: Protein carbonyl levels (PC); C: Ratio between reduced and oxidized glutathione (GSH/GSSG), in *Mytilus galloprovincialis* exposed to different treatments and temperatures (CTL 17, exposure at 17 °C; CTL 21, GO-PEI, GO-PEI+Hg, Hg and RSW, exposure at 21 °C) at the end of the experiment (28 days). Results are mean ± standard deviation (n = 9). Significant differences among the conditions are presented with uppercase letters.

PC levels were significantly higher in mussels exposed to 21 °C compared with mussels under 17 °C. No significant differences were observed among mussels exposed to CTL 21 and RSW as well as between mussels exposed to GO-PEI and GO-PEI+Hg (Figure 3B, Table 3). GSH/GSSG values were significantly lower in mussels exposed to 21 °C compared with mussels under 17 °C. No significant differences were observed among mussels exposed to CTL 21, GO-PEI, GO-PEI+Hg and RSW (Figure 3C, Table 3).

AChE activity was significantly lower in mussels exposed to 21 °C compared with mussels under 17 °C. No significant differences were found among CTL 21, GO-PEI, GO-PEI+Hg and Hg mussels as well as among CTL 21, GO-PEI and RSW mussels (Figure 4, Table 3).

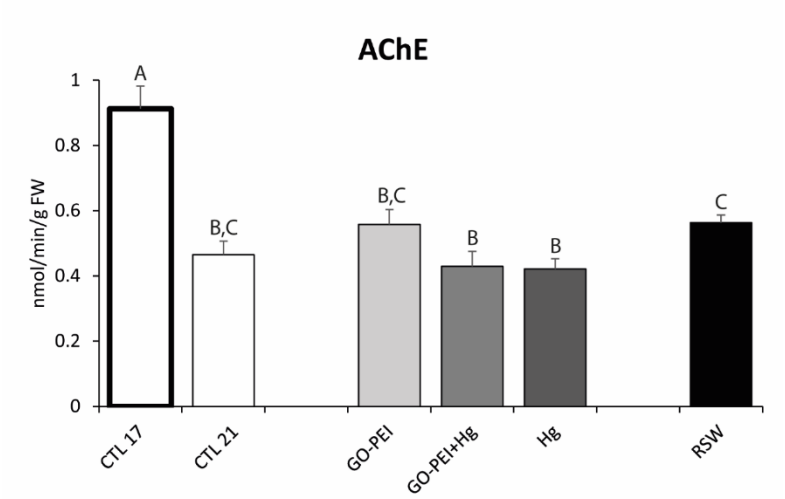

Figure 4. Acetylcholinesterase activity (AChE), in *Mytilus galloprovincialis* exposed to different treatments and temperatures (CTL 17, exposure at 17 °C; CTL 21, GO-PEI, GO-PEI+Hg, Hg and RSW, exposure at 21 °C) at the end of the experiment (28 days). Results are mean ± standard deviation (n = 9). Significant differences among the conditions are presented with uppercase letters.

5.3.3 Biological responses: histopathological measurements

Significantly higher *ih* in gills was observed in organisms exposed to Hg in comparison to mussels exposed to the remaining treatments. No significant differences were found among mussels exposed to GO-PEI, GO-PEI+Hg and RSW (Figure 5A, Table 3).

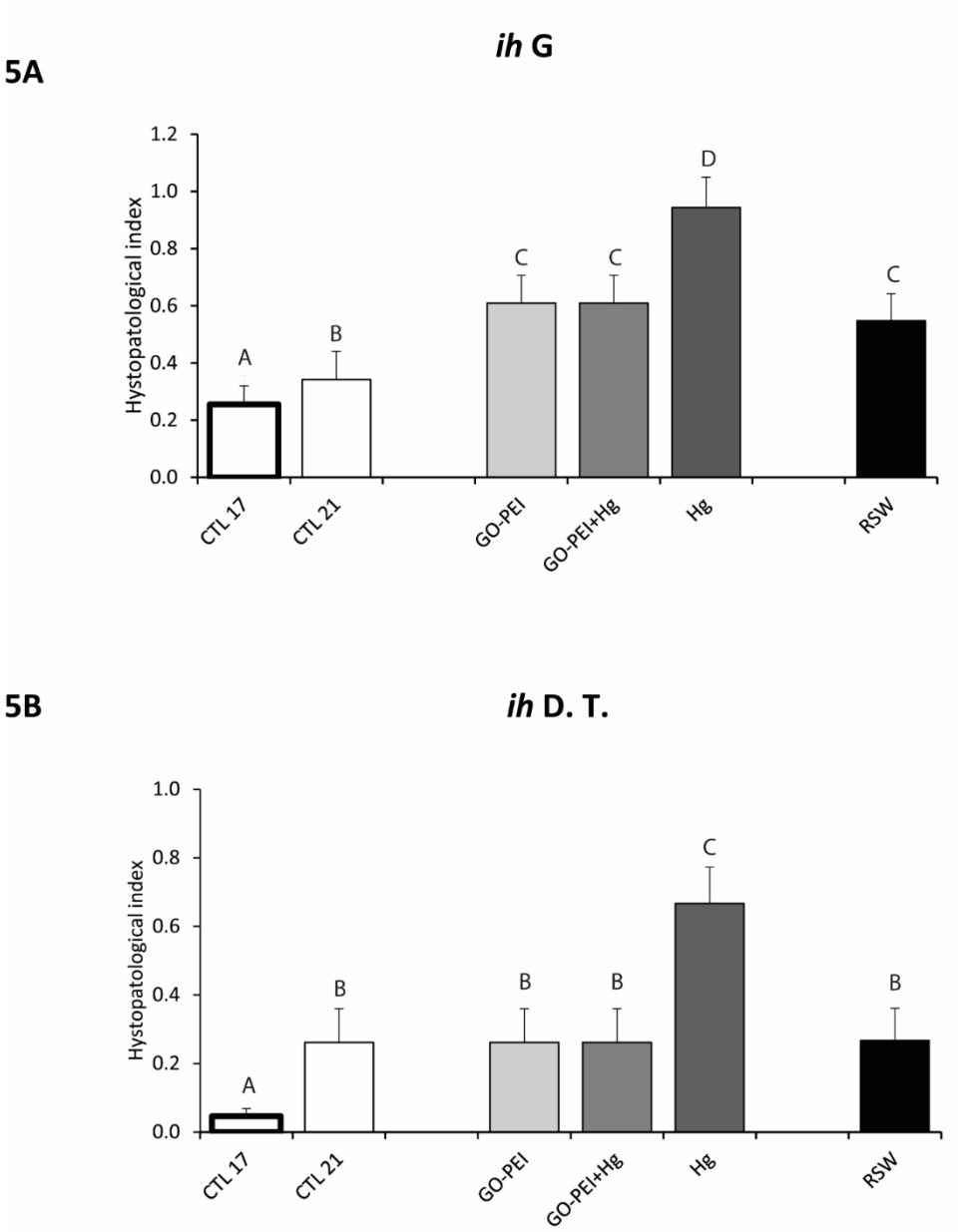

Figure 5. A: Histopathological index in gills (*ih* G); B: Histopathological index in digestive tubule (*ih* D.T.), in *Mytilus galloprovincialis* exposed to different treatments and temperatures (CTL 17, exposure at 17 °C; CTL 21, GO-PEI, GO-PEI+Hg, Hg and RSW, exposure at 21 °C) at the end of the experiment (28 days). Results are mean ± standard deviation (n = 3). Significant differences between conditions are represented with uppercase letters.

Figure 6 shows the gills histopathological alterations among treatments. The haemocytes infiltration (arrows) were found in all mussels' tissues especially in Hg and RSW conditions. Also, evident enlargement of the central vessel (long arrows) was observed in organisms exposed to GO-PEI and RSW. Abundance of lipofuscin aggregates (*) in gills for each condition were observed. In digestive tubules significantly higher *ih* values were observed in organisms exposed to Hg in

comparison to the remaining conditions (Figure 5B). No significant differences were found among mussels exposed to CTL 21, GO-PEI, GO-PEI+Hg and RSW (Figure 5B, Table 3). Figure 6 also shows the digestive glands histopathological alterations among the conditions. The haemocytes infiltration (arrows) were found in all conditions especially in mussels' tissue exposed to Hg. Also, atrophied (a) in digestive glands were showed in RSW and Hg conditions. This last also presented necrosis (n).

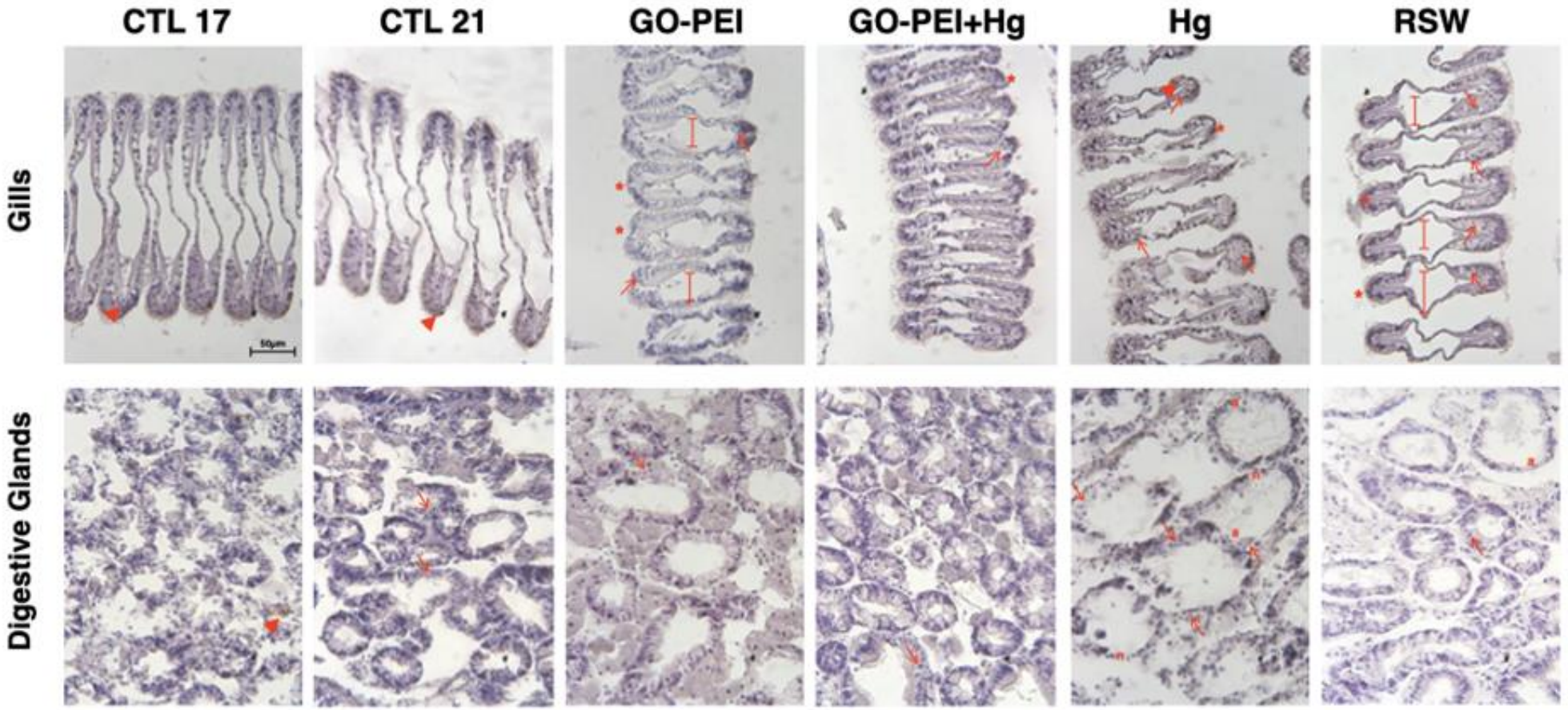

Figure 6. Micrographs of different tissues in *Mytilus galloprovincialis* exposed to different treatments stained with haematoxylin. i) Gills: cilia lost (*), haemocytes infiltration (arrows), evident enlargement of the central vessel (line with straight ends), abundance of lipofuscin aggregates (arrowheads); ii) Digestive glands: haemocytes infiltration (arrows), atrophied (a) and necrosis (n). Scale bar = 50µm.

5.3.4. Integrated Biomarker Response

The highest IBR value (2.09) was found on the mussels exposed to GO-PEI, while the lowest value (0.08) was observed in CTL 21 organisms. Moreover, the results obtained for organism exposed to Hg, GO-PEI+Hg and RSW were 0.92, 0.57, 0.42 (respectively).

5.3.5 Multivariate analysis

The PCO graph based on Hg bioaccumulation in water and mussels, biochemical and histopathological alterations is presented in Figure 7, revealing that PCO axis 1 explained 58.5 % and PCO axis 2 explained 23.1 % of the total variation. PCO1 separated the positive side mussels under control condition (clean seawater at 17 °C) from the remaining conditions on the negative side. PCO2 clearly separated organisms exposed to GO-PEI on the negative side from the Hg, GO-PEI+Hg and RSW in the positive side. Mussels exposed to CTL 17 present high correlation with GSH/GSSG and AChE ($p > 0.9$), while mussels exposed to Hg showed high correlation with histopathological indices and CAT activity ($p > 0.85$). Mussels exposed to GO-PEI presented high correlation with SOD ($p > 0.88$).

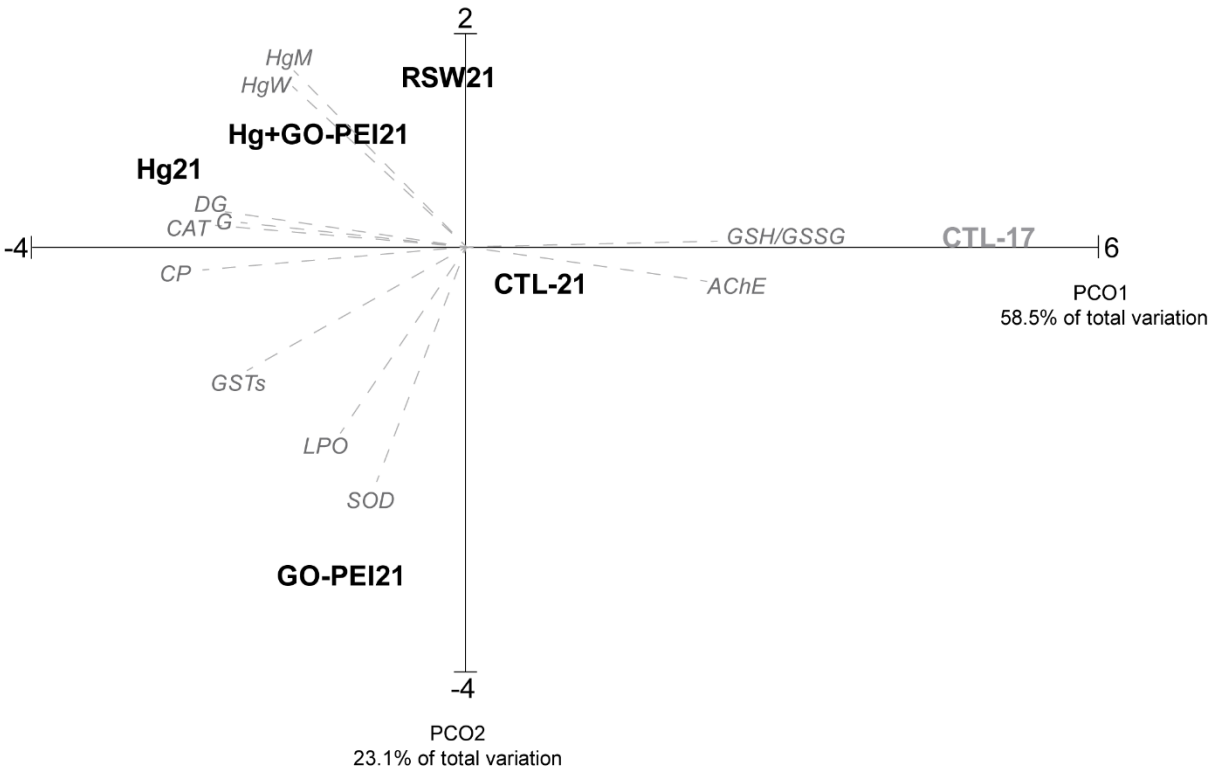

Figure 7. Principal coordinated analyses (PCO) based on Hg concentration, biochemical responses and histological alterations measured in *Mytilus galloprovincialis* exposed to different treatments and temperatures (CTL 17, exposure at 17 °C; CTL 21, GO-PEI, GO-PEI+Hg, Hg and RSW, exposure at 21 °C) at the end of the experiment (28 days). Pearson correlation vectors are superimposed as supplementary variables (r > 0.75): ETS, SOD, CAT, GSTs, LPO, PC, GSH/GSSG, AChE, [Hg] water, [Hg] mussels, G (*ih*) and DT (*ih*).

5.4 Discussion

In aquatic systems, climate warming and the presence of pollutants has been recently a topic of concern due to the scarce information on the combined impacts induced to inhabiting wildlife. Although efforts have been made to reduce the impacts of pollutants, namely through the development of water remediation processes, still little is known on the effects of decontaminated water, especially if considering predicted warming scenarios. To increase the knowledge on this subject, in the present study the impacts of temperature were addressed in non-contaminated and contaminated mussels exposed to contaminated and remediated seawater. The results obtained were discussed comparing biological responses observed in *Mytilus galloprovincialis* exposed to the same treatments but at different temperatures: 17 °C (Coppola et al., 2020a) vs 21 °C (present study). For this, alterations on mussels' metabolic capacity, oxidative and neurotoxic status as well as histopathological alterations were compared in non-contaminated mussels maintained at 17 and 21 °C. Also, the influence of temperature was discussed by comparing the toxicological effects observed at 21 °C in contaminated mussels and mussels exposed to remediated seawater (present study) with the effects induced in mussels maintained at 17 °C (Coppola et al., 2020a) under the same treatments.

5.4.1 Impacts of temperature in mussels exposed to control treatment

Recent studies highlighted the impacts of increased temperature in marine species, namely bivalves (Freitas et al., 2017; Matozzo et al., 2013; Attig et al., 2014; Anacleto et al., 2014; Coppola et al., 2017; Moreira et al., 2017; Fearman and Moltschaniwskyij, 2010; Nardi et al., 2017). The results here presented are in agreement with previous findings, showing that in non-contaminated organisms increased temperature (21 °C) led to oxidative stress and generated neurotoxicity in comparison to mussels at control temperature (17 °C). In particular, activation of antioxidant and biotransformation defences were observed in organisms maintained at 21 °C compared with the ones at 17 °C. Although enhancing their defence mechanisms under increased temperature mussels showed protein damage (represented by higher protein carbonylation) and loss of redox homeostasis (showed by the decrease in the ratio GSH/GSSG) in comparison to organisms at 17 °C. Besides oxidative stress, neurotoxicity was also observed in non-contaminated mussels maintained at increased temperature. Furthermore, histopathological damages in gills and digestive tubules, with accumulation of lipofuscin and haemocytes infiltration, were observed in non-contaminated mussels under 21 °C indicating that the increase of temperature could be responsible for histological alterations. Previous studies also demonstrated that bivalves exposed to warming scenarios enhanced the activity of antioxidant and biotransformation enzymes, although cellular damages still occurred (González Durán et al., 2018; Marigómez et al., 2017; Matoo et al., 2013; Vratsitas et al., 2016). Regarding histopathological effects, studies conducted by Pandey et al. (2016) also showed that temperature increase was responsible for the occurrence of impacts in bivalves, with cilia and haemocytes damage in mussels' gills (*Lamellidens marginalis*).

5.4.2 Impacts of temperature in mussels exposed to Hg treatments

Several studies have been focused on the toxic impact of classical pollutants, namely metals (e.g. Hg), in bivalves (Sonawane, 2015; Coppola et al., 2017; Boukadida et al., 2017; Velez et al., 2015; Pan and Wang, 2011). The present study demonstrated that, under warming conditions, mussels exposed to Hg accumulated similar concentrations of this metal both when exposed to Hg alone or combined with GO-PEI. These findings indicate that under 21 °C the presence of GO-PEI did not avoid the accumulation of the metal. However, previous studies developed by Coppola et al. (2020a) demonstrated that at 17 °C the concentration of Hg in mussels exposed to GO-PEI+Hg (27 ± 5 µg/g) was significantly lower than the concentration of this metal in mussels exposed to Hg alone (42 ± 11 µg/g), indicating that under control temperature the nanomaterials may prevent Hg accumulation. However, the present findings further demonstrated that values of Hg in mussels' tissues were lower at 21 °C (both at GO-PEI+Hg and Hg conditions) in comparison to values found in mussels at the same treatments but at 17 °C (Coppola et al., 2020a). Similarly, Coppola et al. (2017) as well as Coppola et al. (2018) demonstrated that *M. galloprovincialis* exposed to Hg alone under two different temperatures (17 and 21 °C) accumulated lower metal concentration under warming conditions (8.4 ± 1 µg/g at 17°C vs 1.7 ± 0.19 µg/g, (Coppola et al., 2017); 12.9 ± 5.2 µg/g 17 °C vs 8.5 ± 1.4 µg/g at 21 °C). Such findings can indicate that increased temperature may change organism's behavior, enhancing their capacity to avoid Hg entrance through filtration reduction. This may not be the case since both at 21 °C (present study) and 17 °C (Coppola et al., 2020a) the ETS activity was similar in mussels exposed to GO-PEI+Hg and Hg and, thus, mussels might have the same filtration capacity at both temperatures. In fact, the present study demonstrated that in the presence of Hg (both alone or in combination with GO-PEI) mussels were able to maintain their metabolic activity comparing with non-contaminated mussels at the same temperature (21 °C) but also compared to organisms maintained at 17 °C. Although previous studies conducted by Freitas et al. (2017) demonstrated that warmer conditions may decrease ETS activity in Hg contaminated mussels, this study differed from the present one. While in Freitas et al. (2017) mussels were maintained at warming conditions for 14 days after which were contaminated with Hg at the same temperature, in the present study mussels were maintained at warming conditions in the presence of Hg during the entire experimental period (28 days). Therefore, in the previous study (Freitas et al., 2017) the exposure to Hg was in fact shorter and animals were already "acclimated" to increased temperature which may allowed organisms to decrease their metabolism to avoid Hg accumulation. In the present study Hg and increased temperature were tested together for 28 days, which prevented mussels to have the same response. This hypothesis is supported by the results obtained at 17 °C (Coppola et al., 2020a) that reported lower ETS activity in Hg contaminated individuals, indicating that under control temperature mussels may have the capacity to reduce their metabolism to limit Hg accumulation and injuries, which is a strategy no longer valid when the stress is higher, i.e., increased temperature combined with the presence of Hg. Thus, lower Hg concentration at 21 °C may result from alterations on the contaminant behavior rather on the organisms 'response or from other defense mechanisms not evaluated in the present study. This should be further explored. In the present study, the lack of metabolic activation may explain the fact that

mussels did not enhance their antioxidant defense mechanisms in the presence of Hg, presenting similar SOD and CAT activity compared with control mussels at the same temperature (21 °C). On the other hand, the previous study conducted by Coppola et al. (2020a) demonstrated that, although lower ETS activity was observed, under control temperature mussels were able to active their antioxidant defenses, regardless the tested treatment (Hg, GO-PEI+Hg). Nevertheless, for the same treatments (GO-PEI+Hg, Hg) antioxidant and biotransformation enzymes activities were similar at 21 and 17 °C. Also, Morosetti et al. (2020) showed similar CAT and GSTs activities in *M. galloprovincialis* exposed to Hg contamination at control (17 °C) and increase (22 °C) temperatures. The limited antioxidant capacity observed in bivalves exposed to Hg (Hg and GO-PEI+Hg conditions) was associated with a higher cellular damages (high LPO and PC levels) and low GSH/GSSG values (in the case of Hg treatment). In fact, although contaminated mussels tried to eliminate Hg through the activation of GSTs (higher GSTs values were observed in Hg and GO-PEI conditions compared with CTL 21), this detoxification mechanism was not sufficient to avoid cellular damage and the loss of redox balance. Furthermore, in the present study, the exposure to Hg did not enhance the neurotoxic effects, with similar AChE activity in Hg and GO-PEI+Hg exposed mussels compared with the control ones at the same temperature (21 °C) and with Hg contaminated mussels maintained at 17 °C (Coppola et al., 2020a). The oxidative stress observed in contaminated mussels was accompanied by histopathological alterations in gills and digestive tubules, with higher impacts in mussels exposed to Hg alone. These results may indicate that the presence of GO-PEI may prevented histopathological alterations. Comparing the present findings with results obtained under the same treatments but at 17 °C (Coppola et al., 2020a) it is possible to observe that similar responses were observed regardless the temperature, indicating higher impact of Hg than temperature.

5.4.3 Impacts of temperature in mussels exposed to GO-PEI treatment

Recent research has been testing the use of nanomaterials for water remediation, including GO-PEI, with few studies showing the impacts of these materials towards marine wildlife (Coppola et al., 2020a,b,c, 2019; Pan and Wang, 2011; Morosetti et al., 2020). Previous studies (Coppola et al., 2020a,b) already demonstrated that at control temperature (17 °C) GO-PEI (10 mg/L) induced biochemical impacts (metabolism and oxidative status alterations) in mussels and clams, with greater impacts in mussels. The present study further revealed impacts on mussels caused by GO-PEI at 21 °C, leading to oxidative stress and neurotoxic impacts. In this case, although detoxification (GSTs) and antioxidant (SOD) enzymes increased their activities still LPO was observed in mussels exposed to GO-PEI at 21 ˚C, accompanied by lower GSH/GSSG ratio. As a result of higher LPO values, SOD and GSTs activities in mussels exposed to GO-PEI, the highest IBR was obtained in this condition. Comparing the present results with previous ones (Coppola et al., 2020a), we may hypothesis that warming conditions increased the impacts of GO-PEI when acting alone, which may result from the toxicity of the material and/or higher sensitivity of mussels to it. In fact, mussels exposed to GO-PEI under 21 °C showed higher oxidative levels, with higher enzymes activity (SOD, CAT and GSTs

activities of 0.77 ± 0.12, 27.32 ± 1.93 and 0.13 ± 0.01 U/g FW, respectively) when compared with mussels exposed to GO-PEI but at 17 °C (SOD, CAT and GSTs activities of 0.62 ± 0.11, 10.70 ± 3.13 and 0.03 ± 0.003 U/g FW, respectively (Coppola et al., 2020a)), with greater cellular damage at 21 °C (117.6 ± 8.46 nmol MDA/g FW) than at 17 °C (31.42 ± 5.16 nmol MDA/g FW). Also De Marchi et al. (2018) showed increased detoxification capacity in bivalves (*Ruditapes decussatus*) exposed to carbon nanotubes and increased temperature, although in this case no LPO was observed which may be due to shorter exposure period (96 h). Also, Coppola et al. (2020b) demonstrated that for *R. philippinarum* the antioxidant capacity increased in the presence of GO-PEI at warming conditions (22 °C) while no LPO was observed compared to CTL (without GO-PEI). This may indicate that clams might prevent LPO more efficiently than mussels when under the combined effect of temperature and GO-PEI. In the presence of the GO-PEI no neurotoxicity was observed, with similar AChE activity in mussels exposed to GO-PEI and to CTL 21. Especially in gills, histopathological alterations were observed in GO-PEI exposed mussels.

5.4.4 Impacts of temperature in mussels exposed to remediated seawater

Mussels exposed to remediated seawater (RSW) showed the highest ETS activity compared with the other treatments. Since electron transport systems is responsible for the generation of ROS, this could explain higher LPO levels observed in this treatment in comparison to non-contaminated mussels at 21 °C (CTL 21). Nevertheless, mussels exposed to RSW and CTL 21 treatments presented similar CAT, GSTs, PC, GSH/GSSG and AChE levels, indicating that organisms exposed to remediated seawater were experiencing similar stress levels as organisms exposed to non-contaminated water (CTL) at the same temperature (21 °C). Previous studies conducted by Coppola et al. (2020a) also demonstrated that under 17 °C Hg remediated seawater induced similar impacts to mussels as clean seawater (CTL) at 17 °C. However, when comparing the impacts at 21 °C (present study) and 17 °C (Coppola et al., 2020a), organisms exposed to remediated seawater at increased temperature presented higher impacts than organisms at 17 °C. In particular, higher metabolic capacity, antioxidant and biotransformation activity was observed in mussels exposed to RSW at 21 °C comparing with mussels exposed RSW but at 17 °C. These results indicate that predicted warming conditions may enhance the impacts caused by remediated seawater, which can result from changing organisms' sensitivity to pollutants or increasing pollutants toxicity, even at vestigial levels. Considering that previous studies conducted with clams under the same treatments (Coppola et al., 2020b) showed no differences at two different temperatures (17 and 21°C), the present findings may indicate a species-specific response and, therefore, higher influence of temperature in organism sensitivity than on pollutant toxicity. Nevertheless, the results obtained further demonstrated that, even at increased temperature, organisms under RSW presented less alterations than mussels exposed to contaminated conditions (Hg, GO-PEI+Hg), highlighting the positive effects of water remediation processes.

5.5 Conclusion

Overall, comparing temperatures, the results clearly demonstrate that mussels exposed to clean seawater (control treatment) at 21 °C were under higher stressful conditions (i.e., greater oxidative stress and neurotoxicity) than mussels at 17 °C. At 21 °C mussels exposed to remediated seawater (seawater previously contaminated with Hg and decontaminated during 24h with GO-PEI, RSW treatment) presented less biochemical alterations than mussels exposed to Hg (Hg and GO-PEI+Hg treatments). Furthermore, at the same temperature (21 °C) mussels exposed to RSW and control treatments showed similar biochemical and histopathological alterations. Nevertheless, mussels exposed to RSW at 21 °C showed greater metabolic capacity and antioxidant and biotransformation enzymes activity than mussels under the same treatment but maintained at 17 °C, revealing the effect of temperature.

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CHAPTER 6.

