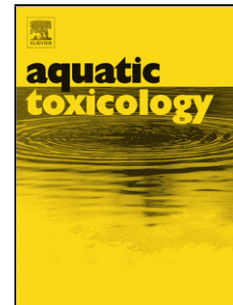


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Ecotoxicological effects of lanthanum in *Mytilus galloprovincialis*: Biochemical
and histopathological impacts

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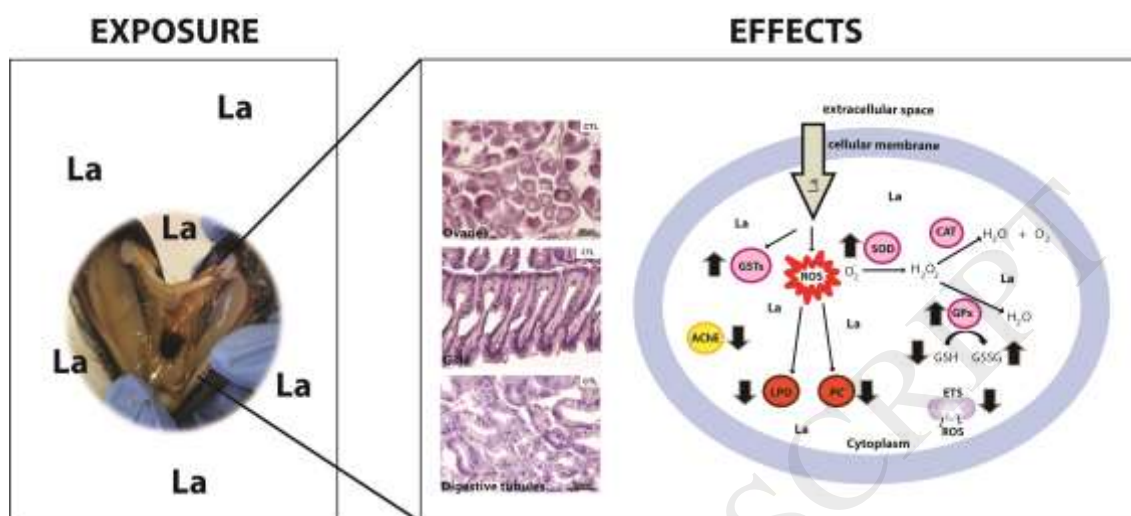
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GRAPHICAL ABSTRACT



Highlights

- *Mytilus galloprovincialis* bioaccumulated lanthanum
- Mussels exposed to Lanthanum decreased their metabolic capacity
- Contaminated mussels activated their antioxidant and biotransformation defences
- Contaminated mussels showed increased lipid peroxidation and lower GSH/GSSG ratio
- Neurotoxicity was induced in contaminated mussels
- Histopathological alterations were induced by lanthanum

Summary

Inappropriate processing and disposal of electronic waste contributes to the contamination of aquatic systems by various types of pollutants such as the rare-earth elements (REE) in which lanthanum (La) is included. Knowledge on the toxicity of these elements in marine organisms is still scarce when compared to other metals such as mercury (Hg) and arsenic (As).

Therefore, this study aims to assess the toxicity of La on the mussel *Mytilus galloprovincialis*, considered a good bioindicator of aquatic pollution, through the analysis of metabolic, oxidative stress, neurotoxicity and histopathological markers. Organisms were exposed to different concentrations of La for a period of 28 days (0, 0.1, 1, 10 mg/L) under controlled temperature ($18^{\circ}\text{C} \pm 1.0$) and salinity (30 ± 1) conditions. La concentrations in mussels increased in higher exposure concentrations. La exposure demonstrated a biochemical response in mussels, evidenced by lowered metabolism and accumulation of energy reserves, activation of the antioxidant defences SOD and GPx as well as the biotransformation enzymes GSTs, especially at intermediate concentrations. Despite oxidative stress being shown by a decrease in GSH/GSSG, oxidative damage was avoided as evidenced by lower LPO and PC levels. Inhibition of the enzyme AChE demonstrated the neurotoxicity of La in this species. Histopathological indices were significantly different from the control group, indicating impacts gonads, in gills and digestive glands of mussels due to La. These results show that La can be considered a risk for marine organisms and as such its discharge in the environment should be supervised.

Keywords: Rare-earth elements; mussels; oxidative stress; metabolism; neurotoxicity; histological alterations.

1. INTRODUCTION

Nowadays, an emerging concern associated with anthropogenic activities is the disposal of electronic waste (e-waste), a consequence of the increasingly rapid technological evolution (Otsuka et al., 2012). Higher demand along with shorter lifespans and early obsolescence for common electronic devices such as mobile phones has led to alarming production of e-waste. To ensure a profitable resource management, e-waste is often collected in order to recover valuable metal resources. However, inappropriate processing and recycling such as dismantling, open storage or open burning can release hazardous substances to the environment (Uchida et al., 2018). The processing of e-waste can therefore represent a source of contamination for aquatic systems mainly through uncontrolled dumping of waste water from treatment facilities as well as leaching from exposed dumping sites (Sepúlveda et al., 2010). E-waste contributes to the environmental contamination not only by classical pollutants as metals or organic compounds (Huang et al., 2014) but also by less known elements such as the rare-earth elements (REEs) (McLellan et al., 2014; Klaver et al., 2014). The groups of REEs encompasses the lanthanides as well as Scandium (Sc) and Yttrium (Y) (IUPAC, 2005), and despite their rare-earth nomination, they are rather abundant in the Earth's crust, although often less concentrated than other elements (USEPA, 2012). Because of their unique physicochemical properties (e.g., naturally coherent behaviour, high lustre and electrical conductivity; Topp, 1965), REEs are widely and intensively applied in numerous high-tech, medical and agricultural products and processes (US EPA, 2012), being potentially released into the aquatic environments worldwide (Khan et al., 2017). Bau and Dulski (1996) studies were among the first ones that related rising levels of REEs in aquatic environments with anthropogenic activities, revealing a positive correlation between REE Gadolinium (Gd) concentration and riverine inputs from densely industrialized areas.

Increased anthropogenic enrichments of other REEs such as lanthanum (La) were reported by Kulaksiz & Bau (2011) and Klaver et al. (2014). Lanthanum is part of the lanthanides group, being characterized by low solubility and a tendency to precipitate or bind to complexing ions (Sneller et al., 2000), with the solubility of these complexes being very low (Herrmann et al., 2016). This element is commonly used in many industrial applications, such as the glass production, where it is used as an additive to alter properties like the glass's colour, refractive index and ability to absorb ultraviolet light. In high-technology industry, it is used in screens colour

control of electronic applications as well as in rechargeable batteries. Lanthanum compounds are also used in fluorescent lights and other uses that require coloured light (Weeden et al., 2015). Natural La concentrations in aquatic systems are highly variable with studies conducted by Elderfield et al. (1990) showing that this element occurs in ranges of 20-600, 5-200 and 5-40 ng/L in rivers, estuaries and coastal areas, respectively. However, anthropogenic activities such as industrial wastewaters from the production of fluid catalytic cracking catalysts (Merschel and Bau, 2015) or acid mine drainage (Olías et al., 2005) can lead to the enrichment of this element in aquatic systems. In fact, contamination of aquatic systems by La is often associated with runoff or discharge of direct effluents. Cases of such contamination have already been studied by different authors, such as Olías et al. (2005), who reported La concentrations up to 0.04 mg/L in a Spanish aquifer contaminated by acid mine drainage. Higher La concentrations of about 0.9 mg/L have also been reported by Åström (2001) in streams affected by acid sulphate soils. Furthermore, different authors also revealed the presence of La in aquatic biota, including the scallop *Chlamys varia* (Bustamante & Miramand.,2005), the amphipod *Paramoera walkeri* (Palmer et al., 2006), blue mussels *Mytilus edulis* (Riget et al., 1996), different snails (*Potamopyrgus antipodarum*; *Lymnaea stagnalis*; *Radix ovata*; *Physella acuta*; *Planorbarius corneus*; *Planorbis planorbis*) and bivalves (*Dreissena polymorpha*; *Corbicula fluminae*) (Weltje et al., 2002).

As a consequence of the presence of La in aquatic systems, the toxicological effects of La in aquatic organisms have been identified, but almost all the studies focus on freshwater species. Xu et al. (2012), demonstrated that antioxidant defences increased in the freshwater plant *Hydrocharis dubia* exposed to 5.56 mg/L of La. Also, damages in cellular structures like mitochondria and nucleus were observed at 11.1 mg/L of La. The freshwater invertebrate *Daphnia magna* showed 50% of mortality when exposed to 0.552 mg/L of La for 14 days (Bogers, 1995). Other studies further demonstrated that La exposure affects the neurotransmitter acetylcholine through the inhibition or activation of the enzyme acetylcholinesterase (Tomlinson et al., 1982; Figueiredo et al., 2018). However, studies assessing the effects induced by La in marine invertebrates, including bivalves, is almost unknown.

Therefore, the present study aimed to assess the impacts of different La concentrations in the mussel *Mytilus galloprovincialis* after 28 days of exposure to this element, evaluating the

biochemical effects in terms of metabolic, oxidative and neurotoxic status but also assessing mussels histopathology in gills, gonads and digestive glands. *M. galloprovincialis* is a bivalve widely recognized as a good bioindicator in monitoring and ecotoxicological studies (Rouane-Hacene et al., 2015; Belivermiş et al., 2016; Azizi et al., 2017), due to its extensive geographical distribution, sedentary nature and capacity to tolerate various environmental and chemical conditions (Wang et al., 1996; Banni et al., 2014a, 2014b; Viarengo et al., 2007). Several studies with *M. galloprovincialis* already demonstrated the sensitivity of this species towards metals (e.g. Coppola et al., 2018; Freitas et al., 2017), pharmaceuticals (e.g. Trombini et al., 2016; Teixeira et al., 2017) and nanoparticles (e.g. Andrade et al., 2018; Taze et al., 2016).

