Environmental Science and Pollution Research
https://doi.org/10.1007/s11356-021-17745-3

RESEARCH ARTICLE

The influence of salinity on the toxicity of remediated seawater

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Received: 12 July 2021 / Accepted: 21 November 2021

Abstract

Mercury (Hg) is one of the most hazardous pollutants, due to its toxicity, biological magnification and worldwide persistence in aquatic systems. Thus, new efficient nanotechnologies (e.g. graphene oxide functionalized with polyethyleneimine (GO-PEI)) have been developed to remove this metal from the water. Aquatic environments, in particular transitional systems, are also subjected to disturbances resulting from climate change, such as salinity shifts. Salinity is one of the most relevant factors that influences the distribution and survival of aquatic species such as mussels. To our knowledge, no studies assessed the ecotoxicological impairments induced in marine organisms exposed to remediate seawater (RSW) under different salinity levels. For this, the focus of the present study was to evaluate the effects of seawater previously contaminated with Hg and remediated with GO-PEI, using the species *Mytilus galloprovincialis*, maintained at three different salinities(30, 20 and 40). The results obtained demonstrated similar histopathological and metabolic alterations, oxidative stress and neurotoxicity in mussels under RSW treatment at stressful salinity conditions (20 and 40) in comparison to control salinity (30). On the other hand, the present findings revealed toxicological effects including cellular damage and histopathological impairments in mussels exposed to Hg contaminated seawater in comparison to non-contaminated ones, at each salinity level. Overall, these results confirm the high efficiency of GO-PEI to sorb Hg from water with no noticeable toxic effects even under different salinities, leading to consider it a promising eco-friendly approach to remediate contaminated water.

Keywords Biomarkers · Mercury · *Mytilus galloprovincialis* · Graphene oxide functionalized with polyethyleneimine · Seawater remediation · Salinity · Bioaccumulation

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 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, 2020). In 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; 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

Responsible editor: Philippe Garrigues.

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Published online: 12 January 2022

bivalves (Belivermiş et al., 2019; Briant et al., 2017; Coppola et al., 2017, 2020a, 2020b; 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 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 and Guha, 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, 2020b) showed that the remediation of seawater using GO-PEI prevented *M. galloprovincialis* and *Ruditapes philippinarum* from biochemical and histopathological impacts induced by Hg.

In coastal and marine environments, 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., 2021). One of the predicted consequences of extreme events, including heavy 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 transitional systems as 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). Furthermore, 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, 2019b), in *Cerastoderma edule* and in *Scrobicularia plana* (Gonçalves et al., 2017); and increase production of essential fatty acids in *C. edule* and *S. plana* (Gonçalves et al., 2017). Also, hyper-salinity induced hemocyte 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 *Crassostrea gigas* (Gagnaire et al., 2006), *Haliotis diversicolor* (Cheng, 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 (lead and arsenic) (Freitas et al., 2016, 2019a, 2019b), 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, metabolic and oxidative status and histopathological alterations induced in *M. galloprovincialis* after exposure to Hg contaminated and remediated seawater and understand the influence of salinity in the toxicity of Hg contaminated and remediated seawater.

Materials and methods

Experimental treatments

Mytilus galloprovincialis mussels were collected in August 2019 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 box was used to transport mussels from the field to the laboratory, where they were depurated for 1 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, photoperiod 12:12 h light/dark 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 (*ad libitum*) with Algamac Protein Plus. During the acclimation week, mussels were divided in three groups: (1) mussels under control salinity (30 ± 1), (2) mussels under salinity 20 ± 1 and (3) mussels under salinity 40 ± 1. During this week, salinity was gradually reduced and/or increased,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, 2018). 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 each 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 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 decrease up to 20 with freshwater purified by reverse osmosis or increase up to 40 by adding synthetic salt, 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, pH, aeration, 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, three mussels from each aquarium (nine per treatment) were sacrificed and frozen with liquid nitrogen for bioaccumulation and biochemical assessment, while one mussel from each aquarium (three per treatment) was fixed in the Davidson solution for histopathological evaluation.

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 s) 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 °C 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.

Mercury quantification

A cold vapour 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) procedure. 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 (mussels' tissue, 0.071 ± 0.007 mg/Kg (total Hg)). The results were expressed in mg/Kg.

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 °C as described in Coppola et al. (2020a).

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.

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.

Antioxidant enzyme 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 $\varepsilon = 6.22$/(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.

Biotransformation enzyme 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.

Cellular damage and Redox balance

Lipid peroxidation levels (LPO) and the ratio between reduced and oxidized glutathione (GSH/GSSG) were considered cellular damage and redox balance indicators, respectively. 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.

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 (2007).

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).

Histopathological analyses

Mussels for histopathological observations were fixed in Davidson solution for 24 h and then washed in ethanol 70%. Daily change of ethanol was performed to remove all fixative's traces. Digestive tubules and gills were dissected afterward, gradually dehydrated in ascending ethanol and cleared 48 h in methyl benzoate. 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 paraffin (Hermanns et al., 2001). Sections 5 µm thick, obtained at microtome (Jung Autocut, Leica), were stained 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, 2020b). The histopathological indices were calculated as described in Leite et al. (2020) in order to evaluate the morphological status of organisms after the exposure, using with the following equation (Costa et al., 2013):

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

where *Ih* is the histopathological index for the individual *h*, *wj* the weight of the *jth* histopathological alteration, *ajh* the score attributed to the hth individual for the *jth* alteration and *Mj* is the maximum attributable value for the *jth* alteration.

Statistical analyses

Concentrations of Hg in the water and mussels' soft tissues, the biochemical parameters (ETS, GLY, SOD, GRed, GSTs, LPO, GSH/GSSG and AChE) as well as histopathological indices were separately submitted to a non-parametric permutational analysis of variance (PERMANOVA Add-on in Primer v7) (Anderson et al., 2008). A one-way hierarchical design was followed in this analysis. 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 with $p < 0.05$ were considered significantly different. For Hg concentration, biomarkers and histopathological indices, the null hypotheses tested were as follows: for each salinity level, no significant differences exist among treatments; 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, significant differences among exposure treatments were represented with different letters in the graphs and with *p*-values in Table 1A–C. For each treatment, differences among salinity levels (30, 20, 40) were represented with *p*-values in Table 2. Values < 0.05 were indicated in bold.

Results

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).

