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Salinity influences on the response of *Mytilus galloprovincialis* to the rare-earth element lanthanum

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ABSTRACT

The multiplicity and wide variety of applications of electrical and electronic equipment has largely increased with the technological and economic progress and, in consequence, the amount of generated waste of electrical and electronic equipment (WEEE). Due to inappropriate processing and disposal of WEEE, different chemical elements and compounds, including rare-earth elements such as Lanthanum (La) have been released in the environment. Nevertheless, the environmental risks resulting from La presence are almost unknown, especially in marine systems, which may be challenged by foreseen climate changes such as water salinity shifts. Within this context, the present study aimed to understand the combined effects of salinity and La by assessing biochemical alterations in mussels *Mytilus galloprovincialis* exposed to La (0 and 10 µg/L) at different salinity levels (20, 30 and 40). A decrease in salinity caused a wide range of biochemical changes to both

non-contaminated and contaminated organisms, such as metabolism, antioxidant and biotransformation defenses activation, associated to hypotonic stress. Furthermore, the decrease in salinity enhanced the effects of La exposure seen as an increase on lipid and protein cellular damage in those exposed, probably due to free metal ions increase at lower salinities, resulting in a higher bioaccumulation and toxicity. In general, La exposure caused cellular damage and inhibition of antioxidant defenses in contaminated mussels when compared to non-contaminated ones, with cellular damages being higher at the lowest salinity. Overall, the present study highlights the need to investigate the presence and impacts of emerging contaminants of WEEE source at environmentally relevant concentrations, not just at present but also under forecasted climate change scenarios, thus providing a more realistic environmental risk assessment.

Keywords: Lanthanum; salinity; oxidative stress; metabolism; neurotoxicity

1. INTRODUCTION

The fast technological development in recent years, associated with the increasing number and diversity of electrical and electronic equipment (EEE) while shortening their life span, has led to a larger amount of discarded products which has not been accompanied by its properly management (Laurenti et al., 2015). The amount of emerging waste of electrical and electronic equipment (WEEE) has been continually increased, with about 52.2 Mt of WEEE being expected in 2021 under an annual growth rate of 3 to 4 % (Baldé et al., 2017). Currently there is a lack of recycling protocols while legal management of WEEE is costly and, thus, scarcely used worldwide (Grant et al., 2013, UNEP et al., 2019). Therefore, specific chemical elements and compounds can be released in the environment, either directly as components of the equipment or released after recycling processes (Grant et al., 2013). In fact, since WEEE is non-biodegradable, its components may accumulate in the environmental compartments: soil, air, water and organisms (UNEP et al., 2019). As an example, rare-earth

elements (REEs) constitute a chemically similar group of substances widely and indispensably used in EEE due to their unique chemical properties. Among REEs, Lanthanum (La) is commonly used in different electronics and optoelectronics to produce luminophores, lasers, catalysts, metal alloys, batteries and communication devices (Palasz et al., 2000; US EPA, 2012) and to stimulate growth in agricultural products as trace fertilizer (Feng et al., 2006). Thus, it is expected to occur in industrial effluents and, consequently, in the environment, namely aquatic systems. For instance, La has been reported at concentrations of 0.0291-12.85 µg/L in surface waters and up to 40.37 µg/L in alluvial aquifers affected by acid mine drainages (Sultan et al., 2019, Olías et al., 2005). Speciation diagrams have nonetheless predicted that La free species precipitate as $LaPO_4$(s) and $La_2(CO_3)_3$ (s) under salinity 32 and pH between 7.5 and 8.4, which resembles oceanic water conditions (Mucci et al., 2020).

The presence of REEs in aquatic environments, such as estuaries, may have an impact towards their biological resources. Among them bivalves are socio-economically important (WHO, 2010) and have been widely recognized as good bioindicators in both monitoring and ecotoxicological studies (Aziz et al., 2018; Belivermiş et al.,2016; Rouane-Hacene et al., 2015). Although the effects of La are still poorly understood, in the Mediterranean mussel *Mytilus galloprovincialis* this element has seen to bioaccumulate and trigger changes in their metabolism, energy reserves content and antioxidant and biotransformation processes (Pinto et al., 2019). Furthermore, this element already revealed to be toxic to embryos of mussels *M. galloprovincialis* (Mestre et al., 2019) and of oysters *Crassostrea gigas* (Moreira et al., 2020). Moreover, recent evidences for freshwater clams *Dreissena polymorpha* and mussels *Corbicula fluminea*, indicate bioaccumulation of La and impacts on the bivalve's metabolism and on oxidative status (Hanana et al. 2017; Zhao and Liu, 2018).

Under natural environmental conditions, marine bivalves may not just be exposed to

pollutants but simultaneously subjected to abiotic variations such as the ones related to Climate Change (CC). For instance, salinity shifts due to an acceleration in the global rainfall and evaporation cycles (Durack, 2015) are among the expected changes due to CC. Salinity variations have already demonstrated to affect bivalves metabolic rate and oxidative status (Coughlan et al., 2009; Gonçalves et al., 2017; Sarà et al., 2008; Velez et al., 2016a,b). Still, little information is available on organisms' responses when exposed to combined factors, namely the presence of pollutants and salinity shifts, which may interact and be synergic or on the contrary mask or prevent some of the effects. Recent studies conducted by Moreira et al. (2016) revealed that the effects of Arsenic on oysters' (*Crassostrea angulata*) oxidative stress appears to be more deleterious under higher salinities (20, 30 and 40), comparing to salinity 10. However, greater As accumulation as well as increased metabolic and antioxidant capacity was observed at salinity 10 which can limit oysters reproductive capacity and growth.

In this way, the present study aims to assess the impacts of the REE La, at a realistic environmental concentration and under different salinities, using the mussel *M. galloprovincialis* as bioindicator species. The biochemical alterations evaluated, after 28 days-exposure period, were related to metabolic, oxidative and neurotoxic pathways. Accordingly, null hypotheses of no effects of La on mussels' biochemical alterations and no influence of salinity on the effects of La were tested.

2. METHODOLOGY

2.1. Sampling and experimental conditions

Mytilus galloprovincialis of similar size (length: 54.4±2.1 mm; width: 33.7±2.3 mm) were collected in November 2019 during low tide at an unpolluted area of the Mira Channel (40° 38′ 31.7″N, 8° 44′ 10.9″W, Ria de Aveiro coastal lagoon, northwest of Portugal). In this area a variation of salinity from 30 to 37 during the fall season is known to happen

(Rodrigues et al., 2012). In the laboratory mussels were allowed to depurate and acclimate for 2 weeks. Organisms were maintained under continuous aeration in synthetic saltwater prepared with deionized water and artificial salt (Tropic Marin® SEA SALT from Tropic Marine Center). During this period, mussels were maintained under control conditions, resembling those at the sampling site: temperature of 17.0±1.0 ºC, pH of 8.0±0.1, salinity of 30±1 (control) and a photoperiod of 12 h light: 12 h dark. A progressive acclimation of mussels to the salinities out of the control (20±1 and 40±1) was performed during the second week and before the experimental period. From the day 5 of arrival, organisms were fed every 2 days with AlgaMac Protein Plus (Aquafauna BioMarine®, CA, USA), a ration constituted by 39 % protein, 20.4 % lipid and 20.6 % carbohydrates, with several heterotrophic and phototrophic species, vitamins, attractants and pigmentation. Seawater was renewed twice in the first week and once in the second week, with conditions being re-established each time.