2. METHODOLOGY

2.1. Sampling and experimental conditions

Mytilus galloprovincialis specimens were collected in June 2018 during the low tide in a subtidal area at the Ria de Aveiro (a coastal lagoon, northwest of Portugal). After sampling, during 15 days, mussels were depurated and acclimated to laboratory conditions. During this period, organisms were maintained at 18.0 ± 1.0 °C; pH 7.80 ± 0.10 , 12h light: 12h dark photoperiod and continuous aeration in synthetic saltwater (salinity 30 ± 1), prepared by using a commercial salt (Tropic Marin® SEA SALT from Tropic Marine Center) and freshwater purified by reverse osmosis. Except for the first 3 days, during this period organisms were fed with Algamac protein plus (150,000 cells/animal) every three days. Seawater was renewed every 2 days during the first week and once during the second week.

For the experimental exposure, mussels were distributed into different glass containers (6 L aquaria filled with synthetic saltwater), with 6 individuals per aquarium and 3 aquaria per condition. Salinity, temperature and pH conditions were the same as those used during acclimation. Exposure lasted for 28 days and the conditions tested were: CTL (La 0 mg); La 0.1 mg/L; La 1 mg/L; La 10 mg/L, using a commercial La salt (Lanthanum(III) chloride heptahydrate, 99% (*Alfa Aesar*)). The selection of the tested concentrations was based on: i) La concentrations present in polluted aquatic environments (Åström, 2001; Olías et al., 2005); ii) and La concentrations that may cause biological effects, considering previously published studies that tested similar concentrations range in invertebrate (Barry and Meehan, 2000; Lürling and Toman, 2010; Hanana et al., 2017) and fish species (Hua et al., 2017).

During the exposure period, organisms were fed with Algamac protein plus (150,000 cells/animal) three times a week. Seawater in each aquarium was continuously aerated and renewed once a week and La concentrations re-established. Lanthanum was quantified in water collected immediately after spiking each aquarium to obtain real exposure concentrations.

After the 28 days of exposure, with the exception of two mussels per aquarium used for histopathological analyses, the remaining organisms were frozen with liquid nitrogen and stored at -80 °C. For biochemical and chemical analyses three frozen mussels per aquarium (nine per condition), which were manually homogenized with a mortar and a pestle under liquid nitrogen and each homogenized individual was divided in 0.5 g aliquots.

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

The quantification of La in seawater samples was achieved through direct analysis of samples by Inductive Coupled Plasma Optical Emission Spectroscopy (ICP-OES) on a Jobin Yvon Activa M spectrometer. The limits of detection and quantification of the method were 1.4 µg/L and 4.0 µg/L, respectively, with an acceptable coefficient of variation among replicates ($n \geq 2$) of 10%. Calibration curve was made with La standards in the range of 4 to 100 µg/L.

For La quantification in mussel's tissue samples, 0.2 g of sample was weighted into a Teflon vessel to which 1 mL of HNO₃ 65% (v/v), 2 mL of H₂O₂ and 1 mL of H₂O were added. Samples were left 15 min in a microwave with increasing temperature up to 190 °C, which was then maintained for 3 min. After cooling, samples were collected to polyethylene flasks, made up to a final volume of 25 mL with ultrapure water and stored at room temperature until quantification. Blanks, fortified samples (samples added with standard solutions in order to evaluate element recovery) and duplicates were analyzed to guarantee a good quality of the results obtained. Blanks were always below the quantification limits of the ICP-OES for La (4 µg/L), recovery tests gave results between 88 and 109 % and the coefficient of variation of samples duplicates varied from 12% to 23%.

2.3. Biological responses: biochemical parameters

In order to evaluate the biochemical alterations the biomarkers related to metabolic changes (electron transport system (ETS) activity), energy reserves (glycogen (GLY) content, total protein (PROT) content), oxidative stress (superoxide dismutase (SOD) activity; catalase (CAT) activity; glutathione peroxidase (GPx) activity; glutathione S-transferases (GSTs) activity; lipid peroxidation (LPO) levels; protein carbonylation (PC) levels; ratio between reduced (GSH) and oxidized (GSSG) glutathione content) and neurotoxicity (Acetylcholinesterase (AChE) activity) were analyzed. For the determination of the biochemical parameters duplicates of each sample were used.

For each biomarker, the extraction was performed with specific buffers using a proportion of 1:2 (w/v) with the homogenized tissue. Initially, tissue samples were homogenized using a TissueLyser II (Qiagen) for 90 s, after which they were centrifuged for 20 min at 10,000 g (3,000

g for ETS) and 4 °C. Supernatants were immediately used or stored at -20 °C. For each biomarker specific buffers were used: potassium phosphate for PROT, GLY, SOD, CAT, GPx, GSTs, PC and AChE; 0.1 mol/L Tris- HCl for ETS; 20% (w/v) trichloroacetic acid (TCA) for LPO; 0.6% (w/v) sulfosalicylic acid in potassium phosphate for GSH and GSSG.

2.4.1. Metabolic capacity and energy reserves

The activity of ETS was measured based on the method of King and Packard (1975) with modifications performed by Coen and Janssen (1997). The absorbance was measured at 490 nm during 10 min with intervals of 25 s. The amount of formazan formed was calculated using the extinction coefficient (ϵ) 15,900 M⁻¹ cm⁻¹. The results were expressed in nmol/min per g fresh weight (FW).

The GLY content was quantified following the sulfuric acid method (Dubois et al. 1956), using 8 glucose standards in the concentration range of 0 to 10 mg/mL in order to obtain a calibration curve. Absorbance was measured at 492 nm after being incubated for 30 min at room temperature. The results were expressed in mg per g FW.

The PROT content was determined according to the Biuret method described by Robinson and Hogden (1940). A stock solution of bovine serum albumin (BSA) was used to prepare 5 standards (0–40 mg/mL) to obtain a calibration curve. After 10 minutes of incubation at 30°C, absorbance was measured at 540 nm. The results were expressed in mg per g FW.

2.4.2. Antioxidant and biotransformation defenses

The activity of SOD was determined according to the method of Beauchamp and Fridovich (1971). For the calibration curve 7 SOD standards (0.25 - 60 U/mL) were used. After 20 min of incubation at room temperature, the absorbance was measured at 560 nm. The activity was expressed in U per g FW, where U corresponds to a reduction of 50% of nitroblue tetrazolium (NBT).

The activity of CAT was quantified according to Johansson and Borg (1988). For the calibration curve 9 formaldehyde standards (0 - 150 μ mol/L) were used. The absorbance was measured at 540 nm and activity expressed in U per g FW, where U represents the amount of enzyme that caused the formation of 1.0 nmol formaldehyde per min.

The activity of GPx was determined following the method of Paglia and Valentine (1967). Absorbance measurements were performed at 340 nm during 5 min in 10 s intervals and the activity was determined using the extinction coefficient (ϵ) $6.22 \text{ mM}^{-1}\text{cm}^{-1}$. Results were expressed in U per g FW, where U corresponds to the quantity of enzyme which catalyzes the conversion of 1 μmol nicotinamide adenine dinucleotide phosphate (NADPH) per min.

The activity of GSTs was quantified based on the method of Habig et al. (1974) with modifications performed by Carregosa et al. (2014). Absorbance was read at 340 nm during 5 min in 10 s intervals and the amount of thioether formed was calculated using the extinction coefficient (ϵ) $9.6 \text{ mM}^{-1} \text{ cm}^{-1}$. The results were expressed in U per g FW, where U corresponds to the quantity of enzyme that causes the formation of 1 μmol of dinitrophenyl thioether per min.

2.4.3. Oxidative damage

LPO levels were determined by the quantification of malondialdehyde (MDA), a by-product of lipid peroxidation according to the method described in Ohkawa et al. (1979). Absorbance was measured at 535 nm and the amount of MDA formed was calculated using the extinction coefficient (ϵ) $156 \text{ mM}^{-1} \text{ cm}^{-1}$. The results were expressed in nmol per g FW.

PC levels were determined according to the DNPH alkaline method described by Mesquita et al. (2014). Absorbance was read at 450 nm and PC levels were determined using the extinction coefficient (ϵ) $0.022 \text{ mM}^{-1} \text{ cm}^{-1}$. The results were expressed in nmol of protein carbonyls groups formed per g FW.

The quantification of GSH and GSSG was performed following the method described in Rahman et al. (2007), using GSH and GSSG as standards (0–90 $\mu\text{mol/L}$). Absorbance was read at 412 nm during 2 min in 30 s intervals and the results expressed in μmol per g FW. The GSH/GSSG ratio was determined [$\text{GSH} / \text{GSSG} = \text{GSH} / (2 * \text{GSSG})$].

2.4.4. Neurotoxicity

The activity of AChE was determined using Acetylthiocholine iodide (ATChI, 470 $\mu\text{mol/L}$) substrates, according to the methods of Ellman et al. (1961) with modification performed by Mennillo et al. (2017). The activity was measured at 412 nm during 5 min and expressed in nmol/min per g FW using the extinction coefficient (ϵ) $13.6 \times 10^3 \text{ M}^{-1}\text{cm}^{-1}$.