The influence of salinity in the toxicity of remediated seawater

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Abstract

Mercury (Hg) is one of the most hazardous pollutants, due to its toxicity, biological magnification and persistence. Its presence in aquatic systems has been described worldwide and new nanotechnologies have been developed with the aim to reduce the presence of this metal in the water. Aquatic environments, and in particular transitional systems, are also subjected to disturbances resulting from climate change, such as rising of temperature and sea-level, as well as extreme weather events, all affecting seawater salinity levels. Salinity range is one of the most relevant factors that condition the distribution and survival of aquatic species. Although previous works showed the efficiency of nanomaterials (e.g. graphene oxide functionalized with polyethyleneimine (GO-PEI)) to decontaminate seawater from toxic metals, no studies assessed the ecotoxicological impairments induced in mussels exposed to remediated seawater under different salinities levels. Therefore, the purpose of this study is to analyse the impacts of seawater previously contaminated with Hg and remediated with GO-PEI under three different salinity levels (20, 30 and 40) in *Mytilus galloprovincialis*. Overall, the results obtained demonstrated similar histopathological and metabolic alterations, oxidative stress, neurotoxicity in mussels under RSW treatment independently form salinity, namely when compared to control condition (salinity 30).

Keywords

Biomarkers, toxicity, *Mytilus galloprovincialis*, GO-PEI, seawater remediation, salinity shifts, bioaccumulation.

6.1 Introduction

The last decades have been characterized by socio-economic, scientific and technological development, which has also led to the increasing of environmental pollution (Appannagari, 2017; Can et al., 2020; Grossman and Krueger 1995; Ongan et al., 2020). In particular, coastal marine habitats are among the most affected by anthropogenic activities such as industrial and agricultural discharges, overpopulation, tourism and ports, with a rise on seawater contaminants (metal(loid)s, pharmaceuticals and personal care products, nanomaterials, pesticides) (Cao et al., 2020; Fu et al., 2003; Kristan et al., 2014; Maanan, 2008; Sauvé and Desrosiers, 2014). Among metals, mercury (Hg) is one of the most hazardous, due to its toxicity, potential for biomagnification, and rapid diffusion by air and in the water column (Liu et al., 2012; Liu et al., 2020). In marine coastal systems, Hg concentrations may range between 0.025 and 0.106 µg/g dw (dry weight) in sediments, while in seawater concentrations vary between 0.26 and 0.72 µg/L (Sun et al., 2020). Considering Hg concentration accumulated in bivalves, published literature showed that values may reach up to 0.35 µg/g dw in mussels (Casas and Bacher, 2006; Costa et al., 2020, Sun et al., 2020). In what regards to toxic effects, several studies highlighted the capacity of Hg to cause biochemical, histopathological and metabolic alterations in bivalves (Belivermiş et al., 2019; Briant et al., 2017; Coppola et al., 2017 and 2020a,b; Kim et al., 2017; Maanan, 2008; Oliveira et al., 2018; Parisi et al., 2021; Sıkdokur et al., 2020). In particular, it was already demonstrated that Hg can induce oxidative stress and lipid peroxidation damage as well as histopathological alterations in *Mytilus galloprovincialis* (Coppola et al., 2017, 2020a; Morosetti et al., 2020) and variations in hemocyte viability and modification of the morphological and cytoskeletal features in this mussel species (Parisi et al., 2021).

Considering the threats resulting from pollution towards aquatic organisms, in the last decades new technologies have been developed to reduce the quantity of pollutants in aquatic systems, including diverse strategies: sorption on nanomaterials, chemical precipitation, nanofiltration, reverse osmosis and ultrafiltration (Ali et al., 2011; Anjum et al., 2019; Aroua et al., 2007; Henke et al., 2001; Matlock et al., 2001; Muthukrishnan et al., 2008; Pugazhenthi et al., 2005). Among these approaches, nanotechnology has shown to be efficient but expensive for the remediation of contaminated water (Araújo et al., 2015; Henriques et al., 2016; Latif et al., 2020; Sánchez et al. 2011). For this reason, low-cost nanostructured materials (NSMs), including graphene oxide (GO), have been successfully applied to remediate waters (Henriques et al., 2016; Tavares et al., 2013). Recently, GO functionalized with a high molecular weight branched polyethyleneimine (PEI) demonstrated to be efficient to remediate seawater previously contaminated with Hg 50 µg/L, with an efficiency of 81 % in removing the contaminant after 6 h (Bessa et al., 2020). Coppola et al. (2020a,b) showed that the remediation of seawater using GO-PEI prevented *M. galloprovincialis* and *Ruditapes philippinarum* from biochemical and histopathological impacts induced by Hg.

In marine environment another source of disturbance is represented by climate change, which is leading to ocean warming and acidification, sea level rise, and the intensification of extreme weather events, with serious consequences on marine biodiversity (IPCC, 2019; Gissi et al., 2020). One of the predicted consequences of extreme events, including extreme rainfalls, is the salinity variation in transitional

ecosystems (Peteiro et al., 2018; Philippart et al., 2011). Also changes in ocean currents induced by wind may lead to changes in the salinity patterns (Gibson and Najjar, 2000; Justić et al. 1996). Salinity has a deep impact on the equilibrium of the estuaries, since it influences water density and therefore its circulation and stratification (Johnson et al. 1991), pH and organic matter solubility (Cai et al. 1998). Wildlife of these ecosystems, including edible species such as bivalves and crustaceans, depend on specific salinity ranges (Gibson and Najjar, 2000; Jackson and Jesien 1996). In the case of bivalves, salinity variation influences their metabolism by increasing energy cell volume to avoid osmotic shock (Berger and Kharazova, 1997; Hauton, 2016; Widdows and Shick, 1985). Moreover, mobilization of reserves leads to an increase in oxygen consumption and oxidative stress (Berger and Kharazova, 1997; Hauton, 2016; Widdows, and Shick, 1985), with consequences in growth performance, reproduction and immune function (Berger and Kharazova, 1997; Beukema et al., 2010; Hauton, 2016; Peteiro et al., 2018; Widdows and Shick, 1985). In bivalves, increasing salinity revealed to promote infections and reduced cell membrane stability in *Ostrea edulis* (Hauton et al., 2000), inhibition of the antioxidant defences in *R. philippinarum* (Freitas et al., 2016; Velez et al., 2016), in *M. galloprovincialis* (Freitas et al., 2019a,b), in *Cerastoderma edule* and in *Scrobicularia plana* (Gonçalves et al., 2017) and increase production of essential fatty acid in *C. edule* and *S. plana* (Gonçalves et al., 2017). Furthermore, hyper-salinity induced hemocytes alterations in *R. philippinarum* (Reid et al., 2003), *Chamelea gallina* (Matozzo et al., 2007) and *M. galloprovincialis* (Malagoli et al., 2007). On the other side, low salinities induced a reduction in the number of hemocytes in *Crassotrea gigas* (Gagnaire et al., 2006), *Haliotis diversicolor* (Cheng et al., 2004) and *M. edulis* (Bussell et al., 2008) and caused an increment of total protein content in the hemolymph of *Pinctada imbricata* (Kuchel et al., 2010; Matozzo and Marin 2011).

Although, a vast literature has been published on the impacts of pollutants in bivalves, with very scarce information on the combined effect of salinity shifts and metal(loid)s (Pb and As) (Freitas et al., 2016, 2019), no studies, to our knowledge, have investigated the ecotoxicological impact caused by the combined effect of salinity variation and Hg. Also, there is nothing published regarding possible effects in organisms exposed to remediate seawater under different salinities. Therefore, the purpose of this study is to investigate the bioconcentration capacity, the metabolic and oxidative status, and the histopathological alterations induced in *M. galloprovincialis* after exposure to Hg contaminated and remediated seawater, and understand the influence of salinity in the toxicity of remediated seawater.

6.2 Materials and methods

6.2.1. Experimental treatments

Mytilus galloprovincialis mussels were collected in August 2018 in the Mira channel (Ria de Aveiro lagoon, Portugal), with a mean length of 5.9 ± 0.5 cm and a mean width of 3.5 ± 0.6 cm. Plastic boxes were used to transport mussels from the field to the laboratory, where they were depurated for one week. During the depuration week the organisms were maintained in artificial seawater with 30 ± 1 salinity (Tropic Marin® SEA SALT from Tropic Marine Center), 17 ± 1 °C temperature, pH 8.0 ± 0.1 and constant aeration. Seawater was changed 2-3 times a week and mussels were not fed during this period. Then, mussels were let to acclimate for one extra week, during which mussels were fed with Algamac Protein Plus (150.000 cells/animal/day) 2-3 times a week. During the acclimation week mussels were divided in three groups: 1) mussels under control salinity (30 ± 1); 2) mussels under salinity 40 ± 1; and 3) mussels under salinity 20 ± 1. During this week, salinity was gradually increased and/or reduced while temperature, pH and aeration were maintained stable. Salinity 30 was selected as the control condition considering the annual mean value found in the sampling area (IPMA, 2017). The lowest and the highest salinities were selected considering extreme weather events, including heavy rainy or long drought periods, which can lead to drastic salinity shifts (Pörtner et al., 2014). Within each salinity level, each group was divided in five different treatments, including control (CTL), graphene oxide functionalized with polyethyleneimine (GO-PEI), mercury (Hg), the mixture of both (GO-PEI+Hg) and remediated seawater (RSW). Three aquaria with six organisms were used for each treatment.

The 50 µg/L Hg selected for this study, was based on the maximum permissible limit in industrial wastewater discharges (Directive, 2013/39/EU, 2013). The concentration of GO-PEI, 10 mg/L, was established based on previous studies that demonstrated the capacity of this nanostructured material to remove Hg from water (Bessa et al., 2020). The remediate seawater (RSW) was obtained through a remediation process during 24 h. In this process, clean seawater at salinity 30 was previously contaminated with Hg (50 µg/L) then remediated by GO-PEI (10 mg/L), and then filtrated. After this, the salinity of RSW was increase up to 40 adding synthetic salt or decrease up to 20 with freshwater purified by reverse osmosis to simulate the discharge of remediated seawater into coastal systems characterized by different salinity conditions. The experiment lasted for 28 days and during this period, temperature, salinities and mussels' mortality were constantly monitored. Organisms were fed with Algamac Protein Plus (150.000 cells/animal/day) 2-3 times a week. During the experiment, for every treatment and for each aquarium, the seawater was weekly changed and treatments reestablished, including different salinity concentrations. To compare real concentrations with nominal ones, seawater was collected from every aquarium for Hg quantification after each water renewal and conditions reestablishment.

At the 28th day 3 mussels from each aquarium (9 per treatment) were sacrificed and frozen with liquid nitrogen for bioaccumulation and biochemical assessment while 1 mussel from each aquarium (3 per treatment) was fixed in the Davidson solution for histopathological evaluation.

6.2.2 Synthesis of nanostructured material

The nanostructured material (GO-PEI) was prepared as previously reported by Bessa et al. (2020). Briefly, a hydrogel was obtained by the rapid shaken (10'') of an aqueous mixture containing graphene oxide (GO) water dispersion (0.4 wt % (weight percent) concentration, from Graphenea, used as received) and polyethyleneimine (PEI) solution 50 % (w/v) in acid water adjusted to 2.0 ± 0.1 pH with 0.1 mol/L HCl solution at a ratio GO/PEI of 24 % v/v. The hydrogel was frozen at -80 ºC and then lyophilized to obtain a 3D porous structure. This 3D structure was washed in MilliQ water for 12 h to remove acidic residues and finally was re-frozen at -80 ºC and lyophilized again to generate the functional macrostructure.

6.2.3. Mercury quantification

A cold vapor atomic fluorescence spectroscopy (CV-AFS), using a PSA 10.025 Millennium Merlin Hg analyser, was used for Hg quantification in seawater samples, weekly collected from each aquarium after water renewal, following Henriques et al. (2019) procedured. Quantification limits obtained through blank measurements (n = 15) were 0.021 µg/L. The results were expressed in µg/L. The thermal decomposition atomic absorption spectrometry with gold amalgamation (LECO model AMA-254) was used for the Hg quantification in mussel's whole soft-tissues (three individuals per aquarium) as described in Costley et al. (2000). Analytical quality control was performed by using Certified Reference Material, ERM-CE278K (mussles tissue, 0.071 ± 0.007 mg/Kg (total Hg)). The results were expressed in mg/Kg.

6.2.4 Biochemical markers

After the exposure period (28 days) three mussels from each aquarium were used for biochemical analyses. Each individual was carefully opened and the soft tissues were separated from the shells to perform the biochemical assays. For this, tissues from each mussel were homogenized individually with liquid nitrogen, divided into 0.5 g fresh weight (FW) aliquots and stored at -80 ºC as described in Coppola et al. (2020a).

6.2.4.1 Metabolic capacity

Electron transport system (ETS) activity was selected to assess mussels' metabolic capacity. Absorbance was measured during 10 min at 490 nm with intervals of 25 s and the extinction coefficient (Ɛ) of 15.9 /(mmol/L)/ cm was used to calculate the amount of formazan formed, following King and Packard (1975) method with modifications by De Coen and Janssen (1997). Results were expressed in nmol per min per g FW.

6.2.4.2 Energy reserve content

Glycogen (GLY) content was quantified to evaluate mussels' energy reserve levels. Quantification was done following sulphuric acid method based on DuBois et al. (1956), using glucose standards (0–10 mg/mL). Absorbance was measured at 492 nm and GLY content was expressed in mg per g FW.

6.2.4.3 Antioxidant enzymes activity

Superoxide dismutase (SOD) and glutathione reductase (GRed) enzymes were chosen to evaluate mussels' antioxidant system. The activity of SOD was determined with the method of Beauchamp and Fridovich (1971) and SOD standards (0.25 – 60 U/mL) were used for the standard curve. After 20 min of incubation at room temperature, absorbance was measured at 560 nm. The activity was expressed in U (one unit: quantity of the enzyme that catalyses the conversion of 1 µmol of substrate per min) per g FW.

The activity of GRed was quantified following Carlberg and Mannervik (1985). Absorbance was measured at 340 nm, and the enzymatic activity was determined using ε=6.22 /(mmol/L)/cm. The results were expressed in U (amount of enzymes necessary to the formation of 1.0 µmol NADPH oxidized per min) per g FW.

6.2.4.4 Biotransformation enzymes activity

Also, glutathione S-transferases (GSTs) activity was selected in order to measure the biotransformation capacity of mussels. Its activity was measured according to Habig et al. (1974). Absorbance was quantified at 340 nm and to determine enzymatic activity the extinction coefficient Ɛ = 9.6 /(mmol/L)/cm was used. The activity was expressed in U per g FW, where U represents the amount of enzyme necessary to catalyse the formation of 1 µmol of dinitrophenyl thioether per min.

6.2.4.5 Cellular damage

Lipid peroxidation levels (LPO) and the ratio between reduced and oxidized glutathione (GSH/GSSG) were considered as cellular damage and redox balance indicators. Following Ohkawa et al. (1979), the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation, was used to determine the levels of LPO. Absorbance was measured at 535 nm. LPO levels were calculated through the extinction coefficient Ɛ = 156 /(mmol/L)/cm and expressed in nmol of MDA formed per g of FW.

6.2.4.6 Redox balance

GSH and GSSG were used as standards (0–60 µmol/L) and absorbance was measured at 412 nm. The ratio between oxidized and reduced glutathione was calculated (GSH/2 ∗ GSSG) according to Rahman et al. (2007).

6.2.4.7 Neurotoxicity

Acetylcholinesterase activity (AChE) was determined for neurotoxicity evaluation. The estimation of AChE activity was obtained using acetylthiocholine iodide (ATChI 5 mmol/L) substrates as described by Ellman et al. (1961). During 5 min at 412 nm, enzyme activities were continuously observed. The activity was calculated using Ɛ =13.6 /(mmol/L)/cm expressed in nmol per min per g FW.

All biochemical parameters were made in duplicate using a microplate reader (Biotek).

6.2.5 Histopathological analyses

Mussel for histopathological observations were fixed in Davidson solution for 24 h, and then washed in ethanol 70 %. Daily changed of ethanol were performed to remove all fixative's traces. Digestive tubules and gills were dissected afterward, gradually dehydrated in ascending ethanol, and cleared 48 h in methylbenozate. After a 20 min in benzene, the tissues were placed in a benzene/paraffin mixture (1:1) for 20 min at 56 ± 2 °C, and then plain paraffin for 1 h in vacuum oven (Heraeus vacutherm) at 56 °C and 0 bar, twice. Subsequently the tissue was embedded in clean paraffine (Hermanns et al., 2001). Sections 5 µm thick, obtained at microtome (Jung autocut, Leica), were stained with haematoxylin. After all, each section was coloured with Mayer's haematoxylin solution to detect the presence of cilia loss, enlargement of the central vessel, hemocyte infiltration, atrophy, necrosis and lipofuscin for gills and digestive tubules (Coppola et al., 2020a,b). The histopathological indices were calculated as described in Leite et al. (2020).

6.2.6 Statistical analyses

Concentrations of Hg in the water and mussels' soft tissues, the biochemical parameters (ETS, GLY, SOD, CAT, GRed, GSTs, LPO and GSH/GSSG) as well as histopathological indices were separately submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in Primer v7) (Anderson, 2008). A one-way hierarchical design was followed in this analysis. The pseudo-F p-values in the PERMANOVA main tests were evaluated in terms of significance. When significant differences were observed in the main test, pairwise comparisons were performed. Values with $p < 0.05$ were considered as significantly different. For Hg concentration, biomarkers and histopathological

indices, the null hypotheses tested were: for each treatment (CTL, GO-PEI, Hg, GO-PEI+Hg and RSW) no significant differences exist among salinity levels (30, 20 and 40); for each salinity level, no significant differences exist among treatments. For each treatment, differences among salinity levels (30, 20, 40) were represented with **p**-values in a table format and values < 0.05 were indicated in bold. For each salinity level, significant differences among exposure treatments were represented with different letters in the graphs.

6.3 Results

6.3.1 Mortality

For 28 days 8% of mortality was detected in mussels exposed to salinity 20 under GO-PEI, Hg and GO-PEI+Hg treatments. Also 8% of mortality was detected in organisms exposed to CTL and GO-PEI at salinity 40. No mortality was recorded in mussels exposed to treatments at salinity 30 as well in organisms exposed to RSW at stressful salinities (20 and 40).

6.3.2 Mercury concentration in seawater and mussels

The Hg quantification in seawater samples collected immediately after spiking confirmed that the entire experiment was conducted with levels close to the nominal concentration of 50 µg/L (56.3 ± 5.1 µg/L) for the contamination with Hg. Regardless the salinity tested, seawater samples from GO-PEI+Hg aquaria presented Hg concentration of 36.7 ± 2.3 µg/L, while in RSW treatment [seawater previously contaminated with Hg (50 µg/L) then remediated by GO-PEI (10 mg/L) during 24 h] the Hg concentration was 10.4 ± 1.6 µg/L. The concentration of Hg in CTL and GO-PEI treatments was below the limit of quantification (≤ 0.021 µg/L).

The Hg quantification in mussels' soft tissues showed significant differences among treatments (GO-PEI+Hg, Hg and RSW) at salinity 30, with the highest concentration in mussels exposed to Hg treatment (14.4 ± 0.4 µg/g), followed by those exposed to GO-PEI+Hg (4.0 ± 0.2 µg/g) and RSW (1.5 ± 0.02 µg/g) (Figure 1). Also, under salinity 20, mussels soft tissue showed significant differences among GO-PEI+Hg, Hg and RSW with the highest concentration in organisms exposed to Hg treatment (9.1 ± 0.4 µg/g), followed by those exposed to GO-PEI+Hg (3.8± 0.1 µg/g) and RSW (0.4 ± 0.03 µg/g) (Figure 1). At salinity 40, significant differences among Hg treatments were found between remediated (RSW) and non-remediated (Hg and GO-PEI+Hg) treatments which presented the highest Hg levels (0.4 ± 0.03, 8.2 ± 0.2 and 8.7 ± 0.8 µg/g, respectively) (Figure 1). Regardless the salinity, the lowest Hg concentrations were always found in mussels exposed to CTL and GO-PEI treatments. Among salinities, significant differences were found in all treatments except between salinities 30 and 20 when comparing mussels exposed to GO-PEI+Hg, and between salinities 40 and 20 for Hg treatment (Table 1).

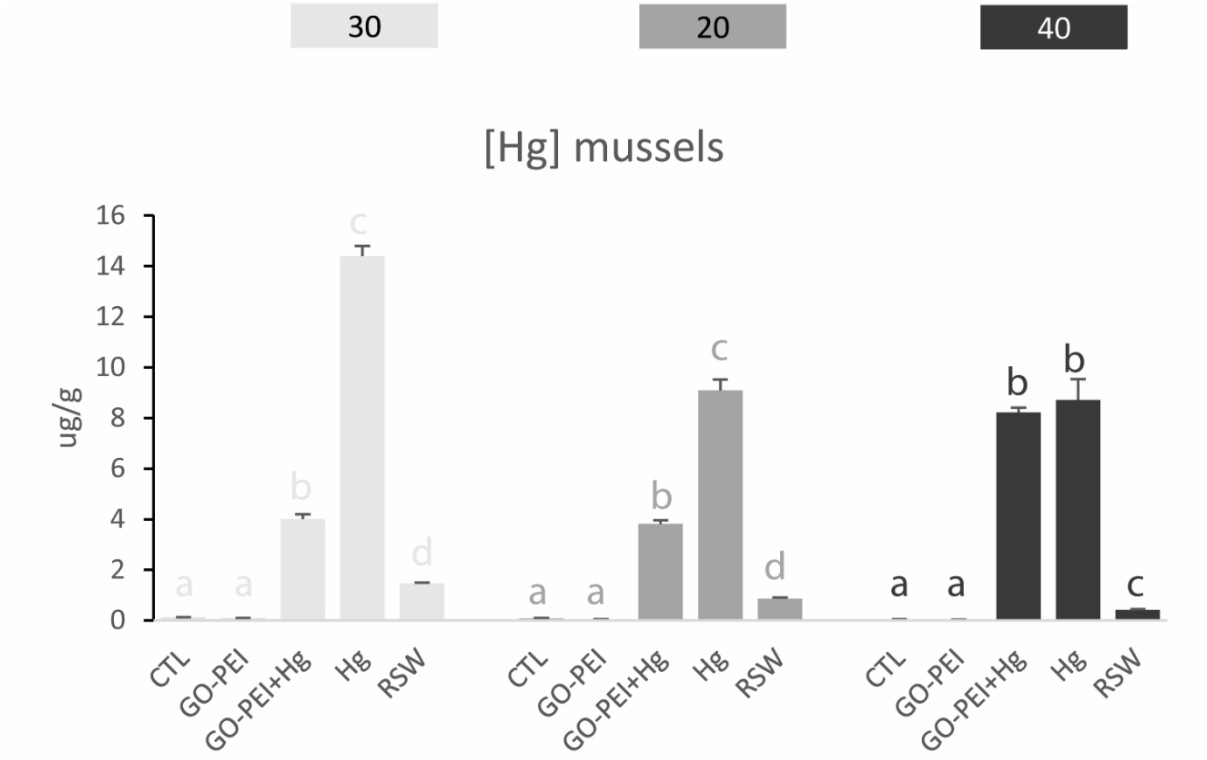


Figure 1. Mercury concentration, in *M. galloprovincialis* soft tissue exposed to different treatments (CTL, GO-PEI, GO-PEI+Hg, Hg and RSW) each under different salinities conditions (30, 20 and 40). Results are the means + standard deviation. Significant differences ($p<0.05$) among conditions are identified with different lowercase letters.

Table 1. *p-values,* obtained by pair-wise comparisons between conditions (30, 20 and 40) of the treatments (CTL, GO-PEI, GO-PEI+Hg, Hg and RSW) for each biomarker: Electron transport system activity (ETS); Glycogen content (GLY); Superoxide dismutase activity (SOD); Glutathione reductase activity (GRed); Glutathione-S-transferases activity (GSTs); Lipid peroxidation levels (LPO); ratio between reduced and oxidized glutathione (GSH/GSSG); Acetylcholinesterase activity (AChE); also for the Hg bioaccumulation in the mussels' tissue and for histopathological index: Digestive Tubules; Gills. Significant differences ($p < 0.05$) are highlighted in bold.

$p \leq 0.05$	**ETS**	**GLY**	**SOD**	**GRed**	**GSTS**	**LPO**	**GSH/GSSG**	**AChE**	**$[Hg]_{tissue}$**	**GILLS**	**DT**
CTL 30 vs CTL 20	**0.07**	0.63	**0.0006**	**0.002**	0.83	0.07	**0.04**	0.58	**0.0002**	0.28	**0.04**
CTL 30 vs CTL 40	0.17	0.83	**0.004**	**0.0001**	**0.0001**	0.47	**0.02**	**0.04**	**0.0001**	0.33	0.26
CTL 20 vs CTL 40	**0.0005**	0.74	0.24	**0.0001**	**0.0001**	0.51	0.06	0.10	**0.0003**	**0.003**	**0.04**
GO-PEI 30 vs GO-PEI 20	**0.003**	**0.01**	**0.0001**	**0.002**	0.08	**0.0004**	0.11	0.19	**0.0001**	**0.01**	0.30
GO-PEI 30 vs GO-PEI 40	0.35	0.21	0.15	**0.0001**	**0.0004**	**0.04**	**0.0001**	0.15	**0.0001**	**0.0005**	**0.0008**
GO-PEI 20 vs GO-PEI 40	**0.002**	**0.04**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	0.69	**0.005**	0.61	**0.03**
Hg+GO-PEI 30 vs Hg+GO-PEI 20	**0.02**	**0.001**	**0.0001**	**0.02**	**0.0005**	**0.0002**	0.79	0.16	0.63	**0.002**	**0.003**
Hg+GO-PEI 30 vs Hg+GO-PEI 40	**0.004**	0.59	**0.0001**	**0.0001**	**0.001**	**0.0001**	0.73	**0.005**	**0.0001**	0.33	0.45
Hg+GO-PEI 20 vs Hg+GO-PEI 40	0.97	**0.0002**	**0.0001**	**0.0001**	**0.04**	0.61	0.93	0.22	**0.0001**	**0.0001**	**0.003**
Hg 30 vs Hg 20	**0.006**	0.01	**0.0001**	**0.005**	0.24	0.41	0.82	0.32	**0.0001**	**0.0001**	0.18
Hg 30 vs Hg 40	0.38	0.21	**0.0001**	**0.0001**	0.46	**0.0002**	**0.001**	0.12	**0.0001**	0.10	**0.038**
Hg 20 vs Hg 40	**0.005**	0.16	0.10	**0.0001**	0.7	**0.002**	**0.0001**	0.54	0.68	0.20	0.91
RSW 30 vs RSW 20	0.80	**0.001**	**0.001**	0.25	**0.01**	**0.04**	**0.03**	0.06	**0.0009**	**0.003**	0.6
RSW 30 vs RSW 40	0.23	**0.0002**	**0.0001**	0.86	**0.0008**	0.06	**0.02**	**0.04**	**0.0001**	**0.003**	0.86
RSW 20 vs RSW 40	0.69	0.15	**0.0002**	0.57	**0.0008**	0.98	**0.0009**	0.68	**0.0001**	0.28	**0.039**

6.3.3 Biochemical markers

6.3.3.1 Metabolic capacity

At salinity 30, significantly higher ETS activity was detected in organisms exposed to RSW and CTL in comparison to the remaining treatments. No significant differences were found among GO-PEI, GO-PEI+Hg and Hg treatments (Figure 2A). At salinity 20, significantly lower ETS activity was observed in organisms exposed to RSW and GO-PEI+Hg compared to the CTL. No significant differences were found between GO-PEI and Hg and the remaining treatments (Figure 2A). At salinity 40, GO-PEI+Hg treated organisms showed significantly higher ETS activity than at CTL, GO-PEI and Hg. No significant differences were found among the remaining treatments (Figure 2A). Among salinities significant differences were observed at CTL, GO-PEI and Hg treatments, with higher ETS capacity at salinity 20. Significant differences were detected also at GO-PEI+Hg treatment, with lower ETS capacity under 30 compared to 20 and 40 (Table 1).

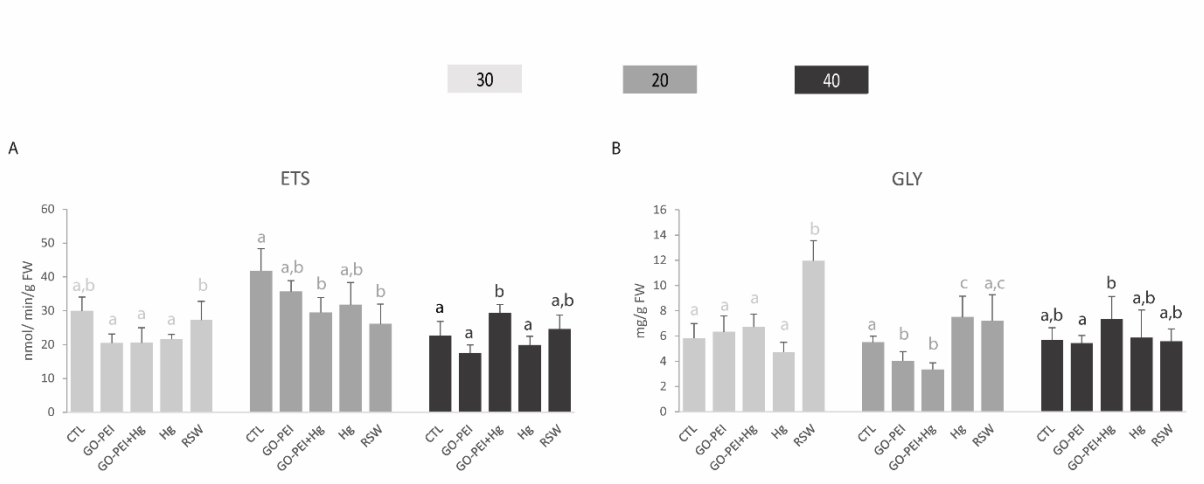


Figure 2. A: Electron transport system activity (ETS) and B: Glycogen content (GLY), in *M. galloprovincialis* exposed to different treatments (CTL, GO-PEI, GO-PEI+Hg, Hg and RSW) each under different salinities conditions (30, 20 and 40). Results are the means + standard deviation. Significant differences ($p<0.05$) among conditions are identified with different lowercase letters.