For the experiment, 5 mussels were placed in each aquarium (filled with 3 L of synthetic seawater), with 3 aquaria assigned to each treatment (15 mussels in total per treatment). The experimental period lasted 28 days and the temperature and pH conditions were those used during the acclimation period. The salinity of 30±1 used in the acclimation was adopted as control and the salinities of 20±1 and 40±1 to resemble rainy and drought periods, respectively. A concentration of 10 µg/L of La was used in the experiments, according to realistic values reported in contaminated aquatic environments (Sultan et al., 2019, Olías et al., 2005) and concentrations previously selected in studies using freshwater and marine bivalves (Hanana et al., 2017, Moreira et al., 2020). A stock solution of 15 mg/L of La was used by dilution of a La commercial solution (1000 mg/L by Inorganic Ventures) in ultrapure water. Six treatments were performed: non-contaminated organisms under salinity 20; non-contaminated organisms under salinity 30 (CTL); non-contaminated

organisms under salinity 40; contaminated organisms under salinity 20; contaminated organisms under salinity 30 and contaminated organisms under salinity 40.

During the experimental period organisms were fed three times per week with the formerly described AlgaMac preparation at a concentration of 150,000 cells/animal per day. Seawater was renewed once a week and the medium conditions (La concentration, salinity, temperature and pH) re-established each time, and feces removed. Seawater samples were collected from each exposure aquaria and blanks (aquaria without organisms under the same conditions) immediately after spiking with La for quantification aiming to obtain the real exposure concentrations (exposure aquaria) and La stability in solution (blanks). Seawater samples were also collected from blanks at 24, 48, 72, 144 and 168 hours after La spiking, including immediately before water renewal to verify the element's stability during one week after spiking.

After the 28 days-exposure period, organisms were sampled and immediately frozen with liquid nitrogen and stored at -80 °C. In the case of biochemical and chemical analyses three frozen mussels per aquarium (nine per treatment) were considered, being the whole soft tissue manually homogenized with liquid nitrogen using a mortar and pestle. Each homogenized tissue was divided in 0.5 g fresh weight (FW) aliquots and stored at -80ºC until further analyses.

2.2. Lanthanum quantification in seawater and mussel's soft tissue

Seawater samples for La quantification were diluted and acidified with HNO_2 2% to pH<2, and measured through inductively coupled plasma mass spectroscopy (ICP-MS), on a Thermo ICP-MS XSeries equipped with a Burgener nebuliser. The limit of quantification (LOQ) was estimated to be of 0.02 µg/L.

Total La concentration in the mussels' tissues was also determined by ICP-MS, after microwave assisted acid digestion. About 200 mg per freeze-dried samples were weighted in

a Teflon vessel being added afterwards 1 mL of HNO_3 65 % (v/v), 2 mL of H_2O_2 and 1 mL of H_2O. Samples were placed in a CEM MARS 5 microwave with increasing temperature up to 170 ºC during 15 min, being then maintained at this temperature for more 5 min. After cooling, samples were transferred to polyethylene flasks which were filled with ultrapure water up to a final volume 25 mL and stored at room temperature until quantification. The coefficient of variation (CV) of La quantification in tissues' sample duplicated varied between 4–12 %. Digested blanks (microwave vessels without sample), duplicates and the certified reference material BCR-668 (Mussel tissue; 80±6 µg/Kg of La) were considered for quality control. Quantification of La in digested blanks gave values below the detection limit of the methodology and recovery values in certified reference material (3 replicates) were between 91-97%, showing a good performance of the digestion and quantification method. The methods' limit of quantification (LOQ) for tissue was of 0.02 µg/L (corresponding to the value of the lowest standard concentration used in the calibration curve).

2.3. Biological responses: biochemical parameters

The biochemical alterations were evaluated through biomarkers related to energy metabolism: electron transport system (ETS) activity; glycogen (GLY) and total protein (PROT) contents; antioxidant enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione reductase (GR); biotransformation enzymes: glutathione S-transferases (GSTs) and carboxylesterases (CbEs) activities; cellular damage: lipid peroxidation (LPO) and protein carbonylation (PC) levels; and neurotoxicity: acetylcholinesterase (AChE) activity. The determination of each biomarker was performed in a total of nine samples per treatment (3 individuals per aquarium and 3 aquaria per treatment, making a total of 9 individuals per treatment) and technical duplicates of each sample were considered in each analysis. Samples extraction was performed using a proportion of 1:2 (w/v) with the adequate buffer for each assay. The homogenization step was done using a TissueLyzer II (Qiagen) set at frequency

20 1/s, during 90 s, centrifuged at 4 ºC and the supernatant collected and stored at -80 ºC, or immediately used. For most parameters (GLY, PROT, SOD, CAT, GR, GSTs, CbEs, PC and AChE) a potassium phosphate buffer was used in the extraction, while 20% (w/v) trichloroacetic acid (TCA) in the case of LPO measures and 0.1 mol/L Tris-HCl were adequate for ETS determination. Extracted samples either with potassium phosphate buffer and TCA were centrifuged at 10,000 *g* for 20 min, while those for ETS measures were centrifuged at 3,000 *g* for the same period.

2.3.1. Metabolic capacity and energy reserves

Metabolic capacity was evaluated by measuring ETS following Packard (1974) methodology, with modifications performed by Coen and Janssen (1997). The reaction was followed in a microplate reader at 490 nm for 10 min at 25 s intervals. The amount of formazan formed in each well was calculated using the extinction coefficient (Ɛ) 15,900 $(mol/L)^{-1}$ cm^{-1} and results were expressed in nmol/min per g FW.

Energy reserves were assessed as GLY content following the sulfuric acid method (Dubois et al., 1956) and the PROT content according to the Biuret method (Robinson and Hogden, 1940). Standards were used for both quantifications, being glucose (0-2 mg/mL) used for GLY content and bovine serum albumin (100 µg/mL) used for PROT content. The final reactions were read at 492 nm (GLY) and 440 nm (PROT). Results were expressed in mg per g of FW.

2.3.2. Antioxidant and biotransformation enzymes

Antioxidant activity was measured by quantifying SOD, CAT and GR following the methods described by Beauchamp and Fridovich (1971), Johansson and Borg (1988) and Carlberg and Mannervik (1985), respectively. Standards for SOD were 0.25-60 U/mL of SOD and 0-150 µmol/L of formaldehyde for CAT were used for quantification. SOD and CAT reactions were read at of 560 nm and 540 nm, respectively, while GR activity was

followed at 340 nm during 5 min in intervals of 15 s using for quantification the extinction coefficient (Ɛ) 6.22 x 10^{-3} $(mol/L)^{-1}$ cm^{-1}. Results of activities were expressed in U per g FW. For SOD one unit (U) of enzyme activity represents a reduction of 50 % of nitroblue tetrazolium (NBT); the formation of 1 nmol formaldehyde per min in the case of CAT, and the quantity of enzyme which catalyzes the conversion of 1 μmol of NADPH per min for GR.