2.4. Biological responses: histopathological measurements

After 28 days of exposure two mussels per aquarium, six per condition (CTL, 0.1, 1.0 and 10.0 mg/L) were fixed in Bouin's solution (composition: 5% of acetic acid, 9% of formaldehyde, 0.9% of picric acid) for 24 h at room temperature. Subsequently, samples were placed in 70-75% ethanol for one month, replacing the ethanol daily until the total removal of the fixative. After this, tissues were dehydrated, placed in xylene and then immersed in paraffin (58°C) in a vacuum stove for 1 h. After this step paraffin was removed and samples were once again embedded in new paraffin. Sections of 7 µm were then placed on slides covered with glycerin/albumin.

The sections were then rehydrated, and half of them were stained with hematoxylin, to assess tissue health. For the remaining sections Toluidine blue 0.2% in Walpole buffer at pH 4.2 was used to identify the relative abundance of hemocytes in each tissue, as described by Gabe (1968).

Histopathological changes were identified in gonads (females and males), gills and digestive glands of mussels, in each condition. For each individual and condition, the individual histopathological condition index (I_h) was estimated per organ (gills and digestive glands), based on Bernet et al. (1999) and modifications performed by Costa et al. (2013). The I_h was estimated according to the concepts of the differential biological significance of each surveyed alteration (weight) and its degree of dissemination (score). The weight ranges between 1 (minimum severity) and 3 (maximum severity) while the score ranges between 0 (none) and 6 (diffuse). The I_h was calculated following the formula:

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

where I_h is the histopathological index for the individual h ; w_j the weight of the j th histopathological alteration; a_{jh} the score attributed to the h th individual for the j th alteration and M_j is the maximum attributable value for the j th alteration. The index was estimated for digestive tubules and gills. The condition weights proposed were based on Costa et al. (2013). Weight for the histopathological alteration considered in this study are reported in Table 1.

2.5. Data analysis

2.5.1 Bioconcentration factor

To evaluate the bioaccumulation of La in mussels' tissues, the bioconcentration factor (BCF) was calculated for each exposure condition. BCF is defined as the ratio of the concentration in the organism and the concentration in the water. The calculation is based on the equation from Arnot and Gobas (2006):

$$BCF = \frac{\text{concentration in the organism}}{\text{concentration in the water}}$$

2.5.2. Statistical and multivariate analyses

Histopathological data, namely values of I_h for gills and digestive tubules obtained for each La concentration (CTL- La 0 mg/L; La 0.1 mg/L; La 1 mg/L; La 10 mg/L) were submitted to a t-test, testing the null hypothesis: for gills and digestive tubules, no significant differences were observed in terms of I_h values between contaminated (La 0.1 mg/L; La 1 mg/L; La 10 mg/L) and control mussels (CTL- La 0 mg/L). Significant differences between CTL and exposed mussels were represented by * if $p \leq 0.05$, ** if $p \leq 0.01$ and *** if $p \leq 0.001$.

Data obtained on La concentrations and biochemical data were submitted to statistical hypothesis testing using permutational multivariate analysis of variance with the PERMANOVA + add-on in PRIMER v6 (Anderson et al. 2008). A one-way hierarchical design was followed in this analysis. The pseudo-F values in the PERMANOVA main tests were evaluated in terms of significance. If significant differences were observed using main test, subsequent pairwise comparisons were performed. Pairwise comparisons were evaluated in terms of significance and significant differences were identified for Monte-Carlo p-values lower than 0.05. The null hypothesis tested was: for each biochemical parameter, no significant differences existed between experimental conditions (CTL- La 0 mg/L; La 0.1 mg/L; La 1 mg/L; La 10 mg/L). The significant differences ($p \leq 0.05$) among conditions were presented with different letters in figures.

The matrix containing histopathological and biochemical results as well as La concentrations in mussels' tissue for each condition was normalised and the Euclidean distance

similarity matrix calculated. This similarity matrix was simplified through the calculation of the distance among centroids based on La concentrations, which was then submitted to ordination analysis, performed by Principal Coordinates Ordination analysis (PCO). In the PCO graph, the variables that best explained the samples spatial distribution ($r > 0.85$) were represented as superimposed vectors.

2.5.3. Integrated biomarker response (IBR)

To integrate results from the different biomarkers and to try to understand the general mussel's biochemical response, the integrated biomarker response (IBR) was used, which was calculated according to Beliaeff and Burgeot (2002). Therefore, results from the studied biomarkers were used in order to evaluate the general response of *M. galloprovincialis* to different La exposure conditions. IBR index was calculated considering: the mean value of each biomarker for each condition (X), general mean (m) and general standard deviation (s) for each biomarker taking into account all conditions; Y and Z values, where $Y = (X - m) / s$ and $Z = Y$ or $-Y$, in the case of activation on inhibition, respectively; S value, calculated as $S = Z + |\text{Min}(Z)|$. Finally, the IBR was calculated as $\text{IBR} = \sum A$, where $A = (S_i S_{i+1})/2$. The IBR calculations were always performed with the same order of parameters for all conditions.

All of the determined biomarkers were used in the calculation of the IBR and they were arranged clockwise in the following order: SOD, CAT, LPO, GLY, GST, GPx, AChE, PROT, CP, ETS and GSH/GSSG. Values were discussed in terms of a general response given by the final IBR value, where higher values correspond to higher mussels' response.

3. RESULTS

3.1 *Lanthanum concentrations in seawater and soft tissues*

The concentration of La in seawater, comparatively with the nominal concentrations, presented maximum deviations that ranged between 13 and 16% in the analyzed conditions (0.1 mg/L, 1 mg/L and 10 mg/L La).

Concentrations of La in mussel's tissues were <0.5 , 30 ± 4 , 119 ± 26 and 383 ± 19 $\mu\text{g/g}$ in organisms exposed to 0.0 (CTL), 0.1, 1.0 and 10.0 mg/L of La, respectively.

BCF values obtained for each exposure condition were 357, 142 and 48 for 0.1, 1.0 and 10.0 mg/L La exposure conditions, respectively.

3.2. *Biological responses: biochemical parameters*

3.2.1. *Metabolic capacity and energy reserves*

The electron transport system (ETS) activity was significantly lower in contaminated mussels compared to control organisms, with the lowest values in mussels exposed to the highest La concentration (Figure 1A).

The glycogen (GLY) content was significantly higher in mussels exposed to concentration 1.0 mg/L, with no significant differences among the remaining conditions (Figure 1B).

Organisms exposed to La showed significantly higher protein (PROT) content in comparison with control organisms, with no significant differences among organisms exposed to La concentrations (Figure 1C).

3.2.2. *Oxidative status*

Enzymatic parameters

Mussels exposed to La showed significantly higher superoxide dismutase (SOD) activity in comparison to control organisms, with no significant differences between organisms exposed to 0.1 and 1.0 mg/L of La as well as between organisms exposed to 0.1 and 10 mg/L (Figure 2A).

No significant differences in terms of catalase (CAT) activity were observed among all the tested conditions (Figure 2B).

The activity of glutathione peroxidase (GPx) was significantly higher in organisms exposed to La compared with organisms under control conditions, with no significant differences among contaminated organisms (Figure 2C).

The activity of glutathione-S-transferases (GSTs) significantly increased in mussels exposed to 1.0 mg/L of La, with no significant differences among the remaining conditions (Figure 3).

Non-enzymatic parameters

Lipid peroxidation (LPO) levels significantly decreased in organisms exposed to La in comparison to control organisms, with the lowest values in mussels exposed to the highest La exposure concentration (Figure 4A).

Protein carbonylation (PC) levels significantly decreased in La contaminated organisms in comparison to control ones, with no significant differences among other La exposure conditions (Figure 4B).

The ratio reduced/oxidized glutathione (GSH/GSSG) was significantly lower in mussels exposed to La in comparison with control organisms, with the lowest values in mussels exposed to the highest La concentration (Figure 4C).

3.2.3. Neurotoxicity

In the presence of La mussels significantly decreased the activity of Acetylcholinesterase (AChE) in comparison to values observed in control organisms, with the lowest values in mussels exposed to 1.0 mg/L of La (Figure 5).

3.3. *Biological responses: histopathological measurements*

In gonads, the stress induced by La exposure leads to the emission of gametes already at the lowest concentration of 0.1 mg/L (Figure 6). Therefore, no enough morphological effects were detectable to calculate the I_h . However, regarding female gonads, comparing CTL organisms with exposed ones, results revealed that at CTL condition ovaries showed abundant vitellogenic oocytes while the ovaries of mussels exposed to different La showed a reduction of oocytes number in a dose depended manner: at 0.1 mg/L it is possible to observe a drastic

reduction of oocytes number; at 1.0 mg/L as well as at 10.0 mg/L there is the presence of apoptotic forms (Figure 6). In male gonads no differences between CTL and contaminated mussels were observed (data no shown).

Mussels' gills (Figure 7) showed several histopathological alterations that were detectable and measurable. In gills filaments of CTL mussels no morphological abnormalities were detected displaying epithelial cells with a regular distribution of lateral frontal cilia, tight central vessel and reduced number of lipofuscin aggregates and hemocytes infiltration. Comparing to control, exposure to La at different concentrations leads to an increase of damage severity in a dose dependent manner with a progressive accumulation of lipofuscin and loss of cilia in the frontal area, while no evident reduction of lateral cilia was observed (Figure 7). Moreover, the enlargement of the central vessel resulted abundant in organisms under 0.1 mg/L while at higher La concentrations was less diffuse, but still appreciable if compared to CTL (Figure 7). From the I_h graphic (Figure 8A) significantly higher values were obtained for contaminated mussels in comparison to control organisms.

