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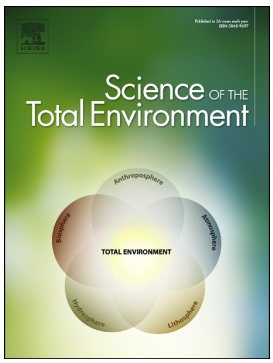

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Can water remediated by manganese spinel ferrite nanoparticles be safe for marine bivalves?

Francesca Coppola[a], Daniela S. Tavares[b,c], Bruno Henriques[b,d], Rui Monteiro[b,d], Tito Trindade[c], Etelvina Figueira[a], Amadeu M.V.M. Soares[a], Eduarda Pereira[b,d], Rosa Freitas[a]*

[a]Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[b]Departamento de Química & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[c]Departamento de Química & CICECO, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[d]Departamento de Química & LAQV-REQUIMTE, Universidade de Aveiro, 3810-193 Aveiro, Portugal

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Corresponding author: Rosa Freitas

Address: Departamento de Biologia, Universidade de Aveiro

Campus Universitário de Santiago

3810-193 Aveiro, Portugal

e-mail address: rosafreitas@ua.pt

ABSTRACT

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

Keywords:

Toxicity; Biomarkers; *Mytilus galloprovincialis*; Mn nanoparticles; Lead contamination.

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1. INTRODUCTION

Several human activities, including mining operations and sludge dumping as well as industrial and agricultural production, have greatly contributed to the increase of the number and concentration of pollutants in costal environments (Alimba and Faggio, 2019; Green-Ruiz and Páez-Osuna, 2001; Morais et al., 2012; Poulos et al., 2000; Prokić et al., 2019; Stara et al., 2020; Yi et al., 2011). In fact, often the final destination of pollutants are coastal aquatic systems, namely lagoons and estuaries, where organisms such as bivalves are continuously exposed to these anthropogenic substances due to their filter-feeding and sedentary behaviour (Capillo et al., 2018; Fattorini et al., 2008; Manzo et al., 2017; Pagano et al., 2017; Schiavo et al., 2018; Ventura-Lima et al., 2009, 2011; Zhang et al., 2015). Among pollutants, published information has already revealed that bioaccumulation of metal(oid)s can lead to toxic effects in bivalves, namely in mussels, including the alteration of their metabolism and redox status (Binelli et al., 2011; Errahmani et al., 2014; Freitas et al., 2018, Manduzio et al., 2005; Savorelli et al., 2017). In particular, studies conducted with metal(loid)s on the top list of the most hazardous materials, such as Mercury (Hg), Arsenic (As), Copper (Cu) and Cadmium (Cd), already showed the capacity of these elements to activate mussel's antioxidant defence mechanisms and decrease their metabolic capacity (Azizi et al., 2018; Coppola et al., 2018a,b; Mubiana and Blust, 2007; Nardi et al., 2017; Raftopoulou et al., 2011). Among the most widely distributed hazardous materials throughout the environment it is identified Lead (Pb) (ATSDR, 2017) due to its use in a vast diversity of anthropogenic activities (e.g. fertilizers, pesticides), being considered as one of the most dangerous metals, especially towards marine and estuarine species (de Souza Machado et al., 2016; Machado et al., 2014; Rzymski et al., 2014; Wang et al., 2009, 2012; Wood et al., 2012a,b). Accordingly, previous studies already showed alteration of bivalve's oxidative status accompanied by metabolic depression after exposure to Pb (Alak et al., 2013; Bocchetti et al., 2008; Freitas et al., 2014; Pirone et al., 2019).

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Due to the constant increase of environmental pollution, especially in aquatic systems, different approaches for water decontamination have been applied in the last decade (Baciocchi et al., 2005; Ballinas et al., 2004; Hansen et al., 2006; Katsoyiannis et al., 2002; Leupin et. al, 2005). Some of these techniques have shown a great potential for removing inorganic pollutants from water, including the use of nanoparticles (NPs) that revealed high effectiveness in removing metal(loid)s from water (Gehrke et al., 2017; Mohan et al., 2007; Paul et al., 2015; Zhang et al., 2010). For example, Mohmood et al. (2016) demonstrated that 10 mg/L of Silica-Coated magnetic NPs (Fe_3O_4@SiO_2-NPs) had the capacity to remove 98% of Hg from water with 0.5 mg/L Hg after 24h. Manganese-ferrite ($MnFe_2O_4$) is a common spinel ferrite material that has also been used to decontaminate water from inorganic pollutants (metal(loid)s) due to its ability to sorb elements such as Pb, and its physical magnetic properties that allows the easy separation from the water at the end (Tavares et al., 2013). It is well recognized that the use of these magnetic NPs for water decontamination is one of the most promising research areas (Aslibeiki et al., 2016; Bahadar et al., 2016; Beij et al., 2010; Coppola et al., 2019; Federici et al., 2007). However, the collateral effects of a remediated water towards organisms inhabiting aquatic environments it is not yet well understood (Bhatt and Tripathi, 2011; Blaise et al., 2008; Coppola et al., 2019, 2020; Lovern and Klaper, 2006; Lovern et al., 2007; Smith et al., 2007; Warheit et al., 2007).

For this reason, the present study was focused on the toxicity of seawater previously contaminated with Pb and remediated by $MnFe_2O_4$, NPs. The laboratory experiment was conducted over 28 days using *Mytilus galloprovincialis* as model organism. This species is worldwide recognized as a good bioindicator due to mussels sedentary and filter-feed behaviour and, consequently, the capacity to accumulate pollutants, showing exposure effects (Attig et al., 2014; Banni et al., 2014a,b; Coppola et al., 2018a,b; Freitas et al., 2018; Hu et al., 2015; Livingstone et al., 2000; Nardi et

al., 2017). The experimental set up included the following treatments: clean seawater; seawater only with Pb 1 mg/L; seawater only with NPs 50 mg/L; seawater with Pb 1 mg/L and 50 mg/L simultaneously; and seawater having initially Pb 1 mg/L and remediated with NPs 50 mg/L during 24 h. Toxicity was evaluated in terms of mussel's Pb accumulation, metabolic capacity and energy reserves content, antioxidant and biotransformation defence capacity, lipids and protein damage, as well as neurotoxic impacts.