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 of 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) (Fig. 1, Table 1A). 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 (0.8 ± 0.03 µg/g) (Fig. 1, Table 1B). 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) (Fig. 1, Table 1C). Regardless of 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 2).

Biochemical markers

Metabolic capacity

At salinity 30, significantly higher ETS activity was detected in organisms exposed to RSW and CTL in comparison to the remaining treatments (Fig. 2A, Table 1A). At salinity 20, significantly lower ETS activity was observed in organisms exposed to RSW and GO-PEI + Hg compared to the CTL (Fig. 2, Table 1B). At salinity 40, GO-PEI + Hg-treated organisms showed significantly higher ETS activity than at CTL, GO-PEI and Hg (Fig. 2A, Table 1C). Comparing 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 salinity 30 compared to 20 and 40 salinities (Table 2).

Energy reserve content

Regarding GLY content, at control salinity (30), mussels exposed to RSW showed significantly higher GLY levels (Fig. 2B, Table 1A). 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 (Fig. 2B, Table 1B). At salinity 40, organisms exposed to GO-PEI + Hg showed significantly higher GLY content in comparison to GO-PEI treatment (Fig. 2B, Table 1C). Among salinities,

Table 1 *p-values* obtained by pair-wise comparisons among treatments (CTL, GO-PEI, GO-PEI + Hg, Hg and RSW) for each salinity: **A** 30, **B** 20 and **C** 40. [Hg]tissue, Hg bioaccumulation in the mussels' tissues; ETS (electron transport system) activity, GLY (glycogen) content, SOD (superoxide dismutase) activity, GRed (glutathione reductase) activity, GSTs (glutathione-S-transferases) activity, LPO (lipid peroxidation) levels, GSH/GSSG ratio between reduced and oxidized glutathione, AChE (acetylcholinesterase) activity; histopathological index- digestive tubules (D.T.), gills. Significant differences ($p < 0.05$) are highlighted in bold

Pairwise comparisons	$[Hg]_{tissue}$	ETS	GLY	SOD	GRed	GSTs	LPO	GSH/GSSG	AChE	D.T.	GILLS
A) Salinity 30											
CTL vs GO-PEI	0.1	0.08	0.55	**0.0007**	0.36	0.33	**0.01**	**0.0001**	**0.04**	**0.0001**	0.62
CTL vs GO-PEI + Hg	**0.0001**	0.08	0.41	0.1	0.41	**0.0001**	**0.03**	**0.0002**	0.99	**0.0002**	**0.0006**
CTL vs Hg	**0.0001**	0.1	0.18	**0.01**	0.68	**0.0001**	0.34	**0.01**	0.88	**0.0001**	**0.0001**
CTL vs RSW	**0.0001**	0.61	**0.001**	0.17	**0.009**	**0.03**	0.46	**0.009**	**0.05**	**0.0003**	**0.0001**
GO-PEI vs GO-PEI + Hg	**0.0001**	0.99	0.72	**0.05**	0.2	0.1	**0.0003**	0.75	**0.003**	**0.03**	**0.02**
GO-PEI vs Hg	**0.0001**	0.53	0.06	0.88	0.3	**0.0001**	**0.0007**	0.30	**0.05**	0.06	**0.0001**
GO-PEI vs RSW	**0.0001**	**0.02**	**0.001**	**0.01**	**0.03**	**0.05**	**0.01**	0.06	**0.003**	0.59	**0.003**
GO-PEI + Hg vs Hg	**0.0001**	0.59	0.06	**0.05**	0.54	**0.0001**	0.12	0.29	0.06	**0.002**	**0.03**
GO-PEI + Hg vs RSW	**0.0001**	**0.03**	**0.0005**	0.58	0.33	**0.0001**	0.26	0.07	**0.04**	0.25	0.56
Hg vs RSW	**0.0001**	**0.02**	**0.0001**	**0.05**	**0.03**	**0.0001**	0.95	0.62	**0.04**	0.52	0.06
B) Salinity 20											
CTL vs GO-PEI	0.1	0.27	**0.02**	**0.007**	0.07	0.16	**0.0008**	**0.0001**	0.43	**0.0003**	**0.0001**
CTL vs GO-PEI + Hg	**0.0001**	**0.02**	**0.0001**	**0.0008**	0.14	**0.0001**	0.06	**0.0001**	0.63	**0.0001**	**0.0001**
CTL vs Hg	**0.0001**	0.06	**0.04**	**0.0009**	0.12	**0.0001**	0.06	**0.0001**	0.52	**0.0004**	**0.0001**
CTL vs RSW	**0.0001**	**0.009**	0.07	0.53	0.44	0.54	0.59	**0.0001**	0.52	**0.0001**	**0.0001**
GO-PEI vs GO-PEI + Hg	**0.0001**	0.22	0.23	**0.05**	0.41	**0.0001**	**0.0002**	0.22	0.72	0.08	**0.0002**
GO-PEI vs Hg	**0.0001**	0.45	**0.003**	**0.05**	0.68	**0.0001**	**0.02**	0.45	0.95	0.07	**0.02**
GO-PEI vs RSW	**0.0001**	0.09	**0.007**	0.08	0.17	0.06	**0.001**	0.07	0.07	0.17	**0.005**
GO-PEI + Hg vs Hg	**0.0001**	0.59	**0.0003**	0.41	0.36	0.74	**0.005**	0.49	0.81	0.41	0.36
GO-PEI + Hg vs RSW	**0.0001**	0.46	**0.001**	**0.007**	0.41	**0.0001**	0.42	0.59	0.2	**0.0002**	**0.0001**
Hg vs RSW	**0.0001**	0.25	0.8	**0.01**	0.22	**0.0001**	0.07	0.14	0.17	**0.009**	**0.0007**
C) Salinity 40											
CTL vs GO-PEI	0.18	0.14	0.69	0.07	**0.0007**	**0.0003**	**0.01**	0.66	0.14	1	**0.0001**
CTL vs GO-PEI + Hg	**0.0001**	**0.016**	0.06	**0.0001**	**0.0001**	**0.002**	0.16	**0.002**	0.73	**0.02**	**0.0001**
CTL vs Hg	**0.0001**	0.29	0.81	0.98	**0.0001**	0.44	**0.04**	**0.0001**	0.61	**0.0002**	**0.0001**
CTL vs RSW	**0.0001**	0.39	0.91	**0.0009**	**0.0002**	**0.002**	0.41	0.26	**0.008**	**0.02**	**0.0001**
GO-PEI vs GO-PEI + Hg	**0.0001**	**0.002**	**0.03**	**0.0001**	**0.002**	0.88	0.09	**0.0001**	0.2	**0.003**	0.63
GO-PEI vs Hg	**0.0001**	0.48	0.6	**0.002**	**0.0001**	**0.008**	0.28	**0.0001**	0.3	**0.0001**	**0.002**
GO-PEI vs RSW	**0.0001**	0.06	0.8	**0.0001**	**0.0001**	0.62	0.14	0.21	0.3	**0.005**	**0.02**
GO-PEI + Hg vs Hg	0.48	**0.002**	0.18	**0.0001**	**0.003**	**0.02**	0.35	**0.008**	0.82	**0.002**	**0.0004**
GO-PEI + Hg vs RSW	**0.0001**	0.07	0.09	0.13	**0.0001**	0.59	0.73	**0.004**	**0.01**	0.44	0.06
Hg vs RSW	**0.0001**	0.06	0.77	**0.0003**	**0.0001**	**0.01**	0.37	**0.0001**	**0.02**	**0.02**	**0.0001**