Biotransformation activity was determined by quantifying GSTs according to Habig et al. (1974) with modifications performed by Carregosa et al. (2014), and CbEs following Hosokawa et al. (2001) methodology adapted by Solé et al. (2018). In the case of CbEs, two different commercial colorimetric substrates, p-nitrophenyl acetate (pNPA) and p-nitrophenyl butyrate (pNPB), were used. The kinetic reactions were read at 340 nm during 5 min in 10 s intervals for GSTs and at 405 nm during 5 min in 25 s intervals for CbEs. For the activity quantification, the amount of thioether formed was calculated for GSTs using the extinction coefficient (Ɛ) 9.6 x 10^{-3} $(mol/L)^{-1}$ cm^{-1} and the formation of p-nitrophenol for CbEs using the extinction coefficient (Ɛ) 18 x 10^{3} $(mol/L)^{-1}$ cm^{-1}. The results were expressed in U per g FW in the case of GSTs, where U indicates the quantity of enzyme that causes the formation of 1 μmol of dinitrophenyl thioether per min. In the case of CbEs results were expressed by μmol per min per g of FW.

2.3.3. Oxidative damage

Oxidative damage of the lipids and proteins was evaluated by assessing LPO levels according to the Ohkawa et al. (1979) method with modifications by Carregosa et al. (2014) and by assessing PC levels following the 2,4-dinitrophenylhydrazine (DNPH) alkaline method described by Mesquita et al. (2014). LPO was determined at 535 nm and PC at 450 nm, using the extinction coefficients for Malondialdehyde (MDA) (1.56 x 10^{5} $(mol/L)^{-1}$ cm^{-1}) and for DNPH (22,308 $(mol/L)^{-1}$ cm^{-1}), respectively. In the case of LPO, results were expressed in nmol MDA equivalents per g of FW, and for PC, in μmol of protein carbonyls

groups formed per g of FW.

2.3.4. Neurotoxicity

Neurotoxicity was evaluated through the activity of AChE using Acetylthiocholine iodide (ATChI 5 mmol/L) as substrate, following Ellman et al. (1961) methodology with modifications performed by Mennillo et al. (2017). The kinetic reaction was set at 412 nm during 5 min at 25 s intervals. The extinction coefficient (Ɛ) 1,36 x 10^4 $(mol/L)^{-1}$ cm^{-1} was used for quantification and the results were expressed in nmol per min per g FW.

2.4. Data analyses

2.4.1. Bioconcentration factor

The bioconcentration factor (BCF) was calculated to evaluate the bioaccumulation of La in mussels' tissues from water. The calculation was performed based on Arnot and Gobas (2006) equation, defined as the ratio between total concentration of chemical in tissue and total concentration of chemical in water.

2.4.2. Statistical and multivariate analyses

The biochemical data (ETS, GLY, SOD, LPO, PC, AChE, SOD, CAT, GR, GSTs, CbEs) from all treatments were submitted to hypothesis testing using a permutational multivariate analysis of variance by using the PERMANOVA add-on in PRIMER v7 (Anderson et al., 2008). Three null hypotheses were tested: 1) no significant differences exist among different salinities for non-contaminated organisms; 2) no significant differences exist among different salinities for La contaminated mussels; 3) no significant differences exist between non- and La contaminated organisms at each salinity. A significance level was established at $p<0.05$ for each hypothesis. In the figures, significant differences were represented with low case letters in the case of the first hypothesis and with capital letters in the case of the second hypothesis. For the third hypothesis, significant differences are represented by an asterisk.

The Euclidean distance similarity matrix was calculated using data assembling for all the biochemical descriptors per treatment. This similarity matrix was submitted to ordination analysis by Principal Coordinates (PCO) after being simplified resorting to the calculation of the distance among centroids based on the treatment: non-contaminated and contaminated organisms under salinity 20 (CTL20, La20), salinity 30 (CTL30, La30) and salinity 40 (CTL40, La40). Pearson correlation vectors of biochemical descriptors with correlation > 75 % were provided as supplementary variables being superimposed on the PCO graph.

3. RESULTS

3.1. Lanthanum concentration in seawater and mussel's tissue

Lanthanum levels in seawater from non-contaminated treatments at all salinities were always below the limit of quantification (<0.02 µg/L). In La exposed treatments, concentrations in seawater collected immediately after spiking ranged between 10.3±0.3 µg/L (at salinity 20), 10.5±0.5 µg/L (at salinity 30) and 10.8±0.5 µg/L (at salinity 40), all values close to the nominal concentration of 10 µg/L. Thus, the levels of La measured in the seawater were those expected, with a maximum deviation to the nominal concentration of 7.5 %.

The evaluation of the La stability in seawater along time, that was performed by collecting water samples from blanks at 0, 24, 48, 72, 144 and 168 hours after La spiking, showed losses that were lower than 10 % for salinity 20, 4 % for salinity 30 and 5 % for salinity 40. For this reason, regardless the salinity tested, La concentration was considered stable after spiking.

Lanthanum was present in mussels' tissues at the end of the 28-day experimental period, regardless of the treatment. In the case of non-contaminated mussels concentrations (in µg/g of dry weight) of 0.094±0.034 µg/g (salinity 20), 0.032±0.001 µg/g (salinity 30) and 0.029±0.006 µg/g (salinity 40) were recorded, with significant differences between salinity

20 and salinities 30 and 40. For contaminated mussels, significantly higher La concentrations were present at salinity 20 (1.6±0.4 µg/g, La20), compared to mussels at salinities 30 (0.75±0.2 µg/g, La30) and 40 (0.47± 0.2µg/g, La40) (Table 1). Thus, La concentration values in contaminated mussels were 17 (salinity 20), 23 (salinity 30) and 23 (salinity 40) times higher than the ones found in non-contaminated mussels.

The bioconcentration factor (BCF) (Table 1) was significantly higher at the lowest salinity (La20) compared to control (La30) and the highest salinity (La40), with no significant differences between the salinities 30 and 40.

3.2. Biological responses: biochemical parameters

3.2.1. Metabolic capacity and energy reserves

In non-contaminated mussels, significant differences in ETS activity were observed among all treatments, with the highest values at salinity 20 and the lowest at salinity 40. The ETS activity in La contaminated mussels was also significantly higher at salinity 20 compared to salinities 30 and 40. When comparing non-contaminated with contaminated mussels at each salinity level, significant differences were observed only at control salinity 30, with higher ETS values in those non-contaminated (Fig. 1A).

Although mussels tended to decrease the GLY content at increasing salinities, no significant differences were observed in either case (exposed or non-exposed) or between them at each salinity (Fig. 1B).

The PROT content showed, by contrast, a significant increase at increasing salinity for both non-contaminated and contaminated organisms, with no significant differences between non-contaminated and contaminated mussels at each salinity (Fig. 1C).

3.2.2. Antioxidant and biotransformation enzymes

Non-contaminated mussels showed significantly higher SOD activity at salinity 20 compared to salinity 30, while in those La exposed SOD activity was significantly higher at

the lowest salinity compared to values registered at salinities 30 and 40. Comparing non-contaminated with contaminated mussels, significant differences were only recorded at salinity 40, with the lowest SOD values at those contaminated (Fig. 2A).