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2. MATERIALS AND METHODS

2.1 Model organisms and experimental setup

Mytilus galloprovincialis was selected as model species to evaluate the toxicological impacts of seawater previously contaminated with Pb and decontaminated by magnetic spinel ferrite NPs ($MnFe_2O_4$).

Organisms were collected in the Ria de Aveiro, Portugal (40°38'51.1"N 8°44'05.5"W), and transported to the laboratory where they were placed in tanks of 50 L of artificial seawater at salinity 30±1, temperature of 17±1ºC (resembling conditions at the sampling area), during 14 days for depuration and acclimation. Artificial seawater was prepared by mixing a commercially available salt mixture (Tropic Marin® SEA SALT from Tropic Marine Center – see Atkinson and Box (2010) for salt composition) with freshwater obtained by reverse osmosis (four stage unit, Aqua-win RO-6080, Thailand). During this period artificial seawater was in continuous aeration (with a weekly renewal) and mussels were fed twice per week with Algamac protein plus (150,000 cells per animal per L).

Organisms with a mean body weight of 21.3±6.61 g, shell length 6.18±0.46 cm and width 3.52±0.27 cm were used for the experimental assays.

After acclimation organisms were exposed to 17.0±1.0ºC; pH 8.0±0.10, 12 light: 12 dark, continuous aeration, in artificial seawater with salinity 30±1, distributed into 7 different treatments (see Table 1), including: CTL (control seawater), treatment A (seawater with Pb levels before remediation); treatment a (seawater with Pb levels after remediation); treatment B (seawater with NPs levels before remediation), treatment b (seawater without NPs, after remediation); treatment C (seawater with Pb and NPs before remediation) and treatment c (seawater with Pb and NPs after remediation). Each treatment was conducted in triplicate with 4 individuals/replicate. Salinity and temperature were kept constant to match those from the acclimation period

(30 and 17°C, respectively) and individuals were fed every 2 days, as previously described.

Lead (Lead nitrate, CAS No: 10099-74-8, EC No: 233-245-9; Sigma-Aldrich) was used to prepare a concentration of 1 mg/L selected for initial exposure as it is considered the maximum Pb concentration permissible in wastewater discharges from industry (Directive, 2013/39/EU, 2013). Treatment a, with 0.02 mg/L of Pb, was considered as remediated seawater since preliminary studies conducted with 1 mg/L in seawater and remediated with $MnFe_2O_4$, NPs (50 mg/L) resulted into 0.02 mg/L of Pb in the medium.

The $MnFe_2O_4$, NPs 50 mg/L was selected according to NPs capacity of removal, which was studied in a previous work (data not shown). Those experiments were performed placing 50 mg/L of $MnFe_2O_4$ NPs in 1 L of an aqueous solution containing 1 mg/L of Pb for 24 hours. After this period, NPs were separated from seawater by applying an external magnetic field using a NdFeB magnet (ferromagnetic behaviour of the MnFe2O4 NPs in well known (Balaji et al. 2002; Thirupathi et al . 2012)) and the residual concentration of Pb in solution was circa 0.02 mg/L. Despite the remarkable reduction in Pb, the remediated water could possibly present some toxicity, due to the amount of Pb remaining in solution, or due to NPs that eventually remained in the solution after separation. Treatement a, i.e., seawater with a concentration of Pb like that achieved after remediation (0.02 mg/L), together with condition b (seawater after 24 h of contact with NPs 50 mg/L, which were then separated)) were used to elucidate this possible issue.

During the 28 days of experiment, seawater was renewed every 7 days, immediately after which all exposure conditions were re-established. During the experiment, water samples were collected immediately after concentrations reestablishment for the quantification of Pb in the solution and identification of real exposure concentrations. During the experimental period mortality was daily checked.

Dead organisms, individuals with open shells and unresponsive to external stimulus, were removed when identified.

After 28 days of experiment, organisms from each aquarium (12 per treatment) were collected and immediately frozen in liquid nitrogen, being preserved at −80°C.

To evaluate mussels' biochemical responses and Pb accumulation the whole soft tissue was removed from the shells and homogenized using a mortar and pestle under liquid nitrogen. Tissue homogenates were distributed in 5 aliquots of 0.5 g fresh weight (FW) each for biochemical analyses, and the remaining tissue was used for Pb quantification. Samples for biochemical parameters and Pb quantification were stored at −80°C.

2.2 Synthesis and characterization of $MnFe_2O_4$ nanoparticles

$MnFe_2O_4$ nanoparticles were synthesized according a previous work (Tavares et al., 2013). The morphological characterization of NPs was confirmed by transmission electron microscopy (TEM) using the Hitachi H-9000 TEM microscope operating at 300 kV. For TEM analysis a drop of sample was dispersed in ethanol on a carbon-coated copper grid and then it was air-dried. Surface area of the NPs was confirmed by N_2 adsorption/desorption on a Gemini V2.0 Micromeritics instrument. The crystalline phase of the NPs was identified by x-ray powder diffraction of the powders using a Philips Analytical PW 3050/60 X'Pert PRO (θ/2θ) diffractometer equipped with an X'Celerator detector and with automatic data acquisition (X'Pert Data Collector v2.0b software) by a monochromatized Cu Kα radiation (λ = 1,54056 Å) at 45 Kv/40 Ma. Fourier Transform Infrared (FT-IR) spectra of the NPs was recorded using a spectrometer Mattson 7000 at 4 cm^{-1} resolution, using a horizontal attenuated total reflectance (ATR) cell.