The analysis of the digestive gland (Figure 9) showed for CTL mussels a normal structure with digestive tubules being constituted by a single layer of cells surrounding a narrow or occluded tubular lumen. In exposed mussel' digestive glands showed a progressive damage increase in a dose dependent manner. In comparison with CTL an increase in atrophy and necrosis was observed among the contaminated conditions: atrophy alterations were already detectable at 0.1 mg/L, while the necrosis appeared at concentrations of 1 and 10 mg/L (Figure 9). Compared to CTL the I_h regarding the digestive gland were significantly higher in La exposed mussels (Figure 8B).

3.4. Multivariate analysis

Results from the PCO analysis are presented in Figure 10. The first principal component axis (PCO1), which represents 76.4% of the variability, was clearly associated with La exposure concentrations, with a clear distinction between individuals exposed to La concentrations (negative side) and individuals under control condition (positive side). PCO2 axis explained 13.6% of the variability, separating organisms under intermediate La concentration (positive side) from organisms exposed to the highest La concentrations (negative side). LPO, PC, ETS, AChE and

GSH/GSSG were the variables presenting higher correlation with PCO1 positive side ($r>0.95$) while SOD, GPx, PROT, I_h for digestive glands (IhDG) were the variables that best correlate with PCO negative side ($r>-0.95$). La concentration was highly associated with C3 condition (10.0 mg/L) ($r>0.75$).

3.5. *Integrated Biomarker Response (IBR)*

IBR values showed the highest score (4.19) for the intermediate concentration of La (1.0 mg/L) which indicates higher impact in organisms under this condition. The results obtained also indicates similar effects level in organisms exposed to the lowest and the highest La concentrations, with IBR values of 3.24 and 3.14, respectively.

4. DISCUSSION

In the present study the impacts induced by La in the species *Mytilus galloprovincialis* were evaluated in terms of mussel's histopathological alterations and biochemical effects, including impacts on organisms metabolism, oxidative and neurotoxic status.

Metabolic capacity an energy reserves

Regarding mussels' metabolic capacity, the electron transport system (ETS) activity was used to estimate the energy consumption at the mitochondrial level and thus obtain a proxy of organisms' metabolic status (Coen and Janssen, 1997; Berridge et al., 2005; Fanslow et al., 2001; García-Martín et al., 2014). The present findings clearly revealed that organisms exposed to La decreased their metabolic capacity avoiding the consumption of GLY. Also, the present results revealed that the PROT content was higher in contaminated organisms which may indicate that these energy reserves were also preserved as a result of lower metabolic activity but may also increase as a result of higher enzymes production. This behavior seems to point out that organisms decreased their metabolism, avoiding energy expenditure, which may be achieved by closing their valves, and reducing their respiration and filtration rates and avoid La accumulation. To corroborate this hypothesis, our findings showed that although La concentrations increased in mussels' tissues with the increasing exposure concentration, BCF values strongly decreased with the increasing exposure concentrations indicating that organisms tended to limit the accumulation of La; i.e., lower BCF values at higher La exposure concentrations. Similarly, Hanana et al. (2017) demonstrated that BCF values decreased along the increasing exposure gradient in *Dreissena polymorpha* mussels exposed to La for 28 days. The reduction of the filtration rate in bivalves exposed to pollutants was already demonstrated, namely by Almeida et al. (2014, 2015) that revealed the capacity of *Ruditapes philippinarum* clams to reduce their filtration rate and decrease their BCF along an increasing exposure gradient of carbamazepine. Although to our knowledge no information is available on the impacts of La in bivalves metabolism and energy reserves, different studies demonstrated that for the same species a similar metabolic depression with no expenditure of energy reserves occurred when *M. galloprovincialis* was exposed to other pollutants, including Ti (Monteiro et al., 2018) and Hg (Coppola et al., 2017, Freitas et al., 2017). Studies on the effects of pollutants in other bivalves, including clams, also demonstrated a similar

pattern, namely in *Macoma balthica* exposed to Cd (Duquesne et al., 2014), and in *R. philippinarum* exposed to As (Velez et al., 2016).

Oxidative status

Besides the reduction of organisms metabolic capacity, the exposure to abiotic changes, such as the presence of pollutants, may cause overproduction of reactive oxygen species (ROS) in marine bivalves that, if not eliminated, will originate oxidative damage of the cellular membranes (Almeida et al., 2017; Freitas et al., 2016; Gomes et al., 2011; Matozzo et al., 2012; Monteiro et al., 2019b, 2019a; Regoli and Giuliani, 2014; Regoli and Principato, 1995). Thus, lipid peroxidation (LPO) may occur when lipid membranes are attacked by ROS, where lipids are oxidized into lipid hydroperoxides (Catalá, 2009; Regoli and Giuliani, 2014). To avoid damages of the lipid membrane organisms are able to increase their antioxidant defenses, namely increasing the activity of SOD, CAT and GPx enzymes (Regoli and Giuliani, 2014). In a similar way to LPO, another process that can result from the effect of ROS is the oxidation of proteins, called Protein Carbonilation (PC) (Suzuki et al., 2010). Some studies have already shown that metals can be strong pro-oxidants in mussels through the formation of protein carbonyls (McDonagh et al., 2006). In the present study mussels exposed to La strongly increased their antioxidant defences, especially evidenced by a significant activity increase of SOD and GPx enzymes in contaminated organisms, indicating that organisms were trying to eliminate the excess of ROS produced to avoid cellular injuries. The activation of SOD under La exposure reflects the organism's first enzymatic defence mechanism, responsible for the dismutation of oxygen radicals into oxygen and hydrogen peroxide (McCord and Fridovich, 1969). Because these sub-products are also toxic, a second defence mechanism often needs to be activated. Although CAT and GPx can both detoxify hydrogen peroxide (Regoli & Principato, 1995), only GPx activation was verified in *M. gallprovincialis* exposed to La. Other studies reveal that the preferential or simultaneous activation of CAT or GPx can vary by species or the type of contaminant (Trevisan et al., 2014; Rocha et al., 2015). Similarly, *M. galloprovincialis* demonstrated the capacity to increase their antioxidant defenses to fight against ROS overproduction and avoid cellular damages. In particular, Rocha et al. (2016) showed higher

antioxidant defenses and lower LPO levels in *M. galloprovincialis* exposed to Cd; and Coppola et al. (2017) demonstrated a similar response when the same species was exposed to Hg.

Along with the activation of antioxidant defenses, mussels exposed to La were able to activate biotransformation enzymes, namely GSTs that are a group of enzymes whose function doubles as a detoxification agent through the reaction of a xenobiotic with GSH (Towsend & Tew, 2003) and the inactivation of lipid peroxidation products through the use of GSH as a reducing agent (Sturve et al., 2008). In the presence of La mussels increased the activity of GSTs up to 1.0 mg/L while at the highest La concentration the activity of these enzymes was similar to control values. Such results may indicate that the highest La concentration may inhibit the activity of this group of enzymes. In agreement with such findings Hanana et al. (2017) also demonstrated that the mussel *Dreissena polymorpha* increased the activity of GSTs in the presence of La with inhibition at the highest tested concentration after 14 days of exposure. Also 28-days exposure to Hg and As led to the activation of this group of enzymes in *M. galloprovincialis* (Coppola et al., 2017).

The activation of antioxidant and biotransformation enzymes with elimination of ROS, associated to lower ETS activity, and therefore generating lower ROS amount, lead to lower LPO and PC levels in mussels exposed to La in comparison to control organisms. A similar pattern was observed in *D. polymorpha* exposed to La during 14 days (Hanana et al., 2017). Nevertheless, although defense mechanisms were activated avoiding cellular damage, oxidative stress was demonstrated by lower GSH/GSSG values in contaminated mussels. GSH is considered one of the most important ROS scavengers. When organisms are under oxidative stress due to ROS increase, GSH can act as antioxidant in the cytoplasm, directly neutralizing ROS, being oxidized to GSSG (Regoli and Giuliani 2014). For this reason, GSH/GSSG ratio has been commonly used as an indication of oxidative stress (e.g. Almeida et al. 2014, Freitas et al. 2015, Coppola et al. 2017). Furthermore, GSH also acts as a co-factor of other antioxidant enzymes such as GPx (Regoli and Giuliani 2014). Therefore, the decrease of GSH/GSSG ratio with the increase of La concentrations, indicates that GSH was oxidized into GSSG which was further associated with increased GPx activity, an enzyme associated with the conversion of GSH to GSSG. Several studies also demonstrated the decrease of GSH/GSSG ratio in bivalves

exposed to pollutants, including studies conducted by Coppola et al. (2017) where *M. galloprovincialis* was exposed to Hg.

Neurotoxicity

In what regards to mussels' neurotoxic status, the present study demonstrated that La strongly induced the inhibition of the AChE enzyme, evidencing a clear neurotoxicity capacity of this pollutant. AChE is an enzyme responsible for the breakdown of acetylcholine and has been widely used as an indicator of neurotoxicity in aquatic ecotoxicology due to its high sensitivity to many neurotoxic compounds such as metals (Maisano et al., 2017). Several studies already demonstrated the neurotoxic capacity of metals by inhibition of AChE in bivalves, including in mussels (Attig et al., 2010; Chalkiadaki et al., 2014) and in clams (Liu et al., 2011; Matozzo et al., 2005).