The activity of CAT in non-contaminated mussels decreased from salinity 20 to 40 with significant differences among all salinities. In contaminated mussels a similar trend was observed with significant differences between the lowest (20) and the other two salinities. Comparing non-contaminated with contaminated mussels, significant differences were found at control salinity (30), with lower CAT activity in those contaminated (Fig. 2B).

The activity of GR showed no significant differences among salinities, regardless of La presence or absence. At each salinity, no significant differences were observed between non-contaminated and contaminated mussels (Fig. 2C).

Non-contaminated mussels showed significantly higher GSTs activity at salinity 20 compared to salinities 30 and 40, while in La exposed mussels the activity of GSTs was significantly different at all salinity levels, with higher values at the lowest salinity. Comparing non-contaminated with contaminated mussels, significant differences were found at salinity 30, with higher GSTs activity in contaminated organisms (Fig. 3A).

Using the substrate *p*NPA, the activity of CbEs showed no significant differences among salinities in non-contaminated mussels, while in La exposed mussels significantly higher CbEs activity was observed at salinity 20 compared to salinities 30 and 40. No significant differences were found between non-contaminated and contaminated mussels regardless of salinity (Fig. 3B). Using the substrate *p*NPB, the activity of CbEs was significantly higher at salinity 20 compared to salinities 30 and 40, both in the absence or presence of La. No significant differences were observed between non-contaminated and contaminated mussels at each salinity (Fig. 3C).

3.2.3. Oxidative damage

The LPO levels showed no significant differences among different salinities in non-contaminated organisms. The same was observable for those contaminated, although there was a decreasing trend with the increase in salinity. When comparing non-contaminated mussels with contaminated ones, at each salinity, contaminated organisms showed significantly higher LPO levels at salinities 20 and 30 (Fig. 4A).

The PC levels showed no significant differences among salinities for non-contaminated mussels, while in contaminated organisms PC levels were significantly higher in mussels under the lowest salinity compared with salinities of 30 and 40. Comparing non-contaminated and contaminated mussels, significant differences in terms of PC levels were revealed at salinity 20, with higher values in those La exposed (Fig. 4B).

3.2.4. Neurotoxicity

In non-contaminated mussels AChE activity did not change among salinities, while in La exposed ones this enzyme was significantly lower at the lowest salinity. No significant differences were found between non-contaminated and contaminated mussels regardless the salinity level (Fig. 5).

3.3. Multivariate Analysis

The results from PCO analysis (Fig.6) revealed that the first principal component (PCO1) explained 72.7 % of total variance among treatments, clearly separating the non-contaminated and La contaminated mussels at the lowest salinity in the positive side and the organisms under the two other salinities (30 and 40) in the negative side. PCO2 explained 13.9 % of total variation, separating the mussels exposed to La at all salinities in the positive side from the non-contaminated mussels under all salinities in the negative side. The biochemical descriptors superimposed on the PCO1 showed that ETS, SOD, CAT, GSTs and CbEs were highly correlated ($p > 0.75$) with the lowest salinity (20) since higher values of these parameters were achieved in all mussels at this salinity. On the other hand, PROT and

AChE were highly correlated with the other two salinities (30 and 40) in the negative side of the axis. LPO and GR were associated to La exposure since higher values were attained in mussels exposed at all salinities.

4. DISCUSSION

In the present study, the influence of salinity on La toxicity was evaluated using the model species *Mytilus galloprovincialis*, by assessing La bioaccumulation and its impacts on mussel's metabolic performance, energy reserves, oxidative status and neurotoxicity.

4.1. Concentration and BCF of La in mussels

Salinity showed to influence La accumulation in *M. galloprovincialis* with higher La presence and BCF in mussels exposed to the lowest salinity, while at increased salinity (40) no influence on La accumulation was revealed in comparison to salinity control (30). Studies have already shown that both marine and freshwater mussels accumulate La after 28 days of exposure (Pinto et al. 2019; Hanana et al. 2017). However, to our knowledge, there is no data on La accumulation under different salinities. In marine mussels the uptake and concentration of other trace metals has shown to increase with decreasing salinities (Ali and Taylor, 2010; Blackmore and Wang, 2003; Moreira et al., 2016; Wright, 1995). This trend has been justified by the fact that a decrease on chloride complexation increases the free metal ions concentration, which has been considered as the main forms that marine invertebrates can uptake metals (Campbell, 1995) and thus facilitating accumulation and related toxicity in bivalves. In the case of the ion La^{3+}, it was described that organisms are able to displace Ca^{2+} from the cell membrane, interfering with Ca-dependent functions (Evans, 1983) and, in mussels *Mytilus edulis* and algae *Chara corallina*, the uptake of La^{3+} into cells was also evidenced (Chassard-Bouchard and Hallegot, 1984; Li et al., 2008). Furthermore, it is known that physiological responses may also depend on the salinity, affecting in turn the metal uptake (Wright, 1995). Recent studies have already demonstrated that low salinity levels

were responsible for higher metabolism, which could have favored contaminant accumulation, such was the case of lead (Freitas et al., 2019). Accordingly, the present study demonstrated that higher metabolism occurred at salinity 20, which is most probably associated to higher filtration rate and, therefore, enhanced La accumulation. Thus, the present findings indicate that higher La accumulation at the lowest salinity may result from changes on the medium properties but also from mussels increased metabolism. Higher accumulation observed at salinity 20 was close related with bivalves' biochemical performance, as we will describe below.

4.2. Metabolic capacity and energy reserves

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

compared to non-contaminated ones. Such results may indicate a physiological trait to prevent La accumulation by reducing their metabolism. It has been repeatedly demonstrated that when under stressful conditions, bivalves may close their valves and reduce their metabolism to avoid the negative consequences of an unfavorable environment (Anestia et al. 2007; Gosling, 2003; Ortmann, 2003). In particular, former studies in bivalves already revealed that in the presence rare earth elements (Gadolinium (Gd) and La) as well as elements such as Hg and As, the strategy to decrease their ETS activity and/or associated filtration capacity, was adopted in an attempt to prevent contaminant accumulation (Henriques et al. 2019; Pinto et al. 2019; Coppola et al., 2017, 2018).

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

4.3. Antioxidant and biotransformation enzymes

Organisms under stressful conditions activate antioxidant defenses in order to

eliminate the excess of ROS produced and prevent cellular damage (Regoli and Giuliani, 2014). Higher antioxidant enzymes activity (SOD and CAT) was equally observed at lower salinity in non-contaminated and contaminated organisms. Similarly, Freitas et al. (2017) observed higher CAT and SOD activities in mussels at low salinity, which was also related with higher metabolic capacity and lower energy reserves. In particular, higher antioxidant defenses at lower salinities has been observed in the clams *R. philippinarum* and in the oysters *C. angulata* (Velez et al. 2016b, Moreira et al., 2016), thus further supporting that hypotonic stress is associated with the activation of antioxidant defenses probable related to increased ROS production and to prevent cellular damage. The particular effect of La at studied salinities took place differently: at salinity 40 for SOD and at salinity 30 for CAT, in both cases being the antioxidant capacity lowered in La exposed mussels. Similarly, CAT inhibition was observed in eels *Anguilla Anguilla* (Figueiredo et al., 2018) and in goldfish *Carassius auratus* (Chen et al., 2000). Overall, as demonstrated by metabolism-related biomarkers, it seems that salinity plays a major role on mussels' antioxidant defenses than La acting alone (i.e., under salinity 30).