2.3 Lead quantification

The quantification of Pb in seawater was made by inductively coupled plasma mass spectrometry (ICP-MS), on a Thermo ICP-MS X Series equipped with a Burgener nebulizer as described by Henriques et al. (2017). The quantification limit of the method was 0.1 µg/L (n =12), with an acceptable relative standard deviation among replicates <10% (n≥3).

The ICP-MS was also used to determinate the total Pb concentration in *M. galloprovincialis* soft tissues, after microwave-assited acid digestion using HNO_3 and H_2O_2, as described by Henriques et al. (2017). The quality control was assured by running blanks (reaction vessels with only HNO_3 and H_2O_2) and certified reference material TORT-3 (Lobster Hepatopancreas; 0.225±0.018 mg/kg Pb) in parallel with samples. Blanks were always below the quantification limit and mean percentage of recovery for Pb in TORT-3 was 110±4% (n = 4).

2.4 Biomarkers

Mussels' whole soft tissues (2 individuals per aquarium, 6 per treatment) prepared for biochemical assays (0.5 g FW aliquots) were used for extractions with different buffer solutions (1 mL). After adding the buffer, samples were extracted through high-speed shaking by tissue lyser, centrifuged (at 10000 g or 3000 g depending on the biomarker, at 4°C), and the supernatants collected and stored at −80°C until analysis.

Biochemical analyses were performed in duplicate, alongside with blanks. A total of 4 extraction buffers were used, depending on the biomarker (see references Andrade et al., 2019; Pirone et al., 2019). All parameters were analysed spectrophotometrically using a multi-detection microplate reader (BioTek Synergy HT).

The biochemical parameters evaluated were: i) metabolism and energy related markers, including electron transport system activity (ETS), glycogen (GLY) and total protein (PROT) concentrations, measured according to King and Packard (1975) and

the modifications performed by De Coen and Janssen (1997), Dubois et al. (1956) and Robinson et al. (1940) methods, respectively; ii) antioxidant enzymes activities, including superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) activities, measured following the methods described by Beauchamp and Fridovich (1971), Johansson and Borg (1988) and Paglia and Valentine (1967), respectively; iii) indicators of cellular damage as lipid peroxidation (LPO) and protein carbonyl (PC) levels, determined following the methods described by Ohkawa et al. (1979) and Levine et al. (1990), respectively; iv) indicators of redox homeostasis as glutathione content ratio (GSH/GSSG) determined according to Rahman et al. (2007); v) neurotoxicity measuring acetylcholinesterase (AChE) activity according to Ellman et al. (1961) and modification by Mennillo et al. (2017). All parameters are described in detail in Coppola et al. (2019) and Pirone et al. (2019).

2.5 Statistical analyses

Biochemical parameters and Pb contamination levels obtained from each tested treatment were submitted to a statistical hypothesis testing using permutational analysis of variance, employing the PERMANOVA+add-on in PRIMER v6 (Anderson et al., 2008). The matrix gathering all biomarkers responses and Pb concentrations per treatment was used to calculate the Euclidean distance similarity matrix. The pseudo-F *p*-values in the PERMANOVA main tests were evaluated in terms of significance. When significant differences were observed in the main test, pairwise comparisons were performed. Values lower than 0.05 ($p \leq 0.05$) were considered as significantly different. The matrix gathering biochemical descriptors per condition were used to calculate the Euclidean distance similarity matrix. The similarity matrix was simplified through the calculation of the distance among centroids matrix, which was then submitted to ordination analysis, performed by Principal Coordinates (PCO). Pearson correlation vectors of biochemical descriptors (correlation > 0.75) were provided.

The null hypotheses (H0) tested were: i) no significant differences exist among control and contaminated treatments (CTL, A, B and C); *p*-values are presented in Table 4, with significant differences highlighted in bold; ii) no significant differences exist among control and remediated treatments (CTL, a, b, and c); *p*-values are presented in Table 4, with significant differences highlighted in bold; iii) no significant differences exist between A vs a, B vs b, C vs c treatments; significant differences between each pair of treatments are represented highlighted in bold in Table 4, with significant differences identified with an asterisk in Figures.

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3. RESULTS

3.1 Mortality

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

3.2 Characterization of $MnFe_2O4$ nanoparticles

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

3.3 Lead concentration in seawater and mussels' tissues

Lead quantification in water samples, at the beginning and immediately after conditions reestablishment showed that differences between measured and nominal concentrations was always below 15%. Also, concentrations of Pb in control condition (CTL) and in condition with NPs (B and b treatments) were very low (Table 2) and not statistically different. The quantification in treatment C were not performed because the sorption of Pb by the NPs is extremely rapid.

Organisms exposed for 28 days to CTL and treatments a and b presented low concentrations of Pb (Table 3). The highest levels of Pb in mussels were observed in treatment A that corresponds to seawater contaminated with Pb initial concentration. For each group of treatments (A vs a, B vs b, and C vs c) significantly higher contents of Pb were recorded in mussels exposed to “non-remediated” conditions (A, B and C) comparatively to “remediated” ones (a, b and c), and to control.

3.3 Biochemical markers

Metabolic capacity and energy reserves

The ETS activity was significantly higher at control (CTL) in comparison to values obtained in mussels exposed to non-remediated (A, B, C) and remediated (a, b, c) seawater (Figure 2A, Table 4). Comparing non-remediated with remediated treatments, B vs b and C vs c, significantly higher values were observed in organisms exposed to remediated seawater (Figure 2A, Table 4). Moreover, significantly lower ETS activity was observed in organisms exposed to B in comparison with A and C treatments (Figure 2A, Table 4). Except a vs b treatments, significant differences were observed among remediated treatments (a vs c, b vs c) (Figure 2, Table 4).

The GLY content was significantly lower at control (CTL) in comparison to values obtained in mussels exposed to non-remediated (A, B, C) and remediated (b, c) seawater (Figure 2B, Table 4). Comparing non-remediated with remediated treatments, A vs a and C vs c, significantly higher values were observed in organisms exposed to non-remediated ones (Figure 2B, Table 4). Significant differences among mussels

exposed to non-remediated treatments were observed between A and C as well as between B and C treatments, while among remediated treatments significant differences were only observed between a and c (Figure 2B, Table 4).