6.3.3.2 Energy reserve content

Regarding GLY content, at control salinity (30) mussels exposed to RSW showed significantly higher GLY levels, while no significant differences were found among the remaining treatments (Figure 2B). At salinity 20, significantly lower GLY content was releveled in GO-PEI and GO-PEI+Hg organisms compared to CTL treatment, while mussels exposed to Hg and RSW showed significantly higher GLY content compared to all treatments (Figure 2B). At salinity 40 organisms exposed to GO-PEI+Hg showed significantly higher GLY content in comparison to GO-PEI and no significant differences were found among the remaining treatments (Figure 2B). Between salinities, significant differences were

observed at GO-PEI and GO-PEI+Hg treatments, with lower GLY content at 20 compared to 30 and 40 salinities, while in RSW treatment organism exposed to 30 salinity showed significantly higher GLY levels compared to 20 and 40 (Table 1).

6.3.3.3 Antioxidant enzymes activity

At salinity 30 significantly higher SOD activity was observed in mussels exposed to GO-PEI and Hg in comparison to organisms under the remaining treatments. No significant differences were found among CTL, GO-PEI+Hg and RSW treatments (Figure 3A). At salinity 20 mussels subjected to GO-PEI+Hg and Hg treatments showed significantly higher SOD activity when compared with the other treatments (Figure 3A). At salinity 40 organisms treated with GO-PEI showed significantly lower SOD activity compared to the remaining treatments with exception to CTL. Moreover, mussels exposed to GO-PEI+Hg and RSW treatments revealed significantly higher SOD activity in comparison to the remaining treatments (Figure 3A). Between salinities, organisms exposed to CTL treatments showed significantly lower SOD activity under 30 compared to 20 and 40 salinities. Moreover, significantly higher SOD activity was showed in GO-PEI treatment under 20 compared to 30 and 40 salinities. Mussels exposed to Hg, GO-PEI+Hg and RSW treatments showed significantly higher SOD activity at salinities 20 and 40 than at salinity 30 (Table 1).

At salinity 30, the activity of GRed was significantly higher in RSW comparing with CTL, GO-PEI and Hg treatments (Figure 3B). At salinity 20, no significant differences were observed among treatments (Figure 3B). At salinity 40, significantly higher and lower GRed activity was observed in mussels exposed to Hg and RSW, respectively (Figure 3B). Considering the different salinities, significant differences were observed at each treatment, with exception to mussels exposed to RSW, with the highest values observed at 40 conditions compared to salinities 30 and 20 (Table 1).

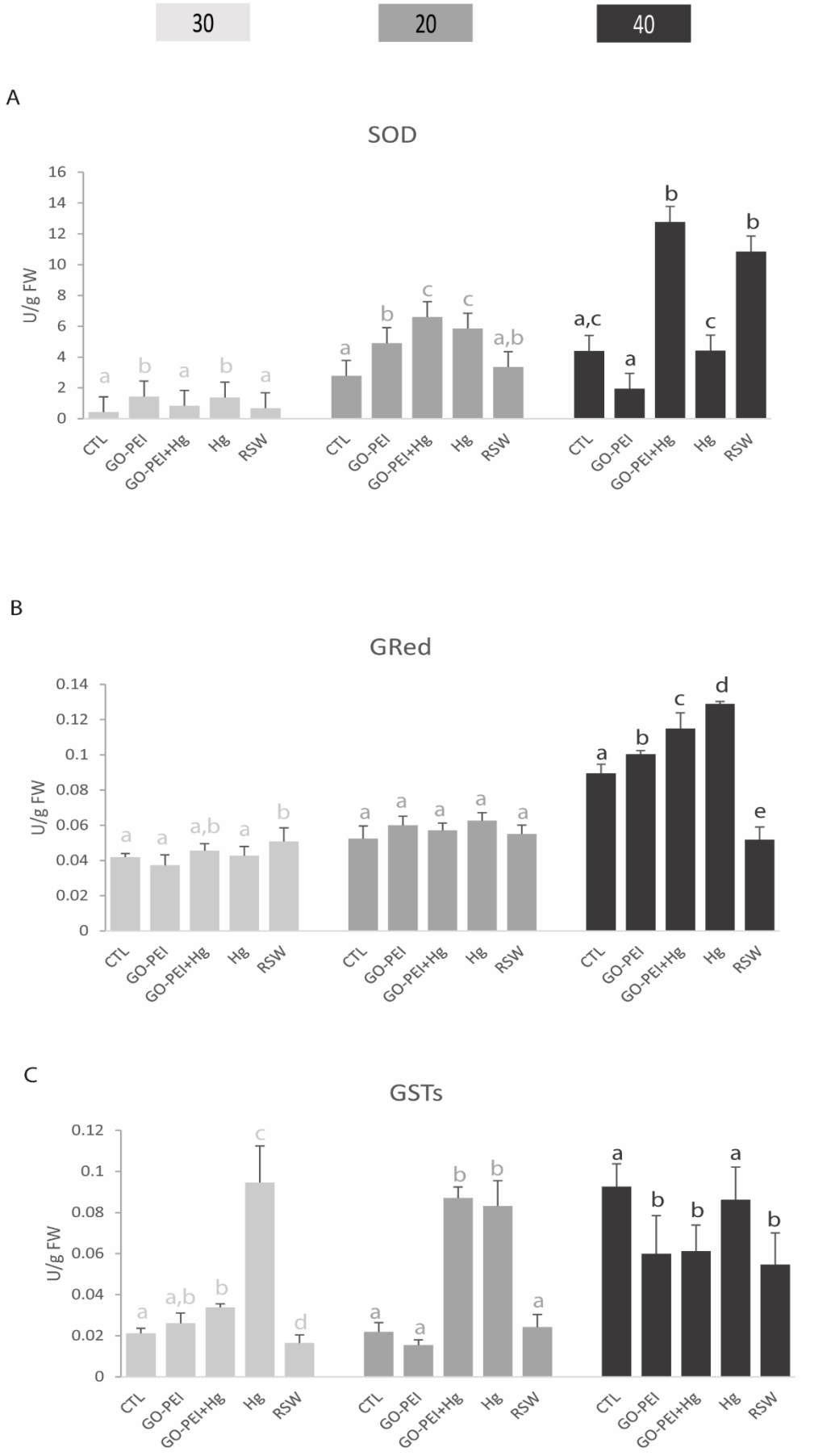


Figure 3. A: Superoxide dismutase (SOD); B: Glutathione reductase (GRed) and C: Glutathione-S-

transferases (GSTs) activities, in *M. galloprovincialis* exposed to different treatments (CTL, GO-PEI, GO-PEI+Hg, Hg and RSW) each under different salinities conditions (30, 20 and 40). Results are the means + standard deviation. Significant differences ($p<0.05$) among conditions are identified with different lowercase letters.

6.3.3.4 Biotransformation enzymes activity

Organisms exposed to Hg and RSW treatments at salinity 30 showed, respectively, significantly higher and lower GSTs activity compared to the other treatments. Significant differences were also observed between CTL and GO-PEI+Hg treatments, with the lowest GSTs values at CTL (Figure 3C). At salinity 20, mussels under GO-PEI+Hg and Hg treatments showed significantly higher GSTs activities compared to the remaining ones. No significant differences were found among the remaining treatments (Figure 3C). Regarding mussels at salinity 40, significantly higher GSTs activities were detected in CTL and Hg treatments compared to the other ones (Figure 3C). Among salinities, at salinity 40 organisms exposed to CTL and GO-PEI treatments revealed significantly higher GSTs activity compared to those exposed at salinities 30 and 20. Furthermore, significant differences were observed among all GO-PEI+Hg and RSW conditions with lower and higher values at 30 and 20 treatments, respectively (Table 1).

6.3.3.5 Cellular damage

At salinity 30, significantly lower and higher LPO levels were detected in mussels exposed to GO-PEI and GO-PEI+Hg treatments, respectively, compared to the other treatments (Figure 4A). Regarding organisms under salinity 20, significantly higher LPO levels were observed for GO-PEI treatment compared to the remaining ones (Figure 4A). At salinity 40 significantly higher LPO levels were observed at CTL compared to GO-PEI and Hg treatments (Figure 4A). Between salinities, significant differences were detected for GO-PEI treatments, with significantly higher and lower cellular damage at 20 and 40 compared to salinity 30. Furthermore, organisms under GO-PEI+Hg treatments showed significantly higher LPO at salinity 30 in comparison to salinities 20 and 40. Significantly higher cellular damages were observed for Hg at salinity 40 compared to the remaining treatments. Lastly, RSW treatment showed significant higher LPO level at 30 compared to remaining salinities (Table 1).

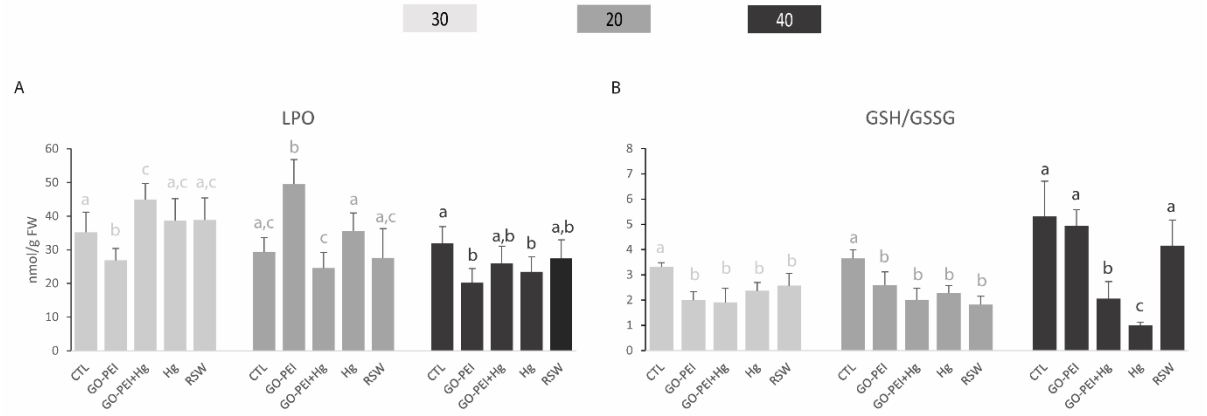


Figure 4. A: Lipid peroxidation levels (LPO) and B: Ratio between reduced and oxidized glutathione (GSH/GSSG), in *M. galloprovincialis* exposed to different treatments (CTL, GO-PEI, GO-PEI+Hg, Hg and RSW) each under different salinities conditions (30, 20 and 40). Results are the means + standard deviation. Significant differences ($p<0.05$) among conditions are identified with different lowercase letters.

6.3.3.6 Redox balance

Regarding GSH/GSSG ratio, at salinity 30 as well as at salinity 20, *M. galloprovincialis* at CTL condition showed significantly higher values than those obtained for the remaining treatments (Figure 4B). Furthermore, at salinity 40, significantly lower GSH/GSSG values were observed in organisms under GO-PEI+Hg and Hg treatments compared to the other ones, with the lowest value at Hg treatment (Figure 4B). Among salinities, significantly lower GSH/GSSG values were observed at salinity 30 compared to salinities 20 and 40 at CTL treatment. Furthermore, mussels exposed to GO-PEI and Hg treatments showed, respectively, significant higher and lower GSH/GSSG values when comparing salinity 40 to salinities 20 and 30. Lastly, higher GSH/GSSG values were detected at salinity 40 for RSW treatment (Table 1).

6.3.3.7 Neurotoxicity

At salinity 30, significantly lower and higher AChE values were detected, respectively, in GO-PEI and RSW treatments, compared to the remaining ones (Figure 5). At salinity 20, no significant differences were observed among treatments (Figure 5). Regarding mussels exposed at salinity 40, significantly higher AChE activity was detected in RSW when compared to the other treatments, with

exception to GO-PEI (Figure 5). Among salinities, significantly higher AChE activity was observed at CTL, GO-PEI+Hg and RSW at salinity 40 compared to salinity 30 (Table 1).

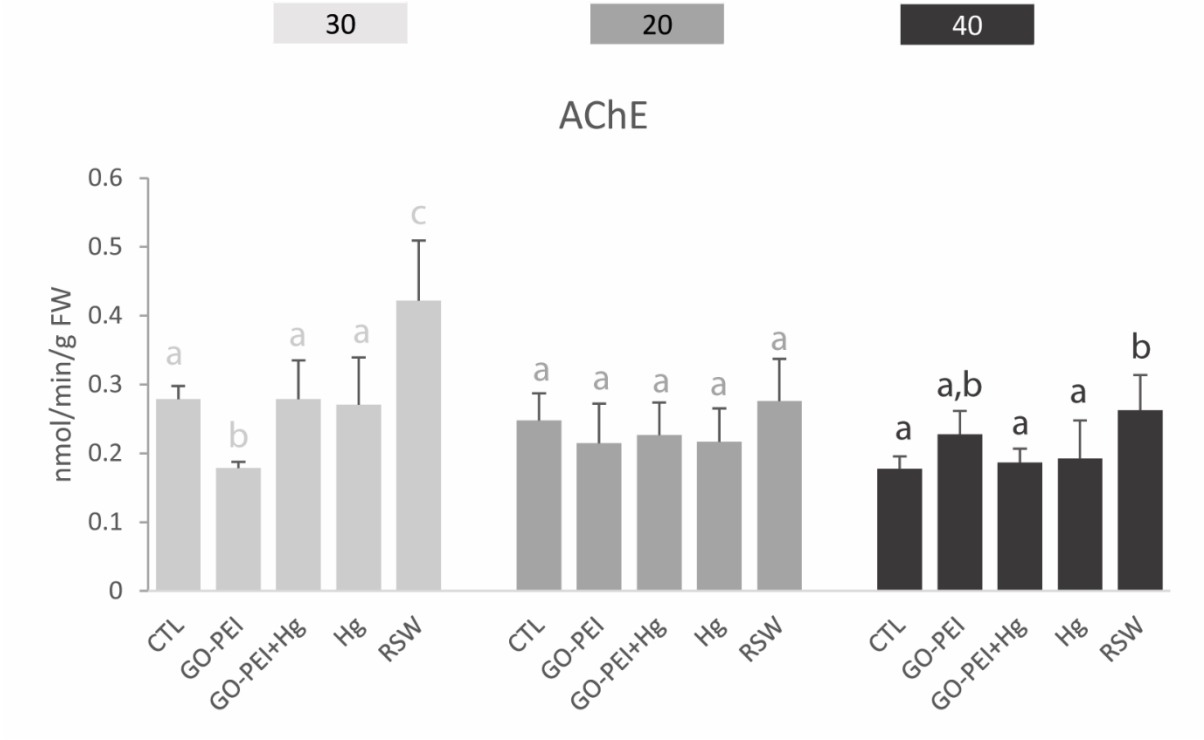


Figure 5. Acetylcholinesterase activity (AChE), in *M. galloprovincialis* exposed to different treatments (CTL, GO-PEI, GO-PEI+Hg, Hg and RSW) each under different salinities conditions (30, 20 and 40). Results are the means + standard deviation. Significant differences ($p<0.05$) among conditions are identified with different lowercase letters.

6.3.4 Histopathological measurements

6.3.4.1 Digestive tubules

At salinities 30 and 20, mussels under CTL condition showed significantly lower histopathological index (I_h) in digestive tubules (D.T.) compared to the other treatments (Figure 6A). At salinity 40, significantly higher histopathological index in digestive tubules was found in mussels under Hg compared to the remaining treatments. In addition, no significant differences were observed between CTL and GO-PEI, as well as between GO-PEI+Hg and RSW (Figure 6A). Examples of histopathological alterations detectable in digestive tubules at salinities 30, 20 and 40 are reported in Figures 7, 8 and 9, respectively. Lipofuscin aggregates were found prevalently at GO-PEI 30, GO-PEI+Hg 20, Hg 30 and 20 conditions, and in all mussel's digestive tubules exposed to salinity 40 treatments. Hemocyte infiltration was detected at GO-PEI 30 and 40 conditions, RSW 30 and 40 conditions, and Hg treatments under all salinities. Atrophy in digestive tubules was observed prevalently

in GO-PEI+Hg and Hg treatments. Necrosis was found in Hg treatments, independently from the salinities.

Among salinities, organisms' digestive tubules at CTL treatment, showed significantly lower I_h levels at salinity 20 in comparison to 30 and 40 salinities. Significantly lower I_h values were detected in mussels exposed to GO-PEI at salinity 40 compared to other two salinities. Moreover, mussels exposed to GO-PEI+Hg at salinity 20 showed significantly higher I_h values in comparison to salinities 30 and 40. Regarding mussels' digestive tubules under Hg treatments, significantly lower I_h values were detected at control salinity compared to salinity 40. In organisms at RSW treatments, significantly lower histopathological index was observed when comparing salinities 20 and 40 (Table 1).

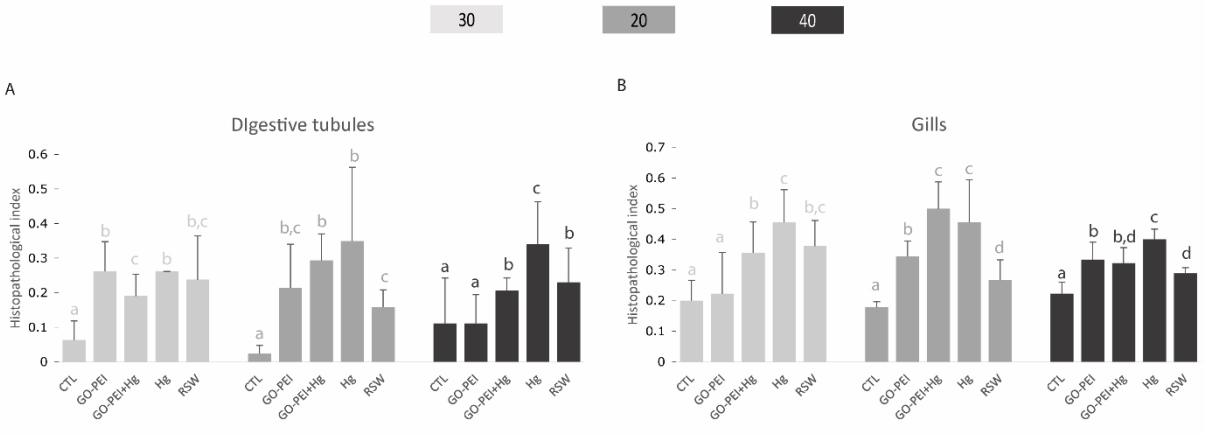


Figure 6. A: Histopathological index in digestive tubule and B: Histopathological index in gills, in *M. galloprovincialis* exposed to different treatments (CTL, GO-PEI, GO-PEI+Hg, Hg and RSW) each under different salinities conditions (30, 20 and 40). Results are the means + standard deviation. Significant differences ($p<0.05$) among conditions are identified with different lowercase letters.

6.3.4.2 Gills

At salinity 30, organisms exposed to CTL and GO-PEI treatments showed significantly lower I_h values in gills compared to values obtained for the remaining treatments (Figure 6B). Regarding gills exposed to salinity 20, significant differences were observed among all treatments, except between GO-PEI+Hg and Hg, with the lowest histopathological index in mussels' gills at CTL condition (Figure 6B). Considering mussels' gills at salinity 40, significant differences were observed among all treatments, except between GO-PEI+Hg and GO-PEI as well as between GO-PEI+Hg and RSW. The lowest and highest I_h in gills were observed in CTL and Hg treatments, respectively (Figure 6B). Histopathological alterations present in gills at salinities 30, 20 and 40 are reported in Figures 7, 8 and 9, respectively. Abundance of lipofuscin aggregates (highlighted with red asterisk) was observed in gills for each treatment, although less in CTL and GO-PEI treatments at salinity 30. The hemocytes infiltration was found in all treatments especially in mussels' gills exposed to GO-PEI+Hg and Hg regardless the salinity and in mussels exposed to GO-PEI at salinities 20 and 40. Also, evident enlargement of the central vessel was observed in organisms exposed to Hg at all salinities. Loss of

cilia was observed prevalently in mussels' gills exposed to Hg conditions, and in mussel exposed to GO-PEI+Hg at salinity 20.

Among salinities, at CTL condition, significantly higher I_h was observed at salinity 40 compared to salinity 20. Furthermore, mussels exposed to GO-PEI treatments, showed significantly lower I_h values at salinity 30 in comparison to salinities 20 and 40. Significantly higher I_h values were found in gills under GO-PEI+Hg at salinity 20 compared to salinities 30 and 40. Also, mussels exposed to Hg and RSW treatments showed, respectively, significantly lower and higher I_h values in gills at salinity 30 compared to the remaining salinities (Figure 6B).

Figure 7.

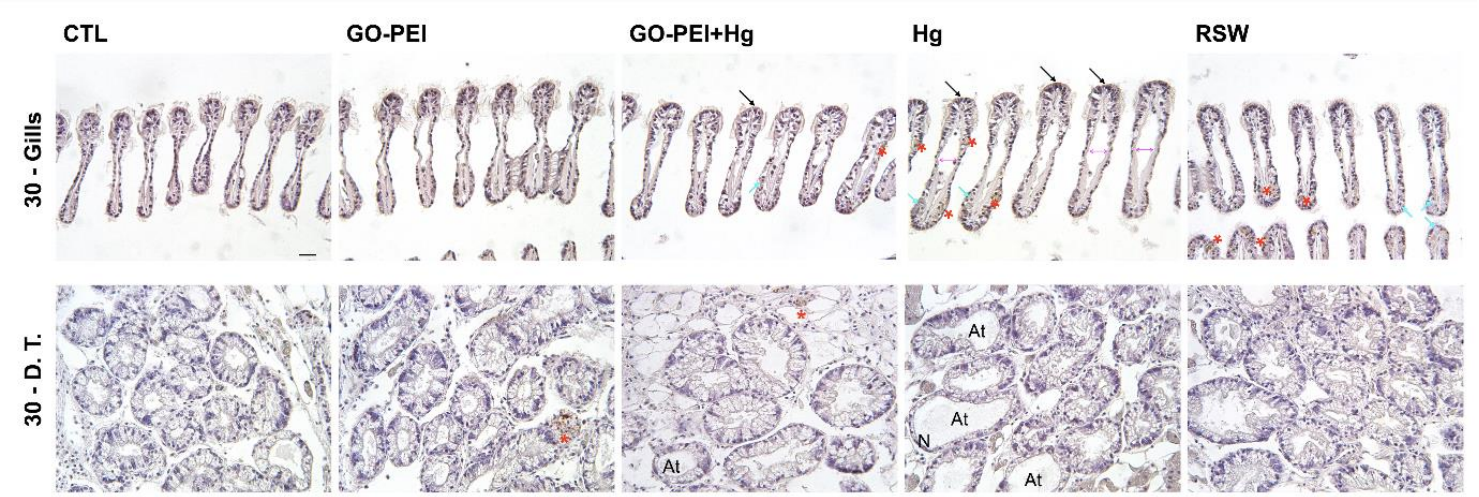


Figure 8.

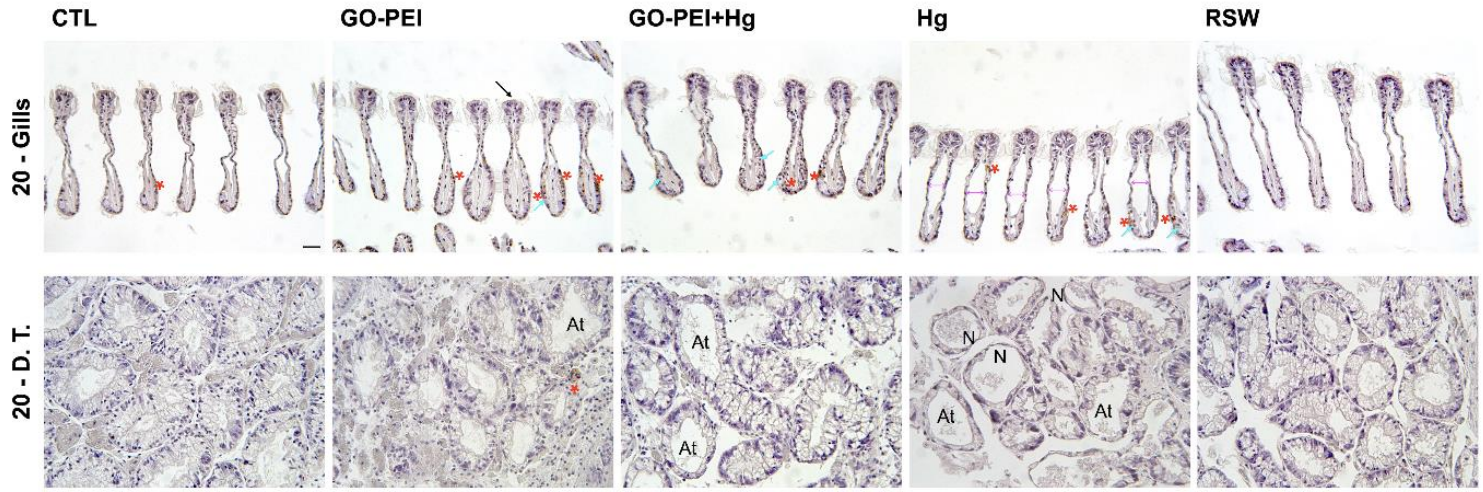
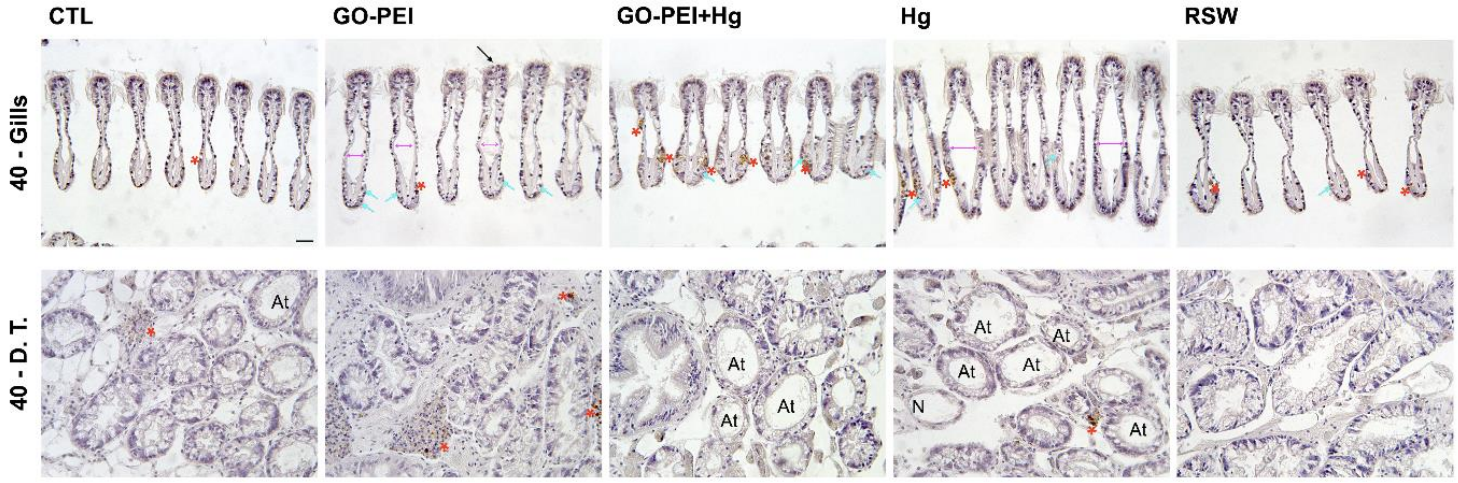


Figure 9.



Figures 7, 8 and 9. Micrographs of different tissues in *M. galloprovincialis* after 28 days-exposure. At 30 salinity, the tested treatments were: CTL, GO-PEI, GO-PEI+Hg, Hg and Remediated seawater (RSW). Digestive tubule (D.T.): lipofuscin aggregates (highlighted with red asterisk), atrophied (At) and necrose (N). Scale bar = 50 and 100µm; Gills: hemocyte infiltration (blue arrows), enlargement of the central vessel (pink arrows), abundance of lipofuscin aggregates (highlighted with red asterisk) and loss of cilia (black arrows).

6.4 Discussion

Under environmental conditions, bivalves may experience hyposaline and hypersaline stress, especially during extreme precipitation events that dilutes seawater and/or due to drought periods, leading to water evaporation and salinity rise (Anestis et al., 2007, Denton and Burdon-Jones, 1981, Gamain et al., 2016, Hamer et al., 2008, Mohammed and Scholz, 2018, Qiu et al., 2002, Rodrigues et al., 2014, Westerbom et al., 2002, Velasco et al., 2019). Accompanying such environmental alterations, different studies have demonstrated the impacts induced towards aquatic organisms, including physiological and behavioural alterations such as valve closure, reduction in feeding activity, slower growth rates and alterations on their endogenous rhythm (measured in oxygen consumption) (among others, Navarro and Gonzalez, 1998, Hamer et al., 2008, Sarà et al., 2008). Also, salinity changes can lead to oxidative stress and histopathological alterations (Matozzo et al., 2007, Munari et al., 2011, Freitas et al., 2020; Freitas et al., 2019ab, Bignell et al., 2008, Kefi et al., 2014). Previous studies also demonstrated that bivalves, including mussels, may alter their biochemical performance when exposed to pollutants as nanomaterials or metal(loid)s, especially under salinity alterations (among others, Pravoni et al., 2006, Delgado and Pérez-Camacho, 2007, Bebiano and Barreira, 2009, Ramos-Gómez et al., 2011, Figueira et al., 2012, Moschino et al., 2012, Figueira and Freitas, 2013, De Marchi et al., 2018 a,b, Velez et al., 2016). Nevertheless, to our knowledge no information is available on how salinity shifts may alter the sensitivity of bivalves when exposed to an Hg remediated seawater. In this way, the present study compared the responses given by *Mytilus galloprovincialis* when exposed to Hg, GO-PEI, GO-PEI+Hg and Hg remediated seawater (RSW) at salinities 30 (control salinity) 20 and 40. For this, metal bioaccumulation, biochemical and histopathological alterations after 28 days of chronic exposure were measured.

6.4.1 Mussels' responses observed under low salinity (20) and salinity control (30) condition

At both salinities, higher Hg concentration was found in mussels exposed to Hg, followed by mussels exposed to Hg combined with GO-PEI, and the lowest Hg values were found in mussels exposed to remediated seawater (RSW), revealing the capacity of *GO*-PEI to reduce the quantity of Hg in seawater and, thus, prevent the accumulation of this metal. These findings are in agreement with studies by Coppola et at. (2020a,b), that demonstrated a reduced accumulation of Hg in mussels and clams under remediated seawater at control salinity, regardless the temperature tested.