In addition to antioxidant enzymes, organisms possess biotransformation enzymes such as GSTs and CbEs which are known to catalyze the conversion of chemicals to facilitate their excretion (Regoli and Giuliani, 2014; Yan, 2014). In the present study, both biotransformation enzymes displayed higher activities at the lowest salinity for non-contaminated as well as La exposed organisms. Former studies have already demonstrated that shifts in abiotic factors, such as temperature, pH and salinity, often induce multifactorial effects on GSTs activity (Park et al. 2020). As an example, Rivera-Ingraham et al. (2016) observed an upregulation of glutathione S-transferase pi 1 (GSTP1) in marine intertidal flatworms at low salinity, justifying that ROS formation under hypoosmotic shock could have triggered GSTs upregulation. Furthermore, these authors also hypothesized this response as a

"preparation for oxidative stress", as a mechanism to overcome upcoming challenges when returning to seawater (i.e., submerged), representing a shift to hypersalinity. The results observed in the present study for GSTs could be related to ROS formation under hypotonic stress and an upregulation of these enzymes to counterattack the effects of salinities shifts. The presence of La had only an increase in GSTs at salinity 30, supporting former evidences by other metabolic enzymes in which salinity has a stronger impact on mussels' biotransformation capacity than the presence of La, probably due to low concentration tested and, on the other hand, suggesting that when salinity is not acting as a confounding factor the presence of La is responsible for the activation of GSTs activity. This last role was recently pointed out by Pinto et al. (2019) assessing the effects on this enzyme in mussels at wider range of La (0.1-10 mg/L). Mussels increased GSTs activity up to 1.0 mg/L of La exposure with similar values to control organisms under the highest concentration tested (10 mg/L), being suggested a possible inhibition of this enzyme under higher concentration. Similarly, Hanana et al. (2007) show a tendency of GSTs to increase in the presence of the lowest concentration of La (10 µg/L) while being inhibited at the highest tested concentration (1250 µg/L).

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

4.3. Oxidative damage

It is known that if the antioxidant defenses are not efficient in eliminating ROS excess production, they may lead to the oxidation of lipids' membranes and proteins in the processes

known as lipid peroxidation (Catalá, 2009; Regoli and Giuliani, 2014) and protein carbonylation (Cattaruzza and Hecker, 2008; Suzuki et al., 2010), thus causing cellular damages. In the present study no cellular damage was observed due to salinity, most probably due to the activation of antioxidant defense mechanisms. However, organisms exposed to La displayed at the lowest salinity increased (but not significant) LPO levels and significantly enhanced PC levels with significantly higher values in contaminated mussels, which may be due to the fact that antioxidant enzymes were not significantly activated in La exposed mussels in comparison to non-contaminated ones. These results further indicate that non-contaminated mussels would be able to better cope with the hypotonic stress than the La exposed ones as two stressing factors may overwhelm their defense mechanisms. Nevertheless, in the freshwater mussel *Dreissena polymorpha*, Hanana et al. (2017) demonstrated a non-significant increase of lipid peroxidation in mussels exposed to 10 µg/L of La after 28 days. The acute exposure study by Moreira et al. (2020), revealed an EC50 of 6.7 (in 24 h) and 36.1 µg/L (in 48 h) of La exposure in *C. gigas* embryos, a more sensitive stage, reveling that low concentrations of La may be enough to cause effects in bivalves.

4.4. Neurotoxicity

In terms of neurotoxicity, AChE has been regarded as a sensitive biomarker easily inhibited by organophosphates or other pesticides and nerve agents (English and Webster, 2012). However, in marine invertebrates, its inhibition has been reported by other compounds as metals and nanoparticles (Brown et al., 2004; De Marchi et al. 2018; Perić et al. 2017), including La in the range of 0.1 to 10 mg/L (Pinto et al., 2019). Regarding the present findings, AChE was not inhibited in non-contaminated organisms under stressful salinities. However, organisms exposed to La at the lowest salinity showed a significant inhibition of AChE compared to the ones under the other two salinities (30 and 40), thus indicating that a concentration of 10 µg/L under low salinity may not just cause cellular damage but

neurotoxicity as well. These results are concomitant to higher concentration of La found in mussels at this condition.

4.5. Integrative PCO analysis

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

5. CONCLUSIONS

Biomarkers revealed that the decreasing of salinity caused an increase in organism responses related to hypotonic stress. They were seen as oxidative stress regardless of La presence or absence, however only those exposed to La suffered an increase of cellular damage and neurotoxicity. Furthermore, organisms exposed to La seemed to have their antioxidant defenses compromised in comparison with non-contaminated ones, regardless of salinity. The present study highlights the possible risks due to the presence of the REE La in coastal systems, wish may be intensified at lower salinities. It is thus stressed the need to assess the consequences of this and possibly other REEs exposure at relevant concentrations and considering additional future climate change related stressors. Their combined action may not just compromise organisms' biochemical processes but, at a higher biological level related to the data obtained in this study, it possibly affects their growth and reproduction

after prolonged exposure with economic consequences.

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Table 1. Lanthanum (La) concentration (µg/g) in mussels' soft tissues and BCF (L/Kg) after 28 days of exposure to seawater spiked with 10 µg/L of La. Different letters represent significant differences among tested concentrations. Results are mean values with standard deviation (±STDEV).

Condition	Mussels tissues (ug/g)	BCF (L/Kg)
La20	1.6 ± 0.4^{a}	155 ± 35^{A}
La30	0.75 ± 0.2^{b}	75 ± 15^{B}
La40	0.47 ± 0.2^{b}	46 ± 16^{B}

Figure 1. A: Electron transport system (ETS); B: Glycogen (GLY) content; C: Protein (PROT) content in *Mytilus galloprovincialis* exposed to different salinity levels (20, 30 and 40) in the absence and presence of lanthanum (La) for 28 days. Results are mean values with standard deviation. Significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). For each salinity level, differences between contaminated and non-contaminated mussels are identified by an asterisk.