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

Antioxidant defences

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

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

mussels exposed to non-remediated treatments were observed between A and C as well as between B and C treatments, while no significant differences were observed among the remediated treatments (Figure 3B, Table 4).

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

Cellular damage

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

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

GSH/GSSG values were significantly higher at control (CTL) in comparison to values obtained in mussels exposed to non-remediated (A, B, C) and remediated (a, b,

c) seawater (Figure 4C, Table 4). Comparing non-remediated with remediated treatments significantly higher GSH/GSSG values were observed in remediated ones (Figure 4C, Table 4). No significant differences were observed among mussels exposed to non-remediated as well as among mussels exposed to remediated treatments (Figure 4C, Table 4).

Neurotoxicity

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

3.4 Multivariate analysis

Principal coordinates analysis (PCO) graph obtained is shown in Figure 6. PCO axis 1 explained 63.3 % total variation, while PCO axis 2 explained 16.6 % (Figure 6). PCO1 separated individuals exposed to A, B and C treatments at the negative side from mussels exposed to CTL, a, b and c in the positive side. PCO2 separated individuals exposed to CTL, a, c and A treatments in the positive side from B, b and C treatments in the negative side. Organisms exposed to C and B treatments were associated to GLY, PROT, SOD and CAT as these markers presented the highest values especially at C treatment. Individuals exposed to CTL, a and c treatments were associated to ETS, AChE and GSH/GSSG. Organisms exposed to A treatment was closely related to LPO and PC, GPx parameters where higher activity of

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these biomarkers were observed. Moreover, these results were confirmed by higher concentration of Pb in samples water and mussels exposed to A treatment.

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DISCUSSION

Nowadays, the increase of nanoparticles (NPs) for different uses can lead to their release and dispersion in the environment with potential toxic effects (Gehrke et al., 2015; Hu et al., 2015; Huang et al., 2016; Keller et al., 2010). Furthermore, published studies already demonstrated that NPs have the capacity to interact with other pollutants altering their potential harmful effects (Gomes et al., 2011; Guan et al., 2018; Pan et al., 2012).

One of the most recent applications of NPs is related to their use for water remediation (Aslibeiki et al., 2016; Davidescu et al., 2015; Gehrke et al., 2015). Although their efficiency in removing pollutants from water has been described (Mehdinia et al., 2014; Mohmood et al., 2016; Ngomsik et al., 2005; Zhang et al, 2015) no studies evaluated the toxicity of the remediated water. This knowledge gap was addressed in the present study, using magnetic manganese spinel ferrite nanoparticles ($MnFe_2O_4$ NPs) that have already demonstrated high capacity to sorb Pb from seawater (Hosseini and Asadnia, 2013). For this, in the present study the bioaccumulation capacity and biochemical performance were evaluated in *Mytilus galloprovincialis* exposed to non-remediated (treatments A, B and C) and remediated (treatments a, b and c) seawater, measuring a set of biomarkers in mussels' tissues after a 28 days exposure period.

In what regards to removal of Pb from seawater, our findings clearly demonstrated that the application of $MnFe_2O_4$, NPs revealed to be a promising procedure, since the concentration of Pb in the water significantly decreased after remediation. These results are in accordance to previous studies conducted by Mohmood et al. (2016), which showed the efficient of Fe_3O_4@SiO_2-NPs to sorb Hg form water.

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This present study clearly demonstrated the capacity of mussels to accumulate Pb, even if present at very low concentrations in the medium (remediated water), which can explain the biochemical alterations observed after a 28 days experimental period. These results agree with previously published studies that demonstrated the toxicity of Pb in mussels (*M. galloprovincialis*, *M. edulis*) even at low but environmentally relevant concentrations (Bocchetti et al., 2008; Fernández et al., 2010; Widdows et al., 2002).

Concerning mussel's biochemical responses, clearly the behaviour observed under non-remediated and remediated seawater differed. These results are highlighted by the PCO analysis that separated remediated and non-remediated waters in positive and negative sides of PCO1, respectively. PCO analysis demonstrated that mussels under non-remediated seawater were characterized by high enzymes activity (high SOD, CAT and GPx activates), cellular damages (high LPO and PC levels) and higher Pb concentrations in mussels tissues, as well as in water.

In detail, the results obtained showed that independently on the tested treatment mussels tended to decrease their metabolic capacity, measured by ETS activity, in comparison to control levels. However, in general, mussels exposed to remediated treatments presents higher ETS activity than mussels exposed to contaminated water, indicating that remediated seawater induced less toxic effects in mussels' metabolic capacity. Furthermore, similar ETS values obtained in remediated treatments (a, b, c) highlight similar toxicity of Pb and NPs, both isolated and in combination. Such results are in accordance with studies already published regarding the toxicity of Pb and NPs in bivalves, revealing that exposure to these type of pollutants (e.g. Pb, Hg, zinc oxide (ZnO_2-NPs), metal oxidase (Me(O)NPs), titanium oxidase (TiO_2-NPs), gold nanoparticles (AuNPs), carbon nanotubes (f-MWCNTs)) resulted in metabolism depression in mussels (*Mytilus edulis, M. coruscus* and *M. galloporivincialis*) and clams *(Mercenaria mercenaria, R. philippinarum* and *Corbicula fluminea)* (Baker et al., 2014; Coppola et al., 2018a,b; De Marchi et al., 2017; Fan et al., 2016; Freitas et al., 2014; Huang et al., 2018; Jaishankar et al., 2014; Li et al., 2018;Tedesco et al., 2010).