Comparing the accumulation of Hg at both salinity levels, the present findings showed higher Hg bioaccumulation in mussels at salinity 30 than those exposed to salinity 20. These results are not related with mussels' metabolic capacity since higher metabolism was observed at salinity 20 and, thus, increased filtration may be associated with low salinity, which could lead to higher Hg accumulation. Furthermore, lower Hg concentration at salinity 20 was not explained by higher detoxification capacity,

since GSTs activity was similar at both salinities. Thus, what we can hypothesise is that under greater stressful conditions (low salinity and the presence of Hg) mussels might develop strategies that limit Hg accumulation, which were not measured in the present study. Such strategies may include phase I detoxification enzymes, such are carboxylesterases, not evaluated in this study and that already showed high capacity to eliminate different pollutants (Solé et al., 2018).

In what concerns mussels' biochemical performance, in the present study higher electron transport system (ETS) activity was observed in bivalves at salinity 20 comparing to those at salinity 30 regardless the treatment, with exception to mussels exposed to remediated seawater (RSW). These results highlight the similar behaviour of mussels at RSW treatment at both salinities, while at the remaining treatments increased metabolic activity at salinity 20 may indicate that mussels were trying to re-establish their biochemical performance under stressful conditions induced by low salinity, independently on the presence or absence of pollutants. Similarly, Velez et al. (2016) showed that when the clam *Ruditapes decussatus* was exposed to salinity 14, the ETS activity was enhanced in comparison to clams exposed to salinity 28. Also the oyster *Crassostrea angulata* revealed increased ETS activity at low salinity (20) in comparison to salinity 30 (Moreira et al., 2016). Hamer et al. (2008) demonstrated that oxygen consumption rate of *M. galloprovincialis* acclimated to decreased salinities increased considerably to about 51 and 65 % in 28 salinity compared to control mussels (salinity 37). Furthermore, Kim et al. (2001) observed increased oxygen consumption in *R. philippinarum* clams exposed to salinity stress (15), indicating a metabolic adjustment to hypoosmotic stress. Such results demonstrate the capacity of bivalves to activate their metabolism when under low salinity conditions, possibly due to the need to fuel defence mechanisms. Higher metabolic potential at salinity 20 was accompanied by higher energetic expenditure, manifested by reduced GLY concentration at most of the treatments at salinity 20 in comparison to salinity 30, highlighting the need of mussels to use their energy reserves when metabolically active.

As demonstrated by other authors (e.g., Amiard-Triquet et al., 2012, Batley and Simpson, 2016, Regoli and Giuliani et al., 2014), in the present study the increased metabolism and expenditure of GLY in mussels exposed to the lowest salinity was associated with the activation of antioxidant defense mechanisms, especially SOD activity, in an attempt to prevent cellular damages. Also Carregosa et al. (2014) observed that at low salinity (14) clams significantly increased their SOD activity contributing to the strong decrease of the LPO levels at this salinity. Moreover, Zaccaron da Silva et al. (2005) showed that antioxidant enzyme activities in the oyster *C. rhizophorae* were higher at a salinity of 9 in comparison to increased salinity (15, 25 and 35). Furthermore, at both tested salinities, the present findings demonstrated that higher antioxidant defences were observed in mussels exposed to GO-PEI and Hg treatments, while mussels exposed to RSW presented enzyme activities similar to control mussels.

As for mussels' biotransformation capacity, increased GSTs activity was observed in mussels exposed to Hg treatments (GO-PEI+Hg and Hg), indicating the role of this enzyme in Hg elimination and protection of the organisms against products of oxidative stress (Hoarau et al., 2002). Again, at both salinities, similar GSTs values were observed between CTL and RSW mussels, highlighting the similar performance of mussels at clean and remediated seawater, independently on the salinity tested.

Regarding AChE activity, the present study showed no differences between salinities, indicating a low influence of salinity on mussels' neurotoxicity. De Marchi et al. (2018a,b) showed similar results when comparing the AChE activity in marine organisms (*R. philippinarum* and *Hediste diversicolor)* exposed to low (21) and control (28) salinity.

In the literature there is no information regarding the histological effects in bivalves when exposed to Hg and different salinity levels. However, the results obtained here in mussels' tissues showed an evident enlargement of the central vessel, abundance of lipofuscin aggregates in gills as well as atrophied and necrose in digestive tubules exposed to contaminated treatments independently on the salinity tested (30 or 20). At control salinity, several authors (e.g., Amachree et al., 2014, Cappello et al., 2013, Cuevas et al., 2015, Fasulo et al., 2012, Leite et al., 2020, Maisano et al., 2017) showed alterations with loss of structural integrity, lipofuscin aggregates, lip extensive areas denuded of cilia, and intense hemocytic infiltration in mussels' gills and digestive tubules exposed to pollutants. In what concerns to the histopathological index in mussels' digestive tubules under RSW and salinity 20, no lipofuscin aggregates, atrophied and necrosis was observed as well in the same tissue from organisms under RSW 30, CTL 20 and CTL 30 treatments. Moreover, mussels exposed to RSW at salinity 20 presented similar gills structure when compared with organisms under CTL 30. These results highlight that remediated seawater generated similar histopathological alterations at both salinities.

6.4.2 Mussels' responses observed under high salinity 40 and salinity control condition (30)

At salinities 30 and 40, higher Hg concentration was found in mussels exposed to Hg in comparison to the remaining treatments, with the lowest values found in RSW treatment followed by CTL. Again, these results indicate the capacity of GO-PEI to reduce the quantity of Hg in seawater and, thus, prevent the accumulation of this metal. The results presented here demonstrated that mussels exposed to Hg at salinity 40 showed lower metal concentration than organisms under salinity 30. Lower Hg accumulation at salinity 40 was not related with mussels' metabolic capacity since, except at GO-PEI+Hg treatment, similar ETS activity was obtained in mussels exposed to salinities 30 and 40. Nevertheless, higher ETS activity with GO-PEI+Hg at salinity 40 may explain higher Hg concentration at this treatment compared to mussels exposed to GO-PEI+Hg at salinity 30. Previous studies developed by Freitas et al. (2018) and Moreira et al. (2016) showed a decreased ETS activity in bivalves (mussel *M. galloprovincialis* and oyster *C. angulata*) under high salinity than the ones at control. The present findings further demonstrated that at each salinity similar ETS values were found between CTL and RSW treatments, and between salinities no differences were observed at RSW and CTL treatments. Similar metabolic capacity in mussels exposed to both salinities was, in general, accompanied by similar GLY content. These results may be justified by the fact that mussels can tolerate an increase in salinity since the Ria de Aveiro mussels can be found at a salinity range between 30 and 37 (IPMA, 2017). Therefore, the ETS activity increase when the organisms were under GO-PEI+Hg at salinity 40 in comparison to those at 30 condition, is probably associated with the variation of abiotic factors (in this case salinity increase)

which modify the behaviour of the Hg as well as its interaction with GO-PEI and at the same time can increase organisms' sensitivity. This possible synergistic effect lead to higher redox state impact under this condition. Published studies already demonstrated that the presence of pollutants, as metals, but also changes on salinity levels, may induce oxidative stress in marine invertebrates, with alterations on antioxidant enzymes activities, occurrence of cellular damage and loss of redox balance (among others, Franzellitti et al., 2013; Zuccato et al., 2006; Gonzalez-Rey and Bebianno, 2014; Carregosa et al., 2014; Freitas et al., 2019a,b). In the present study, although alterations on mussels' metabolic capacity and energy reserve were low, the increase of antioxidant enzymes activity at salinity 40 compared to salinity 30 could explain lower cellular damage at higher salinity with values similar to control salinity. An increase of antioxidant defences was observed by Matozzo et al. (2013) in the gills and digestive gland of *M. galloprovincialis* exposed to salinities 34 and 40 in comparison to control (28). Also, Gonçalves et al. (2017) showed an increase of SOD activity at salinity 35 in relation to control values (25 and 30). As demonstrated by Freitas et al. (2019), mussels *M. galloprovincialis* exposed to high salinity (35) showed an increase of SOD activity if compared to those under control condition (30). Regarding mussels exposed to RSW at salinity 40, the present results showed an increase of SOD accompanied by a decrease of GRed activity when compared to mussels under CTL 40 as well with the same treatments under salinity 30. These findings might explain lower cellular damage in mussels under RSW 40 in comparison to RSW 30. When comparing organisms under RSW and CLT 40 treatments the ratio GSH/GSSG was similar. Furthermore, the ratio was lower in mussels under Hg treatments than those exposed to RSW at the same salinity (40), showing the reduction of toxicity effects due to remediated seawater. Nevertheless, at salinity 40 higher ratio GSH/GSSG was observed in mussels exposed to Hg in comparison to the ones maintained at 30 condition, which is associated with higher GRed activity at this salinity (responsible for the reduction of GSSG to GSH), revealing a general increase of the oxidative status in *M. galloprovincialis* exposed to combination of pollutants and climatic changes.

The present findings evidenced a general inhibition of AChE activity in mussels exposed to salinity 40 compared to the ones at salinity 30, regardless the treatment, with exception for RSW conditions. In particular, organisms under RSW 40 salinity showed similar AChE activity than those at CTL 30 highlighting the limited influence of salinity on the neurotoxic potential of remediated seawater. Different authors also showed the inhibition of AChE in bivalves under combined stressors (Attig et al., 2010; Chalkiadaki et al., 2014; Freitas et al., 2018; Coppola et al., 2020; Morozesk et al., 2018), indicating that in this case high salinity and pollutants may greatly affect AChE.

The present study further demonstrated an increase of histopathological index when mussels are exposed to contaminated treatments under salinity 40 in comparison to those under salinity 30. A study conducted by Pagano et al. (2016) observed histological alterations in the gills and in digestive cells of *M. galloprovincialis* when exposed to quaternium-15 (is a quaternary ammonium salt used as a surfactant and preservative in many cosmetics and industrial substances) under high salinity (37) in comparison to mussels at non contaminated area (with salinity 30). Moreover, similar histopathological alterations were observed in mussels exposed to RSW at both salinities, revealing that salinity does not affect the toxicity of remediated seawater.

6.5 Conclusion

The present findings demonstrated that mussels exposed to salinity shifts (20 to 40) combined with Hg altered the impacts compared with both stressors acting alone. In particular, mussels exposed to lower salinity 20 increased their metabolic capacity in comparison to organisms under 30 condition, which may indicate a protective behaviour, associated with the activation of defence mechanisms under stress exposure, including the increase of antioxidant enzymes. On the other hand, mussels under higher salinity (40) presented similar metabolic capacity, energy reserve and cellular damage when comparing to control condition (30). However, organisms at 40 salinity showed a greater activation of antioxidant and biotransformation mechanisms in comparison to those under control condition. Nevertheless, mussels under RSW and CTL treatments, regardless the salinity tested, presented similar responses indicating the low toxic effect of remediated seawater at each of the salinities tested.

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CHAPTER 7.

Remediation of arsenic from contaminated seawater using manganese spinel ferrite nanoparticles: Ecotoxicological evaluation in *Mytilus galloprovincialis*

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Abstract

In the last decade different approaches have been applied for water remediation purposes, including the use of nanoparticles (NPs) to remove metals and metalloids from water. Although studies have been done on the toxic impacts of such NPs, very scarce information is available on the impacts of water after decontamination when discharged into aquatic environments. As such, in the present study we aimed to evaluate the ecotoxicological safety of seawater previously contaminated with arsenic (As) and remediated by using manganese-ferrite ($MnFe_2O_4$-NPs) NPs. For this, mussels *Mytilus galloprovincialis* were exposed for 28 days to different conditions, including clean seawater (control), As (1000 µg L^{-1}) contaminated and remediated (As 70 µg L^{-1}) seawater, water containing $MnFe_2O_4$-NPs (50 mg L^{-1}) with and without the presence of As. At the end of exposure, concentrations of As in mussels tissues were quantified and biomarkers related to mussels' metabolism and oxidative stress status were evaluated. Results revealed that mussels exposed to water contaminated with As and to As + NPs accumulated significantly more As (between 62% and 76% more) than those exposed to remediated seawater. Regarding biomarkers, our findings demonstrated that in comparison to remediated seawater (conditions a, b, c) mussels exposed to contaminated seawater (conditions A, B, C) presented significantly lower metabolic activity, lower expenditure of energy reserves, activation of antioxidant and biotransformation defences, higher lipids and protein damages and greater AChE inhibition. Furthermore, organisms exposed to As, NPs or As + NPs revealed similar biochemical effects, both before and after water decontamination. In conclusion, the present study suggests that seawater previously contaminated with As and remediated by $MnFe_2O_4$-NPs presented significantly lower toxicity than As contaminated water, evidencing the potential use of these NPs to remediate seawater contaminated with As and its safety towards marine systems after discharges to these environments.

Keywords

Oxidative stress, toxicity, mussels, magnetic spinel ferrite nanoparticles, nanosorbents, metalloids, bioaccumulation.

7.1 Introduction

The increment of pollutants in aquatic environments is closely related with the growth of the world population (Zhang et al., 2015). Studies demonstrated that intense urbanization and industrial activities, with the associated effluents, result in an increase of pollution in the aquatic systems, especially in marine environments (Nardi et al., 2017; Belivermiş et al., 2016; Oliveira et al., 2015; Ventura-Lima et al., 2011). Often, the final destination of pollutants are lagoons and estuaries (Zhang et al., 2015), with tendency to be accumulated not only in sediments but also by organisms inhabiting these areas (Buffet et al., 2014; Ventura-Lima et al., 2009, 2011). Among the most common pollutants in aquatic environments is arsenic (As), a naturally occurring element (ATSDR, 2015; Saxe et al., 2006) released by natural activities, such as volcanism, dissolution of minerals (particularly into groundwater), but principally by human activities, such as mining, metal smelting, combustion of fossil fuels, agricultural pesticide production and use, remobilization of historic sources, including mine drainage water (WHO, 2010; Mandal and Suzuki, 2002; Bhattacharya et al., 2007; Matschullat, 2000; Jang et al., 2016). As a results of its high toxicity, even at trace levels, As presents environmental concerns (IARC, 2012; Quasimeme, 2003; Fattorini et al., 2006). For this reason, currently As is considered the most priority hazardous substance in the environment based on the combination of substance frequency, toxicity and human exposure potential (ATSDR, 2015; Khan et al., 2010). In particular, the presence of As in aquatic systems has already proven to induce toxic impacts in a diversity of species, namely in bivalves, including physiological and biochemical impairments in clams (Freitas et al., 2018) and mussels (Coppola et al., 2018). Because of aquatic pollution and associated concerns, nowadays an important research topic is the development of new technologies for wastewater decontamination (Gehrke et al., 2015; Davidescu et al., 2015). Different methodologies have been developed to remove pollutants from waters, including oxidation/precipitation (Leupin and Hug, 2005; Dutta et al., 2005; Lee and Choi, 2002), coagulation/co-precipitation (Hansen et al., 2006; Kumar et al., 2004), sorption, ion-exchange (Baciocchi et al., 2005; Kim and Benjamin, 2004), membrane technologies (Kim et al., 2006; Ballinas et al., 2004), solvent extraction and bioremediation (Kordmostafapour et al., 2006; Iberhan and Wisniewski, 2003; Katsoyiannis et al., 2002). Some of these techniques have shown a great potential for removing inorganic pollutants from water (Gehrke et al., 2015; Mohan et al., 2006). Among the innovative techniques, one of the most promising approaches to decontaminate water is based on the use of nanoparticles (NPs), with some laboratory studies evidencing their high effectiveness in the removal of metal(loid)s (Tavares et al., 2013; Mohan and Pittman, 2007). In particular, manganese-ferrite ($MnFe_2O_4$) NPs, a common spinel ferrite material has shown to be very effective in decreasing inorganic pollution (including metals and metalloids) in freshwater and seawater (Zhang et al., 2010; Tavares et al., 2013; Jang et al., 2016; Santhosh et al., 2014). However, although the use of $MnFe_2O_4$-NPs for water decontamination is undoubtedly one of the most challenging research areas, important aspects are still missing, such as the potential toxicity of these NPs and the ecotoxicological evaluation of the remediated water (Bhatt and Tripathi, 2011; Lovern and Klaper, 2006; Lovern et al., 2007; Smith et al., 2007; Warheit et al., 2007). Together with decontaminated water or resulting from leaching of chemical elements, after application these NPs can end up in aquatic environments, making crucial the assessment of decontaminated water potential impacts towards inhabiting organisms. Until now,

different studies have already demonstrated the impacts induced directly by $MnFe_2O_4$-NPs in algae, crustaceans and fish, revealing their potential hazard to different aquatic species (Bahadar et al., 2016; Beji et al., 2010; Aslibeiki et al., 2016; Federici et al., 2007). Nevertheless, no studies have been carried out to evaluate the toxicity of water decontaminated by these NPs. To evaluate the impacts of the presence of pollutants, including NPs, in the aquatic environment, benthic species are a good biological model as they accumulate and reflect the impacts of different substances (Velez et al., 2015; Attig et al., 2014; Banni et al., 2014; Hu et al., 2015; Nardi et al., 2017; Coppola et al., 2018; Freitas et al., 2018). Among these species is the mussel *Mytilus galloprovincialis*, identified by several authors as a good bioindicator with the capacity to respond to environmental disturbances, presenting a wide spatial distribution and economic relevance (Coppola et al., 2017; Richir and Gobert, 2014; Freitas et al., 2017; Kristan et al., 2015; Mejdoub et al., 2017). This bivalve is a sedentary filter-feeder and has a large capacity to accumulate pollutants (Coppola et al., 2018; Livingstone, 2001; Selvin et al., 2000). Thus, by the above-mentioned, an important topic of research is to understand if the application of NPs to decontaminate seawater still constitutes a threat to aquatic environment, affecting negatively the inhabiting organisms. For this reason, the present study aimed to evaluate the toxicity induced in the mussel *M. galloprovincialis* exposed to seawater previously contaminated with As and decontaminated with $MnFe_2O_4$-NPs. After exposure to decontaminated seawater, biomarkers related to mussels' metabolic, oxidative stress and neurotoxic status were evaluated.

7.2 Materials and methods

7.2.1 Experimental conditions

The Mediterranean mussel *Mytilus galloprovincialis* was selected as biological model for this study (e.g. Coppola et al., 2018; Della Torre et al., 2015; Gomes et al., 2012a). Organisms were collected in November 2017, at the Ria de Aveiro lagoon (Portugal), with a mean body weight of 21.3 ± 6.6 g, fresh weight (FW). Bivalves were transported from the field to the laboratory in plastic containers, where they were placed in aquaria for depuration and acclimation to laboratory conditions for 2 weeks. To simulate field conditions, in the laboratory organisms were exposed to: temperature 17.0 ± 1.0 °C; pH 8.0 ± 0.1, photoperiod 12 h light and 12 h dark, and continuous aeration, in artificial seawater (salinity 30 ± 1) (Tropic Marin® SEA SALT from Tropic Marine Centre). Seawater was renewed daily during the first week and then every three days until the end of the acclimation period. After the acclimation period organisms were distributed in different aquaria according to the conditions described in Table 1. Seven different conditions were evaluated, with 3 aquaria (containing 3 L of seawater each) per condition and 4 individuals per aquarium/replicate (12 individuals per condition). Decontaminated seawater was obtained by adding 50 mg L^{-1} of $MnFe_2O_4$-NPs to water previously contaminated with 1000 µg L^{-1} of As. The NPs were removed from seawater after 24 h by applying a magnetic field (although a non-quantifiable residual amount of NPs may hypothetically remain in water) as described by Mohmood et al. (2016). During the experimental period (28 days), water medium was changed weekly and exposure conditions completely re-established, including contaminants concentrations and seawater characteristics (salinity, pH, temperature). Every week, immediately after medium renewal, samples of seawater were collected from each aquarium for As quantification. The concentration of As, 1000 µg L^{-1}, was selected according to the emission limit value for this element in wastewater discharges (Decree-Law No. 236/98, in Portuguese), while 70 µg L^{-1} is the residual concentration of As reached in seawater after decontamination with $MnFe_2O_4$-NPs (data from preliminary experiments, not shown). During the entire experimental period (28 days) aquaria were continuously aerated, with a 12 light: 12 dark photoperiod. As for the acclimation, temperature (17 ± 1.0 °C), pH (8.0 ± 0.1) and salinity (30 ± 1) values were selected considering measurements done at the sampling site (data not provided), and were daily checked and adjusted if necessary. During the experimental period organisms were fed with Algamac protein plus (150,000 cells/animal) twice a week. Mortality was also daily checked, with 100% of survival recorded during the experimental period. At the end of the exposure period, organisms were frozen individually with liquid nitrogen and stored at −80 °C, until homogenization of each individual soft tissue using a mortar and a pestle under liquid nitrogen. Each homogenized organism was divided into aliquots (0.5 g each) for biomarkers analyses and As quantification.

Table 1. Experimental conditions.

CONDITIONS		DESCRIPTION
CTL		Seawater with As 0 µg L^{-1} + NPs 0 mg L^{-1}
Water before As decontamination	**A**	Seawater with As 1000 µg L^{-1}
	B	Seawater with NPs 50 mg L^{-1}
	C	Seawater with As 1000 µg L^{-1} and NPs 50 mg L^{-1}
Water after As decontamination	**a**	Seawater with As 70 µg L^{-1}
	b	Seawater after 24 h in contact with NPs (50 mg L^{-1}), which were afterwards separated from seawater
	c	Seawater previously contaminated with As (1000 µg L^{-1}), then remediation using NPs (50 mg L^{-1}) during 24 h (which were afterwards separated from seawater).

7.2.2 Synthesis and characterization of $MnFe_2O_4$ nanoparticles

$MnFe_2O_4$-NPs were prepared by the chemical oxidative hydrolysis of a mixture of $FeSO_4.7H_2O$ and $MnSO_4.H_2O$ in alkaline conditions. Different techniques were applied to perform the chemical, physical and structural characterization of NPs. The morphology and particle size of the NPs were confirmed by transmission electron microscopy (TEM) using the Hitachi H-9000 TEM microscope operating at 300 kV. For TEM analysis, one drop of sample dispersed in ethanol was placed onto carbon-coated copper grid and then let the solvent evaporate. The surface area of the NPs was determined by N_2 adsorption/desorption on a Gemini V2.0 Micromeritics instrument. The crystalline phase of the NPs was identified by x-ray powder diffraction of the powders using a Philips Analytical PW 3050/60 X'Pert PRO (θ/2θ) diffractometer equipped with an X'Celerator detector and with automatic data acquisition (X'Pert Data Collector v2.0b software) by a monochromatized Cu Kα radiation (λ=1,54,056 Å) at 45 Kv/40 Ma. The NPs Fourier- Transform Infrared (FT-IR) spectrum was recorded on a Mattson 7000 spectrometer, at 4 cm^{-1} resolution, using a horizontal attenuated total reflectance (ATR) cell. The average size distribution of $MnFe_2O_4$-NPs in water at salinity 30 were measured by Dynamic Light Scattering (DLS) at T0 (immediately injected into seawater media), T1 (after 1 h) and T24 (after 24 h) (Table 2). These time periods were selected based on previous studies (Yao et al., 2014; Yang et al., 2012; Aubery et al., 2011) that showed aggregation and precipitation of different Fe-NPs within 24 h. DLS measurements were performed on a Delsa Nano C from Beckman Coulter, Inc. (Fullerton, CA) equipped with a laser diode operating at 658 nm. Scattered light was detected at 165° angle and analysed by using a log correlator over 120 accumulations, for 1.0 mL of sample in a UV cuvette semi-micro. Each sample was reproducibly shaken before analysis and exposed to the minimum of DLS measurements needed to obtain at least three valid data. The calculation of particle size distribution and distribution averages was performed by using CONTIN particle size distribution analysis routines through Delsa Nano 3.73 software. The hydrodynamic radius and polydispersity index

of the analysed dispersions were calculated on three replicates of each sample by using the cumulant method. Undetected colloidal material at the end of each measurement is indicated as Invalid data (I.d.).

Table 2. Average size distribution of $MnFe_2O_4$-NPs in seawater (nm), at the beginning of the experiment (T0) and after 1 h and 24 h (T1 and T24, respectively). Standard deviation (SD) of replicates and coefficient of variation (CV) are also displayed.

Time (hours)	MEAN (nm)	SD	CV%
T0	3987	614	15
T1	14045	498	35
T24	67013	152	23

7.2.3 Arsenic quantification

The quantification of As in water samples collected from each condition was performed by inductively coupled plasma mass spectrometry (ICP-MS), on a Thermo ICP-MS X Series equipped with a Burgener nebulizer. The quantification limit of the method was 1.5 µg L^{-1} (n=12), with an acceptable relative standard deviation among replicates (n≥2) < 5%. Total As concentrations in *M. galloprovincialis* whole soft tissues were quantified by ICP-MS, after microwave assisted acid digestion. Samples with 100–200 mg (freeze-dried) were digested in a CEM MARS 5 microwave, firstly with 2 mL of HNO3 (70%) at 170 °C for 15 min, followed by a second identical microwave cycle with 0.5 mL of H_2O_2 (30%). After addition of H2O2, the mixture was allowed to stand for 15 min so that the microwave reaction was not as violent. The obtained digests were transferred into 25 mL polyethylene vessels and the volume made up with ultrapure water. The quality control was assured by running procedural blanks (reaction vessels with only HNO_3 and H_2O_2) and certified reference material TORT-2 (Lobster Hepatopancreas; 21.6 ± 1.8 mg kg^{-1} As) in parallel with samples. Blanks were always below the quantification limit and mean percentage of recovery for As was 110 ± 4% (n=4).

7.2.4 Biomarkers evaluation

The whole tissue of mussels was used for biomarkers determination (see section 2.1). For each parameter, 0.5 g of tissue per organism was used, with 2 individuals per replicate (6 per condition). For each condition, metabolic capacity (electron transport system activity, ETS), energy-related (glycogen content, GLY; total protein content, PROT), antioxidant defence (superoxide dismutase activity, SOD; glutathione peroxidase activity, GPx; glutathione S-transferases activity, GSTs), oxidative damage (lipid peroxidation levels, LPO; protein carbonyl levels, PC; glutathione content ratio, GSH/GSSG) and neurotoxicity (Acetylcholinesterase activity, AChE) biomarkers were assessed. Each sample was performed at least in duplicate. All measurements were done using a microplate reader (BioTek, Synergy HT). The extraction for each biomarker was performed with specific buffers: phosphate buffer for SOD, GPx, GSTs, PROT, GLY, CP and AChE; magnesium sulphate buffer for ETS; trichloroacetic

acid buffer for LPO and KPE buffer for GSH/GSSG. Each sample was sonicated for 15 s at 4 °C and centrifuged for 25 min (or 15 min for GSH/GSSG) at 10,000 *g* (or 3000 *g* for ETS) (Coppola et al., 2018; De Marchi et al., 2018; Freitas et al., 2018). Supernatants were stored at −20 °C and used within a maximum period of 3 weeks.

7.2.4.1 Metabolic capacity and energy-related biomarkers

ETS activity was measured based on King and Packard (1975) and the modifications performed by De Coen and Janssen (1997). The absorbance was measured at 490 nm during 10 min with intervals of 25 s. The amount of formazan formed was calculated using ε=15,900$M^{-1}cm^{-1}$ and the results expressed in nmol min^{-1} per g of fresh weight (FW). For GLY quantification the sulphuric acid method was used, as described by (Dubois et al., 1956). A calibration curve was obtained using glucose standards prepared in concentrations between 0 and 10 mg mL^{-1}. Absorbance was measured at 492 nm and the results were expressed in mg per g FW. The PROT content was determined following the spectrophotometric method of Biuret (Robinson and Hogden, 1940), and bovine serum albumin (BSA) was used as standard (0–40 mg mL^{-1}) to obtain a calibration curve. Absorbance was measured at 540 nm. Concentrations of PROT were expressed in mg per g FW.

7.2.4.2 Antioxidant defences biomarkers

The activity of SOD was quantified following the method of Beauchamp and Fridovich (1971) and was performed with a calibration curve using SOD standards between 0.25 and 60 U mL^{-1}. The absorbance was measured at 560 nm and the results were expressed in U per g FW, where U represents the quantity of the enzyme that catalyses the conversion of 50% of nitroblue tetrazolium (NBT). The activity of GPx was determined following the method of Paglia and Valentine (1967). Absorbance measurements were performed at 340 nm during 5 min in 10 s intervals and the activity was determined using the extinction coefficient of ε=6.22mM^{-1} cm^{-1}. Results were expressed in U per g FW, where U corresponds to the quantity of enzyme which catalyses the conversion of 1 µmol nicotinamide adenine dinucleotide phosphate (NADPH) per min. GSTs activity was determined according to Habig et al. (1974). The absorbance was measured at 340 nm. The activity of GSTs was determined using ε=9.6mM^{-1} cm^{-1}. The enzymatic activity was expressed in U per g FW where U is defined as the amount of enzyme that catalysis the formation of 1 µmol of dinitrophenyl thioether per min.

7.2.4.3 Oxidative damage biomarkers

LPO was determined following the method described by Ohkawa et al. (1979). LPO levels were measured trough the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation. Absorbance was measured at 532 nm (ε=156mM^{-1} cm^{-1}). LPO levels were expressed in nmol of MDA per g FW. PC content was obtained following Levine et al. (1990). Absorbance of samples was

measured at 450 nm and the carbonyl content was calculated using an absorption coefficient Ɛ=0.022mM^{-1} cm^{-1}. Results were expressed in nmol of PC groups formed per g FW. GSH and GSSG glutathione contents were measured at 412 nm (Rahman et al., 2007) and used as standards (0–60 µmol L^{-1}) to obtain a calibration curve. Absorbance was measured at 412 nm, for both assays. The results were expressed as nmol per g FW. The ratio GSH/GSSG was determined taking in account the number of thiol equivalents (GSH/2 ∗ GSSG).

7.2.4.4 Neurotoxicity biomarker

Acetylthiocholine iodide (ATChI, 5 mM) substrates were used for the determination of Acetylcholinesterase (AChE) following the methods of Ellman et al. (1961) and modification by Mennillo et al. (2017). Enzyme activity was recorded continuously for 5 min at 412 nm and expressed in nmol per g FW.