Table 2 *p-values* obtained by pair-wise comparisons between salinity levels (30 vs 20; 30 vs 40; 20 vs 40) and each treatment (CTL, GO-PEI, GO-PEI + Hg, Hg and RSW). [Hg]tissue, Hg bioaccumulation in the mussels' tissues; ETS (electron transport system) activity, GLY (glycogen) content, SOD (superoxide dismutase) activity, GRed (glutathione reductase) activity, GSTs (glutathione-S-transferases) activity, LPO (lipid peroxidation) levels, GSH/GSSG ratio between reduced and oxidized glutathione, AChE (acetylcholinesterase) activity; histopathological index- digestive tubules (D.T.), gills. Significant differences ($p < 0.05$) are highlighted in bold

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

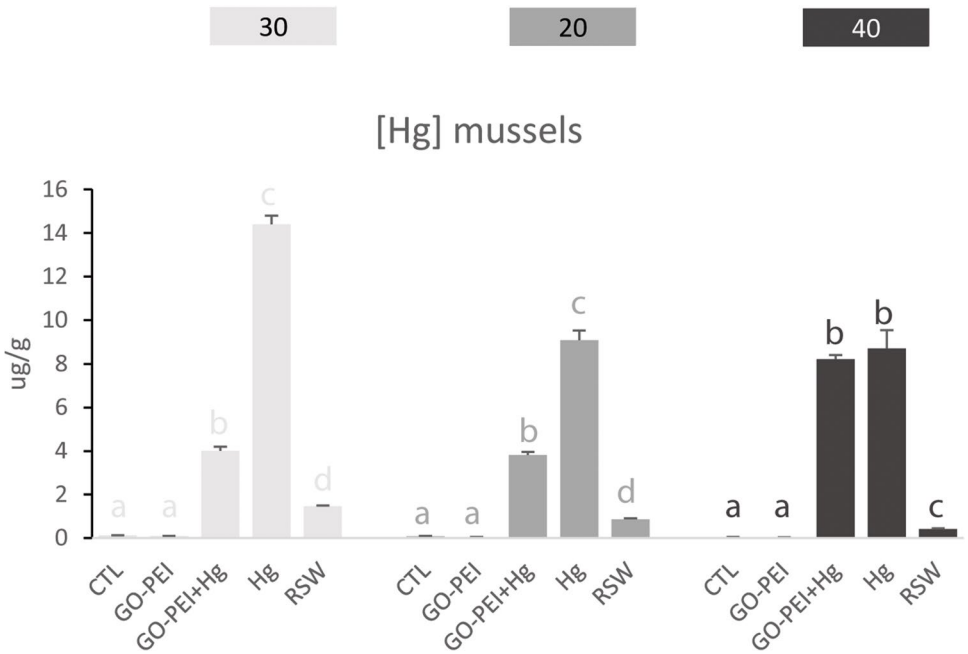
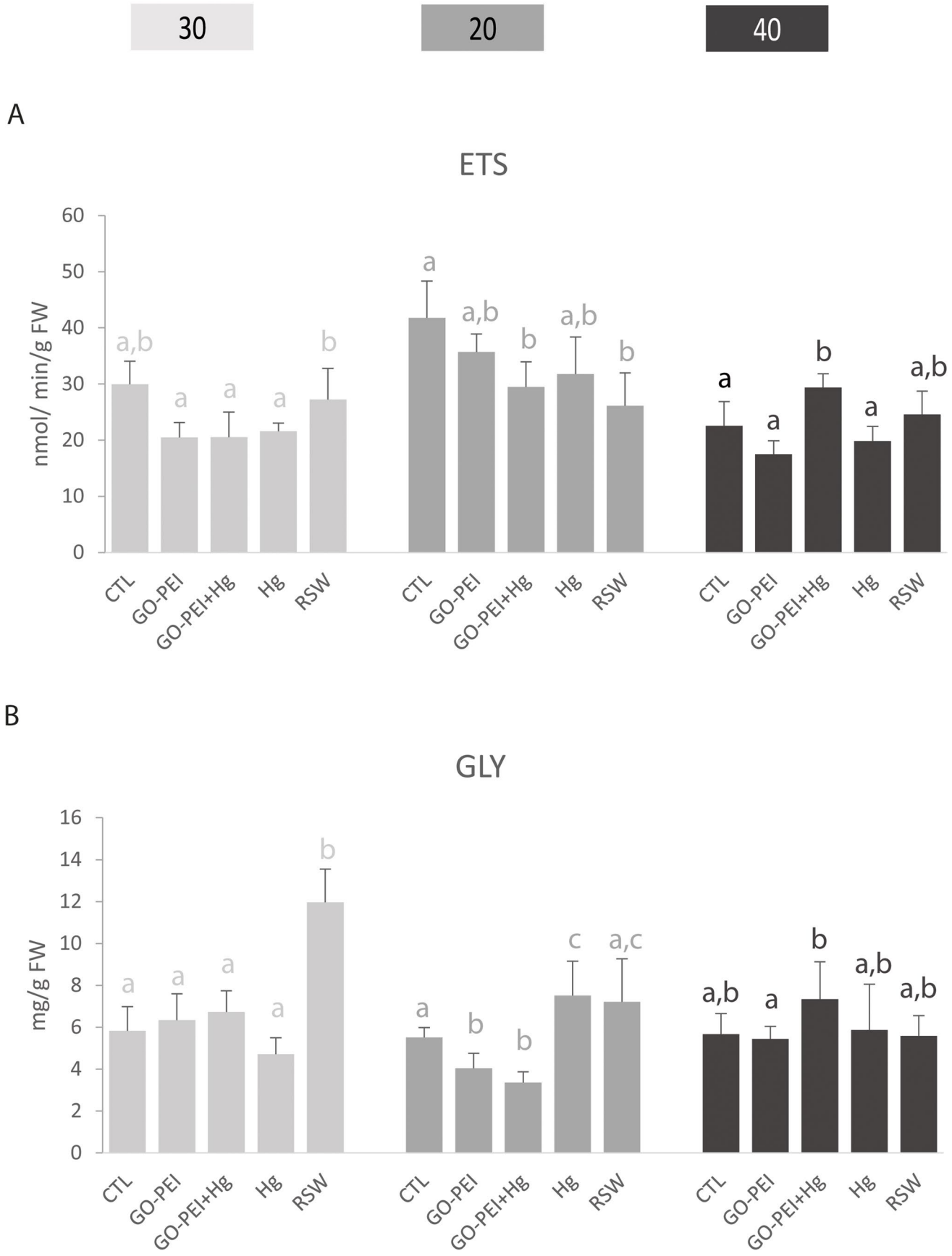