Histopathological alterations

Using classical histology techniques, this work clearly showed that there is a degree of histopathological alterations under different La concentrations at the level of gonads, gills and digestive tubules in mussels. In particular, the results obtained showed an increase in the damages induced by La in a dose-dependent manner, in all analyzed tissues. In what regards to gonads, the histological results revealed that five out of six samples from the control group did not emit eggs during the experiment and their oocytes shown a regular shape, while within treated mussels' groups, already at the lower concentrations, no eggs were found except in one sample at the lower La concentration where the few oocytes left resulted necrotic. These findings clearly suggest that La represent a stressful event that induced female spawning just like other stressful events. To date, no other studies evaluated mussels' histological alterations due to La contamination, namely on the reproductive capacity of *M. galloprovincialis* females. Nevertheless, alterations induced in female gonads indicate that mussel's reproductive capacity is greatly affected by La, that will result into important ecological consequences for population maintenance. Furthermore, histopathological injuries detected in gills and digestive tubules clearly reveal the capacity of La to compromise mussels health and growth capacity. Studies conducted by Hua et al. (2017) in the fish *Gobiocypris rarus* exposed to La also demonstrated histopathological

changes in gill and liver tissues. Also, studies assessing impacts of metals and metalloids already demonstrated histological alterations of gills, gonads and digestive tubules in mussels (Amachree et al., 2014; Cuevas et al., 2015; Sonawane, 2015; Sunila, 2011). Gills present a large surface in direct contact with xenobiotics present in water during mussel's filtration and respiration processes, being for this reason considered a sensitive organ to changes in water quality. Considering that gills alterations usually lead to hypoxia, respiratory failure and ionic and acid–base imbalances the present findings clearly indicate that impacts of La can seriously compromise mussel's growth rate and general health condition namely in what regards to mussel's condition index. Nevertheless, to date, no studies evaluated mussels' histological alterations after contamination by La.

CONCLUSIONS

The integration of all responses (La concentrations in mussels tissues, biochemical parameters and histopathological Indices), as shown by the PCO analyses, clearly reveal that: i) La is bioaccumulated by organisms, with increasing tissue concentration along with the increase of exposure concentration; ii) La accumulated in mussels tissues induced not only biochemical impacts but also histopathological alterations. Nevertheless, integrating the biochemical parameters in a join analyses, here accessed by IBR values, it is possible to observed that a stronger response was given by mussels at an intermediate concentration, which is explained by greater alterations at this condition for some of the biochemical parameters measured (higher GLY, lower AChE activity, higher GSTs activity). These results may indicate that: i) at the lowest concentration there was no stress enough to activate mussels responses and, ii) on the other hand, at the highest concentration, organisms are no longer able to continue to increase their responses and activation of defense mechanisms due to extreme stressful conditions. Still, in what regards to La accumulation, the decrease of BCF values with the increase of La concentration indicates that with the increase of exposure concentrations mussels decrease their accumulation rate, probably maintaining their valves closed for longer periods to avoid the entrance of La into the organism. Nevertheless, accumulation of La in mussels increased along the exposure concentration increasing gradient which could explain the limited response of mussels at the highest exposure concentration. The present study further reveal that the

histopathological alterations followed a dose-response pattern which was not observed in all the biochemical markers analyzed. Such results can be explained by the fact that biochemical analyses were conducted in mussels whole soft tissue, giving a response related to the general status of the organism, while histopathological responses are tissue specific. Considering the effects observed on gonads, reproductive organs, and gills, primary sites for oxygen uptake, it may be hypothesized that mussel's reproduction and respiration can be greatly affected by La. In relation to this topic, further research focused on the effects of rare earth elements on mussel's reproductive rates are mandatory for the management of endangered marine ecosystems.

Overall, histopathological data demonstrated significant alterations induced by La, which is in accordance with La bioaccumulation, metabolic depression, oxidative stress and neurotoxicity identified in contaminated mussels. Such findings indicate that La, even at environmentally relevant concentrations, will negatively affect mussels, with consequences not only on their biochemical performance but also on their reproductive capacity and growth rate.

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Figure captions

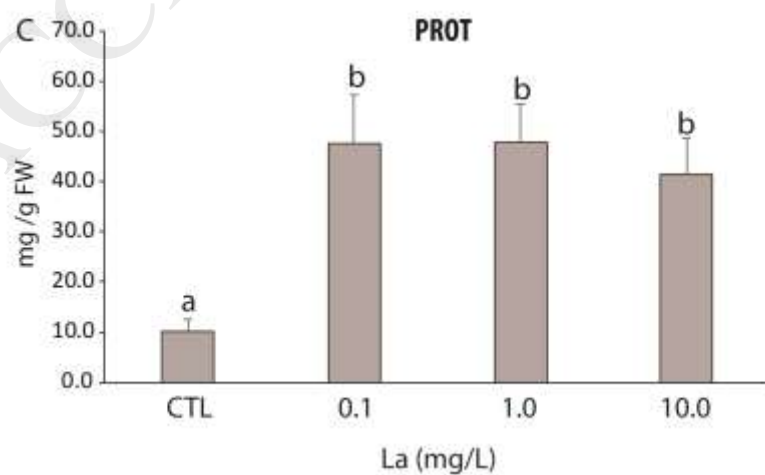
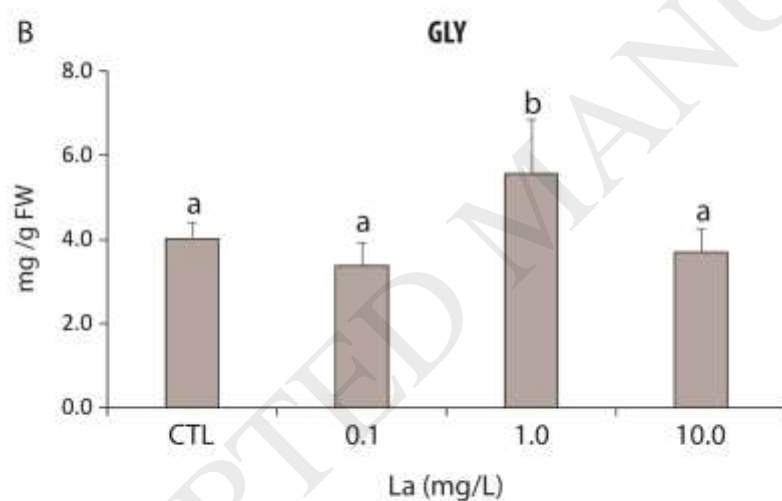
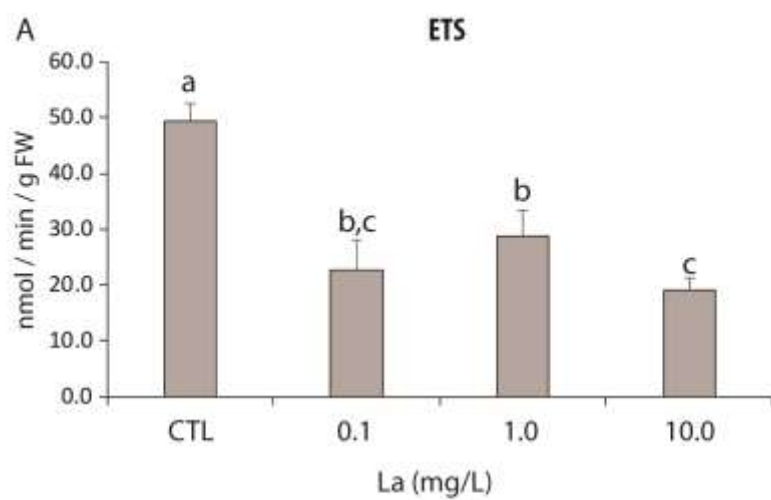


Figure 1. A: Electron transport system activity (ETS), B: Glycogen content (GLY) and C: Protein content (PROT), in *Mytilus galloprovincialis* exposed to different conditions (CTL-0.0 mg/L, 0.1, 1.0 and 10.0 mg/L of La). Values are presented as mean + standard deviation. Significant differences ($p \leq 0.05$) among conditions are represented with different letters.