7.2.5 Integrated biomarker response (IBR)

The integrated biomarker response (IBR) was calculated according to Beliaeff and Burgeot (2002) aiming to evaluate the general mussels' biochemical response among 6 conditions. All biomarkers determined were used in the calculation of the IBR and they were arranged clockwise in the following order: ETS, GLY, PROT, SOD, GPx, LPO, CP, GSH/GSSG, GST, AChE. Values were discussed in terms of a general response given by the final IBR value, where higher values correspond to higher mussels' response.

7.2.6 Statistical analyses

All the biochemical results (ETS, GLY, PROT, SOD, GPx, GSTs, LPO, PC, GSH/GSSG and AChE) and As concentrations in mussels tissues, obtained from each condition, were submitted to statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA+ add-on in PRIMER v6 (Anderson et al., 2008). The pseudo-F *p*-values in the PERMANOVA main tests were evaluated in terms of significance. When significant differences were observed in the main test, pairwise comparisons were performed. Values lower than 0.05 ($p \leq 0.05$) were considered as significantly different. For each biomarker, *p*-value obtained for pair-wise comparisons are represented with *p*-value in Table 5. For As concentrations and each biomarker, the null hypotheses (H0) tested were: i) no significant differences exist among CTL and all the contaminated conditions (CTL, A, B and C). *p*-values are presented in Table 5, with significant differences highlighted in bold; ii) no significant differences exist among decontaminated conditions (CTL, a, b, and c). *p*-values are presented in Table 5, with significant differences highlighted in bold; iii) no significant differences exist between A vs a, B vs b, C vs c conditions. Significant differences between each pair of conditions are represented with an asterisk in figures.

7.3 Results

7.3.1 Characterization *of $MnFe_2O_4$ nanoparticles*

$MnFe_2O_4$-NPs showed a spheroidal morphology (Figure 1) with a mean diameter and standard deviation of 75 ± 15 nm. The infrared spectrum of the NPs displayed a characteristic band at 537 cm^{-1} related to metal-O stretching vibration of the $MnFe_2O_4$-NPs (Bellusci et al., 2009; Mehran et al., 2016; Tavares et al., 2013). The band at 1107 cm^{-1} was attributed to metal-OH and to metal-OH_2 stretching vibrations, which correspond to water sorption on oxide, while 1635 cm^{-1} band is due to H-O-H bending and corresponds to molecular water adsorbed or incorporated into the crystalline lattice (Bellusci et al., 2009). The broad band at 3309 cm^{-1} corresponds to symmetric and asymmetric stretching of OeH bond (Margabandhu et al., 2016). Powder X-ray diffraction (XRD) pattern show peaks that are characteristics of the presence of $MnFe_2O_4$ with the spinel structure (JCPDS–International centre diffraction data, PDF card 01-071-4919). In seawater, an aggregation of the NPs was observed by DLS, reaching an average size of approximately 60000 nm, after 24 h. The presence of As in water did not influence NPs aggregation since sizes in conditions A, B, a and b, after 24 h, the average sizes were indistinguishable (data not shown).

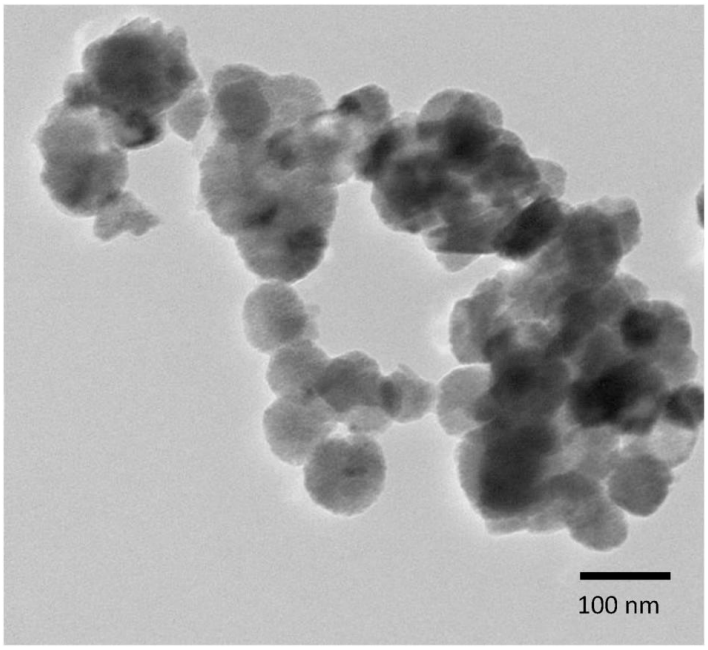

Figure 1. Transmission Electronic Microscopy image of $MnFe_2O_4$-NPs

7.3.2 Arsenic quantification in seawater and mussels' tissues

Concentrations of As in water samples revealed that real and nominal concentrations were similar, both for A and a conditions. In water samples from conditions without As (B and b) the concentrations of this metalloid were lower than the quantification limit (1.5 µg L^{-1}). Concentration of As

in water after decontamination was 55 ± 13 µg L^{-1}. Because sorption of As by the NPs is extremely rapid, As was not possible to quantify in water from condition C (Table 3). The results obtained from As quantification in mussels showed a significant difference between organisms exposed to CTL and those exposed to A and C conditions (Table 4). No significant differences were found between *M. galloprovincialis* submitted to CTL and the organisms exposed to conditions a, b and c (Table 4). Significant differences were observed in terms of As concentrations between mussels exposed to initial (before decontamination) and final (after decontamination) conditions (A vs a and C vs c) (Table 4). Organisms exposed to condition A accumulated more 76% of As than those exposed to condition a, while the contents of As in the mussels exposed to condition C were 62% higher than those in condition c.

Table 3. Arsenic concentration (µg L−1) measured in water samples collected immediately after the weekly water renewal. Results correspond to the mean value and standard deviation of the four weeks.

[As] water µg L^{-1}		
CTL		<1.5
As	**A**	947 ± 17
	a	82 ± 15
NP	**B**	<1.5
	b	<1.5
As + NP	**C**	*
	c	55 ± 13

*Because sorption of As by the NPs is extremely rapid, its quantification in this condition was not performed.

Table 4. Arsenic concentration in mussels (mg kg^{-1}), 28 days after the beginning of the experiment. Concentrations were measured in organisms from different conditions: (CTL, a, A, b, B, c and C). Asterisks represent differences between A vs a, B vs b and C vs c conditions, while different lowercase letters represent differences between CTL vs a, CTL vs b, CTL vs c and uppercase CTL vs A, CTL vs B, CTL vs C conditions.

As concentration (mg kg^{-1})			
CTL		7.4 ± 1.5A,a	
As	**A**	12 ± 2.6B	*
	a	6.8 ± 2.2a	
NP	**B**	5.2 ± 0.9A	
	b	4.4 ± 0.2a	
As + NP	**C**	11 ± 2.7B *	*
	c	6.8 ± 2.2a	

7.3.3 Biochemical markers

7.3.3.1 Metabolic capacity and energy-related biomarkers

The ETS activity was significantly higher at control (CTL) in comparison to the values obtained in mussels exposed to contaminated seawater (conditions A, B, C; resembling initial concentrations, measured before decontamination), with the lowest values at condition A. ETS activity was significantly higher at control (CTL) in comparison to the values obtained in mussels exposed to decontaminated seawater (conditions a, b, c) (Figure 2A, Table 5). The ETS activity was significantly higher in organisms exposed to decontaminated seawater (conditions a, b, c) in comparison to organisms exposed to contaminated seawater (conditions A, B, C) (Figure 2A). The GLY content was significantly lower in mussels exposed to control (CTL) in comparison to the values observed in mussels exposed to contaminated seawater (conditions A, B, C) (Figure 2B, Table 5). Significantly lower GLY content was obtained in organisms exposed to decontaminated seawater (conditions a, b, c) in comparison to organisms exposed to contaminated seawater (conditions A, B, C) (Figure 2B). The PROT content was significantly lower in mussels exposed to control (CTL) in comparison to values observed in mussels exposed to contaminated seawater (conditions A, B, C), (Figure 2C, Table 5). The PROT content was significantly lower in organisms exposed to decontaminated seawater (conditions a, b, c) in comparison to organisms exposed to contaminated seawater (conditions A, B, C) (Figure 2C).

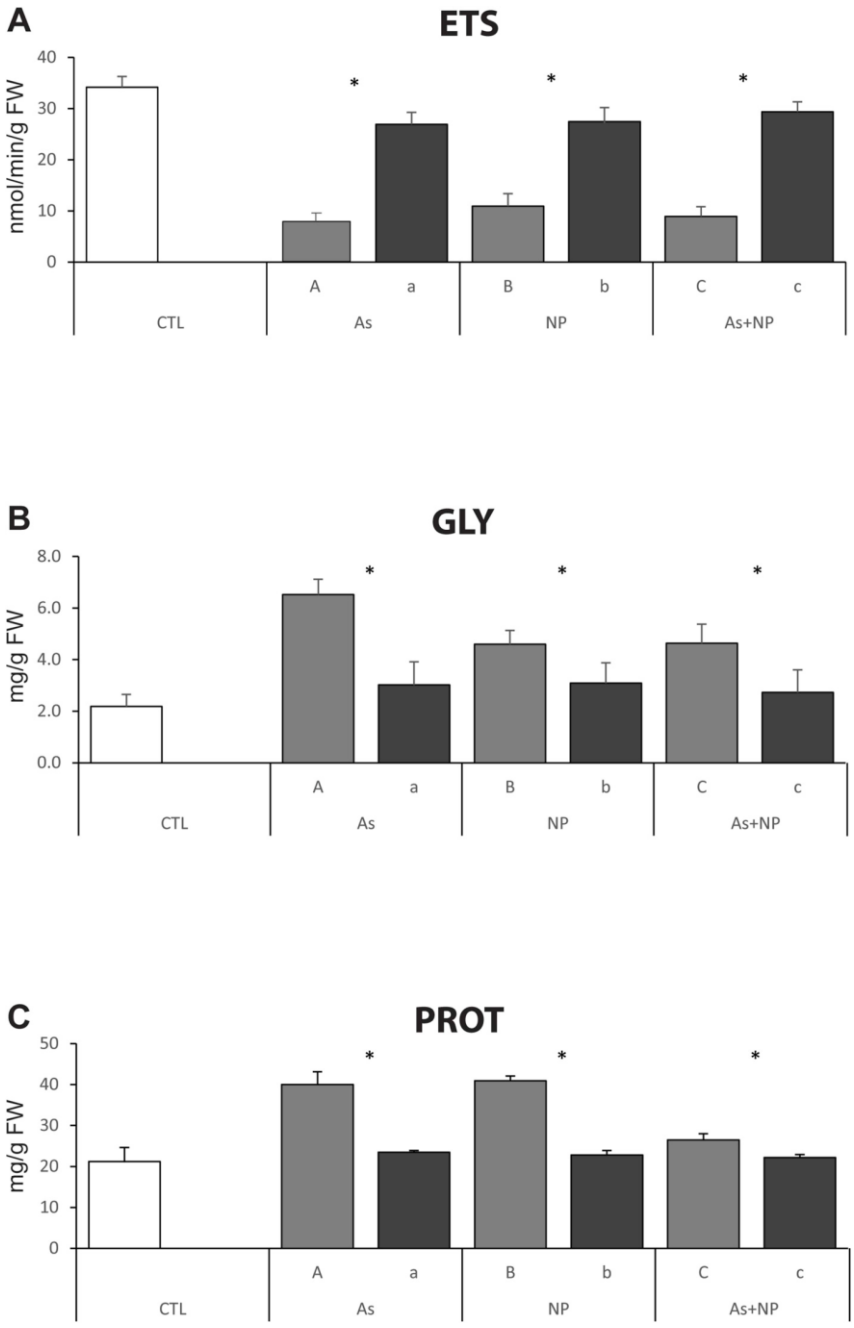

Figure 2. A: Electron transport system activity (ETS); B: Glycogen content (GLY); C: Total protein content (PROT) in *Mytilus galloprovincialis* exposed to different conditions (CTL, a, A, b, B, c and C) at the end of the experiment. Results are mean + standard deviation. Significant differences between conditions A vs a, B vs b, C vs c are presented with asterisks.

Table 5. *p*-values obtained by pair-wise comparisons between conditions (CTL vs A, CTL vs B, CTL vs C, CTL vs a, CTL vs b, CTL vs c, A vs B, A vs C, B vs C, a vs b, a vs c, and b vs c) for each biomarker: ETS, electron transport system activity; GLY, glycogen content; PROT, total protein content; SOD, superoxide dismutase activity, GPx, glutathione peroxidase activity; GSTs, glutathione S-transferases activity; LPO, lipid peroxidation levels; PC, protein carbonyl levels; GSH/GSSG, glutathione ratio; AChE, acetylcholinesterase activity. Significant differences ($p \leq 0.05$) are highlighted in bold.

	ETS	*GLY*	*PROT*	*SOD*	*GPx*	*GSTs*	*LPO*	*PC*	*GSH/GSSG*	*AChE*
CTL vs A	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0001**
CTL vs B	**0.0001**	**0.0001**	**0.0047**	**0.0019**	**0.0001**	**0.0001**	**0.0018**	**0.0001**	**0.0001**	**0.0001**
CTL vs C	**0.0001**	**0.0001**	**0.0003**	**0.0420**	**0.0001**	**0.0001**	**0.0023**	**0.0001**	**0.0001**	**0.0001**
CTL vs a	**0.0081**	0.0966	0.0821	0.2022	**0.0003**	0.0663	0.6358	**0.0293**	**0.0045**	**0.0093**
CTL vs b	**0.0376**	0.0598	0.1077	0.2325	**0.0168**	0.8804	**0.0001**	**0.0448**	**0.3016**	**0.0018**
CTL vs c	**0.0257**	0.1936	0.0609	0.1342	**0.0001**	**0.0057**	0.0512	0.2694	**0.0026**	**0.0043**
A vs B	0.1146	**0.0001**	0.9018	**0.0002**	0.1127	**0.0001**	0.1012	**0.0001**	0.2629	0.8423
A vs C	0.1934	**0.0017**	**0.0032**	**0.0009**	**0.0055**	0.3649	0.0638	0.8632	0.0868	0.9424
B vs C	0.3265	0.9497	**0.0331**	0.4324	0.1733	**0.0001**	0.7600	**0.0001**	0.9478	0.7912
a vs b	0.8776	0.8939	0.3760	0.7590	0.1757	**0.0016**	**0.0027**	0.8243	**0.0298**	0.3500
a vs c	**0.0438**	0.6053	0.1222	0.4063	**0.0384**	0.0675	0.3987	0.1524	0.9786	0.4114
b vs c	0.5399	0.4136	0.4084	0.2369	**0.0006**	**0.0001**	**0.0001**	0.2086	**0.0188**	0.9893

7.3.3.2 Antioxidant defence biomarkers

The SOD activity was significantly lower at CTL in comparison to values obtained in mussels exposed to contaminated seawater (A, B, C). Significantly higher values were obtained in mussels exposed to condition A in comparison to organisms exposed to conditions B and C (Figure 3A, Table 5). The SOD activity was significantly lower in organisms exposed to decontaminated seawater (conditions a, b, c) in comparison to organisms exposed to contaminated seawater (conditions A, B, C) (Figure 3A). The activity of GPx was significantly lower at CTL in comparison to values obtained in mussels exposed to contaminated seawater (A, B, C). Significant differences were observed between organisms exposed to A and C conditions. Regarding to organisms exposed to decontaminated seawater, significantly higher GPx activity was observed at a, b and c conditions in comparison to control (CTL). No significant differences were observed between organisms exposed to conditions a and b (Figure 3B, Table 5). The GPx activity values were significantly lower in organisms exposed decontaminated seawater (conditions a, b, c) in comparison to organisms exposed to contaminated seawater (conditions A, B, C) (Figure 3B). The GSTs activity was significantly lower at CTL in comparison to values obtained in mussels exposed to contaminated seawater (A, B, C). No significant differences were observed between organisms exposed to A and C conditions. Organisms under control (CTL) conditions showed significantly lower GSTs activity than those exposed to decontaminated seawater (condition c). No significant differences were observed between organisms exposed to a and c conditions (Figure 3C, Table 5). The GSTs activity values were significantly lower in organisms exposed to decontaminated seawater (conditions a, b, c) comparatively to organisms exposed to contaminated seawater (conditions A, B, C) (Figure 3C).

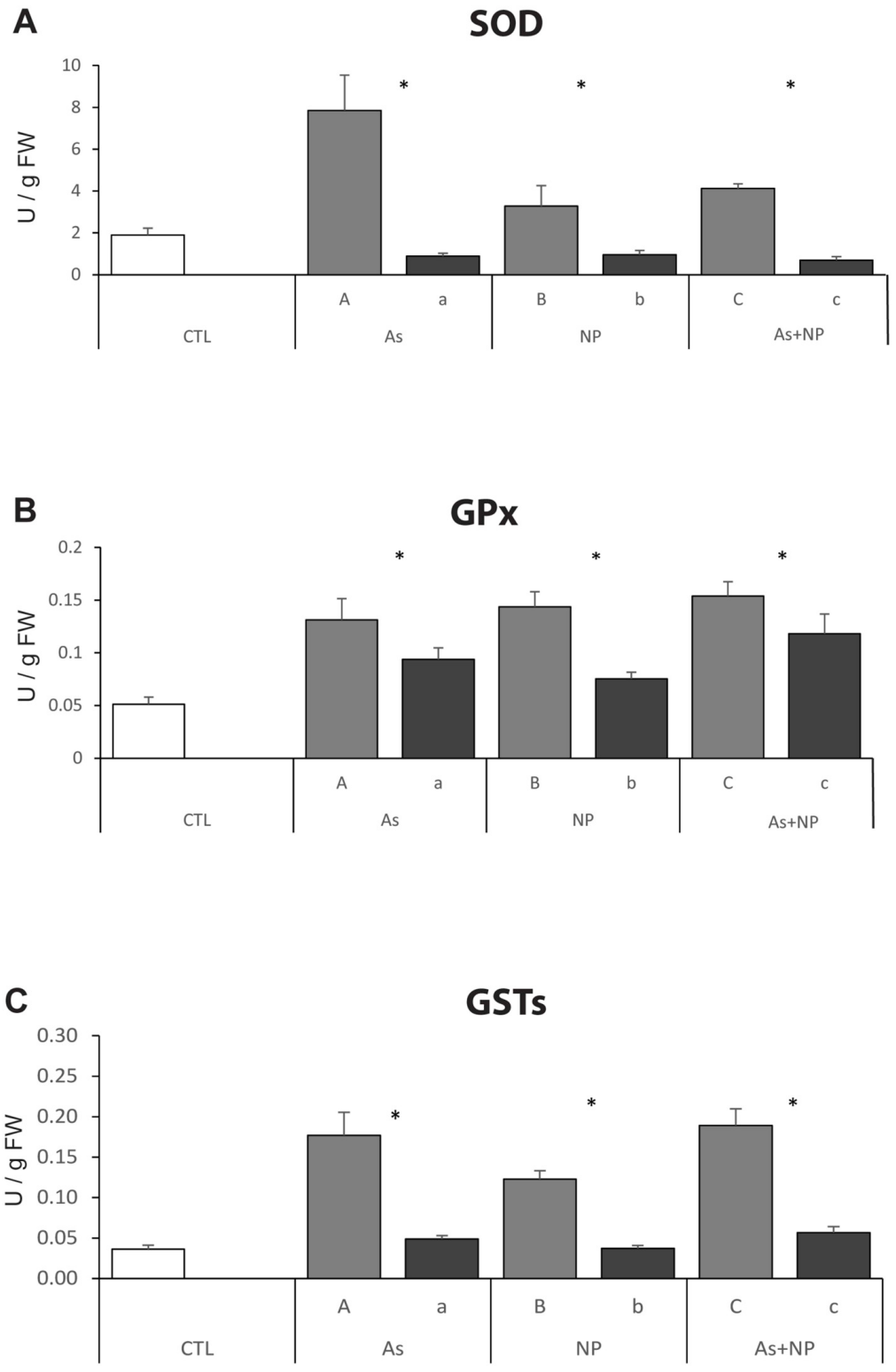

Figure 3. A: Superoxide dismutase activity (SOD); B: glutathione peroxidase activity (GPx); C: Glutathione S-transferases activity (GSTs), in *Mytilus galloprovincialis* exposed to different conditions (CTL, a, A, b, B, c and C) at the end of the experiment. Results are mean + standard deviation. Significant differences between conditions A vs a, B vs b, C vs c are presented with asterisks.

7.3.3.3 Cellular damage biomarkers

The LPO levels were significantly lower at control (CTL) in comparison to values obtained in mussels exposed to contaminated seawater (conditions A, B, C). Significantly higher LPO levels were observed in organisms exposed to CTL compared to organisms exposed to condition b. No significant differences were observed between organisms exposed to a and c conditions (Figure 4A, Table 5). The LPO levels were significantly lower in organisms exposed to decontaminated seawater (conditions a, b, c) in comparison to organisms exposed to contaminated seawater (conditions A, B, C) (Figure 4A). The PC levels were significantly lower in mussels exposed to control (CTL) in comparison to values observed in mussels exposed to contaminated seawater (conditions A, B, C). The PC levels in mussels exposed to control (CTL) were significantly lower than those observed in mussels exposed to conditions a and b. No significant differences were observed among organisms exposed to a, b and c conditions (Figure 4B, Table 5). The PC levels were significantly lower in organisms exposed to decontaminated (conditions a, b and c) seawater comparatively to organisms exposed to contaminated seawater (conditions A, B, C) (Figure 4B). The GSH/GSSG values were significantly higher in mussels exposed to control (CTL) in comparison to values observed in mussels exposed to contaminated seawater (conditions A, B, C). Significantly higher GSH/GSSG values were observed in mussels exposed to control (CTL) in relation to the values observed in mussels exposed to decontaminated seawater (conditions a, c). No significant differences were observed between organisms exposed to a and c conditions (Figure 4C, Table 5). The GSH/GSSG ratio was significantly higher in organisms exposed to decontaminated seawater (conditions a, b, c) than in organisms exposed to contaminated seawater (conditions A, B, C) (Figure 4C).

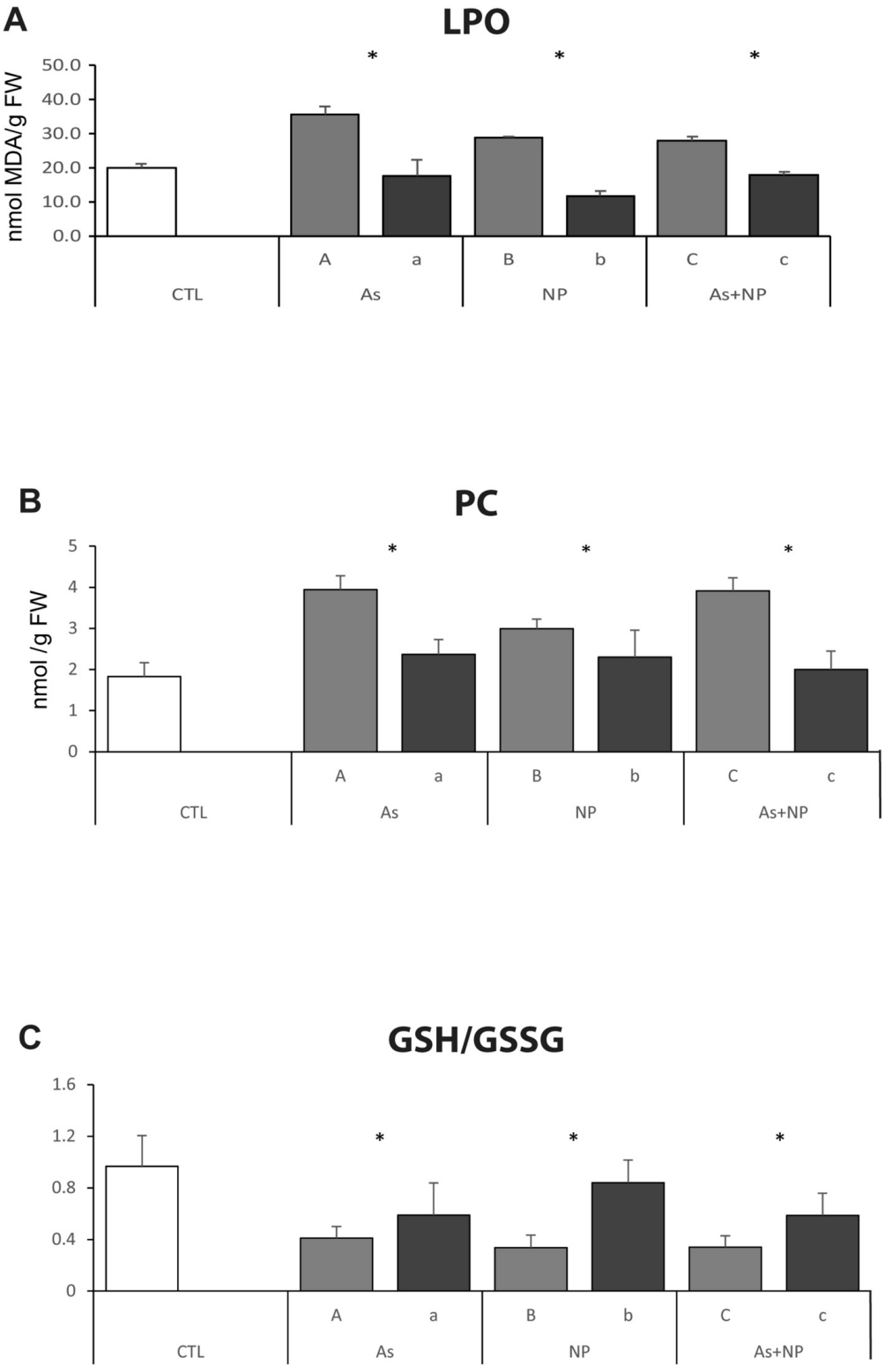

Figure 4. A: Lipid peroxidation levels (LPO); B: protein carbonyl levels (PC); C: ratio between reduced and oxidized glutathione (GSH/GSSG), in *Mytilus galloprovincialis* exposed to different conditions (CTL, a, A, b, B, c and C) at the end of the experiment. Results are mean + standard deviation. Significant differences between conditions A vs a, B vs b, C vs c are presented with asterisks.

7.3.3.4 Neurotoxicity biomarker

The AChE activity was significantly higher in mussels exposed to control (CTL) in comparison to the values observed in mussels exposed to contaminated seawater (conditions A, B, C). Significantly higher AChE values were observed in mussels exposed to control (CTL) in comparison to those observed in mussels exposed to decontaminated seawater (conditions a, b, c) (Figure 5, Table 5). Significantly higher AChE values were observed in organisms exposed to decontaminated seawater (conditions a, b, c) than in organisms exposed to contaminated seawater (conditions A, B, C) (Figure 5).

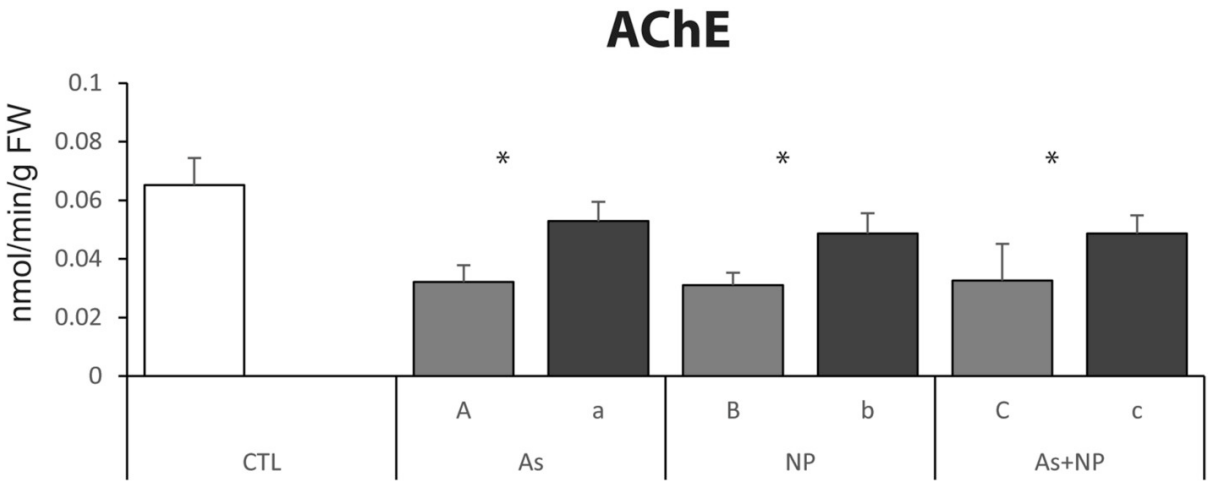

Figure 5. Acetylcholinesterase activity (AChE), in *Mytilus galloprovincialis* exposed to different conditions (CTL, a, A, b, B, c and C) at the end of the experiment. Results are mean + standard deviation. Significant differences between conditions A vs a, B vs b, C vs c are presented with asterisks.

7.3.4 Integrated biomarker response (IBR)

Organism exposed to condition c showed the lowest IBR values (1.57), with higher values for conditions A, a, B, b and C (7.23, 2.23, 18.96, 5.05, 9.70, respectively).

7.4 Discussion

The wide proliferation of NPs for different uses may result in their release and dispersion in the environment with potential harmful effects (Katuli et al., 2014; Keller et al., 2010; Guan et al., 2018; Hanna et al., 2013). Moreover, when in the environment, the capacity of NPs to interact with other pollutants may influence their toxicity (Moore, 2006; Fabrega et al., 2011). Studies focused on the toxicity of NPs in aquatic environment showed that their toxicity depends on their nature, exposure concentration, shape, size, and surface charge (Sun et al., 2016; Jastrzębska and Olszyna, 2015) as well as on the time of exposure, medium composition, route of particle administration and target species (Khosravi-Katuli et al., 2017). Regarding the use of NPs for water decontamination, the available information is very scarce, in particular the one devoted to the possible toxic impacts of remediated water, resulting from incomplete removal of contaminants, prevalence of NPs residues, or other changes in water physicochemical properties induced by the remediation condition. Although the adsorption capacity for both organic and inorganic toxicants of various nanoparticles was evaluated and summarized by several authors (Davidescu et al., 2015; Gehrke et al., 2015; Mohan and Pittman, 2007; Aslibeiki et al., 2016), no knowledge on the possible toxicity of the decontaminated water is available. In the present study we assessed the toxicity of magnetic manganese spinel ferrite nanoparticles ($MnFe_2O_4$-NPs) which have high capacity to adsorb As from seawater, as well as the efficiency of the treatment from an ecotoxicological point of view, assessing the toxicity of the remediated seawater towards the mussels *Mytilus galloprovincialis*.