Fig. 1 Mercury concentration in *M. galloprovincialis* soft tissue ([Hg] mussels) exposed to different treatments (CTL, GO-PEI, GO-PEI + Hg, Hg and RSW) each under different salinities(30, 20 and 40). Results are the means + standard deviation. Significant differences ($p < 0.05$) among treatments are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

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 2).

Antioxidant enzyme 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 (Fig. 3A, Table 1A). At salinity 20, mussels subjected to GO-PEI + Hg and Hg treatments showed significantly higher


30
20
40
A
ETS
nmol/ min/g FW
60
50
40
30
20
10
0
a,b
a
a
a
b
a
a,b
b
b
a,b
b
a
a
b
a
a,b
CTL
GO-PEI
GO-PEI+Hg
Hg
RSW
B
GLY
mg/g FW
16
14
12
10
8
6
4
2
0
a
a
a
a
b
a
b
b
c
a,c
a,b
a
b
a,b
a,b
CTL
GO-PEI
GO-PEI+Hg
Hg
RSW

◂**Fig. 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(30, 20 and 40). Results are the means + standard deviation. Significant differences ($p < 0.05$) among treatments are identified with different lowercase letters

SOD activity when compared with the other treatments (Fig. 3A, Table 1B). 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 (Fig. 3A, Table 1C). Among salinities, organisms exposed to CTL showed significantly lower SOD activity at 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 2).

At salinity 30, the activity of GRed was significantly higher in RSW comparing with CTL, GO-PEI and Hg treatments (Fig. 3B, Table 1A). At salinity 20, no significant differences were observed among treatments (Fig. 3B, Table 1B). At salinity 40, significantly higher and lower GRed activity was observed in mussels exposed to Hg and RSW, respectively (Fig. 3B, Table 1C). 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 salinity compared to salinities 30 and 20 (Table 2).

Biotransformation enzyme 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 (Fig. 3C, Table 1A). At salinity 20, mussels under GO-PEI + Hg and Hg treatments showed significantly higher GSTs activities compared to the remaining ones (Fig. 3C, Table 1B). Regarding mussels at salinity 40, significantly higher GSTs activities were detected in CTL and Hg treatments compared to the other ones (Fig. 3C, Table 1C). 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 2).

Cellular damage

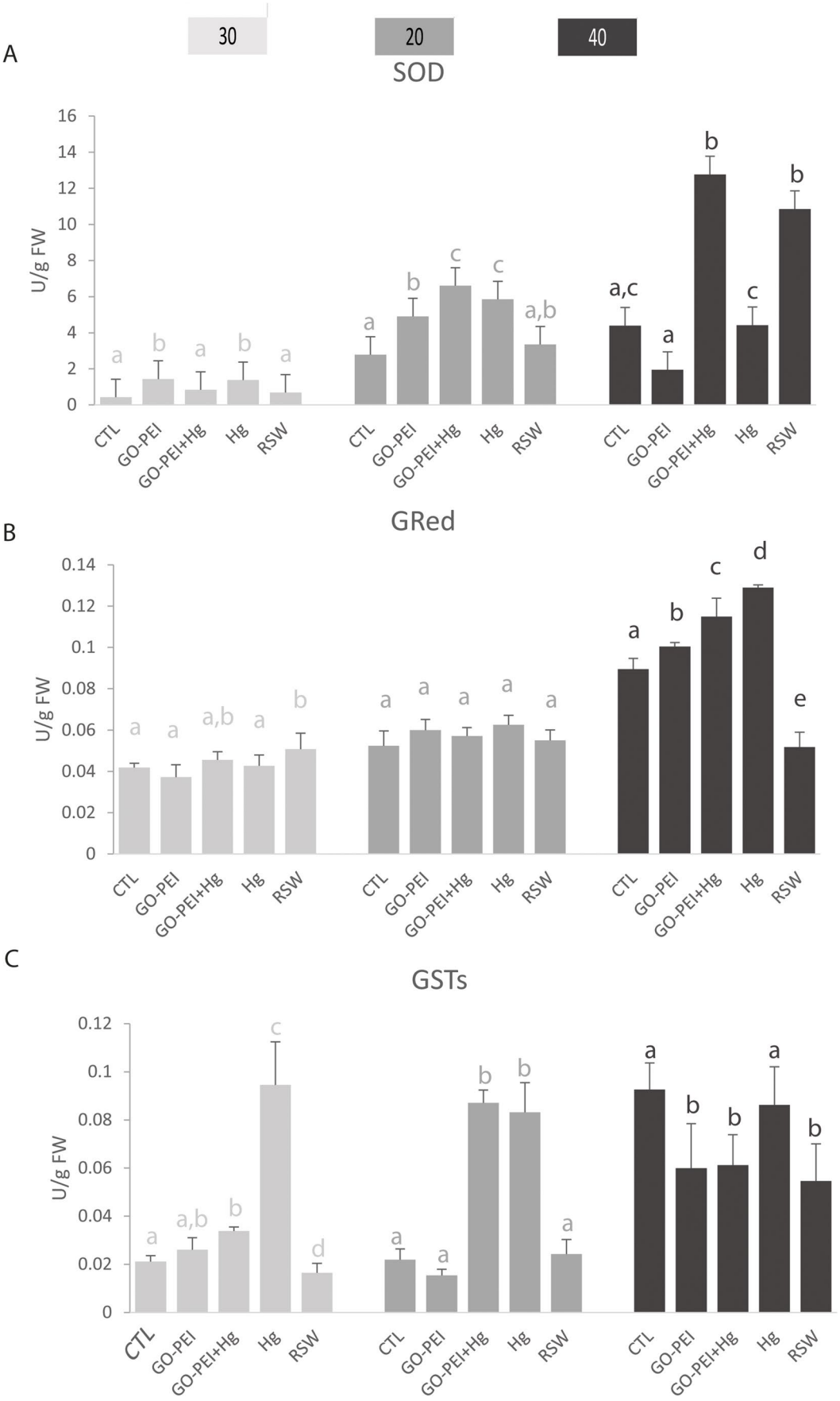
At salinity 30 significantly lower and higher LPO levels were detected in mussels exposed to GO-PEI and GO-PEI + Hg treatments, respectively, compared to the other treatments (Fig. 4A, Table 1A). Regarding organisms under salinity 20, significantly higher LPO levels were observed for GO-PEI treatment compared to the remaining ones (Fig. 4A, Table 1B). At salinity 40, significantly higher LPO levels were observed at CTL compared to GO-PEI and Hg treatments (Fig. 4A, Table 1C). Among salinity levels, 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 lower 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 2).