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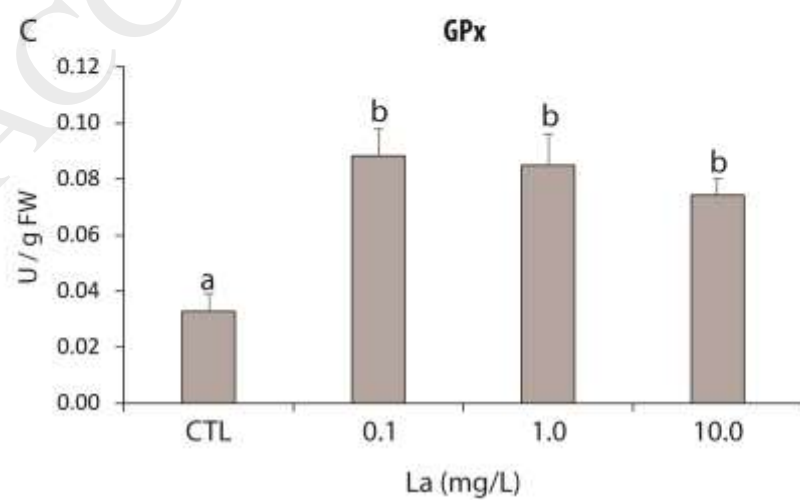
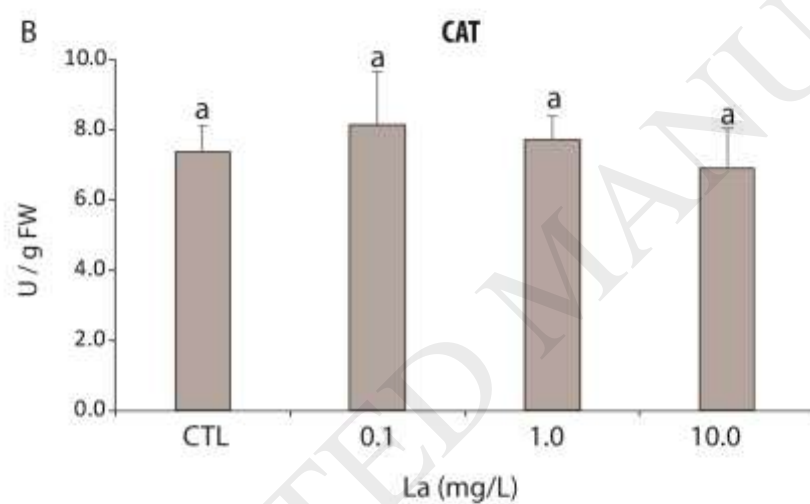
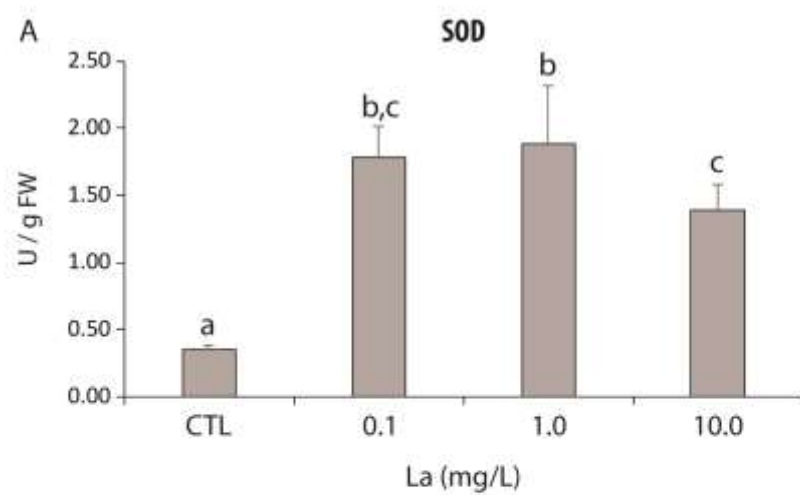


Figure 2. A: Superoxide dismutase activity (SOD); B: Catalase activity (CAT); and C: Glutathione peroxidase activity (GPx), in *Mytilus galloprovincialis* exposed to different conditions (CTL-0.0 mg/L, 0.1, 1.0 and 10.0 mg/L of La). Values are presented as mean + standard deviation. Significant differences ($p \leq 0.05$) among conditions are represented with different letters.

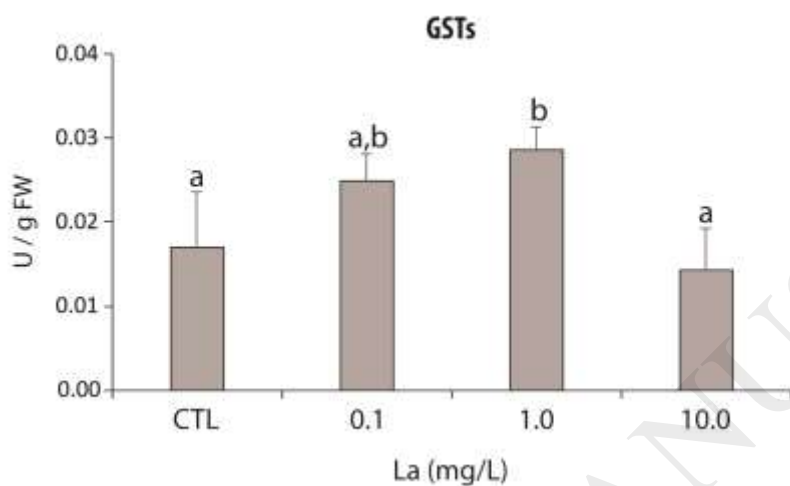


Figure 3. Glutathione S-transferases activity (GSTs), in *Mytilus galloprovincialis* exposed to different conditions (CTL-0.0 mg/L, 0.1, 1.0 and 10.0 mg/L of La). Values are presented as mean + standard deviation. Significant differences ($p \leq 0.05$) among conditions are represented with different letters.

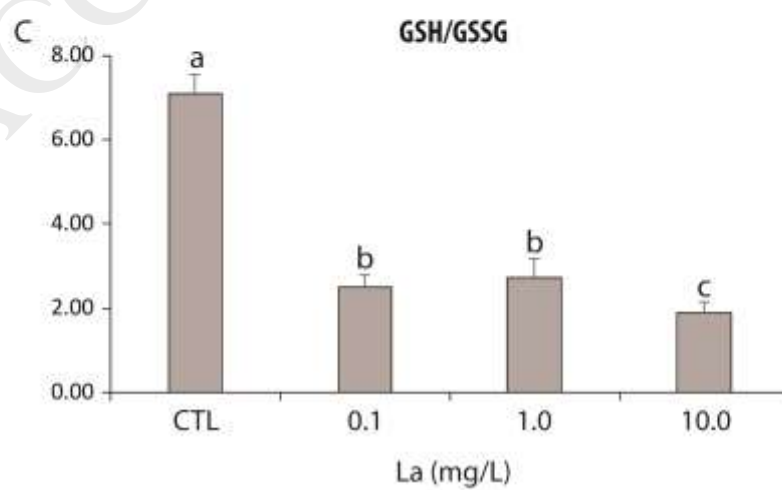
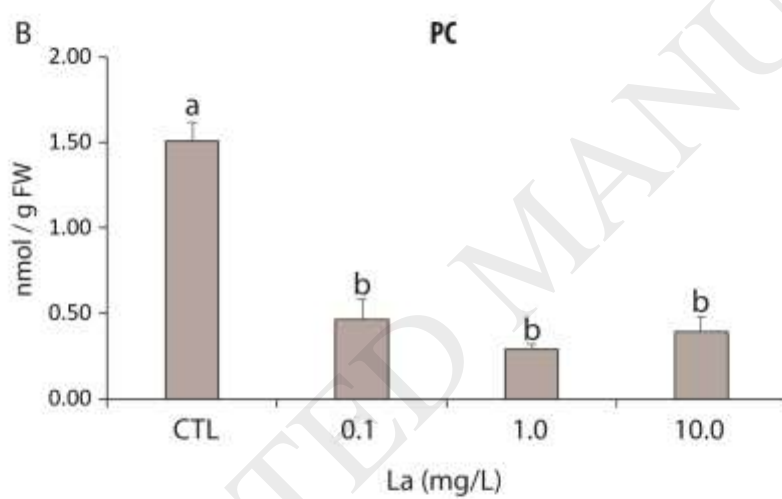
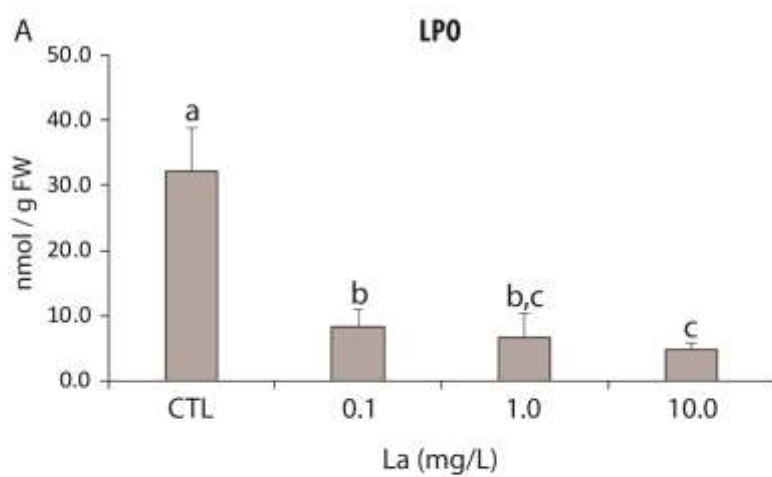


Figure 4. A: Lipid peroxidation levels (LPO); B: Protein carbonylation levels (PC); and C: reduced/oxidised glutathione ratio (GSH/GSSG), in *Mytilus galloprovincialis* exposed to different conditions (CTL-0.0 mg/L, 0.1, 1.0 and 10.0 mg/L of La). Values are presented as mean + standard deviation. Significant differences ($p \leq 0.05$) among conditions are represented with different letters.

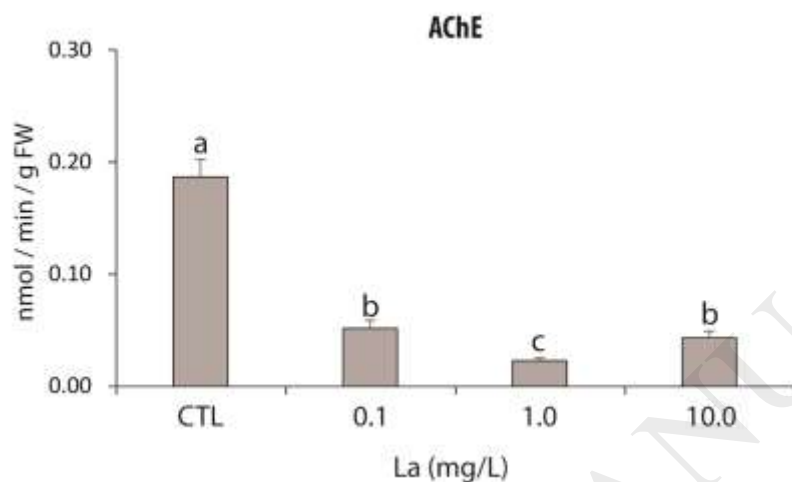


Figure 5. Acetylcholinesterase activity (AChE), in *Mytilus galloprovincialis* exposed to different conditions (CTL-0.0 mg/L, 0.1, 1.0 and 10.0 mg/L of La). Values are presented as mean + standard deviation. Significant differences ($p \leq 0.05$) among conditions are represented with different letters.