7.4.1 Impact of As single exposure, before and after decontamination (conditions A and a)

Regarding As bioaccumulation in the whole soft tissues, the present study demonstrated that the highest As content was found in the mussels exposed to condition A (1000 μg L^{-1}). Previous studies also demonstrated a direct relationship between As exposure and element accumulation (Velez et al., 2015; Hsiung et al., 2004; Celia et al., 2009). As a consequence of As exposure and bioaccumulation, higher cellular alterations were observed in mussels exposed to the highest As concentration. In particular, the present findings clearly evidenced that mussels exposed to As at a concentration equal to the maximum permissible value for wastewater discharges (1000 μg L^{-1} of As, condition A, previous to 24 h decontamination process) strongly decreased their metabolic capacity (preventing energy expenditure), while increasing their antioxidant defences, cellular damages and neurotoxicity. Furthermore, at a smaller scale, the results also demonstrated that mussels exposed to As in a concentration of 70 μg L^{-1} (which corresponds to the value achieved by the NPs-based decontamination methodology – condition a), still presented an oxidative stress and neurotoxic status, with inefficient antioxidant capacity that led to observable protein damages and reduced GSH/GSSH ratio. In particular, the present study demonstrated that seawater contaminated with As at initial (condition A) and final

(condition a) concentration levels (1000 and 70 µg L^{-1}, respectively) induced biochemical alterations in mussels that resulted in a general oxidative and neurotoxic status, with higher impacts when organisms were exposed to the highest As concentration (condition A). Mussels exposed to As initial concentration (1000 µg L^{-1}) clearly reduced their metabolism, preventing the use of energy reserves. However, when exposed to seawater at As concentration equal to that of decontaminated seawater (70 µg L^{-1}), albeit minor, the organisms had a metabolic capacity close to those of control indicating that higher impacts on mussels metabolism result from the exposure to the highest As concentration. The decrease of mussels metabolism may be related to the capacity of bivalves to close their valves and reduce their filtration and respiration rates when exposed to contaminants (Gosling, 2003; Ortmann and Grieshaber, 2003). Previous studies also demonstrated that metals and metalloids even at lower exposure concentrations induced similar metabolic depression in bivalves (Errahmani et al., 2014; Liu et al., 2012; Velez et al., 2015). The present results also demonstrated that mussels exposed to As 1000 µg L^{-1} contaminated seawater strongly increased their antioxidant defences, which may result from the overproduction of reactive oxygen species due to the stress induced by As. Nevertheless, at this condition, mussels clearly revealed oxidative damages with lower GSH/GSSG ratio compared to control organisms and damages in lipids and proteins with higher LPO and PC values compared to control organisms. Under decontaminated seawater (condition a) no cellular damages were observed, evidenced by lower LPO levels in comparison to control values, which may be explained by higher antioxidant (GPx activity) and biotransformation (GSTs activity) defence capacities at this condition. Nevertheless, still oxidative stress was observed in decontaminated seawater, identified by lower GSH/GSSG values in organisms exposed to condition a in comparison to control. These findings are in accordance with previous studies that demonstrated induced of oxidative stress and metabolic depression in bivalves exposed to pollutants (Freitas et al., 2016; Velez et al., 2016b; Moreira et al., 2016a, 2016b; Mejdoub et al., 2017; Coppola et al., 2018; Jaishankar et al., 2014; Mandal and Suzuki, 2002). In what regards to the neurotoxic impacts, both conditions A and a inhibited AChE activity, with higher injuries when organisms were exposed to the highest As concentration (contaminated seawater). Rajkumar (2013) also showed that As concentrations (80 µg L^{-1}) induced neurotoxicity in mussels. A similar pattern was shown by other authors with clams (e.g. *Ruditapes decussatus* and *R. philippinarum*) and oysters (e.g. *Crassostrea gigas* and *C. angulata*) exposed to As contamination (Velez et al., 2015; Freitas et al., 2012; Moreira et al., 2016a,b).

7.4.2 Impact of $MnFe_2O_4$ nanoparticles single exposure, before and after decontamination (conditions B and b)

In what regards to $MnFe_2O_4$-NPs exposure conditions, the present study demonstrated that seawater contaminated with NPs at initial (condition B, 50 mg L^{-1}, previous to decontamination process) and final (condition b, NPs residuals in non-quantifiable concentration, after decontamination process) concentrations induced biochemical alterations in mussels that resulted in metabolism depression and

a general oxidative and neurotoxic status, with higher impacts when organisms were exposed to the highest NPs concentration (condition B). In particular, the present findings demonstrated that mussels decreased their metabolic capacity and reduced energy expenditure when exposed to NPs concentration of 50 mg L^{-1}, probably because of valves closure to prevent bioaccumulation of NPs and higher injuries, a behaviour observed in bivalves when exposed to stressful conditions (Anestis et al., 2007; Gosling, 2003). Nevertheless, when mussels were exposed to NPs at final concentration ETS activity and energy reserves concentrations were closer to control condition evidencing the capacity of organisms to maintain their metabolism at lower NPs concentrations. No previous studies evaluated the metabolic impacts derived from exposure to $MnFe_2O_4$-NPs, although some works already demonstrated that other NPs (titanium (TiO_2), gold (Au) and copper (CuO)) decrease bivalves' metabolism (Xia et al., 2017; Cid et al., 2015; Teles et al., 2016; Gomes et al., 2012a). Our results also demonstrated that mussels exposed to NPs increased their antioxidant enzymes activity, a response to higher ROS production due to the presence of NPs. It is known that the presence of NPs (TiO_2, Au and CuO) increases the production of ROS, which leads to the activation of antioxidant enzymes in bivalves (Xia et al., 2017; Cid et al., 2015; Gomes et al., 2012b; Pan et al., 2012). As a result of increased antioxidant defences in mussels exposed to NPs at concentration of 50 mg L^{-1} damages of the cellular membrane were limited. Nevertheless, at this condition, mussels clearly revealed oxidative damages with lower GSH/GSSG ratio compared to control organisms and damages in lipids and proteins revealed by higher LPO and PC values compared to control organisms. When organisms were exposed to residual levels of NPs (condition b) still protein damages were observed, with mussels revealing a limited capacity to eliminate the excess of ROS. Such limited antioxidant capacity may result from lower toxicity induced by condition b in comparison to NPs at initial concentration (condition B). These results agree with studies conducted by Tedesco et al. (2010), which also showed that AuNPs (20 mg L^{-1}) induced lipid damage in mussels. Regarding the neurotoxic impacts, both NPs conditions (B and b) led to the inhibition of AChE activity, with higher injuries when organisms were exposed to higher NPs concentration (condition B). These results are in line with different studies conducted with diverse NPs: TiO_2, 0.4–10 mg L^{-1}, Au 80 µg L^{-1} -100 mg L^{-1} (Guan et al., 2018, Pan et al., 2012; Teles et al., 2016; Gomes et al., 2012a).

7.4.3 Impact of As and $MnFe_2O_4$ nanoparticles combined exposure before decontamination (conditions C)

Concerning the impacts derived from the combined exposure to As and NPs (condition C), the present study demonstrated that initial concentrations of As and NPs (1000 µg L^{-1} and 50 mg L^{-1}, respectively) reduced mussels' metabolism, increased oxidative stress and neurotoxicity compared to control organisms. In particular, organisms exposed to condition C decreased their metabolic capacity while increasing their energy reserves and increased their antioxidant defences, which were not enough to prevent cellular damages, with lower GSH/GSSG ratio and higher PC values in comparison to control

values. This response pattern was similar to those observed in organisms at single exposures (conditions A and B), revealing that the combination of contaminant and nanoparticles did not induce an additive or synergetic response. Although no previous studies showed biochemical stress induced by the combination of As and $MnFe_2O_4$-NPs in bivalves, former works demonstrated that NPs and metal(loid)s (such as As) had similar impacts, including metabolism alteration and increased antioxidant defences when bivalves were exposed to combination of both pollutants (De Marchi et al., 2018; Velez et al., 2016a; Monteiro et al., 2019; Della Torre et al., 2015). The present results are also in agreement with studies conducted by Freitas et al. (2018), which showed that functionalized NPs (MWCNTs, 0.1 mg L^{-1}) in combination with As (1000 µg L^{-1}) induced reduction of metabolic capacity, increase of oxidative stress and lipid damage in mussels, with a similar effect when organisms were exposed to As and NPs separately. Conversely, results obtained from a study conducted with Au-NPs and cadmium chloride ($CdCl_2$) on *M. edulis* by Tedesco et al. (2010) showed the highest oxidative stress and cellular damage in organism when exposed to these NPs and CdCl2 contamination. Regarding the neurotoxicity activity, As + NPs (condition C) inhibited AChE activity, which is in accordance with former studies that analysed this biomarker in different invertebrates' species after exposure to different pollutants such as metals and NPs (Monteiro et al., 2019; Fan et al., 2018; Freitas et al., 2018; Xia et al., 2017; Xiong et al., 2011).

7.4.4 Impact of As and $MnFe_2O_4$ nanoparticles acting in combination after seawater decontamination (condition c)

The present study demonstrated that organisms exposed to the decontaminated water (condition c, As 70 µg L^{-1} and non-quantifiable concentration of NPs) changed their biochemical performance in comparison to control organisms, namely reducing their metabolism, increasing their oxidative stress and neurotoxic status. In comparison to organisms exposed to conditions a and b, where each contaminant was acting individually, the impacts induced were similar, with no significant differences for most of the biomarkers analysed among conditions (a, b, c). Nevertheless, the impacts induced in organisms exposed to decontaminated seawater (condition c) were significantly lower than the impacts observed in organisms exposed to both contaminants at initial concentrations (condition C). In fact, organisms exposed to the decontaminated seawater presented higher metabolism than organisms exposed to the water enriched with As + NPs (condition C). Higher metabolic capacity did not result into higher antioxidant capacity, which probably was not activated due to low stress induced at this condition, originating in turn higher LPO levels and lower GSH/GSSG values at this condition. Furthermore, greater inhibition of AChE was observed when organisms were exposed to condition C compared to condition c, indicating the highest neurotoxic potential of As + NPs initial conditions.

7.5 Conclusion

The present study demonstrated that As decontaminated seawater (condition c) still generates oxidative stress in mussels, with increased cellular damage and oxidative stress in comparison with the control conditions, but contaminated conditions A, B and C clearly caused higher oxidative stress than the decontaminated seawater (conditions a, b and c) with higher increase in antioxidant defences, neurotoxicity and reduction in metabolism followed by increase of energy reserves. Overall, these results are innovative since, up to our knowledge, no published information is available on the toxic effects induced in mussels when exposed to As contaminated seawater remedied by $MnFe_2O_4$-NPs.

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CHAPTER 8.

Can water contaminated with Pb be safe for marine bivalves after remediation with manganese spinel ferrite nanoparticles?

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Abstract

In the last few years the use of nanoparticles (NPs) such as the manganese spinel ferrite ($MnFe_2O_4$) has been increasing, with a vast variety of applications including water remediation from pollutants as metal(oid)s. Although an increasing number of studies already demonstrated the potential toxicity of NPs towards aquatic systems and inhabiting organisms, there is still scarce information on the potential hazard of the remediated water using NPs. The present study aimed to evaluate the ecotoxicological safety of Pb contaminated seawater remediated with $MnFe_2O_4$, NPs, assessing the toxicity induced in mussels *Mytilus galloprovincialis* exposed to contaminated seawater and to water that was remediated using $MnFe_2O_4$, NPs. The results obtained demonstrated that seawater contaminated with Pb, NPs or the mixture of both (Pb + NPs) induced higher toxicity in mussels compared to organisms exposed to Pb, NPs and Pb + NPs after the remediation process. In particular, higher metabolic depression, oxidative stress and neurotoxicity were observed in mussels exposed to contaminated seawater in comparison to mussels exposed to remediated seawater.

Keywords

Toxicity, biomarkers, *Mytilus galloprovincialis,* Mn nanoparticles, lead contamination

8.1 Introduction

Several human activities, including mining operations and sludge dumping as well as industrial and agricultural production, have greatly contributed to the increase of the number and concentration of pollutants in costal environments (Alimba and Faggio, 2019; Green-Ruiz and Páez-Osuna, 2001; Morais et al., 2012; Poulos et al., 2000; Prokić et al., 2019; Stara et al., 2020; Yi et al., 2011). In fact, often the final destination of pollutants are coastal aquatic systems, namely lagoons and estuaries, where organisms such as bivalves are continuously exposed to these anthropogenic substances due to their filter-feeding and sedentary behaviour (Capillo et al., 2018; Fattorini et al., 2008; Manzo et al., 2017; Pagano et al., 2017; Schiavo et al., 2018; Ventura-Lima et al., 2011; Zhang et al., 2015). Among pollutants, published information has already revealed that bioaccumulation of metal(oid)s can lead to toxic effects in bivalves, namely in mussels, including the alteration of their metabolism and redox status (Binelli et al., 2011; Errahmani et al., 2014; Freitas et al., 2018; Manduzio et al., 2005; Savorelli et al., 2017). In particular, studies conducted with metal(loid)s on the top list of the most hazardous materials, such as Mercury (Hg), Arsenic (As), Copper (Cu) and Cadmium (Cd), already showed the capacity of these elements to activate mussel's antioxidant defence mechanisms and decrease their metabolic capacity (Azizi et al., 2018; Coppola et al., 2018a, 2018b; Mubiana and Blust, 2007; Nardi et al., 2017; Raftopoulou and Dimitriadis, 2011). Among the most widely distributed hazardous materials throughout the environment it is identified Lead (Pb) (ATSDR, 2017) due to its use in a vast diversity of anthropogenic activities (e.g. fertilizers, pesticides), being considered as one of the most dangerous metals, especially towards marine and estuarine species (de Souza Machado et al., 2016; Machado et al., 2014; Rzymski et al., 2014; Wang et al., 2009, 2012; Byrne et al., 2012). Accordingly, previous studies already showed alteration of bivalve's oxidative status accompanied by metabolic depression after exposure to Pb (Alak et al., 2013; Bocchetti et al., 2008; Freitas et al., 2014; Pirone et al., 2019). Due to the constant increase of environmental pollution, especially in aquatic systems, different approaches for water decontamination have been applied in the last decade (Baciocchi et al., 2005; Ballinas et al., 2004; Hansen et al., 2006; Katsoyiannis et al., 2002; Leupin et al., 2005). Some of these techniques have shown a great potential for removing inorganic pollutants from water, including the use of nanoparticles (NPs) that revealed high effectiveness in removing metal(loid)s from water (Gehrke et al., 2015; Mohan and Pittman, 2007; Paul et al., 2015; Zhang et al., 2010). For example, Mohmood et al. (2016) demonstrated that 10 mg/L of Silica-Coated magnetic NPs (Fe_3O_4@SiO_2-NPs) had the capacity to remove 98% of Hg from water with 0.5 mg/L Hg after 24 h. Manganese-ferrite ($MnFe_2O_4$) is a common spinel ferrite material that has also been used to decontaminate water from inorganic pollutants (metal(loid)s) due to its ability to sorb elements such as Pb, and its physical magnetic properties that allows the easy separation from the water at the end (Tavares et al., 2013). It is well recognized that the use of these magnetic NPs for water decontamination is one of the most promising research areas (Aslibeiki et al., 2016; Bahadar et al., 2016; Beji et al., 2010; Coppola et al., 2019; Federici et al., 2007). However, the collateral effects of a remediated water towards organisms inhabiting aquatic environments it is not yet well understood (Bhatt and Tripathi, 2011; Blaise et al., 2008; Coppola et al., 2019, 2020; Lovern and Klaper, 2006; Lovern et al., 2007; Smith et al., 2007;

Warheit et al., 2007). For this reason, the present study was focused on the toxicity of seawater previously contaminated with Pb and remediated by $MnFe_2O_4$, NPs. The laboratory experiment was conducted over 28 days using *Mytilus galloprovincialis* as model organism. This species is worldwide recognized as a good bioindicator due to mussels sedentary and filter feed behaviour and, consequently, the capacity to accumulate pollutants, showing exposure effects (Attig et al., 2014; Banni et al., 2014a, 2014b; Coppola et al., 2018a, 2018b; Freitas et al., 2018; Hu et al., 2015; Livingstone et al., 2000; Nardi et al., 2017). The experimental set up included the following treatments: clean seawater; seawater only with Pb 1 mg/L; seawater only with NPs 50 mg/L; seawater with Pb 1 mg/L and NPs 50 mg/L simultaneously; and seawater having initially Pb 1mg/L and remediated with NPs 50mg/L during 24 h. Toxicity was evaluated in terms of mussel's Pb accumulation, metabolic capacity and energy reserves content, antioxidant and biotransformation defence capacity, lipids and protein damage, as well as neurotoxic impacts.

8.2 Materials and methods

8.2.1 Model organisms and experimental setup

Mytilus galloprovincialis was selected as model species to evaluate the toxicological impacts of seawater previously contaminated with Pb and decontaminated by magnetic spinel ferrite NPs ($MnFe_2O_4$). Organisms were collected in the Ria de Aveiro, Portugal (40°38′51.1″N 8°44′05.5″W), and transported to the laboratory where they were placed in tanks of 50 L of artificial seawater at salinity 30 ± 1, temperature of 17 ± 1 °C (resembling conditions at the sampling area), during 14 days for depuration and acclimation. Artificial seawater was prepared by mixing a commercially available salt mixture (Tropic Marin® SEA SALT from Tropic Marine Center – see Atkinson and Box (2010) for salt composition) with freshwater obtained by reverse osmosis (four stage unit, Aqua-win RO-6080, Thailand). During this period, artificial seawater was in continuous aeration (with weekly renewal) and mussels were fed twice per week with Algamac protein plus (150,000 cells per animal per L). Organisms with a mean body weight of 21.3 ± 6.61 g, shell length 6.18 ± 0.46 cm and width 3.52 ± 0.27 cm were used for the experimental assays. After acclimation, organisms were exposed to 17.0 ± 1.0 °C; pH 8.0 ± 0.10, 12 light:12 dark, continuous aeration, in artificial seawater with salinity 30 ± 1, distributed into 7 different treatments (see Table 1), including: CTL (control seawater), treatment A (seawater with Pb levels before remediation); treatment a (seawater with Pb levels after remediation); treatment B (seawater with NPs levels before remediation), treatment b (seawater without NPs levels after remediation); treatment C (seawater with Pb and NPs levels before remediation) and treatment c (seawater with Pb and NPs levels after remediation).

Table 1. Experimental conditions.

TREATMENTS	DESCRIPTION
CTL	Seawater with Pb 0 mg/L + NPs 0 mg/L
A	Seawater with Pb 1 mg/L
a	Seawater with Pb 0.02 mg/L
B	Seawater with NPs 50 mg/L
b	Seawater after 24 h of contact with NPs 50 mg/L (NPs were separated)
C	Seawater with Pb 1 mg/L and NPs 50 mg/L
c	Seawater having initially Pb 1 mg/L and remediated with NPs 50 mg/L during 24h.

Each treatment was conducted in triplicate with 4 individuals/ replicate. Salinity and temperature were kept constant to match those from the acclimation period (30 and 17 °C, respectively) and individuals were fed every 2 days, as previously described. Lead (Lead nitrate, CAS No: 10099-74-8, EC No: 233-245-9; Sigma- Aldrich) was used to prepare a concentration of 1 mg/L selected for initial exposure as it is considered the maximum Pb concentration permissible in wastewater discharges from industry

(Directive, 2013/39/EU, 2013). Treatment a, with 0.02 mg/L of Pb,was considered as remediated seawater since preliminary studies conducted with 1 mg/L in seawater and remediated with $MnFe_2O_4$, NPs (50 mg/L) resulted into 0.02 mg/L of Pb in the medium. The $MnFe_2O_4$, NPs 50 mg/L was selected according to NPs capacity of removal, which was studied in a previous work (data not shown). Those experiments were performed placing 50 mg/L of $MnFe_2O_4$, NPs in 1 L of an aqueous solution containing 1 mg/L of Pb for 24 h. After this period, NPs were separated from seawater by applying an external magnetic field using a NdFeB magnet (ferromagnetic behaviour of the $MnFe_2O_4$, NPs in well known (Balaji et al., 2002; Thirupathi et al., 2012)) and the residual concentration of Pb in solution was circa 0.02 mg/L. Despite the remarkable reduction in Pb, the remediated water could possibly present some toxicity, due to the amount of Pb remaining in solution, or due to NPs that eventually remained in the solution after separation. Treatment a, i.e., seawater with a concentration of Pb like that achieved after remediation (0.02 mg/L), together with condition b (seawater after 24 h of contact with NPs 50 mg/L, which were then separated) were used to elucidate this possible issue. During the 28 days of experiment, seawater was renewed every 7 days, immediately after which all exposure conditions were reestablished. During the experiment, water samples were collected immediately after concentrations reestablishment for the quantification of Pb in the solution and identification of real exposure concentrations. During the experimental period mortality was daily checked. Dead organisms, individuals with open shells and unresponsive to external stimulus, were removed when identified. After 28 days of experiment, organisms from each aquarium (12 per treatment) were collected and immediately frozen in liquid nitrogen, being preserved at −80 °C. To evaluate mussels' biochemical responses and Pb accumulation the whole soft tissue was removed from the shells and homogenized using a mortar and pestle under liquid nitrogen. Tissue homogenates were distributed in 5 aliquots of 0.5 g fresh weight (FW) each for biochemical analyses, and the remaining tissue was used for Pb quantification. Samples for biochemical parameters and Pb quantification were stored at −80 °C.

8.2.2 Synthesis and characterization of $MnFe_2O_4$ nanoparticles

$MnFe_2O_4$ nanoparticles were synthesized according to a previous work (Tavares et al., 2013). The morphological characterization of NPs was confirmed by transmission electron microscopy (TEM) using the Hitachi H-9000 TEM microscope operating at 300 kV. For TEM analysis a drop of sample was dispersed in ethanol on a carbon-coated copper grid and then it was air-dried. Surface area of the NPs was confirmed by N_2 adsorption/ desorption on a Gemini V2.0 Micromeritics instrument. The crystalline phase of the NPs was identified by X-ray powder diffraction of the powders using a Philips Analytical PW 3050/60 X'Pert PRO (θ/ 2θ) diffractometer equipped with an X'Celerator detector and with automatic data acquisition (X'Pert Data Collector v2.0b software) by a monochromatized Cu Kα radiation (λ = 154,056 Å) at 45 kV/40 Ma. Fourier Transform Infrared (FT-IR) spectra of the NPs was recorded using a spectrometer Mattson 7000 at 4 cm^{-1} resolution, using a horizontal attenuated total reflectance (ATR) cell.

8.2.3 Lead quantification

The quantification of Pb in seawater was made by inductively coupled plasma mass spectrometry (ICP-MS), on a Thermo ICP-MS X Series equipped with a Burgener nebulizer as described by Henriques et al. (2017). The quantification limit of the method was 0.1 µg/L (n=12), with an acceptable relative standard deviation among replicates b 10% (n ≥ 3). The ICP-MS was also used to determinate the total Pb concentration in *M. galloprovincialis* soft tissues, after microwave-assisted acid digestion using HNO_3 and H_2O_2, as described by Henriques et al. (2017). The quality control was assured by running blanks (reaction vessels with only HNO_3 and H_2O_2) and certified reference material TORT-3 (Lobster Hepatopancreas; 0.225 ± 0.018 mg/kg Pb) in parallel with samples. Blanks were always below the quantification limit and mean percentage of recovery for Pb in TORT-3 was 110 ± 4% (n= 4).

8.2.4 Biomarkers

Mussels' whole soft tissues (2 individuals per aquarium, 6 per treatment) prepared for biochemical assays (0.5 g FW aliquots) were used for extractions with different buffer solutions (1 mL). After adding the buffer, samples were extracted through highspeed shaking by tissue lyser, centrifuged (at 10000g or 3000g depending on the biomarker, at 4 °C), and the supernatants collected and stored at −80 °C until analysis. Biochemical analyses were performed in duplicate, alongside with blanks. A total of 4 extraction buffers were used, depending on the biomarker (see references Andrade et al., 2018; Pirone et al., 2019). All parameters were analysed spectrophotometrically using a multi-detection microplate reader (BioTek Synergy HT). The biochemical parameters evaluated were: i) metabolism and energy related markers, including electron transport system activity (ETS), glycogen (GLY) and total protein (PROT) concentrations, measured according to King and Packard (1975) and the modifications performed by De Coen and Janssen (1997), Dubois et al. (1956) and Robinson and Hogden (1940) methods, respectively; ii) antioxidant enzymes activities, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx), measured following the methods described by Beauchamp and Fridovich (1971), Johansson and Borg (1988) and Paglia and Valentine (1967), respectively; iii) indicators of cellular damage as lipid peroxidation (LPO) and protein carbonyl (PC) levels, determined following the methods described by Ohkawa et al. (1979) and Levine et al. (1990), respectively; iv) indicators of redox homeostasis as glutathione content ratio (GSH/GSSG) determined according to Rahman et al. (2007); v) neurotoxicity measuring acetylcholinesterase(AChE) activity according to Ellman et al. (1961) and modification by Mennillo et al. (2017). All parameters are described in detail in Coppola et al. (2019) and Pirone et al. (2019).

8.2.5 Statistical analyses

Biochemical parameters and Pb contamination levels obtained from each tested treatment were submitted to a statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA + add-on in PRIMER v6 (Anderson et al., 2008). The matrix gathering all biomarkers responses and Pb concentrations per treatment was used to calculate the Euclidean distance similarity matrix. The pseudo-F and *p*-values in the PERMANOVA main tests were evaluated in terms of significance. When significant differences were observed in the main test, pairwise comparisons were performed. Values lower than 0.05 ($p \leq 0.05$) were considered as significantly different. The matrix gathering biochemical descriptors per condition were used to calculate the Euclidean distance similarity matrix. The similarity matrix was simplified through the calculation of the distance among centroids matrix, which was then submitted to ordination analysis, performed by Principal Coordinates (PCO). Pearson correlation vectors of biochemical descriptors (correlation < 0.75) were provided. The null hypotheses (H0) tested were: i) no significant differences exist among control and contaminated treatments (CTL, A, B and C); *p*-values are presented in Table 4, with significant differences highlighted in bold; ii) no significant differences exist among control and remediated treatments (CTL, a, b, and c); *p*-values are presented in Table 4, with significant differences highlighted in bold; iii) no significant differences exist between A vs a, B vs b, C vs c treatments; significant differences between each pair of treatments are represented highlighted in bold in Table 4, with significant differences identified with an asterisk in Figures.

8.3 Results

8.3.1 Mortality

After 28 days of exposure, at control (CTL) and treatment b (seawater with NPs levels after remediation) no mortality was observed. The lowest mortality (17%) was observed in organisms exposed to treatments C and c, corresponding to water with Pb + NPs before and after remediation. Organisms exposed to Pb before remediation (treatment A) presented the highest mortality level (50%), while organisms exposed to Pb at concentration levels after remediation (treatment a) and exposed to NPs levels before remediation (treatment B) presented 25% of mortality.

8.3.2 Characterization of $MnFe_2O_4$ nanoparticles

A detailed characterization of $MnFe_2O_4$, NPs has been reported previously by Tavares et al. (2013). Briefly, TEM image shows spherical nanoparticles with a mean diameter of 75 ± 15 nm (Figure 1). The results of FT-IR analysis confirm the presence of a characteristic band at 537 cm^{-1} related to metal-O stretching vibration of the $MnFe_2O_4$, NPs (Bellusci et al., 2009; Tavares et al., 2013). The band at 1107 cm^{-1} was attributed to metal-OH and to metal-OH_2 stretching vibrations, which correspond to water sorption on oxide, while 1635 cm^{-1} band is due to H-O-H bending and corresponds to molecular water adsorbed or incorporated into the crystalline lattice (Bellusci et al., 2009). The broadband at 3309 cm^{-1} corresponds to symmetric and asymmetric stretching of O-H bond (Margabandhu et al., 2016). Powder X-ray diffraction (XRD) pattern show peaks that are characteristics of the presence of $MnFe_2O_4$ with the spinel structure (JCPDS–International center diffraction data, PDF card 01-071-4919).

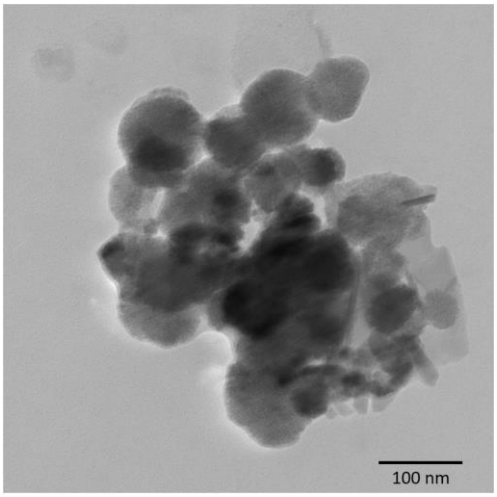


Figure 1. Transmission electronic microscopy image of $MnFe_2O_4$ nanoparticles. Amplification 50,000×.

8.3.3 Lead concentration in seawater and mussels' tissues

Lead quantification in water samples, at the beginning and immediately after conditions reestablishment showed that differences between measured and nominal concentrations were always below 15%. Also, concentrations of Pb in control condition (CTL) and in condition with NPs (B and b treatments) were very low (Table 2) and not statistically different. The quantification in treatment C was not performed because the sorption of Pb by the NPs is extremely rapid. Organisms exposed for 28 days to CTL and treatments a and b presented low concentrations of Pb (Table 3). The highest levels of Pb in mussels were observed in treatment A that corresponds to seawater contaminated with Pb initial concentration. For each group of treatments (A vs a, B vs b, and C vs c) significantly higher contents of Pb were recorded in mussels exposed to “non-remediated” conditions (A, B and C) comparatively to “remediated” ones (a, b and c), and to control.