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In this study the metabolic decrease in mussels exposed to contaminated water (treatments A, B and C) resulted in lower energy expenditure, showing an increase of PROT and GLY content in comparison with mussels exposed to remediated seawater (treatments a, b and c), which presented an energy reserve content similar to control values. In accordance with these results it was already demonstrated by other authors (Coppola et al., 2017; Della Torre et al., 2017; Guan et al., 2018; Jung et al., 2006) that bivalves under pollutants exposure avoid the expenditure of their energy reserves. In fact, previous studies have showed and justified that a consequence of metabolic decrease in mussels under stressful metal exposure is the low energy expenditure with an increase of energy reserves content (Coppola et al., 2017; Freitas et al., 2018; Pirone et al., 2019). Avoiding energy reserves expenditures different physiological processes may be affected, namely reproductive success and growth rate (Berthelin et al., 2000; Dridi et al., 2006; Ojea et al., 2004; Pouvreau et al., 2006).

It is well known that when bivalves are exposed to pollutants the production of reactive oxygen species (ROS) may increase which, as a consequence, results into the activation of antioxidant enzymes (Regoli and Giuliani, 2014) or, it may also result in the inhibition of these enzymes in the case of extreme stressful conditions (Company et al., 2004; Maria and Bebianno, 2011; Monari et al., 2015). The obtained results showed a clear activation of SOD, CAT and GPx enzymes in mussels exposed to non-remediated seawater, independently on the treatment (A, B or C), while in mussels exposed to remediated seawater (treatments a, b and c) there was no effective increase in antioxidant defenses with enzymes activity values closer to control. These findings evidence the toxic impacts of Pb and NPs, with a clear induction of mussel's antioxidant defense mechanisms when exposed to contaminated seawater. As described previously, the increased activity of antioxidant enzymes such as SOD, CAT and GPx may result from the overproduction of ROS due to the presence of pollutants,

namely metals. In particular, similar activation of antioxidant enzymes was observed by Freitas et al. (2014) in the clam *R. decussatus* exposed for 96 h days to Pb, while other authors showed a similar response in mussels exposed to the same metal (Alka et al., 2013). Also, studies on the impacts of NPs in bivalves showed that antioxidant defences were activated in mussels *M. galloprovincialis* exposed to TiO_2-NPs or ZnO NPs (100 mg Zn/L), and in clams *R. philippinarum* exposed to f-MWCNTs (Li et al., 2018; De Marchi et al., 2017; Monteiro et al., 2019).

Under stressful conditions bivalves may prevent the occurrence of cellular damage if antioxidant defences are efficient in eliminating ROS, avoiding a general oxidative status (Regoli and Giuliani, 2014). The present findings clearly demonstrated that organisms exposed to non-remediated seawater (treatments A, B and C) presented higher lipids damage in comparison to mussels exposed to remediated seawater (treatments a, b and c), indicating that although antioxidant defences were activated in mussels exposed to non-remediated seawater these mechanisms were not enough to prevent cellular damage and oxidative stress was observed. On the other hand, although a limited activation of antioxidant enzymes was observed in organisms exposed to remediated seawater (treatments a, b and c) lower cellular damages were observed indicating that these conditions were less toxic to mussels than contaminated seawater. Previous studies also showed the increase of LPO when bivalves (mussels and clams) were exposed to Pb (Alak et al., 2013; Menzi et al., 2017; Pirone et al 2019) and a similar response was observed in mussels *M. galloprovincialis and M. coruscus* and clams *R. philippinarum* exposed to different NPs (TiO_2, f-MWCNTs, ZnO_2) (De Marchi et al., 2017; Huang et al., 2018; Mezni et al., 2017).

Another consequence of ROS overproduction is the oxidation of proteins, identified by protein carbonylation (PC) (Patetsini et al., 2013). The PC levels in mussels exposed to remediated seawater (treatments a, b and c) were close to CTL values and lower than in mussels exposed to contaminated seawater (A, B, C),

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evidencing higher protein damage in mussels exposed to contamination. These results are in accordance with recent studies by Freitas et al. (2019), which showed an increase of PC levels when the mussels *M. galloprovincialis* was exposed to Pb. Also, different authors revealed an increase of PC values in bivalve species after exposure to metals and NPs (Baker et al., 2014; De Marchi et al., 2018; Fan et al., 2016 and 2017; Pirone et al., 2019; Sellami et al., 2014; Valavanidis et al., 2006).

Under a stressful condition, organisms tend to increase oxidized glutathione (GSSG) content while decreasing the amount of reduced glutathione (GSH), decreasing their GSH/GSSG ratio in comparison to control or less stressful conditions (Regolli and Giuliani, 2014). In the present study, although the ratio GSH/GSSG decreased in all treatments in comparison to CTL, the lowest values were observed in organisms under non-remediated seawater (treatments A, B and C). These results evidence lower redox homeostasis in organisms exposed to non-remediated seawater (treatments A, B and C) in comparison to the ones exposed to remediated seawater (treatments a, b and c). Similarly, previous studies also with bivalves showed similar responses with higher GSH/GSSG values at the least stressful conditions (Coppola et al., 2018 a, b; De Marchi et al., 2017).

In marine bivalves it is well described the neurotoxic impacts of different pollutants, evidenced by the decrease on AChE activity due to its high affinity for many neurotoxic compounds such as metals and NPs (Maisano et al., 2017; Wang et al., 2009). Our results showed that organisms tends to decrease the activity of AChE both in remediated and non-remediated seawater compared to CTL, but especially in the presence of Pb and NPs (treatment C). In general, the AChE activity in mussels under remediated seawater were higher than contaminant treatments and this demonstrate that even after removal of Pb and NPs from the water still neurotoxic impacts were induced in mussels exposed to remediated seawater, evidencing the high neurotoxic capacity of Pb and NPs. These findings are in accordance with previous results by De Marchi et al. (2018) which showed a decrease in AChE activity when the clams *R.*

philippinarum were exposed to MWCNTs (Nf- and f- NPs). Also, study conducted by Freitas et al. (2019) showed a decrease of AChE activity when mussels *M. galloprovincialis* were exposed to Pb concentration.