Redox balance

Regarding GSH/GSSG ratio, at salinity 30 as well as at salinity 20, *M. galloprovincialis* at CTL showed significantly higher values than those obtained for the remaining treatments (Fig. 4B, Tables 1A and B). 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 (Fig. 4B, Table 1C). 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 2).

Neurotoxicity

At salinity 30 significantly lower and higher AChE values were detected, respectively, in GO-PEI and RSW treatments, compared to the remaining ones (Fig. 5, Table 1A). At salinity 20 no significant differences were observed among treatments (Fig. 5, Table 1B). Regarding mussels exposed at salinity 40, significantly higher AChE activity was detected in RSW when compared to the other treatments,


A
30
20
40
SOD
U/g FW
CTL
GO-PEI
GO-PEI+Hg
Hg
RSW
B
GRed
U/g FW
C
GSTs
U/g FW

◂**Fig. 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(30, 20 and 40). Results are the means + standard deviation. Significant differences ($p < 0.05$) among treatments are identified with different lowercase letters

with exception to GO-PEI (Fig. 5, Table 1C). Among salinities, significantly lower AChE activity was observed at CTL, GO-PEI + Hg and RSW at salinity 40 compared to salinity 30 (Table 2).

Histopathological analyses

Digestive tubules

At salinities 30 and 20, mussels under CTL showed significantly lower histopathological index (I_h) in digestive tubules (D.T.) compared to the other treatments (Fig. 6A, Tables 1A and B). At salinity 40, significantly higher histopathological index in digestive tubules was found in mussels under Hg compared to the remaining treatments (Fig. 6A, Table 1C). Examples of histopathological alterations detectable in digestive tubules at salinities 30, 20 and 40 are reported in Figs. 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 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 2).