Table 2. Lead concentration (µg/L) measured in water samples collected immediately after the weekly water renewal, from different treatments (CTL, A, a, B, b, C and c). Results correspond to the mean value and standard deviation of the four weeks. Asterisks represent differences between A vs a, B vs b and C vs c conditions, while different uppercase letters represent differences among contaminated treatments and lowercase represent differences among remediated treatments.

Pb water concentration (µg/L)		
CTL	$0.52 \pm 0.11^{A,a}$	
Pb	**A**	853 ± 281^{B}
	a	15 ± 1.9^{b}
NPs	**B**	0.58 ± 0.15^{A}
	b	0.47 ± 0.18^{a}
Pb + NPs	**C**	**
	c	115 ± 13^{c}

** Because the sorption of Pb by the NPs is extremely fast, the quantification of Pb in water was not performed.

Table 3. Lead concentration in mussels (mg/kg), 28 days after the beginning of the experiment. Concentrations were measured in organisms from different treatments (CTL, A, a, B, b, C and c). Asterisks represent differences between A vs a, B vs b and C vs c conditions, while different uppercase letters represent differences among contaminated treatments and lowercase represent differences among remediated treatments.

Pb mussels concentration (mg/kg)			
CTL	$0.6 \pm 0.1^{A,a}$		
Pb	**A**	700 ± 232^{B}	*
	a	1.2 ± 0.2^{b}	
NPs	**B**	6.5 ± 2.5^{C}	*
	b	1.8 ± 1.5^{b}	
Pb + NPs	**C**	53 ± 29^{D}	*
	c	16 ± 13^{c}	

8.3.4 Biochemical markers

8.3.4.1 Metabolic capacity and energy reserves

The ETS activity was significantly higher at control (CTL) in comparison to values obtained in mussels exposed to non-remediated (A, B, C) and remediated (a, b, c) seawater (Figure 2A, Table 4). Comparing non-remediated with remediated treatments, B vs b and C vs c, significantly higher values were observed in organisms exposed to remediated seawater (Figure 2A, Table 4). Moreover, significantly lower ETS activity was observed in organisms exposed to B in comparison with A and C treatments (Figure 2A, Table 4). Except a vs b treatments, significant differences were observed among remediated treatments (a vs c, b vs c) (Figure 2, Table 4). The GLY content was significantly lower at control (CTL) in comparison to values obtained in mussels exposed to non-remediated (A, B, C) and remediated (b, c) seawater (Figure 2B, Table 4). Comparing non-remediated with remediated treatments, significantly higher values were observed in organisms exposed to non-remediated ones (A vs a and C vs c) (Figure 2B, Table 4). Significant differences among mussels exposed to non-remediated treatments were observed between A and C as well as between B and C treatments, while among remediated treatments significant differences were only observed between a and c (Figure 2B, Table 4). The PROT content at control (CTL) was significantly lower in comparison to values obtained in mussels exposed to non-remediated seawater (A, B, C), while was similar to that observed in mussels exposed to remediated sweater (a, b and c) (Figure 2C, Table 4). Comparing non-remediated with remediated treatments (A vs a, B vs b and C vs c) significantly higher values were observed in organisms exposed to non-remediated treatments (Figure 2C, Table 4). Significant differences among mussels exposed to non-remediated treatments were observed between A and C as well as between B and C treatments, while no significant differences were observed among the remediated treatments (Figure 2C, Table 4).

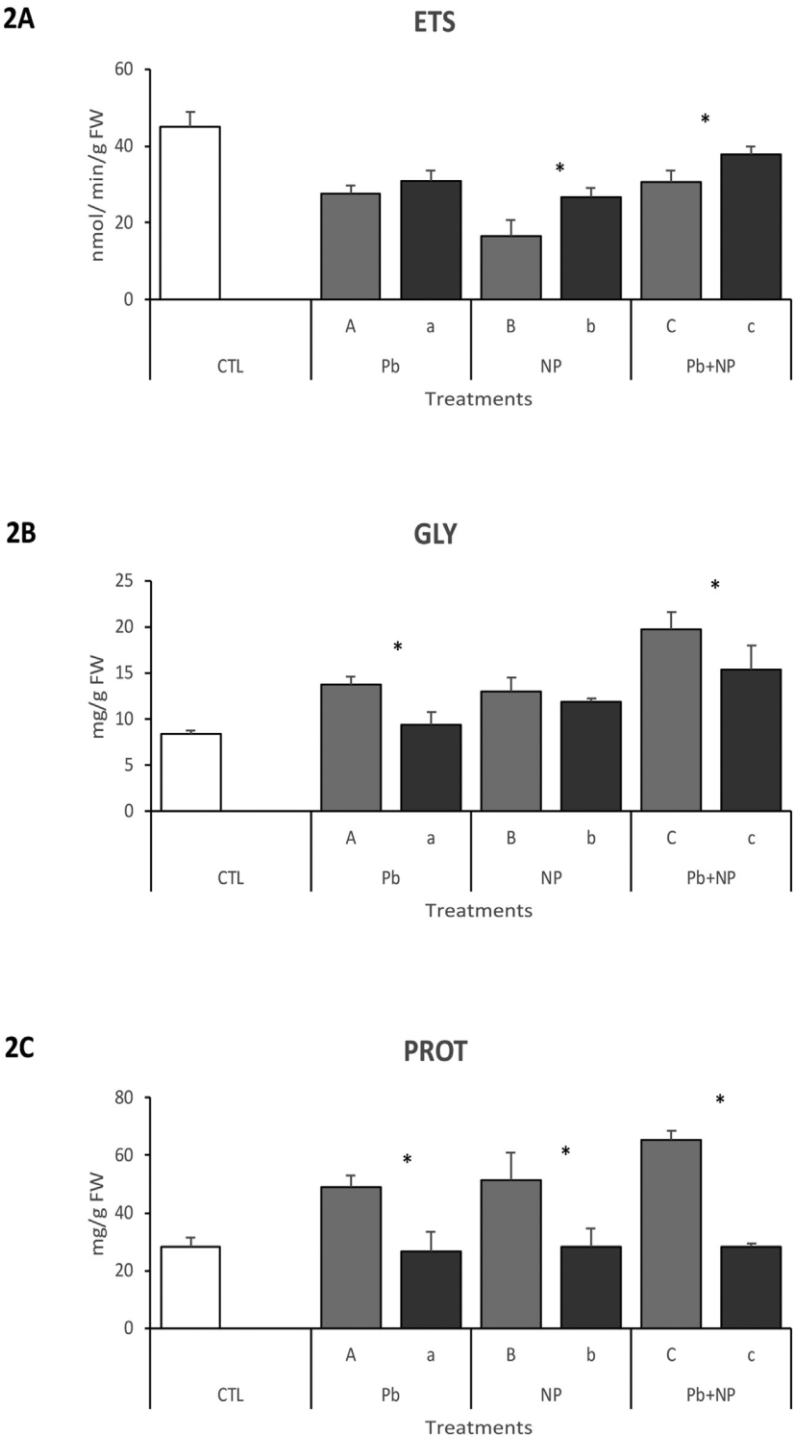


Figure 2. A: Electron transport system activity (ETS); B: glycogen content (GLY); C: total protein content (PROT) in *Mytilus galloprovincialis* exposed to different treatments (CTL, A, a, B, b, C, c) at the end of the experiment. Results are mean + standard deviation. Significant differences between conditions A vs a, B vs b, C vs c are presented with asterisks.

Table 4. *p*-values obtained by pair-wise comparisons between conditions (CTL vs A, CTL vs B, CTL vs C CTL vs a, CTL vs b, CTL vs c, A vs a, B vs b, C vs c, A vs B, A vs C, B vs C a vs b, a vs c and b vs c) for each biomarker: ETS, electron transport system activity; GLY, glycogen content; PROT, total protein content; SOD, superoxide dismutase activity; CAT, catalase activity; GPx, glutathione peroxidase activity; LPO, lipid peroxidation levels; PC, protein carbonyl levels; GSH/GSSG, glutathione ratio; AChE, acetylcholinesterase activity. Significant differences ($p \leq 0.05$) are highlighted in bold.

	ETS	GLY	PROT	SOD	CAT	GPx	LPO	PC	GSH/GSSG	AChE
CTL vs A	**0.0001**	**0.0003**	**0.0001**	**0.0001**	**0.0005**	**0.0001**	**0.0001**	**0.0001**	**0.0023**	**0.0117**
CTL vs B	**0.0001**	**0.0490**	**0.0016**	**0.0001**	**0.0006**	**0.0001**	**0.0015**	**0.0001**	**0.0009**	**0.0071**
CTL vs C	**0.0012**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0020**	**0.0022**	**0.0003**	**0.0016**	**0.0003**
CTL vs a	**0.0002**	0.3271	0.7835	**0.0100**	0.5680	**0.0021**	**0.0001**	0.7876	**0.0238**	**0.0025**
CTL vs b	**0.0001**	**0.0264**	0.9700	**0.0010**	0.2066	**0.0001**	0.8090	0.3325	**0.0150**	**0.0240**
CTL vs c	**0.0099**	**0.0061**	0.9976	**0.0001**	0.7891	**0.0012**	**0.0027**	**0.0001**	**0.0162**	0.1182
A vs a	**0.0910**	**0.0001**	**0.0020**	**0.0002**	**0.0047**	**0.0028**	**0.0224**	**0.0001**	**0.0015**	0.7846
B vs b	**0.0050**	0.6304	**0.0011**	**0.0001**	**0.0098**	0.3309	**0.0044**	**0.0001**	**0.0001**	0.1630
C vs c	**0.0393**	**0.0455**	**0.0001**	**0.0001**	**0.0001**	**0.0235**	**0.0382**	**0.0033**	**0.0002**	**0.0048**
A vs B	**0.0018**	0.7245	0.6388	**0.0090**	0.2371	0.0434	**0.0067**	**0.0464**	0.2404	0.9018
A vs C	0.3092	**0.0001**	**0.0002**	0.0867	**0.0001**	0.0982	0.7361	0.4576	0.3164	**0.0023**
B vs C	**0.0021**	**0.0093**	**0.0147**	0.0549	**0.0020**	0.5118	0.1661	0.6072	0.7502	**0.0002**
a vs b	0.0631	0.0575	0.7503	0.3488	0.5040	0.6976	**0.0001**	0.5074	0.3418	**0.0122**
a vs c	**0.0032**	**0.0095**	0.7383	0.0751	0.6710	0.7813	**0.0132**	**0.0002**	0.4531	0.2883
b vs c	**0.0009**	0.1224	0.9587	0.4154	0.2299	0.4267	**0.0104**	**0.0007**	0.9585	0.861

8.3.4.2 Antioxidant defences

The activity of SOD was significantly lower at control (CTL) in comparison to values obtained in mussels exposed to remediated (a, b, c) and particularly with non-remediated (A, B, C) seawater (Figure 3A, Table 4). Comparing non-remediated with remediated treatments (A vs a, B vs b and C vs c) significantly higher values were observed in organisms exposed to non-remediated (Figure 3A, Table 4). Significant differences among mussels exposed to non-remediated treatments were observed between A and B, while no significant differences were observed among the remediated treatments (Figure 3A, Table 4). The activity of CAT was significantly lower at control (CTL) in comparison to values obtained in mussels exposed to non-remediated (A, B, C) seawater (Figure 3B, Table 4). No significant differences in CAT activity was observed comparing CTL with remediated treatments (a, b and c) (Figure 3B, Table 4). Comparing non-remediated with remediated treatments (A vs a, B vs b and C vs c) significantly higher values were observed in non-remediated ones (Figure 3B, Table 4). Significant differences among mussels exposed to non-remediated treatments were observed between A and C as well as between B and C treatments, while no significant differences were observed among the remediated treatments (Figure 3B, Table 4). The activity of GPx was significantly lower at control (CTL) in comparison to values obtained in mussels exposed to remediated (a, b, c) and non-remediated (A, B, C) seawater (Figure 3C, Table 4). Comparing non-remediated with remediated treatments (A vs a and C vs c), significantly higher values were observed in non-remediated ones (Figure 3C, Table 4). No significant differences were observed among mussels exposed to non-remediated treatments as well as among mussels exposed to remediated treatments, except between treatments A and B (Figure 3C, Table 4).

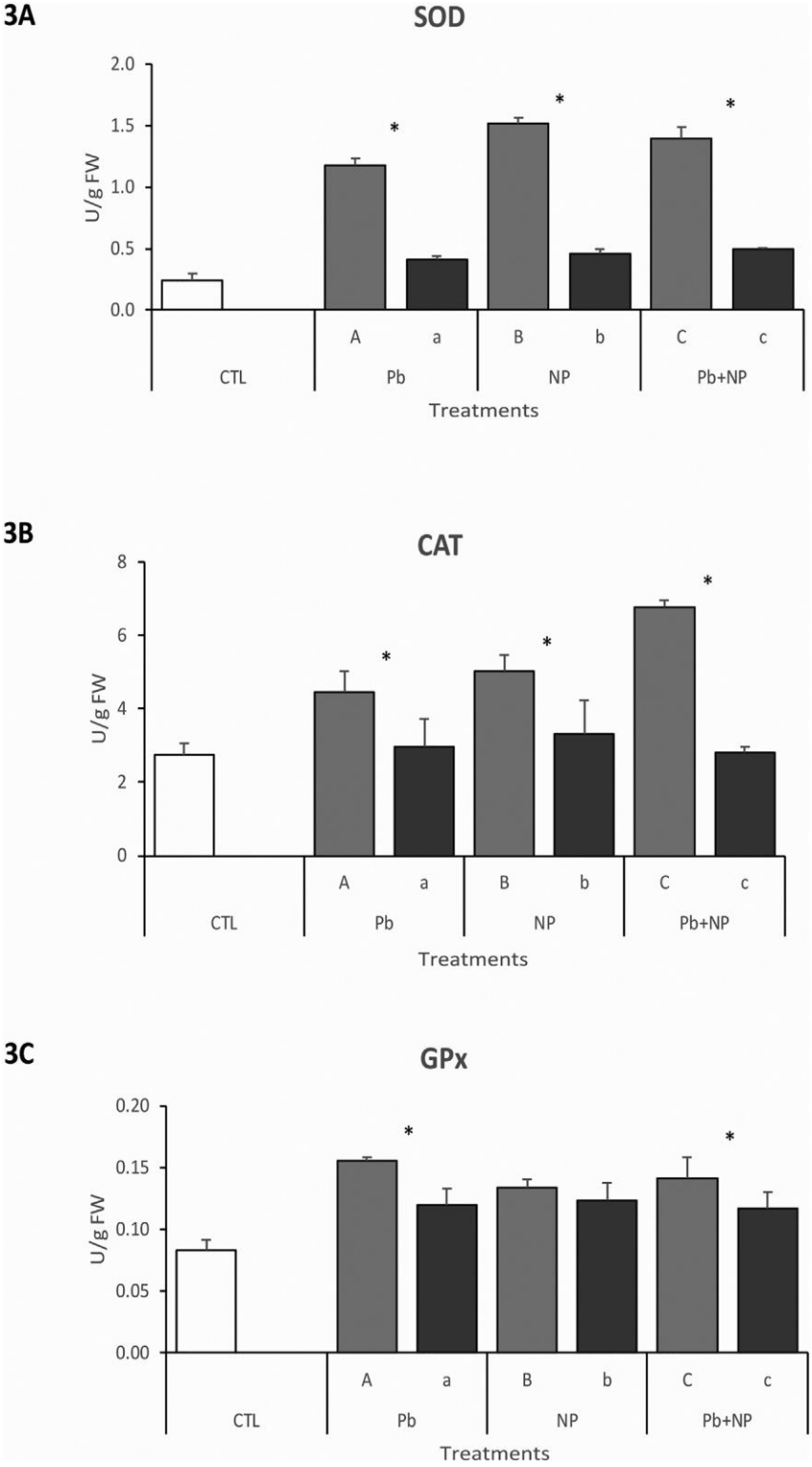
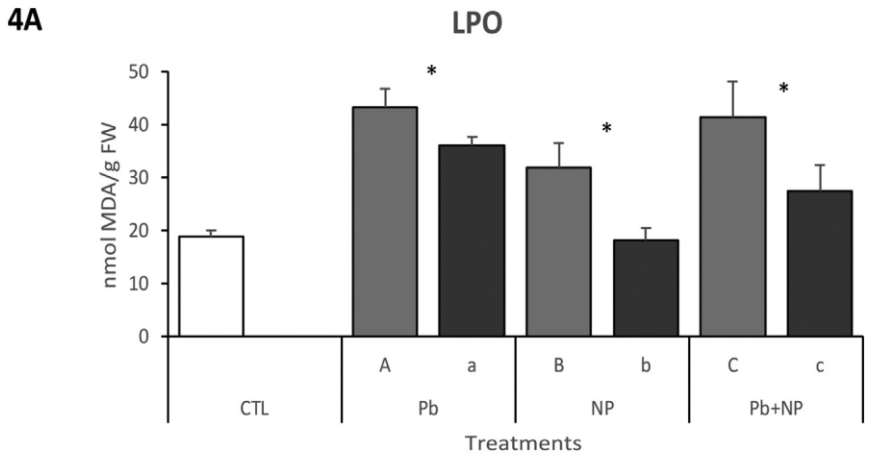
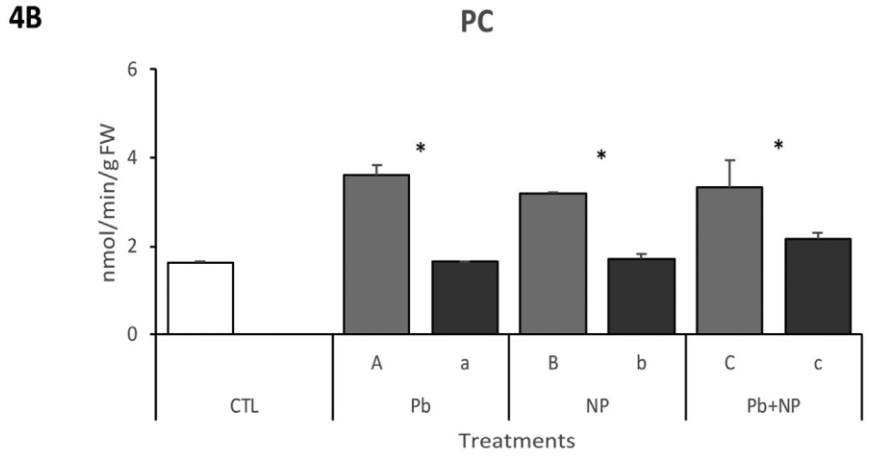


Figure 3. A: Superoxide dismutase activity (SOD); B: catalase activity (CAT); C: glutathione peroxidase activity (GPx), in *Mytilus galloprovincialis* exposed to different treatments (CTL, A, a, B, b, C, c) at the end of the experiment. Results are mean + standard deviation. Significant differences between conditions A vs a, B vs b, C vs c are presented with asterisks.

8.3.4.3 Cellular damage

LPO levels were significantly lower at control (CTL) in comparison to values obtained in mussels exposed to non-remediated (A, B, C) and remediated (a, c) seawater (Figure 4A, Table 4). Comparing non-remediated with remediated treatments (A vs a, B vs b and C vs c) significantly higher LPO values were observed in non-remediated ones (Figure 4A, Table 4). No significant differences were observed among mussels exposed to non-remediated treatments except A vs B, while among remediated seawater, LPO levels were significantly different (Figure 4A, Table 4). PC levels were significantly lower at control (CTL) in comparison to values obtained in mussels exposed to non-remediated (A, B, C) seawater and treatment c (Figure 4B, Table 4). Comparing non-remediated with remediated treatments (A vs a, B vs b and C vs c) significantly higher PC values were observed in non-remediated ones (Figure 4B, Table 4). No significant differences were observed among mussels exposed to non-remediated, except between A vs B, as well as between a vs c and b vs c remediated treatments (Figure 4B, Table 4). GSH/GSSG values were significantly higher at control (CTL) in comparison to values obtained in mussels exposed to non-remediated (A, B, C) and remediated (a, b, c) seawater (Figure 4C, Table 4). Comparing non-remediated with remediated treatments significantly higher GSH/GSSG values were observed in remediated ones (Figure 4C, Table 4). No significant differences were observed among mussels exposed to non-remediated as well as among mussels exposed to remediated treatments (Figure 4C, Table 4).





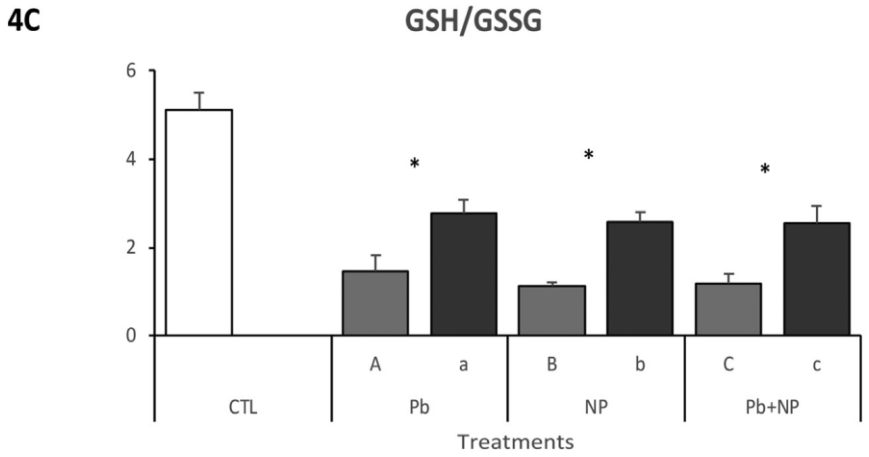


Figure 4. A: Lipid peroxidation levels (LPO); B: Protein carbonyl levels (PC); C: ratio between reduced and oxidized glutathione (GSH/GSSG), in *Mytilus galloprovincialis* exposed to different treatments (CTL, A, a, B, b, C, c) at the end of the experiment. Results are mean + standard deviation. Significant differences between conditions A vs a, B vs b, C vs c are presented with asterisks.

8.3.4.4 Neurotoxicity

AChE activity was significantly higher at control (CTL) in comparison to values obtained in mussels exposed to non-remediated (A, B, C) and remediated (a, b) seawater (Figure 5, Table 4). Comparing non-remediated with remediated treatments significantly higher values were observed in treatments containing Pb + NPs (C vs c) (Figure 5, Table 4). Significant differences among mussels exposed to non-remediated treatments were observed between A and C as well as between B and C treatments, while no significant differences were observed among mussels exposed to remediated treatments, except between a and b (Figure 5, Table 4).

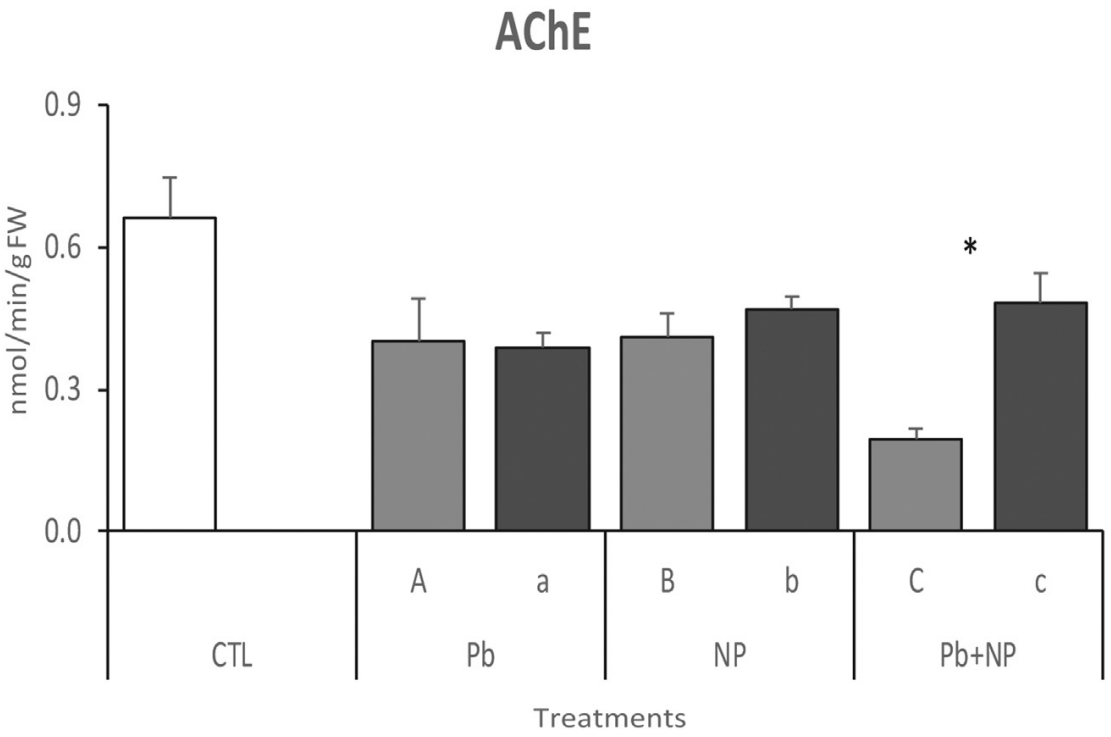


Figure 5. Acetylcholinesterase activity (AChE), in *Mytilus galloprovincialis* exposed to different treatments (CTL, A, a, B, b, C, c) at the end of the experiment. Results are mean + standard deviation. Significant differences between conditions A vs a, B vs b, C vs c are presented with asterisks.

8.3.5 Multivariate analysis

Principal coordinates analysis (PCO) graph obtained is shown in Figure 6. PCO axis 1 explained 63.3% total variation, while PCO axis 2 explained 16.6% (Figure 6). PCO1 separated individuals exposed to A, B and C treatments at the negative side from mussels exposed to CTL, a, band c in the positive side. PCO2 separated individuals exposed to CTL, a, c and A treatments in the positive side from B, b and C treatments in the negative side. Organisms exposed to C and B treatments

were associated to GLY, PROT, SOD and CAT as these markers presented the highest values especially at C treatment. Individuals exposed to CTL, a and c treatments were associated to ETS, AChE and GSH/GSSG. Organisms exposed to A treatment was closely related to LPO and PC, GPx parameters where higher activity of these biomarkers were observed. Moreover, these results were confirmed by higher concentration of Pb in samples water and mussels exposed to A treatment.

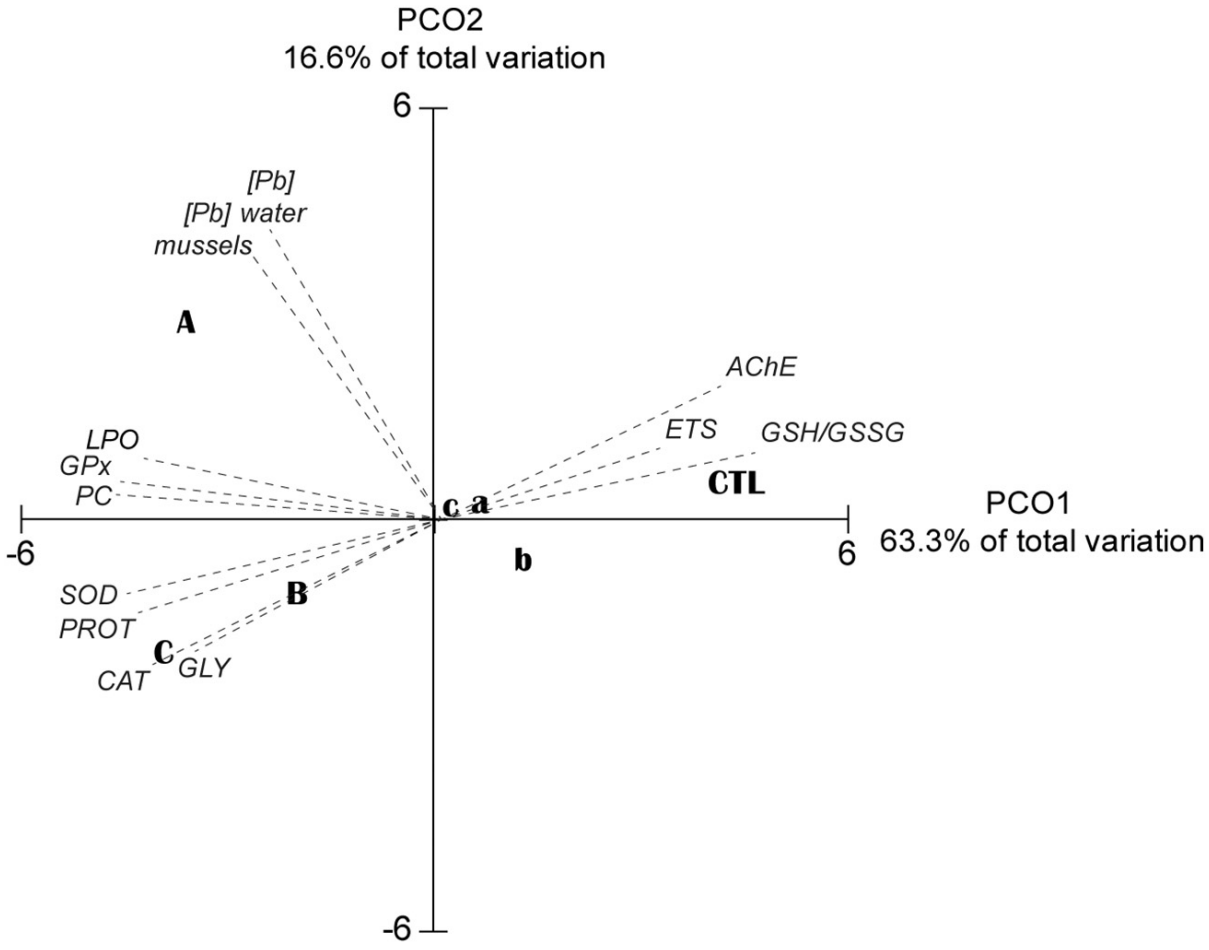


Figure 6. Principal coordinated analyses (PCO) based on biochemical parameters, measured in *Mytilus galloprovincialis* exposed to different treatments (CTL, A, a, B, b, C, c). Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data (r N 0.75): PROT; GLY; CP; CAT; SOD; GPx; LPO, ETS; AChE, GSH/GSSG, [Pb] in organisms and seawater.