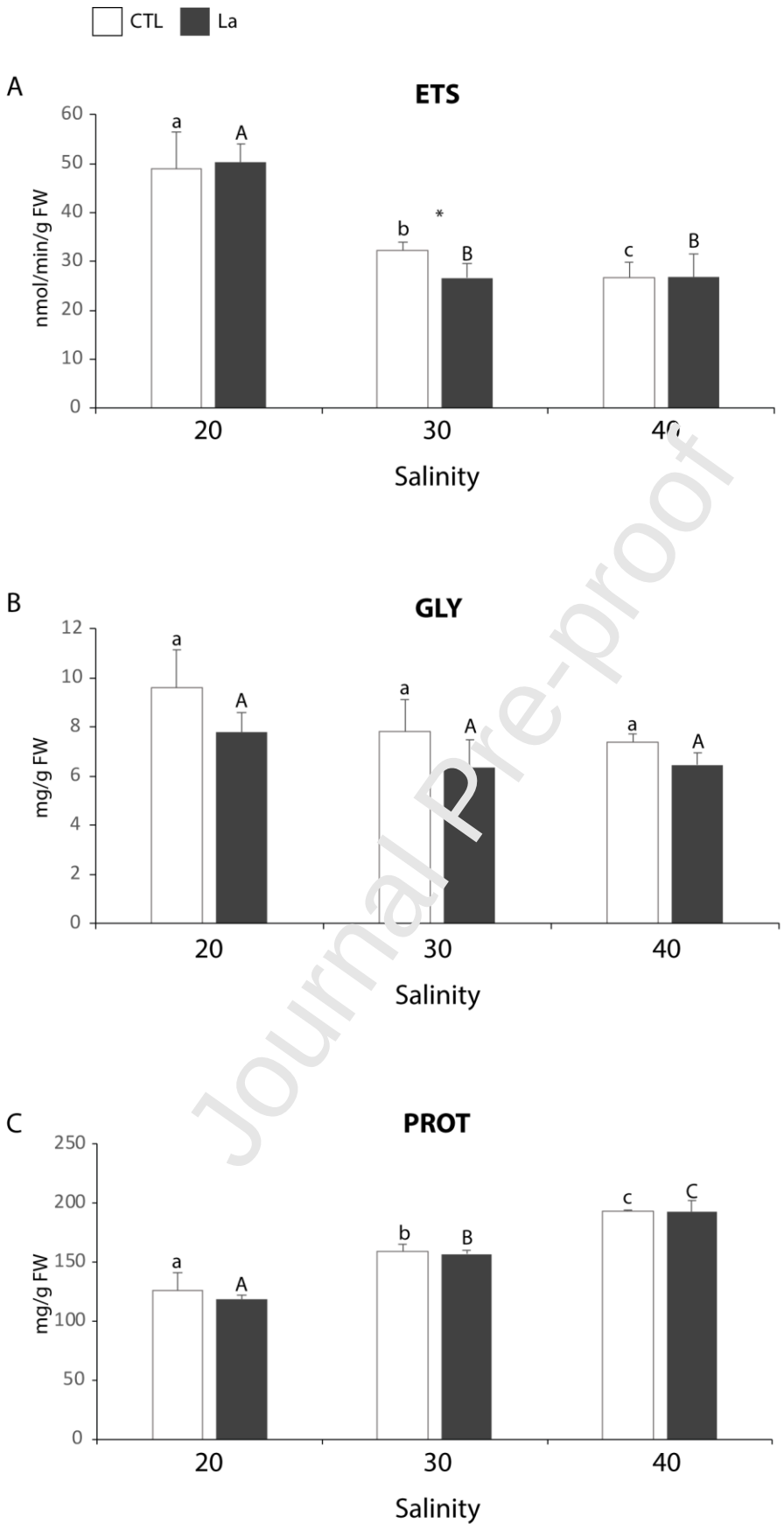


Figure 2. A: Superoxide dismutase (SOD) activity; B: Catalase (CAT) activity; C:

Glutathione reductase (GR) activity in *Mytilus galloprovincialis* exposed to different salinity levels (20, 30 and 40) in the absence and presence of lanthanum (La) for 28 days. Results are mean values with standard deviation. Significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). For each salinity level, differences between contaminated and non-contaminated mussels are identified by an asterisk.

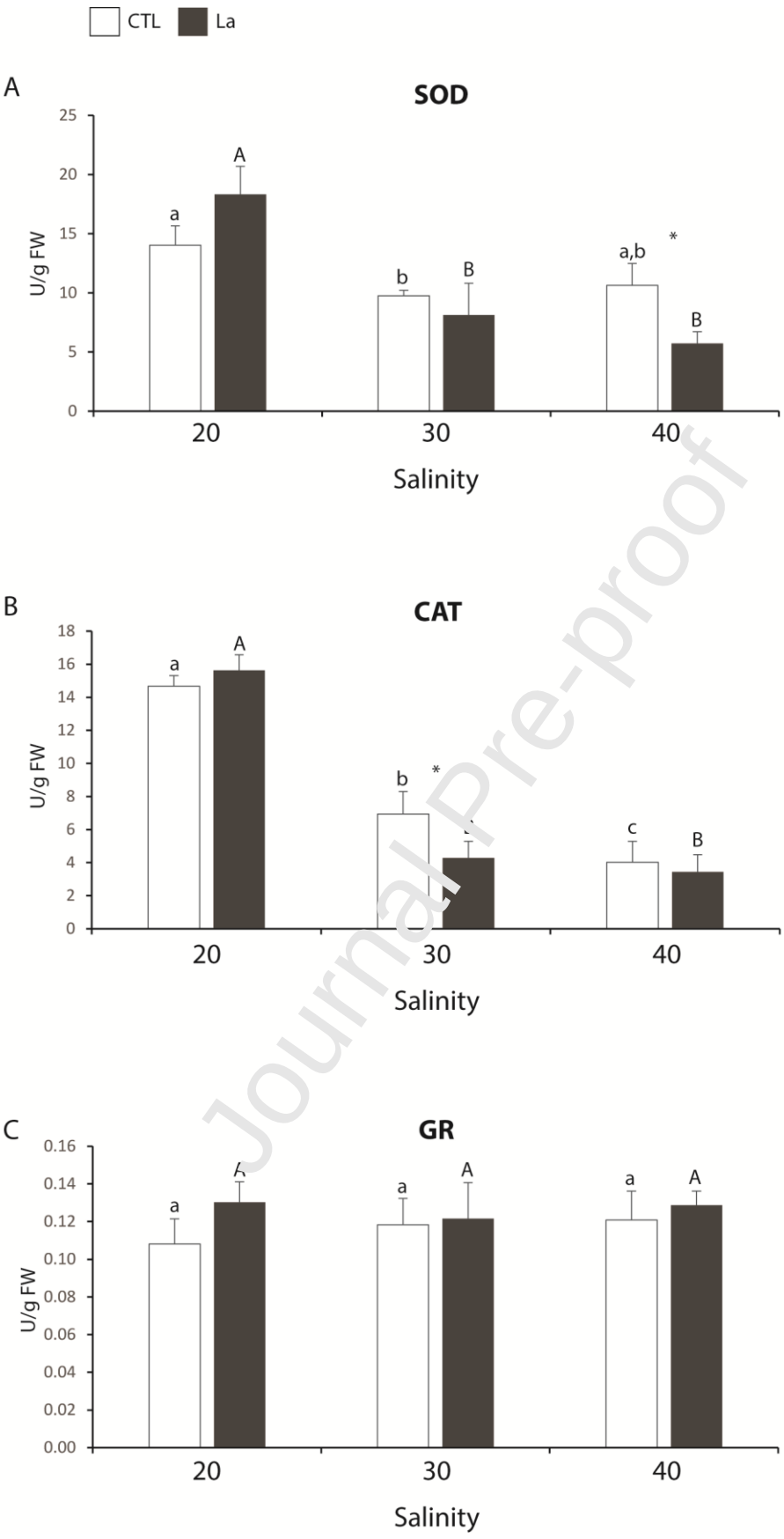


Figure 3. A: Glutathione S-transferase (GSTs) activity; B: Carboxylesterases (CbEs) activity

with *p*NPA substrate; C: Carboxylesterases (CbEs) activity with *p*NPB substrate in *Mytilus galloprovincialis* exposed to different salinity levels (20, 30 and 40) in the absence and presence of lanthanum (La) for 28 days. Results are mean values with standard deviation. Significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). For each salinity level, differences between contaminated and non-contaminated mussels are identified by an asterisk.