CONCLUSION

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

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Credit Authors statment

Rosa Freitas, Etelvina Figueira and Eduarda Pereira are supervisors of the PhD students Francesca Coppola, Daniela Tavares and Rui Monteiro

Rosa Freitas and Eduarda Pereira gave the idea of this study to the students that accepted this challenge and performed all the analyses in the lab. Eduarda Pereira is the responsible for the laboratory where chemical quantification were done. Rosa Freitas and Amadeu Soares are the responsible persons for the labs where biomarkers were determined. Eduarda Pereira, Rosa Freitas, Tito Trindade and Amadeu Soares funded this study.

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Conflict of Interest

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 non-financial interest (such as personal or professional relationships, affi liations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

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Table 1. Experimental conditions.

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

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

Pb water concentration (µg/L)			
CTL		$0.52\pm0.11^{A,a}$	
Pb	**A**	853 ± 281^{B}	*
	a	15 ± 1.9^{b}	
NPs	**B**	0.58 ± 0.15^{A}	
	b	0.47 ± 0.18^{a}	
Pb + NPs	**C**	**	
	c	115 ± 13^{c}	

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

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

	Pb mussels concentration (mg/Kg)		
CTL		$0.6\pm0.1^{A,a}$	
Pb	**A**	700 ± 232^{B}	*
	a	1.2 ± 0.2^{b}	
NPs	**B**	6.5 ± 2.5^{C}	*
	b	1.8 ± 1.5^{b}	
Pb+NPs	**C**	53 ± 29^{D}	*
	c	16 ± 13^{c}	

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

	ETS	GLY	PROT	SOD	CAT	GPx	LPO	PC	GSH/GSSG	AChE
CTL vs A	**0.0001**	**0.0003**	**0.0001**	**0.0001**	**0.0005**	**0.0001**	**0.0001**	**0.0001**	**0.0023**	**0.0117**
CTL vs B	**0.0001**	**0.0490**	**0.0016**	**0.0001**	**0.0006**	**0.0001**	**0.0015**	**0.0001**	**0.0009**	**0.0071**
CTL vs C	**0.0012**	**0.0001**	**0.0001**	**0.0001**	**0.0001**	**0.0020**	**0.0022**	**0.0003**	**0.0016**	**0.0003**
CTL vs a	**0.0002**	0.3271	0.7835	**0.0100**	0.5680	**0.0021**	**0.0001**	0.7876	**0.0238**	**0.0025**
CTL vs b	**0.0001**	**0.0264**	0.9700	**0.0010**	0.2066	**0.0001**	0.8090	0.3325	**0.0150**	**0.0240**
CTL vs c	**0.0099**	**0.0061**	0.9976	**0.0001**	0.7891	**0.0012**	**0.0027**	**0.0001**	**0.0162**	0.1182
A vs a	0.0910	**0.0001**	**0.0020**	**0.0002**	**0.0047**	**0.0028**	**0.0224**	**0.0001**	**0.0015**	0.7846
B vs b	**0.0050**	0.6304	**0.0011**	**0.0001**	**0.0098**	0.3309	**0.0044**	**0.0001**	**0.0001**	0.1630
C vs c	**0.0393**	**0.0455**	**0.0001**	**0.0001**	**0.0001**	**0.0235**	**0.0382**	**0.0033**	**0.0002**	**0.0048**
A vs B	**0.0018**	0.7245	0.6388	**0.0090**	0.2371	**0.0434**	**0.0067**	**0.0464**	0.2404	0.9018
A vs C	**0.3092**	**0.0001**	**0.0002**	0.0867	**0.0001**	0.0982	0.7361	0.4576	0.3164	**0.0023**
B vs C	**0.0021**	**0.0093**	**0.0147**	0.0549	**0.0020**	0.5118	0.1661	0.6072	0.7502	**0.0002**
a vs b	0.0631	0.0575	0.7503	0.3488	0.5040	0.6976	**0.0001**	0.5074	0.3418	**0.0122**
a vs c	**0.0032**	**0.0095**	0.7383	0.0751	0.6710	0.7813	**0.0132**	**0.0002**	0.4531	0.2883
b vs c	**0.0009**	0.1224	0.9587	0.4154	0.2299	0.4267	**0.0104**	**0.0007**	0.9585	0.8618

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Figure 1. Transmission Electronic Microscopy image of $MnFe_2O_4$ nanoparticles. Amplification 50000x.

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

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

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

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

Figure 6. . Principal coordinated analyses (PCO) based on biochemical parameters, measured in *Mytilus galloprovincialis* exposed to different treatments (CTL, A, a, B, b,

C, c). Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data ($r > 0.75$): PROT; GLY; CP; CAT; SOD; GPx; LPO, ETS; AChE, GSH/GSSG, [Pb] in organisims and seawater.

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Highlights

- Pb contaminated seawater was successfully remediated by $MnFe_2O_4$ NPs
- Mussels exposed to remediated seawater accumulated less Pb
- Mussels exposed to Pb and/or NPs decreased their metabolic capacity
- Mussels exposed to non-remediated treatments activated their antioxidant defences
- Non-remediated treatments showed increased damage cell and lower GSH/GSSG ratio