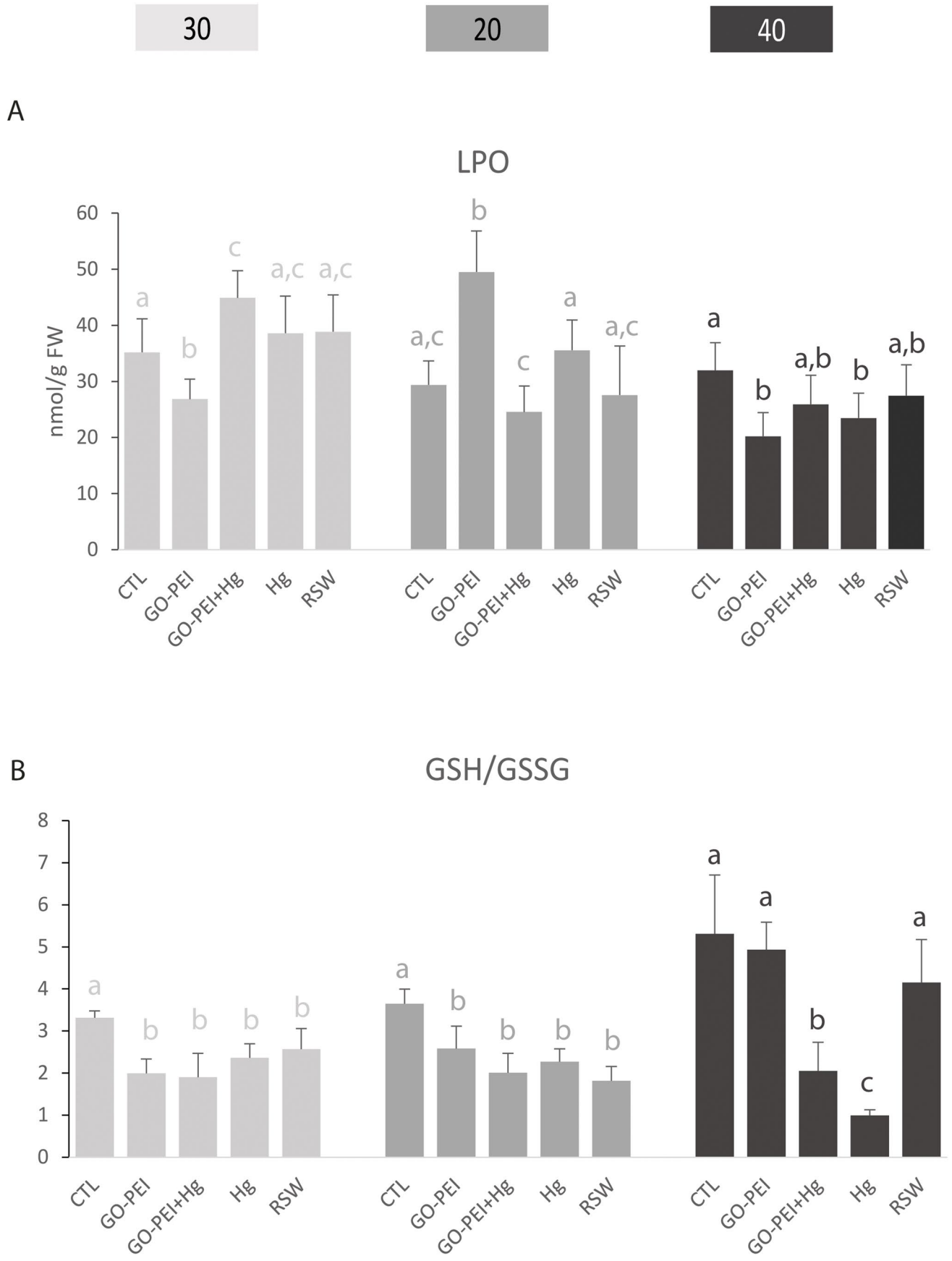
Gills

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 (Fig. 6B, Table 1A). 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 (Fig. 6B, Table 1B). 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 (Fig. 6B, Table 1C). Histopathological alterations present in gills at salinities 30, 20 and 40 are reported in Figs. 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 hemocyte infiltration was found in all treatments especially in mussels' gills exposed to GO-PEI + Hg and Hg regardless of 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 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 RSW treatment showed,significantly higher $I_h$values in gills at salinity 30 compared to the remaining salinities (Table 2).

Discussion

Under environmental conditions, bivalves may experience hyposaline and hypersaline stress, especially during extreme precipitation events that dilute 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 those 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., 2019a, 2019b, Bignell et al., 2008,


30
20
40
A
LPO
nmol/g FW
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10
20
30
40
50
60
a
b
c
a,c
a,c
a,c
b
c
a
a,c
a
b
a,b
b
a,b
CTL
GO-PEI
GO-PEI+Hg
Hg
RSW
B
GSH/GSSG
0
1
2
3
4
5
6
7
8
a
b
b
b
b
a
b
b
b
b
a
a
b
c
a
CTL
GO-PEI
GO-PEI+Hg
Hg
RSW

◂**Fig. 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(30, 20 and 40). Results are the means + standard deviation. Significant differences ($p < 0.05$) among treatments are identified with different lowercase letters

Kefi et al., 2014). Previous studies also demonstrated that bivalves, including mussels, may alter their biochemical performance when exposed to pollunts as nanomaterials or metal(loid)s, especially under salinity alterations (among others, Pranovi 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., 2018a, 2018b, 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 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 and biochemical and histopathological alterations after 28 days of chronic exposure were measured.

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

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 al. (2020a, 2020b) 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 did not result into 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 hypothesize 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 (including CTL), with exception to mussels exposed to remediated seawater (RSW) that presented a similar response at both salinities. These results highlight that, with exception of mussels exposed to RSW, increased metabolic activity at salinity 20 could indicate that mussels were trying to reestablish their biochemical performance under stressful conditions induced

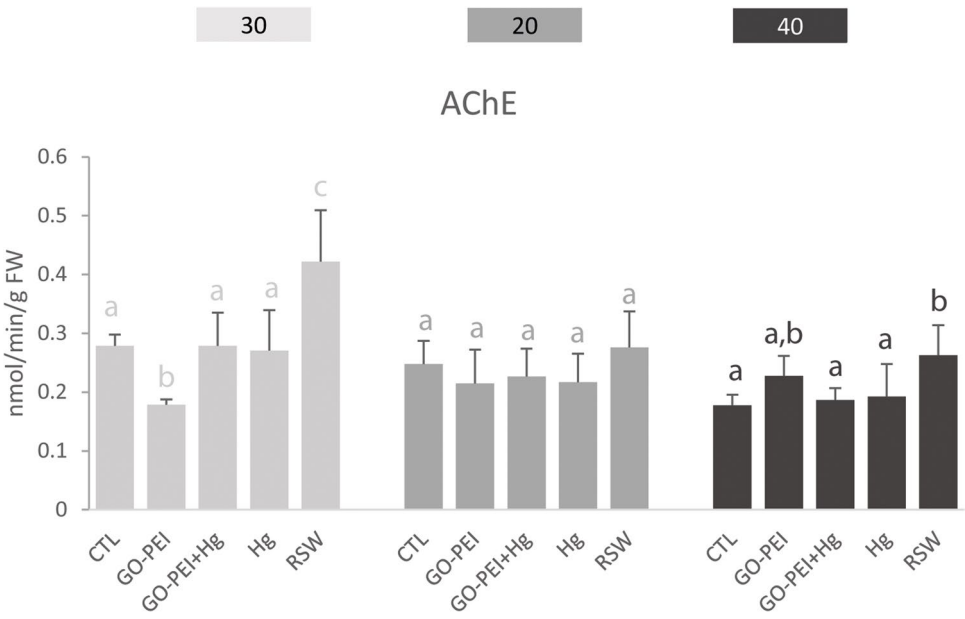
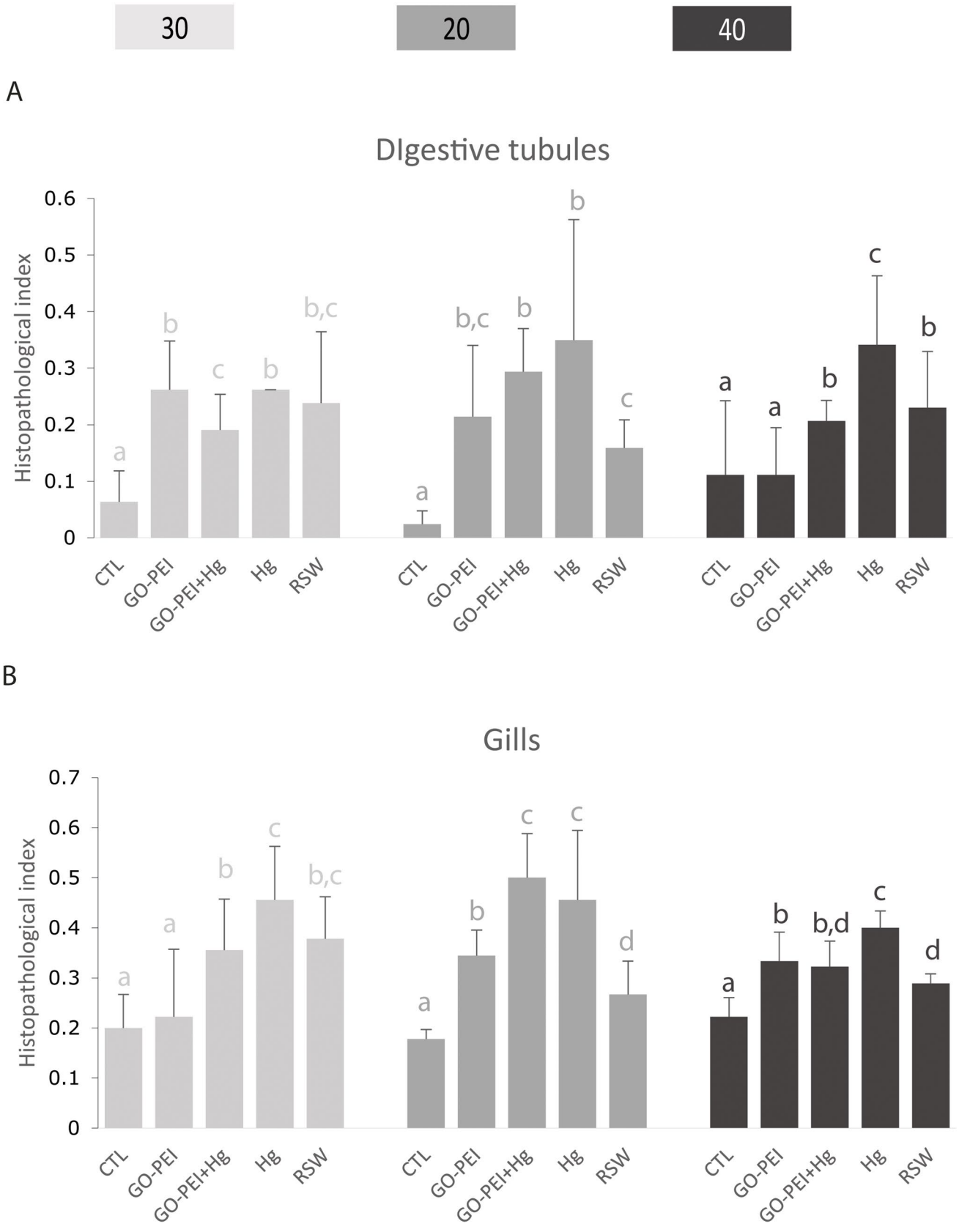