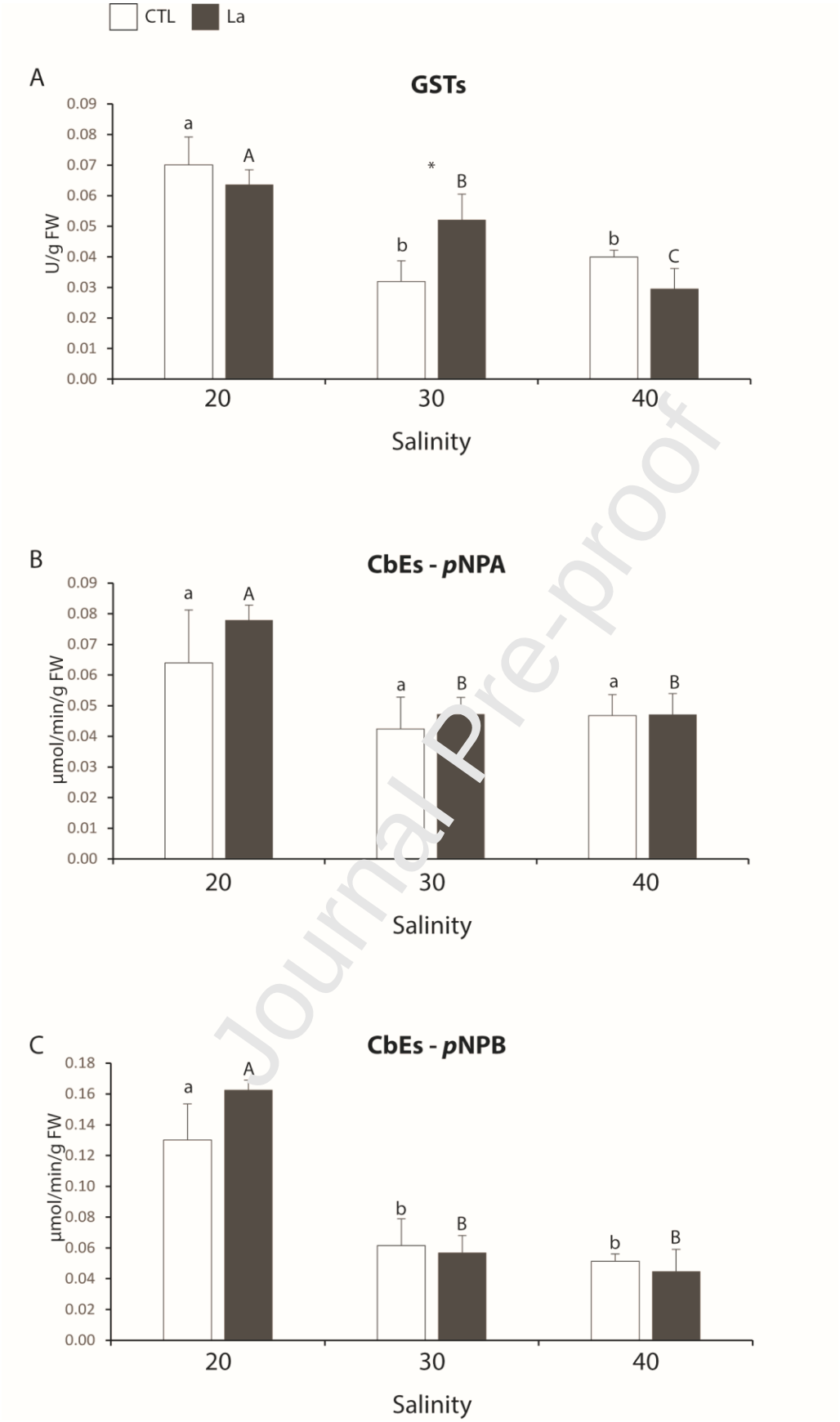


Figure 4. A: Lipid peroxidation (LPO) levels; B: Protein carbonylation (PC) levels in *Mytilus*

galloprovincialis exposed to different salinity levels (20, 30 and 40) in the absence and presence of lanthanum (La) for 28 days. Results are mean values with standard deviation. Significant differences ($p < 0.05$) among concentrations are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). For each salinity level, differences between contaminated and non-contaminated mussels are identified by an asterisk.

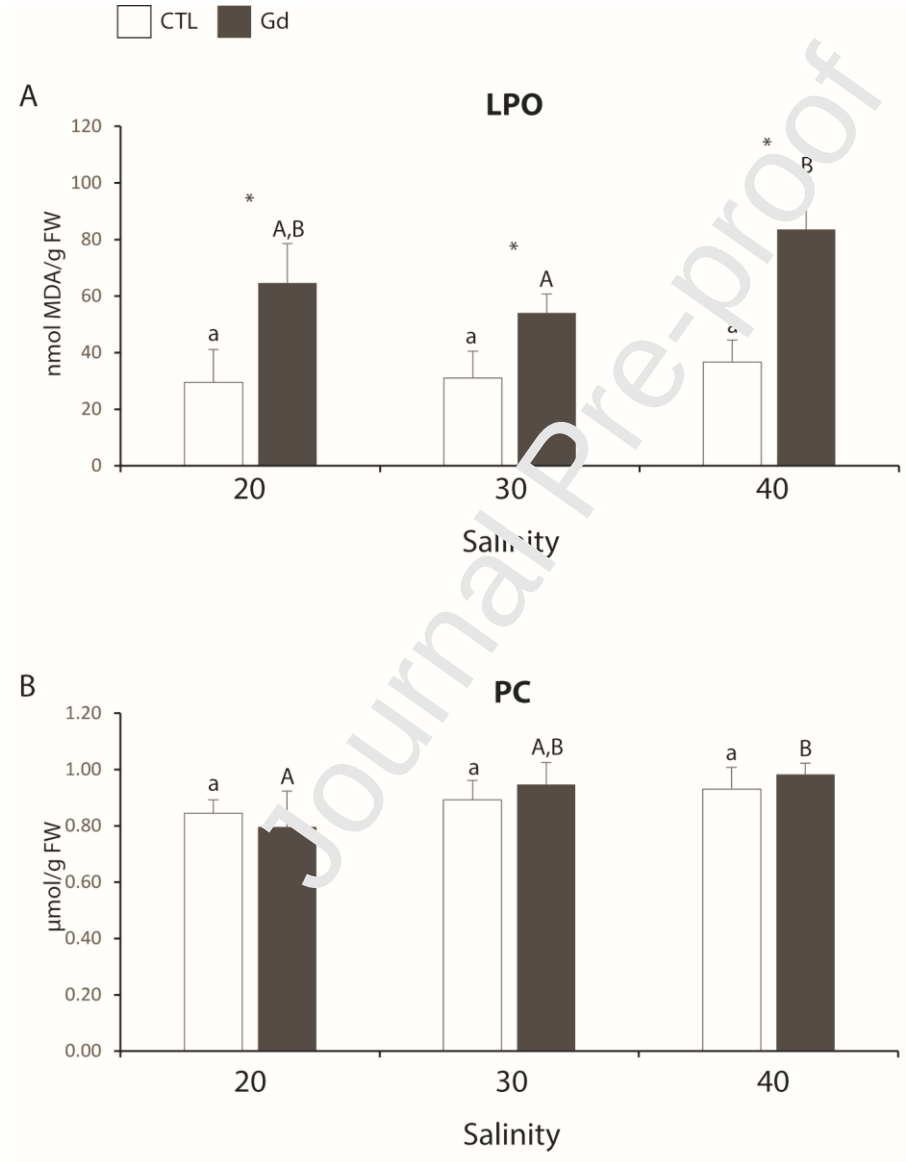


Figure 5. Acetylcholinesterase (AChE) activity in *Mytilus galloprovincialis* exposed to different salinity levels (20, 30 and 40) in the absence and presence of lanthanum (La) for 28 days. Results are mean values with standard deviation. Significant differences ($p < 0.05$)