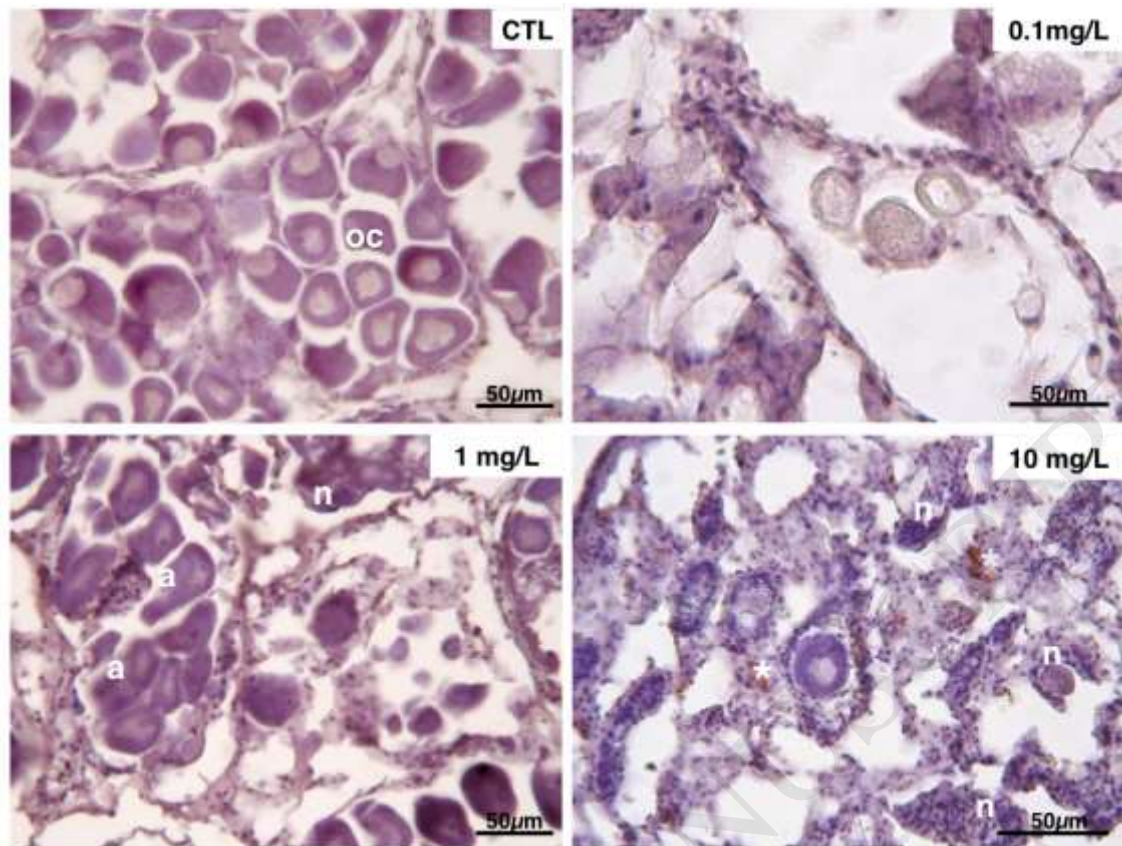


Figure 6. Micrographs of histopathological alterations observed in the ovaries of *Mytilus galloprovincialis* exposed to different La concentrations stained with hematoxylin: CTL exhibited normal ovarian follicles containing maturing oocytes (oc); Follicles after gametes release in mussels exposed to La at 0.1 mg/L and 10 mg/L. Atrophied follicles of a female mussel exposed to La at 1 mg/L, revealing many apoptotic (a) and necrotic (n) oocytes. Lipofuscin aggregates (*) can be observed in the adipogranular tissue of mussel exposed to 10 mg/L La. Scale bars = 50 µm.

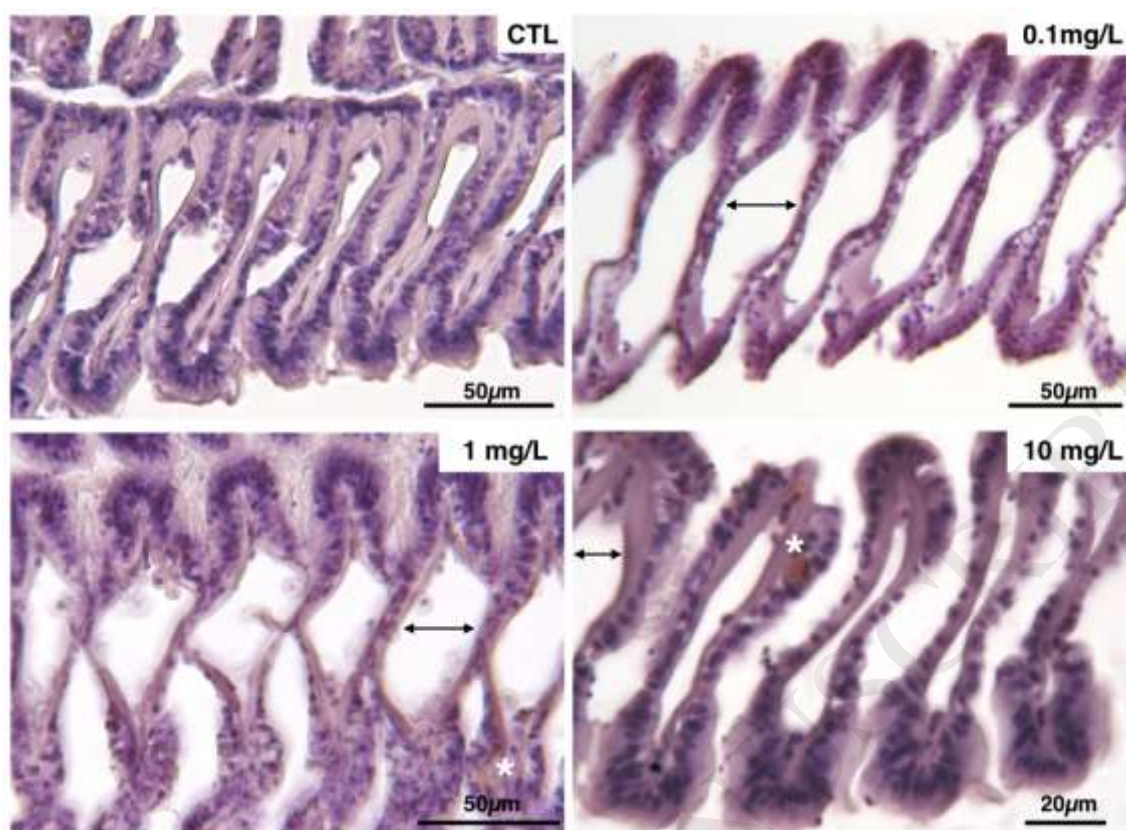


Figure 7. Micrographs of histopathological alterations observed in the gills of *Mytilus galloprovincialis* exposed to different Lan concentrations stained with hematoxylin: CTL mussel gills with frontal and lateral cilia; evident enlargement of the central vessel in mussel exposed to La at 0.1 mg/L; hemocytes infiltration in mussel exposed to La at 1 mg/L; abundance of lipofuscin aggregates (*) in mussel exposed to La at 10 mg/L. Scale bar 50 μm and 20 μm .