8.4 Discussion

Nowadays, the increase of nanoparticles (NPs) for different uses can lead to their release and dispersion in the environment with potential toxic effects (Gehrke et al., 2015; Hu et al., 2015; Huang et al., 2018; Keller et al., 2010). Furthermore, published studies already demonstrated that NPs have the capacity to interact with other pollutants altering their potential harmful effects (Gomes et al., 2011; Guan et al., 2018; Pan et al., 2012). One of the most recent applications of NPs is related to their use for water remediation (Aslibeiki et al., 2016; Davidescu et al., 2015; Gehrke et al., 2015). Although their efficiency in removing pollutants from water has been described (Mehdinia et al., 2014; Mohmood et al.,2016; Ngomsik et al., 2005; Zhang et al., 2015) no studies evaluated the toxicity of the remediated water. This knowledge gap was addressed in the present study, using magnetic manganese spinel ferrite nanoparticles ($MnFe_2O_4$, NPs) that have already demonstrated high capacity to sorb Pb from seawater (Hosseini and Asadnia, 2013). For this, in the present study the bioaccumulation capacity and biochemical performance were evaluated in *Mytilus galloprovincialis* exposed to non-remediated (treatments A, B and C) and remediated (treatments a, band c) seawater, measuring a set of biomarkers in mussels' tissues after a 28 days exposure period. In what regards to removal of Pb from seawater, our findings clearly demonstrated that the application of $MnFe_2O_4$, NPs revealed to be a promising procedure, since the concentration of Pb in the water significantly decreased after remediation. These results are in accordance to previous studies conducted by Mohmood et al. (2016), which showed the efficient of Fe_3O_4@SiO_2-NPs to sorb Hg form water. This present study clearly demonstrated the capacity of mussels to accumulate Pb, even if present at very low concentrations in the medium (remediated water), which can explain the biochemical alterations observed after a 28 days experimental period. These results agree with previously published studies that demonstrated the toxicity of Pb in mussels (*M. galloprovincialis, M. edulis*) even at low but environmentally relevant concentrations (Bocchetti et al., 2008; Fernández et al., 2010; Widdows et al., 2002). Concerning mussel's biochemical responses, clearly the behaviour observed under non-remediated and remediated seawater differed. These results are highlighted by the PCO analysis that separated remediated and non-remediated waters in positive and negative sides of PCO1, respectively. PCO analysis demonstrated that mussels under non-remediated seawater were characterized by high enzymes activity (high SOD, CAT and GPx activates), cellular damages (high LPO and PC levels) and higher Pb concentrations in mussels tissues, as well as in water. In detail, the results obtained showed that independently on the tested treatment mussels tended to decrease their metabolic capacity, measured by ETS activity, in comparison to control levels. However, in general, mussels exposed to remediated treatments presents higher ETS activity than mussels exposed to contaminated water, indicating that remediated seawater induced less toxic effects in mussels' metabolic capacity. Furthermore, similar ETS values obtained in remediated treatments (a, b, c) highlight similar toxicity of Pb and NPs, both isolated and in combination. Such results are in accordance with studies already published regarding the toxicity of Pb and NPs in bivalves, revealing that exposure to these type of pollutants (e.g. Pb, Hg, zinc oxide (ZnO_2-NPs), metal oxidase (Me(O)NPs), titanium oxidase (TiO_2-NPs), gold nanoparticles (AuNPs), carbon nanotubes (f-MWCNTs)) resulted in metabolism depression in

mussels (*Mytilus edulis, M. coruscus* and *M. galloporivincialis*) and clams (*Mercenaria mercenaria, R. philippinarum* and *Corbicula fluminea*) (Baker et al., 2014; Coppola et al., 2018a, 2018b; De Marchi et al., 2017; Fan et al., 2016; Freitas et al., 2014; Huang et al., 2018; Jaishankar et al., 2014; Li et al., 2018;Tedesco et al., 2010). In this study the metabolic decrease in mussels exposed to contaminated water (treatments A, B and C) resulted in lower energy expenditure, showing an increase of PROT and GLY content in comparison with mussels exposed to remediated seawater (treatments a, b and c), which presented an energy reserve content similar to control values. In accordance with these results, it was already demonstrated by other authors (Coppola et al., 2017; Della Torre et al., 2015; Guan et al., 2018) that bivalves under pollutants exposure avoid the expenditure of their energy reserves. In fact, previous studies have showed and justified that a consequence of metabolic decrease in mussels under stressful metal exposure is the low energy expenditure with an increase of energy reserves content (Coppola et al., 2017; Freitas et al., 2018; Pirone et al., 2019). Avoiding energy reserves expenditures different physiological processes may be affected, namely reproductive success and growth rate (Berthelin et al., 2000; Dridi et al., 2007; Ojea et al., 2004; Pouvreauet al., 2006).It is well known that when bivalves are exposed to pollutants the production of reactive oxygen species (ROS) may increase which, as a consequence, results into the activation of antioxidant enzymes(Regoli and Giuliani, 2014) or, it may also result in the inhibition of these enzymes in the case of extreme stressful conditions (Company et al., 2004; Maria and Bebianno, 2011; Monari et al., 2005). The obtained results showed a clear activation of SOD, CAT and GPx enzymes in mussels exposed to non-remediated seawater, independently on the treatment (A, B or C), while in mussels exposed to remediated seawater (treatments a, b and c) there was no effective increase in antioxidant defences with enzymes activity values closer to control. These findings evidence the toxic impacts of Pb and NPs, with a clear induction of mussel's antioxidant defence mechanisms when exposed to contaminated seawater. As described previously, the increased activity of antioxidant enzymes such as SOD, CAT and GPx may result from the overproduction of ROS due to the presence of pollutants, namely metals. In particular, similar activation of antioxidant enzymes was observed by Freitas et al. (2014) in the clam *R. decussatus* exposed for 96 h days to Pb, while other authors showed a similar response in mussels exposed to the same metal (Alak et al., 2013). Also, studies on the impacts of NP sin bivalves showed that antioxidant defences were activated in mussels *M. galloprovincialis* exposed to ZnO NPs (100 mg Zn/L), and in clams *R. philippinarum* exposed to f-MWCNTs (1 mg/L) (Li et al., 2018; De Marchi et al., 2017). Under stressful conditions bivalves may prevent the occurrence of cellular damage if antioxidant defences are efficient in eliminating ROS, avoiding a general oxidative status (Regoli and Giuliani, 2014).The present findings clearly demonstrated that organisms exposed to non-remediated seawater (treatments A, B and C) presented higher lipids damage in comparison to mussels exposed to remediated seawater(treatments a, b and c), indicating that although antioxidant defences were activated in mussels exposed to non-remediated seawater these mechanisms were not enough to prevent cellular damage and oxidative stress was observed. On the other hand, although a limited activation of antioxidant enzymes was observed in organisms exposed to remediated seawater (treatments a, b and c) lower cellular damages were observed indicating that these conditions were less toxic to mussels than contaminated seawater. Previous studies also showed the increase of LPO when bivalves

(mussels and clams) were exposed to Pb (Alak et al., 2013; Pirone et al., 2019) and a similar response was observed in mussels *M. galloprovincialis* and *M. coruscus* and clams *R. philippinarum* exposed to different NPs (TiO_2, f-MWCNTs, ZnO_2) (De Marchi et al., 2017; Huang et al., 2018; Mezni et al., 2017). Another consequence of ROS overproduction is the oxidation of proteins, identified by protein carbonylation (PC) (Patetsini et al., 2013). The PC levels in mussels exposed to remediated seawater (treatments a, b and c) were close to CTL values and lower than in mussels exposed to contaminated seawater (A, B, C), evidencing higher protein damage in mussels exposed to contamination. These results are in accordance with recent studies by Freitas et al. (2019), which showed an increase of PC levels when the mussels *M. galloprovincialis* was exposed to Pb. Also, different authors revealed an increase of PC values in bivalve species after exposure to metals and NPs (Baker et al., 2014; De Marchi et al., 2018; Fan et al., 2016, 2017; Pirone et al., 2019; Valavanidis et al., 2006). Under a stressful condition, organisms tend to increase oxidized glutathione (GSSG) content while decreasing the amount of reduced glutathione (GSH), decreasing their GSH/GSSG ratio in comparison to control or less stressful conditions (Regoli and Giuliani, 2014). In the present study, although the ratio GSH/GSSG decreased in all treatments in comparison to CTL, the lowest values were observed in organisms under non-remediated seawater (treatments A, B and C). These results evidence lower redox homeostasis in organisms exposed to non-remediated seawater (treatments A, B and C) in comparison to the ones exposed to remediated seawater (treatments a, b and c). Similarly, previous studies also with bivalves showed similar responses with higher GSH/GSSG values at the least stressful conditions (Coppola et al., 2018a, 2018b; De Marchi et al., 2017). In marine bivalves it is well described the neurotoxic impacts of different pollutants, evidenced by the decrease on AChE activity due to its high affinity for many neurotoxic compounds such as metals and NPs (Maisano et al., 2017; Wang et al., 2009). Our results showed that organisms tended to decrease the activity of AChE both in remediated and non-remediated seawater compared to CTL, but especially in the presence of Pb and NPs (treatment C). In general, the AChE activity in mussels under remediated seawater were higher than contaminant treatments, demonstrating that even after removal of Pb and NPs from the water still neurotoxic impacts were induced in mussels exposed to remediated seawater, which evidences the high neurotoxic capacity of Pb and NPs. These findings are in accordance with previous results by De Marchi et al. (2018) which showed a decrease in AChE activity when the clams *R. philippinarum* were exposed to MWCNTs (Nf- and f- NPs). Also, study conducted by Freitas et al. (2019) showed a decrease of AChE activity when mussels *M. galloprovincialis* were exposed to Pb concentration.

8.5 Conclusion

In conclusion, the present findings clearly demonstrated that organisms exposed to non-remediated seawater presented greater alterations on their biochemical performance, with higher metabolism depression, oxidative stress and neurotoxicity than mussels exposed to remediated seawater. It was also demonstrated that impacts induced by Pb and NP acting individually or as a mixture induced similar oxidative stress levels but the combination of pollutants induced greater neurotoxicity than acting individually. Overall, the present study evidenced lower toxic impacts of remediated seawater in comparison with non-remediated seawater, showing the potential use of manganese spinel ferrite nanoparticles to remediate water contaminated with metals, and the safety of remediated water towards aquatic systems. However, an ex-situ decontamination is recommended as NPs showed to induce low level toxicity by itself.

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CHAPTER 9.

General discussion and conclusion

9.1 Discussion

The main goal of the present study was to investigate the efficiency of newly developed remediation strategies, by assessing the toxicity of remediated seawater towards different bivalve species, considering seawater previously contaminated with different metal(loid)s and testing at the same time the influence of predicted climate changes as the temperature rise and salinity shifts. Therefore, the following discussion will mainly address the toxicity of remediated seawater, comparing the effects induced before and after remediation. Clams (*Ruditapes philippinarum*) from North Pacific (China) and North Atlantic (Portugal) as well as mussels (*Mytilus galloprovincialis*) from the North Atlantic (Portugal) were used as model species and the responses after chronic exposures will be here briefly compared and discussed.

The studies here discussed include the metal(loid)s mercury (Hg), arsenic (As) and lead (Pb), selected considering their wide occurrence in different environmental matrices (Angel et al., 2016; Azad et al., 2019; Costa et al., 2020; González et al., 2021; Neff, 2002; Nguyen et al., 2007; Portela et al., 2020; Prato et al., 2019; Romano et al., 2013; Sarasiab et al., 2004; Soldi et al, 1996; Spada et al., 2013; Velez et al., 2015; Wan et al., 2009) and their toxicity towards wildlife, including bivalves (among others, Berlin et al., 2007; Chiesa et al., 2018; Khan et al., 2006; Guinot et al., 2012; Prato et al., 2019). Among the impacts caused, published literature includes oxidative stress and metabolic alterations as well cellular damage in marine bivalves exposed to these metal(loid)s, including in oysters (*Crassostrea angulata* and *C. gigas*), clams (*R. decussatus, R. philippinarum and Venerupis corrugata*) and mussels (*M. galloprovincialis* and *M. edulis*) (Bocchetti et al., 2008; Chen et al., 2018; Coppola et al., 2018a; Fernández et al., 2010; Freitas et al., 2018, 2019; Moreira et al, 2016, 2018; Pirone et al., 2019; Prato et al., 2019; Velez et al., 2015, 2016; Widdows et al., 2002). In the case of Hg, studies also demonstrated its capacity to induce histopathological alterations in the clams *R. philippinarum* (Chen et al., 2014; Coppola et al., 2020b; Pan and Wang, 2011), as well as in the mussel *M. galloprovincialis* (Coppola et al., 2020a,c; Maisano et al., 2017; Pagano et al., 2016).

To limit the inputs and impacts of pollutants in aquatic systems, several approaches have been developed including the use of nanomaterials (Chen and Mao, 2007; Mohan and Pittman, 2007; Jackson et al., 2012; Paul et al., 2015; Vilela et al., 2016). However, still limited information is available on the toxicity that might be induced by those nanomaterials or even by water remediated by them. In the present study the nanomaterials graphene oxide functionalized with polyethyleneimine (GO-PEI) and manganese spinel ferrite nanoparticles ($MnFe_2O_4$-NPs) were used, considering previous studies that demonstrated their ability to remove Hg, Pb and As from water (Bessa et al., 2020; Tavares et al., 2013). Although, different studies showed their efficiency (Coppola et al., 2019, 2020a,b,c, 2021a,b,c), still very scarce information is available in what regards to their safety towards aquatic environment. Previous studies demonstrated that graphene oxide (GO) can induce oxidative stress and cellular damage to the polychaete species *Diopatra neapolitana* exposed to 1.0 mg/L of GO after a chronic exposure (28 days) (De Marchi et al., 2017a), while magnetite ferrite nanoparticles induced mortality in the small planktonic crustacean *Daphnia magna* after short-term exposure (48h) at 50 mg/L (Gökçe et al., 2020).

Apart from pollutants, coastal areas have been also subjected to climate changes. Recent studies already demonstrated that the frequency and intensity of extreme weather events are increasing, including longer drought and heat periods (Donnucci et al., 2012; Cardoso et al., 2008; Grilo et al., 2011; IPCC, 2018, 2019; IPMA, 2017), which may negatively impact organisms' survival, reproduction, growth, population abundance and biochemical performance (Hamer et al., 2008; Sarà et al., 2008; Matoo et al., 2013; Matozzo et al., 2013; Munari et al., 2011; Pörtner et al., 2007). Moreover, different studies demonstrated that climate change related factors may alter the behaviour and toxicity of metals, together with changes on organisms' sensitivity, which may enhance the toxic impacts (e.g., Delgado and Pérez-Camacho, 2007; Izagirre et al., 2014; Mubiana and Blust, 2007; Múgica et al., 2015; Nardi et al., 2017; Ramos-Gómez et al., 2011; Sokolova and Lannig, 2008). The effects of nanomaterials may be also altered when organisms are exposed to environmental changes, including temperature and salinity shifts (e.g., Amorim et al., 2018; Andrade et al., 2019; Bebianno and Barreira, 2009; De Marchi et al. 2017b, 2018, 2019a,b). In particular, the effects of metal(loid)s in bivalves under warming scenarios can accelerate their metabolic rates, impair mitochondrial function, induce oxidative stress, and cause damages to lysosomal system and DNA (Cherkasov et al., 2006, 2007; Izagirre et al., 2014; Guinot et al., 2012; Mubiana and Blust, 2007; Nardi et al., 2017; Sokolova and Lannig, 2008). Also, previous studies demonstrated that mussels and clams may alter their biochemical performance, leading to increased antioxidant defenses, cellular damage and metabolic alterations when exposed to pollutants as NPs or metal(loid)s under different salinity levels (among others, Batley et al., 2013; De Marchi et al., 2018; Figueira et al., 2012; Figueira and Freitas, 2013; Moschino et al., 2012; Velez et al., 2016a).

9.1.1 Comparison between clams' populations responses

Ruditapes philippinarum collected from the North Pacific (China) and subjected to Hg treatment showed similar Hg accumulation at 17 and 22 ºC (3.2 and 3.0 µg/g, respectively) but higher than those at control treatments (17 ºC: 0.021 and 22 ºC: 0.019 µg/g). A similar pattern was found in the population from North Atlantic Ocean (Portugal), with higher concentrations of Hg in clams exposed to Hg (17 ºC: 9.1 and 21 ºC: 12 µg/g) in comparison to non-contaminated clams (17 ºC: 0.18; 22 ºC: 0.29 µg/g). These findings are in accordance with previous studies that already demonstrated the capacity of this species to accumulate metals, namely Hg (Coppola et al., 2018a,b; Izagirre et al., 2014; Moreira et al., 2017; Nardi et al., 2017; Velez et al., 2015). The accumulation of Hg may result from bivalves' filtration activity and can be associated with increased metabolic capacity (assessed by ETS activity) necessary to fuel up defense mechanisms. As a consequence, increased metabolic capacity will lead to reactive oxygen species (ROS) overproduction. The obtained results showed that although high antioxidant enzymes activity was observed in both populations exposed to Hg in comparison to control clams, this strategy was not able to detoxify the excess of ROS produced and lipid peroxidation occurred. Previous studies also demonstrated that, in bivalves, increased metabolism was associated with Hg accumulation (Coppola et al., 2020b, 2021; Leite et al., 2020).

Regarding the influence of temperature on Hg accumulation, it was observed that in clams from Portugal differences were observed between temperatures, with higher concentration observed at increased temperature. Such increased concentration was associated with increased metabolic capacity in clams exposed to warming conditions, probably associated with higher filtration and respiration rates and, consequently, higher Hg ingestion. According to these findings, Coppola et al. (2018a) detected higher accumulation of As in mussels (*M. galloprovincialis*) followed by an increase of LPO levels under temperature rise in comparison to control temperature. Also Freitas et al. (2017) demonstrated the increase of enzymatic scavengers (CAT and SOD) in *M. galloprovincialis* under Hg and warming scenario compared to mussels under control temperature.

The data obtained further demonstrated that clams from the North Atlantic accumulated higher Hg concentrations than clams from the North Pacific, regardless the temperature. Such results may be explained by the capacity of clams to protect themselves when living in contaminated areas, which is the case of the population from China, collected from an area where the concentration of metals is higher than concentrations found at the Ria the Aveiro sampling site (Costa et al., 2020; Jiang et al., 2019; Zhang, 2001). A previous study by Freitas et al. (2015), demonstrated that lower accumulation of the carbamazepine (a pharmaceutical drug) associated with high GSTs activity was observed in *Scrobicularia plana* clams collected from contaminated areas than those from non-contaminated sites and exposed to the same drug concentration.

When considering remediated seawater, once again, differences were observed between both populations: clams from North Pacific accumulated lower Hg concentrations (17 ºC: 1.8; 22 ºC: 0.19 µg/g) than clams from the North Atlantic (17 ºC: 4.7; 22 ºC: 2.9 µg/g), which may be explained, once again, by the capacity of bivalves to reduce the accumulation of contaminants in their tissues as a defence mechanism when living in areas highly polluted (Freitas et al., 2015). Nevertheless, values of Hg in clams from both populations subjected to remediated seawater were significantly lower than those found in clams exposed to Hg treatment, and closer to control values. These results are related with low Hg concentration in seawater which lead to a lower accumulation in comparison with organisms exposed to seawater before decontamination, highlighting the efficiency of the remediated approach. These findings are in accordance with previous studies that already demonstrated a positive linear correlation between the concentration of the contaminants and the organisms' capacity to accumulate them (Coppola et al., 2016; Henriques et al., 2019; Leite et al., 2020; Pinto et al., 2019; Velez et al., 2016a). Low concentration of Hg found in both populations subjected to remediated seawater was accompanied by limited biochemical impacts, with similar oxidative stress levels and metabolic capacity in clams from remediated seawater and control treatments.

9.1.2 Comparison between clams and mussels responses

The *R. philippinarum* (collected in Portugal) subjected to Hg treatment showed higher Hg accumulation at 22 ºC in comparison to 17 ºC (12 and 9.1 µg/g, respectively), which was higher than those found in non-contaminated clams (22 ºC: 0.29 and 17 ºC: 0.18 µg/g). In the mussel *M. galloprovincialis* also Hg

accumulation was observed after exposure to Hg (17º C: 42 and 21 ºC 16.2 µg/g) in comparison to control organisms (17 ºC: 0.17 and 21 ºC: 0.08 µg/g) but in this case lower Hg concentration was found at warming conditions. As previously mentioned, the accumulation of metals, including Hg, was previously found in different bivalve species (Coppola et al., 2018a,b; Izagirre et al., 2014; Moreira et al., 2017; Nardi et al., 2017; Velez et al., 2015).

The results obtained further demonstrated that mussels accumulated higher Hg content than clams at both temperatures, which is in accordance to a previous study conducted by Kehrig et al. (2006) showing that mussels have a higher capacity for Hg accumulation compared to other bivalve's species, such as oysters and clams. Higher Hg concentration in mussels exposed to 17 ºC resulted into higher cellular damage in mussels in comparison to clams exposed to Hg at the same temperature.

Also, while clams showed small differences in terms of Hg concentrations between both temperatures, mussels revealed higher differences in terms of accumulation, with higher Hg concentration at control temperature. Although similar metabolic and detoxification capacity was observed in mussels exposed to both temperatures, differences in terms of accumulation, with high Hg content at control temperature was previously observed by Coppola et al. (2017) when exposing *M. galloprovincialis* for 28 days to 0.25 mg/L of Hg. Furthermore, Velez et al. (2015) also demonstrated that *R. philippinarum* and *R. decussatus* accumulated similar Hg concentrations regardless the temperature tested.

When considering remediated seawater, differences between clams and mussels were reduced, with values ranging from 3.4 to 6.1 µg/g in mussels exposed to 17 and 21 ºC respectively, while in clams' values of Hg ranged between 4.7 and 2.9 µg/g at 17 and 22 ºC, respectively. Similar concentrations found in clams and mussels exposed to remediated seawater are associated with lower Hg concentration found in water after remediation in comparison to organisms exposed to Hg treatment. However, higher cellular damage was observed in mussels which could be associated with higher ETS activity, the principal generator of ROS.

Nevertheless, although values of Hg in both species subjected to remediated seawater were still higher than values found in control organisms, the impacts generated in bivalves subjected to these two treatments (control and remediated seawater) were similar regardless the species. In particular, similar cellular damage and oxidative stress as well as histopathological alterations were observed in bivalves exposed to control and remediated seawater treatments, highlighting again the low toxicity of remediated seawater.

9.1.3 The influence of climate change related factors on mussels responses

The experiments conducted under different salinities revealed that mussels tended to accumulate higher Hg concentrations at salinity 30 (14.4 µg/g) in comparison to salinities 20 and 40 (9.1 and 8.7 µg/g, respectively), leading to higher cellular damage at control salinity although greater detoxification was observed at this condition (presence of Hg at salinity 30). As described above, under different temperatures the work conducted showed that higher Hg accumulation was observed at control temperature (17 ºC, 42 µg/g) in comparison to mussels maintained at 21 ºC (16.2 µg/g). Also in this

case higher lipid peroxidation was observed at control temperature compared to 21 ºC. As mentioned before, previous studies already demonstrated a close relationship between Hg accumulation and cellular damage (Coppola et al., 2018; Geret, and Bebianno, 2004; Moreira et al., 2016; Pirone et al., 2019; Velez et al., 2016c).

Lower Hg concentration found in mussels exposed to salinities 20 and 40 in comparison to control salinity (30) could be related with: i) mechanisms to prevent osmosis when exposed to low salinities. It was demonstrated for the clam species *Mercenaria mercenaria* (vulgarly called Hard Clam) that when the salinity of saltwater decline, cells allow free amino acids to leak out but ions remain in the cell at normal concentrations, while total concentration of dissolved molecules (ions plus free amino acids) declines along with the external salinity, preventing gain of water by osmosis and swelling of the cells (Baker et al., 2010). Such behaviour may explain the limited accumulation of contaminants; ii) higher Hg complexation at higher salinity leading to lower metal availability. Similarly, previous studies also demonstrated that the accumulation of metals in bivalves was influenced by the level of salinity (Velez et al., 20116b; Kumar et al., 2015; Moreira et al., 2016). As an example, Moreira et al. (2016) justified lower As accumulation in oysters at salinity 40 by higher complexation of As and, consequently, lower bioavailability.

Regarding remediated seawater, mussels exposed to different salinity levels showed lower Hg accumulation at 20 and 40 (0.87 and 0.42 µg/g, respectively) than those under salinity 30 (1.5 µg/g). Under different temperatures, higher Hg values were observed in organisms at 21 ºC (6.1 µg/g) in comparison to those at 17 ºC (3.4 µg/g). Higher accumulation of Hg observed in mussels exposed to remediated seawater at 21 ºC and salinity was associated to higher ETS values observed in this treatment in comparison to all the other ones. Such response may indicate that under a limit stress (low exposure concentration and temperature rise) organisms may be able to increase their metabolic capacity to fuel up defence mechanisms, resulting into higher filtration and respiration rates and, thus, higher accumulation of Hg.

Nevertheless, when comparing the impacts induced in mussels subjected to remediated seawater and control at different salinities and temperatures the impacts were similar, highlighting that the toxic effects of remediated seawater are similar to the ones observed at control conditions, regardless the climate change scenario tested.

9.1.4 Comparison of mussels responses when exposed to Arsenic and Lead contamination

The results obtained demonstrated that, when exposed to the same concentration (1 mg/L) mussels accumulated higher Pb content than those exposed to As contamination. Furthermore, higher BCF was obtained for Pb exposed mussels (0.82) than for mussels exposed to As (0.013). Published studies demonstrated that *M. galloprovincialis* mussels, exposed to As (1000 µg/L) presented a BCF of 0.002 (Coppola et al., 2018a) while under Pb (50 µg/L) the BCF was 0.032 (Freitas et al., 2019). Differences in terms of concentration and BCF values could be explained by the efforts of mussels to limit the

entrance of contaminants, especially noticed in the presence of As, with lower ETS values in mussels exposed to this metaloid.

When considering mussels exposed to remediated seawater, regardless the metal(oid) higher BCF values were obtained in comparison to control mussels but similar values were found between mussels exposed to As and Pb (As: BCF 0.12; Pb: BCF 0.14). Such findings indicate the low contamination of remediated seawater which lead to similar biochemical impacts in mussels regardless the contaminant of exposure and similar impacts when compared with non-contaminated mussels. Once again, low impacts highlights to the low toxicity of remediated seawater.

9.2 Contribution of the presented research to the United Nations Sustainable Development Goals

The main aim of the present research was to improve the knowledge on the impacts of metal(oid)s towards marine bivalves and understand the potential toxic effects caused by remediated seawater. Also, the influence of climate change related factors was evaluated, trying to assess how predicted environmental changes may alter the toxicity caused by decontaminated seawater. The knowledge obtained will greatly contribute to meet the objectives of different international strategies for environment protection, namely the 2030 Agenda of the United Nations (UN, 2021). In particular, the results obtained will contribute to achieve some of UN the sustainable development goals, including: i) **SDG3 (good health and well-being),** promoting the reduction of contamination levels in aquatic systems and, consequentially, in inhabiting species, namely those associated with human consumption; ii) **SDG6 (Clean water and sanitation)**, by ensuring the environmental safety of water after decontamination processes, promoting the application of new strategies towards the decontamination of water; iii) **SDG9 (industry, innovation and infrastructure),** fostering innovative recycling industry; iv) **SDG11 (sustainable cities and communities),** developing strategies to remediation of wastewater limiting contaminants (metal(loid)s) from the wastes and consequently their toxic effects in the environment; v) **SDG13 (Climate action)**, identifying how climate changes as temperature rise and salinity shifts affects marine environments already under pressure; vi) **SDG14 (Life below water)**, revealing the impacts caused by different contaminants and remediation approaches to marine species, towards the protection marine ecosystems; vii) **SDG17 (partnerships for the goals)**, creating the collaborations between universities and industries to apply these new remediation methods studied on a large scale.

Results obtained from the studies developed under this thesis will also meet the National Strategy for the Sea objectives. In particular, the research conducted will greatly contribute to the objectives 1 (combat climate change and pollution, restore ecosystems), 4 (investing in the guarantee of sustainability and food safety) and 9 (encourage scientific knowledge, technological development and blue innovation) since the research conducted will allow to identify the impacts of pollutants towards marine species and the safety of seawater after remediation. Also, the results obtained will allow to understand how climate change related factors will influence the response of organisms to contamination.

Aiming to critically discuss the results obtained, a SWOT analysis was conducted, highlighting the strengths (S), weaknesses (W), opportunities (O) and threats (T) of research conducted, allowing do identify future work on this field. In wat regards to **Strengths**, the results obtained clearly demonstrated the low toxicity of the remediated seawater, demonstrating the safety of the wastewater if discharged to the environment. As main **Weaknesses** the results obtained showed that climate change related factors may influence the toxicity of remediated seawater especially under rise temperature, in comparison to control treatment; moderate to high toxicity of the nanomaterials tested. This research field represents an **Opportunity** not only in terms of environmental protection but also in terms of economic potential, promoting industries development and employment. Different **Threats** can be identified as increasing of extreme weather events that may clearly influence the toxicity of remediated seawater but also the sensitivity of organisms, development of more sustainable and environmental friendly processes of water remediation, including the use biological sorbents.

Considering the SWOT analysis, it is possible to identify that future research should address the challenge to develop and synthesize cheaper and safer nanomaterials with high capacity to remove mixture of metals from wastewater in the shortest possible time. Moreover, as proved by the present thesis, remediated seawater may influence bivalves' performance as those exposed to control treatments, which highlights the need for future studies on its relevance in the evaluation of different environmental stressors.

9.3 Conclusions

In conclusion, the present thesis shows that bivalves exposed to metal(loid)s treatments presented higher impacts than the ones under control and remediated seawater, with greater activation of antioxidant, cellular damage, and decrease of metabolic capacity as well as histopathological alteration in gills and digestive tubules. The results further evidenced the potential use of nanomaterials to remediate the contaminated water with metal(olid)s, and the safety of remediated water towards aquatic systems. Although climate changes may slightly influence the toxicity of remediated seawater, *Mytilus galloprovincialis* result the most tolerant species.

CHAPTER 10.

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