among concentrations are identified with different lowercase letters (non-contaminated mussels) or uppercase letters (contaminated mussels). For each salinity level, differences between contaminated and non-contaminated mussels are identified by an asterisk.

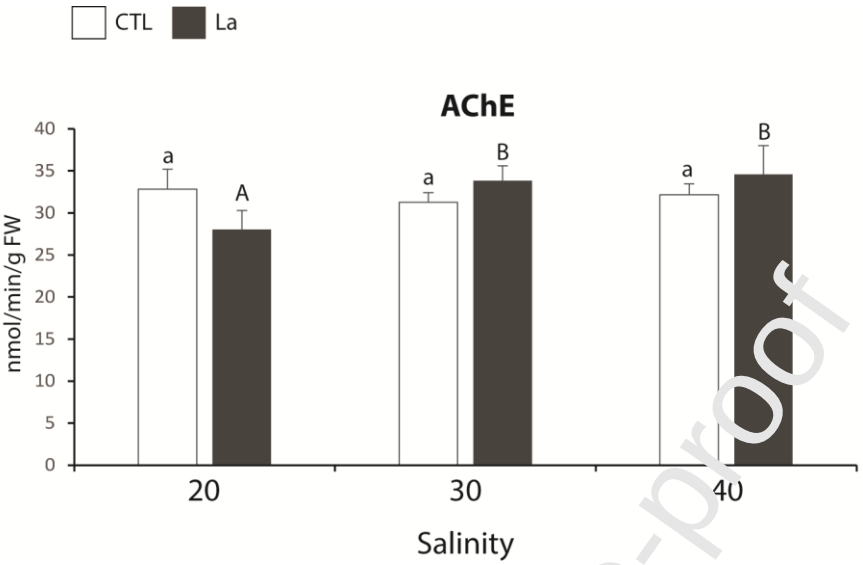
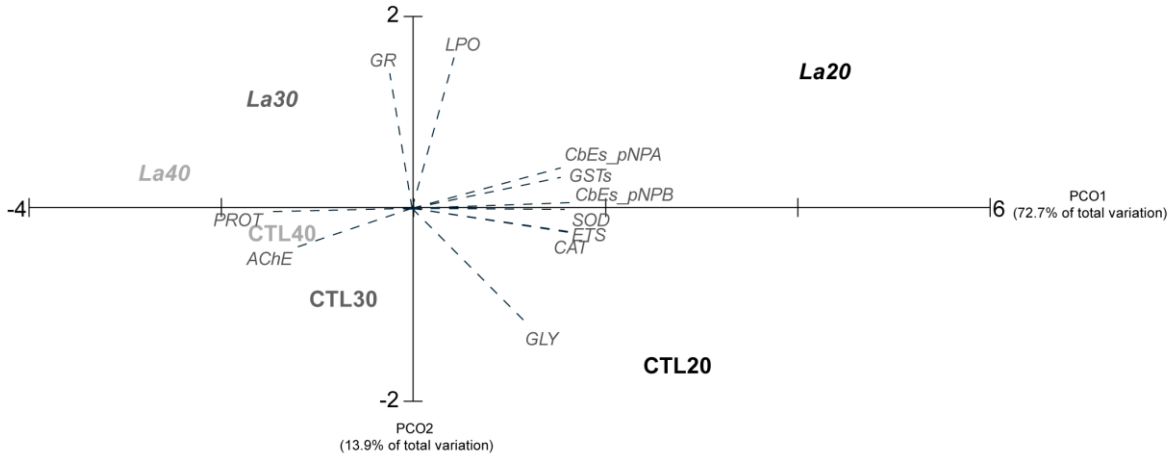


Figure 6. Centroids ordination diagram (PCO) based on the tested conditions and biochemical markers measured in Mytilus galloprovincialis. The following conditions are presented: CTL20, CTL30, CTL 40 (non-contaminated organisms under different salinities of 20, 30 and 40) and La20, La30, La40 (organisms exposed to La under different salinities of 20, 30 and 40). Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data ($r > 0.75$): ETS, PROT, GLY, LPO, AChE, GSTs, CbEs_*p*NPA, CbEs_*p*NPB.



Conflict of interests

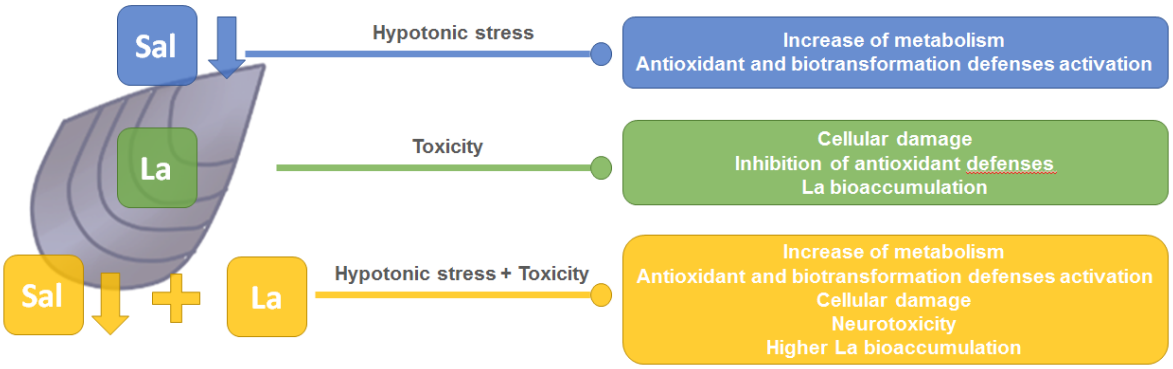
The Authors whose names are listed immediately below certify that they have NO affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or nonfinancial /interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Credit Author Statement

Madalena Andrade: Bibliographic search, data analyses, ms writting and editing

Rosa Freitas, Amadeu M.V.M. Soares, Eduarda Pereira, Montserrat Solé: supervision of data analyses (La quantifications and biochemical data), paper editing and revision

Graphical abstract



Highlights

- Lower salinity caused greater biochemical alterations related to hypotonic stress.
- Lower salinity caused oxidative stress regardless of La presence or absence.
- Lower salinity and La in combination caused cellular damage and neurotoxicity.
- La compromised antioxidant and biotransformation enzymes in mussels.
- Salinity was the most relevant factor in the modulation of biomarker's response.