Fig. 5 Acetylcholinesterase activity (AChE) in *M. galloprovincialis* exposed to different treatments (CTL, GO-PEI, GO-PEI + Hg, Hg and RSW) each under different salinities (30, 20 and 40). Results are the means + standard deviation. Significant differences ($p < 0.05$) among treatments are identified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


30
20
40
A
DIgestive tubules
Histopathological index
0.6
0.5
0.4
0.3
0.2
0.1
0
a
b
c
b
b,c
a
b,c
b
b
c
a
a
b
c
b
CTL
GO-PEI
GO-PEI+Hg
Hg
RSW
B
Gills
Histopathological index
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
a
a
b
c
b,c
a
b
c
c
d
a
b
b,d
c
d
CTL
GO-PEI
GO-PEI+Hg
Hg
RSW

◂**Fig. 6** **A** Histopathological index in digestive tubules 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(30, 20 and 40). Results are the means + standard deviation. Significant differences ($p < 0.05$) among treatments are identified with different lowercase letters

by low salinity. 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 salinity 28 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, 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 defence 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 higher salinities (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 CTL 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, 2018b) 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

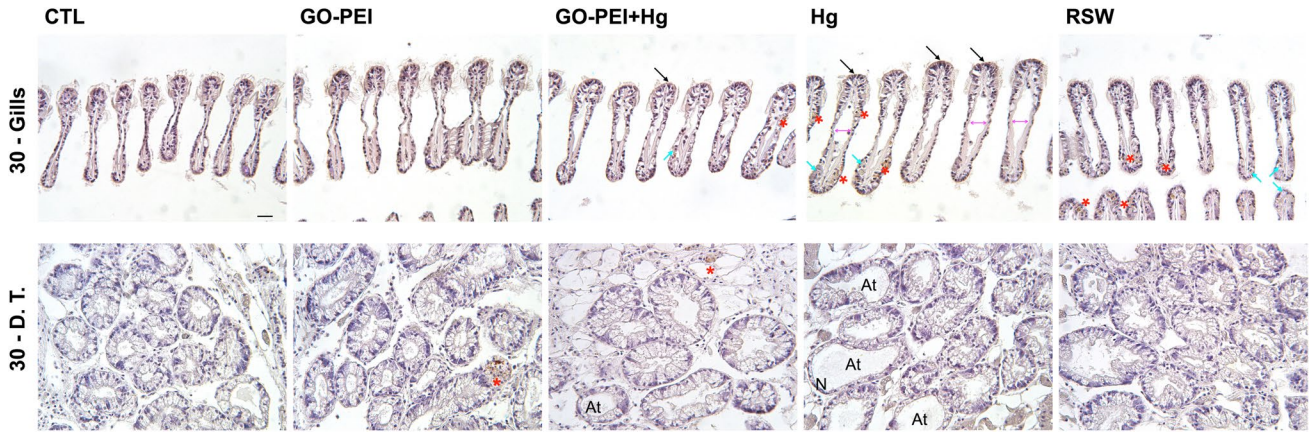


Fig. 7 Micrographs of different tissues in *M. galloprovincialis* after 28-day exposure. At 30 salinity, the tested treatments were CTL, GO-PEI, GO-PEI + Hg, Hg and RSW. Digestive tubule (D.T.): lipofuscin aggregates (highlighted with red asterisk), atropy (At) and necrosis (N). Scale bar = 50 and 100 µm; 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)