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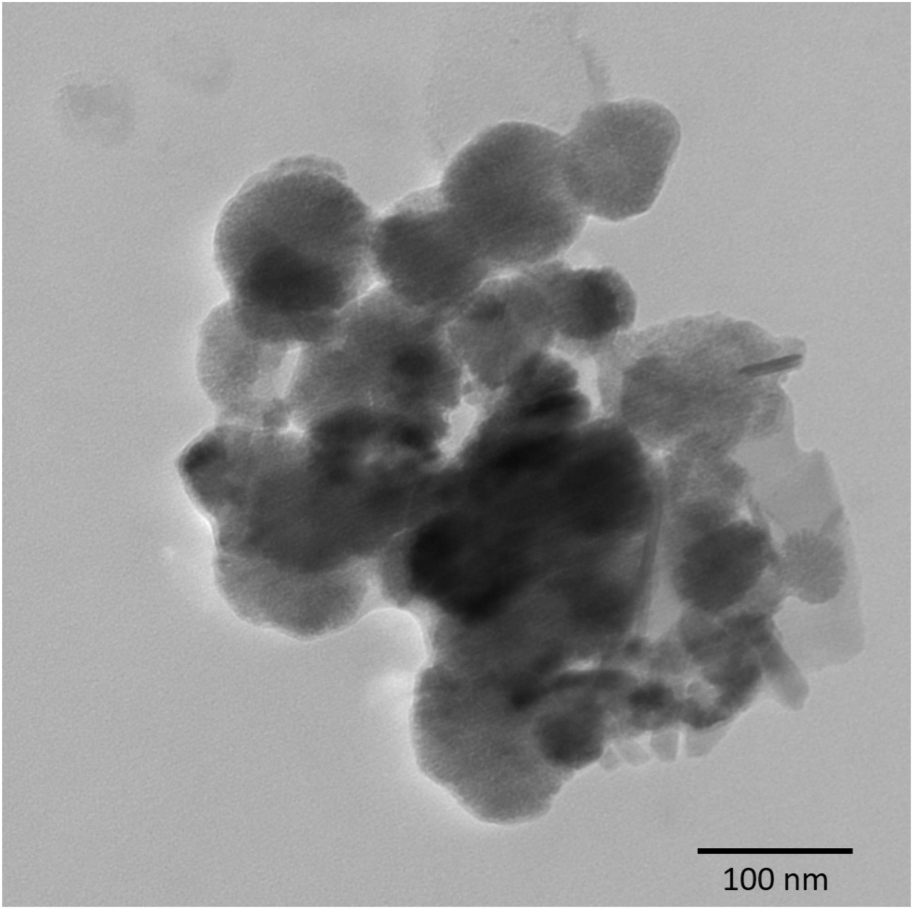

Figure 1

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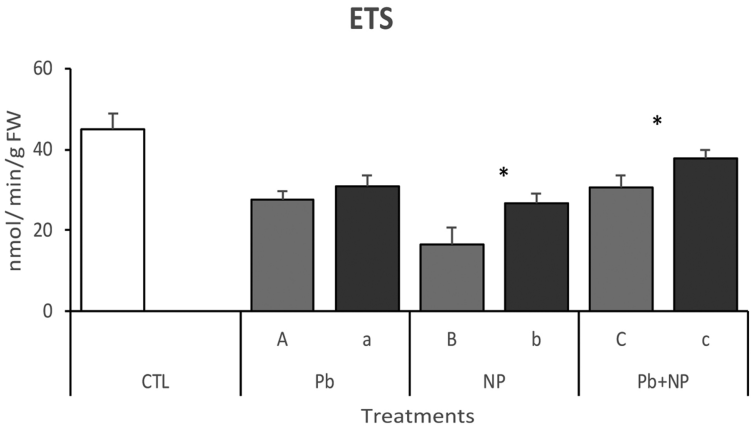

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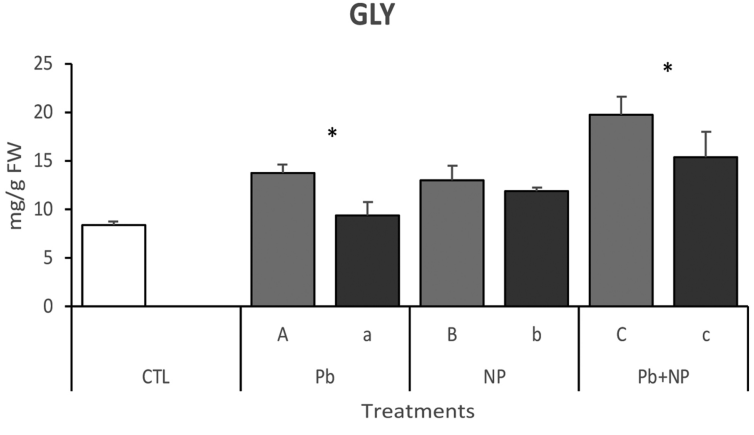

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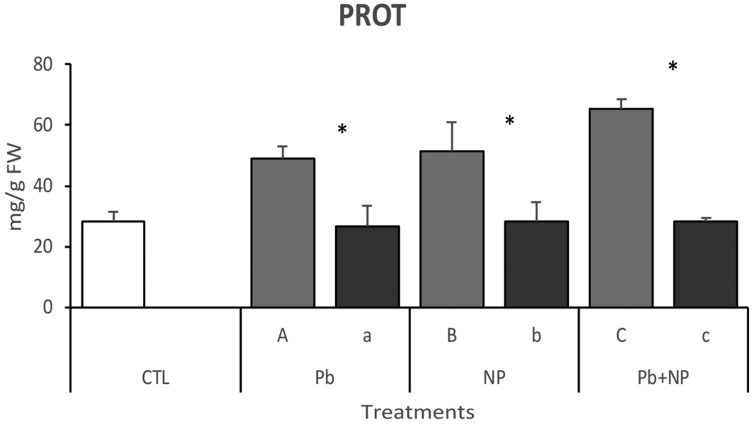
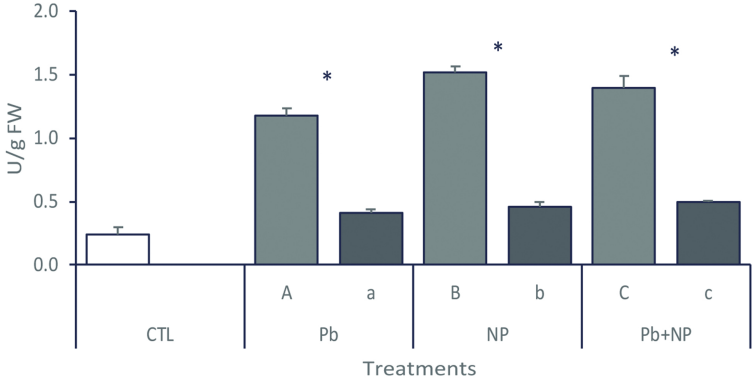