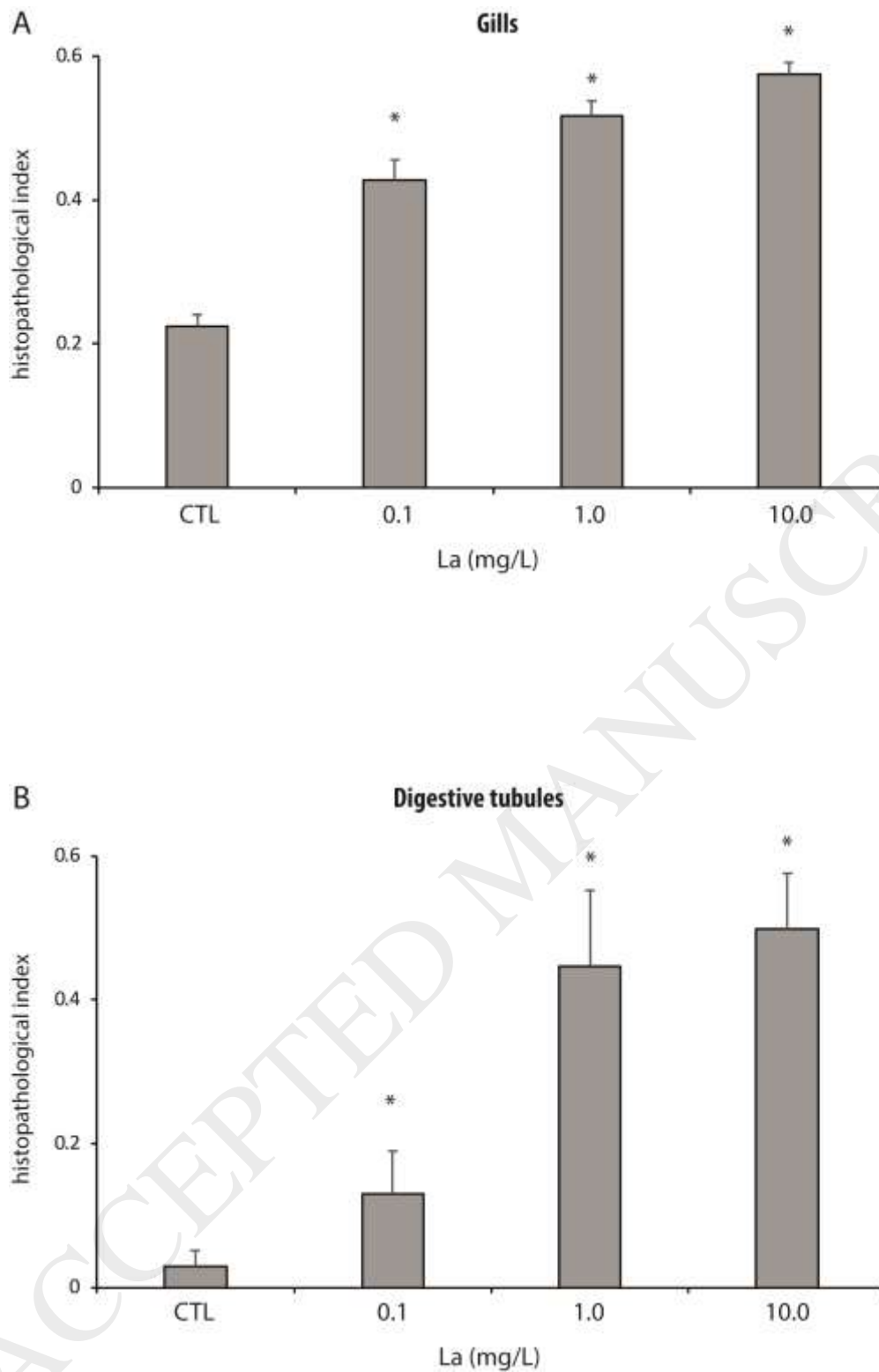


Figure 8. A: Histopathological index in gills (IhG); B: Histopathological index in digestive tubules (IhDG) in *Mytilus galloprovincialis*. Results are mean + standard deviation. Significant Differences between CTL and exposed mussels were represented by * if $p < 0.05$, ** if $p < 0.01$ and *** if $p < 0.001$.

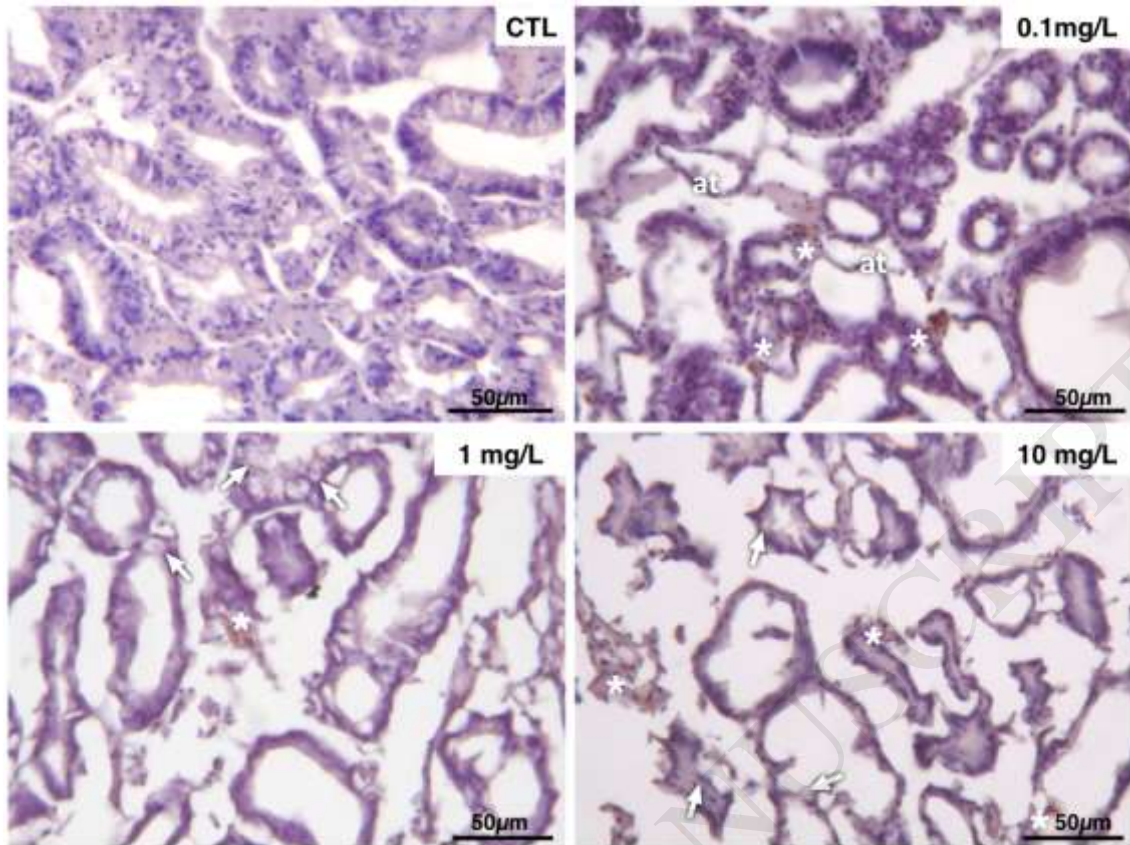


Figure 9. Micrographs of histopathological alterations observed in the digestive tubules of *Mytilus galloprovincialis* exposed to different La concentrations stained with hematoxylin: CTL digestive tubules showing a normal structure; evident lipofuscin accumulation (*) and atrophied digestive tubules (at) showing large lumen and thin epithelium in mussel exposed to La at 0.1 mg/L; degeneration of the digestive tubules and cell loss (arrows) due to necrosis in mussel exposed to La at 1 mg/L and 10 mg/L. Scale bar = 50 μm.

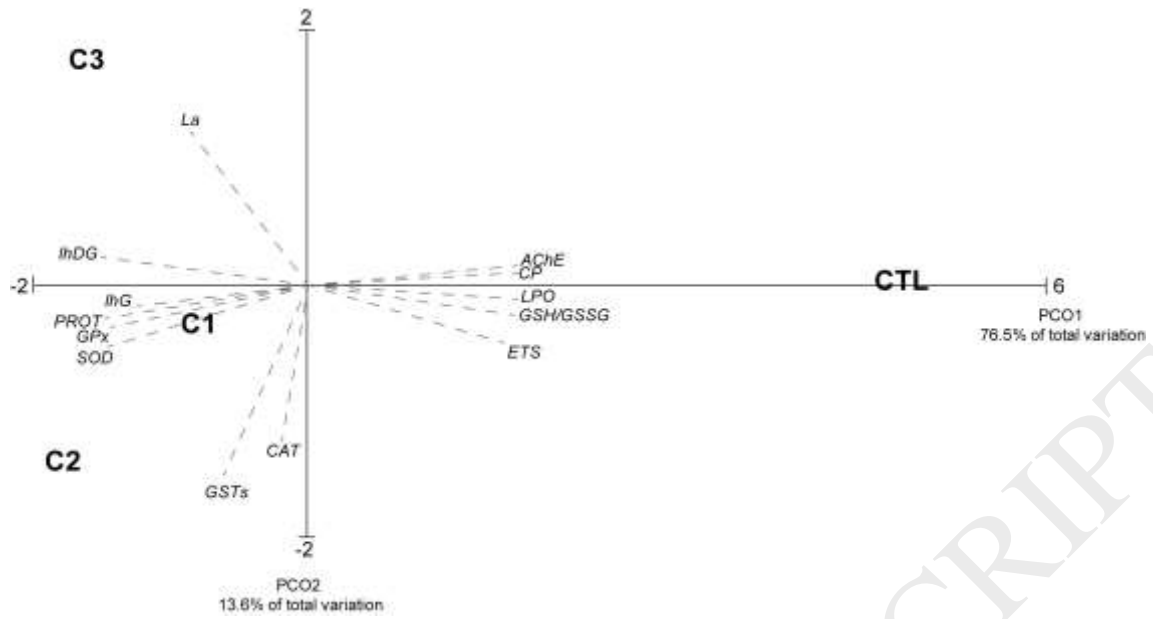


Figure 10. Centroids ordination diagram (PCO) based on biochemical and histopathological parameters, measured in *Mytilus galloprovincialis* exposed to different conditions (CTL-0.0 mg/L, 0.1, 1.0 and 10.0 mg/L of La). Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data ($r > 0.75$): La, PROT; ETS; LPO; PC; GSH/GSSG; AChE; SOD; CAT; GPx; GSTs; IhDG; IhG.

Table 1: Gills and digestive tubules of *Mytilus galloprovincialis* exposed for 28 days under different La concentrations

Tissue	Histopathological alteration	Weight (w)
Gills	Lipofuscin aggregates	1
	Loss of cilia	2
	Enlarged central vessel	1
	Heamocyte infiltration	1
Digestive tubules	Lipofuscin aggregates	1
	Heamocyte infiltration	1
	Atrophy	2
	Necrosis	3