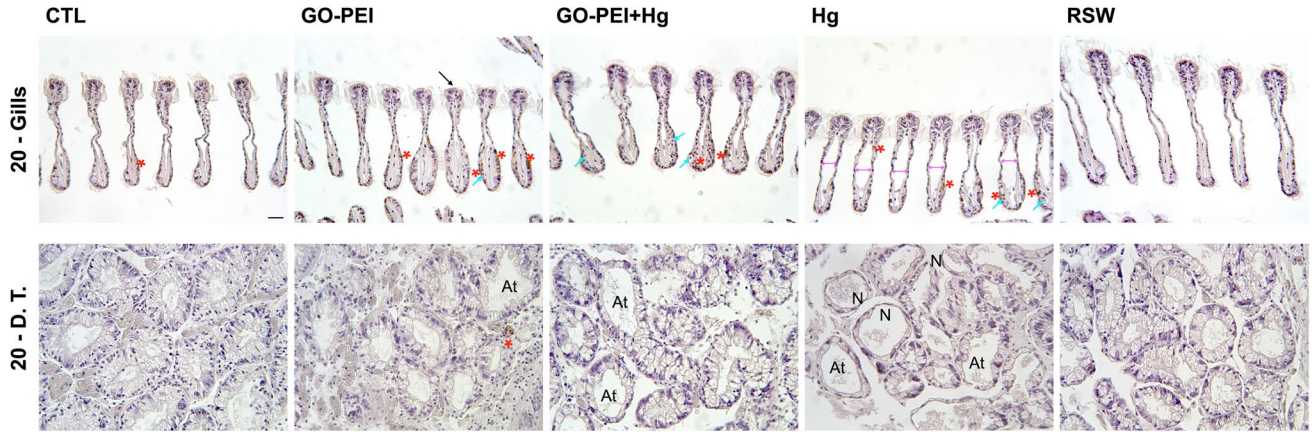


Fig. 8 Histopathological alterations present in gills at salinity 20. Digestive tubule (D.T.): lipofuscin aggregates (highlighted with red asterisk), atrophy (At) and necrosis (N). Scale bar = 50 and 100 μm; Gills: hemocyte infiltration (blue arrows), enlargement of the central vessel (pink arrows), abundance of lipofuscin aggregates (highlighted with red asterisk) and loss of cilia (black arrows)

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, 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 were 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 and between CTL and RSW treatments regardless the salinity tested.

Mussels' responses observed under high salinity 40 and salinity control (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

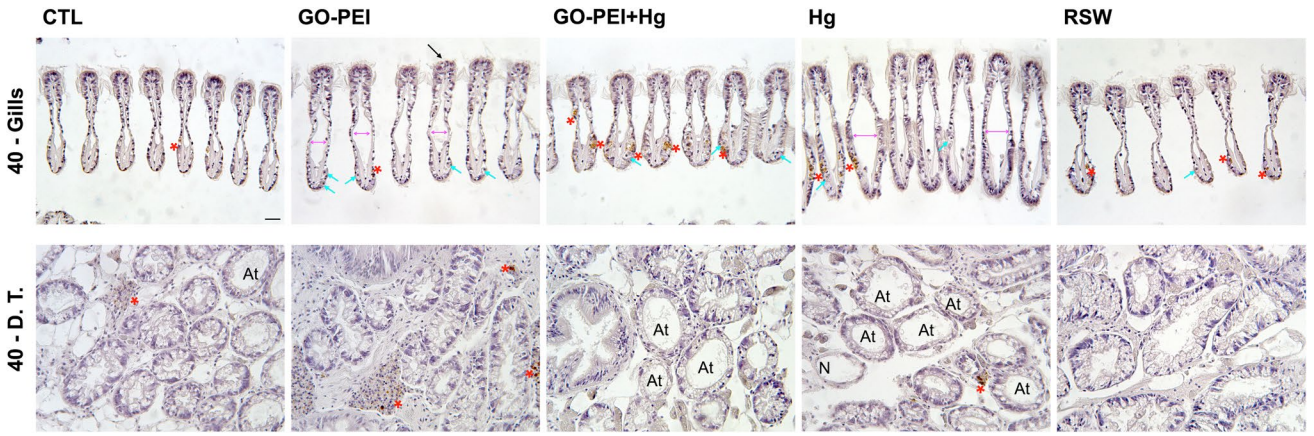


Fig. 9 Histopathological alterations present in gills at salinity 40. Digestive tubule (D.T.): lipofuscin aggregates (highlighted with red asterisk), atrophy (At) and necrosis (N). Scale bar = 50 and 100 μm; Gills: hemocyte infiltration (blue arrows), enlargement of the central vessel (pink arrows), abundance of lipofuscin aggregates (highlighted with red asterisk) and loss of cilia (black arrows)

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, 2018). 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 leads 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, 2019b). 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. (2019a, 2019b), 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 CTL 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., 2020a, 2020b; 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 the toxicity of remediated seawater was not influenced by salinity changes.

Conclusions

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 of the salinity tested,

presented similar responses indicating the low toxic effect of remediated seawater at each of the salinities tested.

Acknowledgements Thanks are due for the financial support to CESAM, REQUIMTE and TEMA (UIDB/50017/2020 + UIDP/50017/2020 and UID/QUI/50006/2013, UIDB/00481/2020 + UIDP/00481/2020, respectively) and to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020.

Author contribution Rosa Freitas and Gianluca Polese supervised the students (Francesca Coppola, Tania Russo) in all biochemical and histopathological assays. Eduarda Pereira and Paula Marques supervised the student Francesca Coppola in all Hg quantifications and synthesis and characterization of nanomaterial. Coppola Francesca performed all laboratory exposures and chemical, biochemical and histopathological analyses. Amadeus M.V.M. Soares financed the resources.

Funding This work was funded by national funds (OE), through FCT – Fundação para a Ciência e a Tecnologia, I.P., in the scope of the framework contract foreseen in the numbers 4, 5 and 6 of the article 23, of the Decree-Law 57/2016, of August 29, changed by Law 57/2017, of July 19. This work was also financially supported by the project BISPECIAl: BIvalveS under Polluted Environment and ClImate chAnge (POCI-01–0145-FEDER-028425) funded by FEDER, through COMPETE2020—Programa Operacional Competitividade e Internacionalização (POCI), and by national funds (OE), through FCT/MCTES. This work was also supported by the budgets of the Regional Operational Program of the Center and national funds (CENTRO-01–0145-FEDER-030513) in its FEDER component and the Foundation for Science and Technology in its State Budget, PTDC/NAN- MAT/30513/2017 (project H2OValue). Francesca Coppola benefited from a PhD grant (SFRH/BD/118582/2016) given by the National Funds through the Portuguese Science Foundation (FCT), supported by FSE and Programa Operacional Capital Humano (POCH) e da União Europeia.

Data Availability All data generated or analysed during this study are included in this published article (and its supplementary information files).

Declarations

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

Competing interests The authors declare no competing interests.

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