Figure 2

3A



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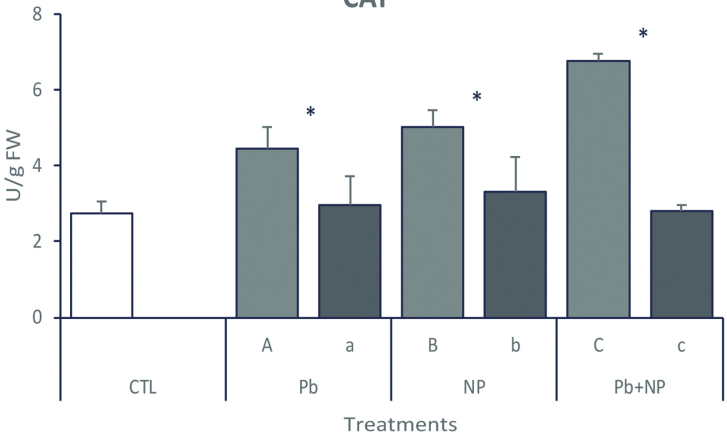

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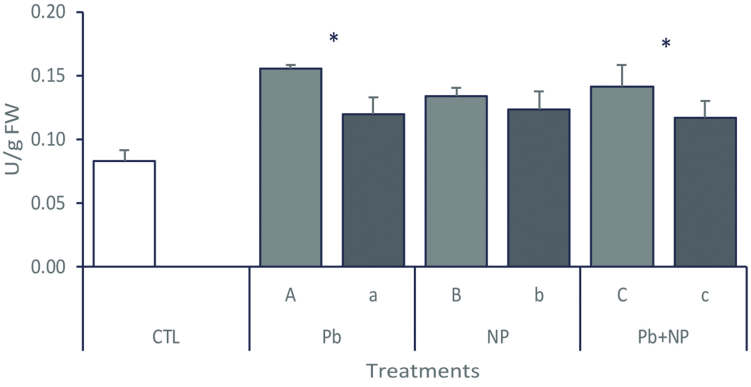
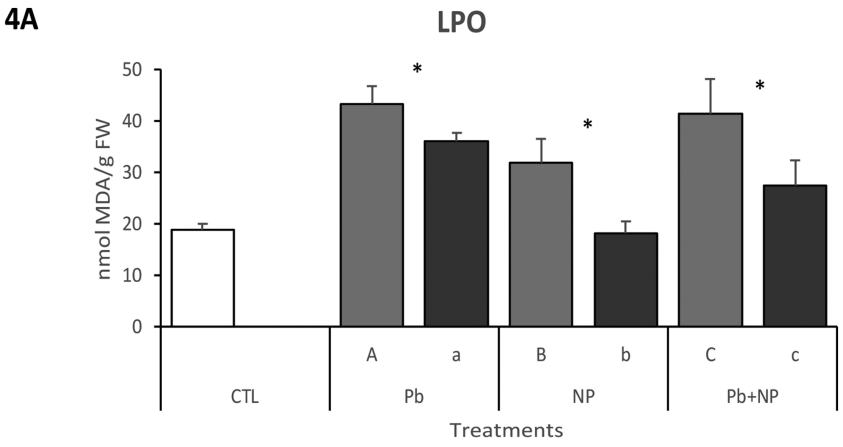


Figure 3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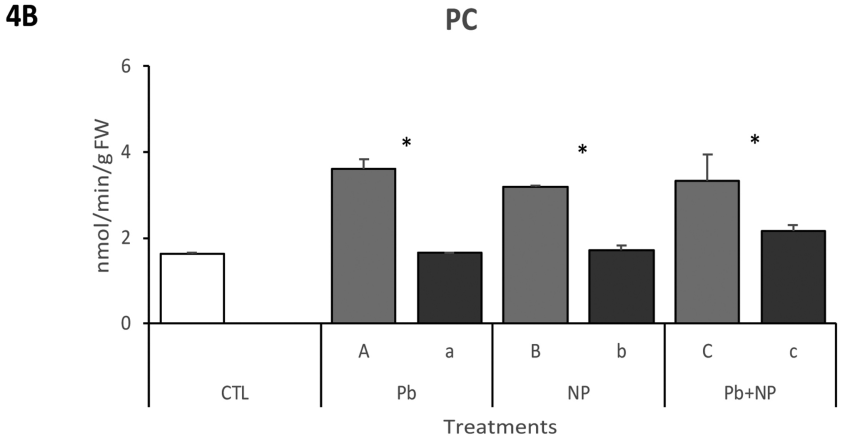
Treatments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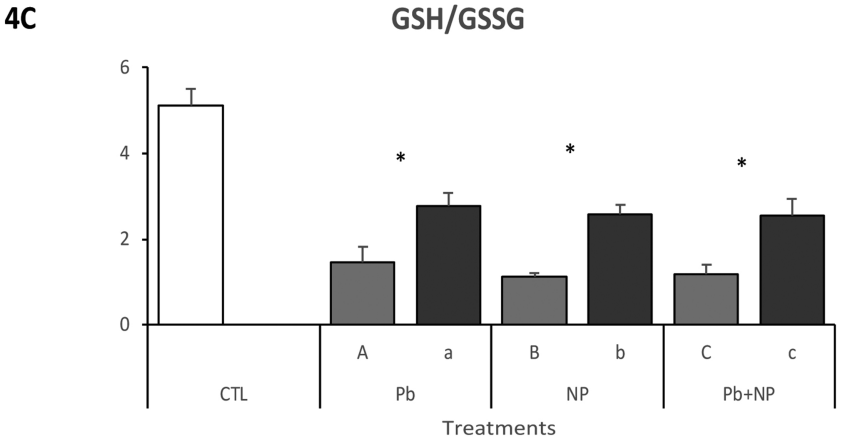
Treatments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*
*
*
A
a
B
b
C
c
CTL
Pb
NP
Pb+NP
Treatments

Figure 4

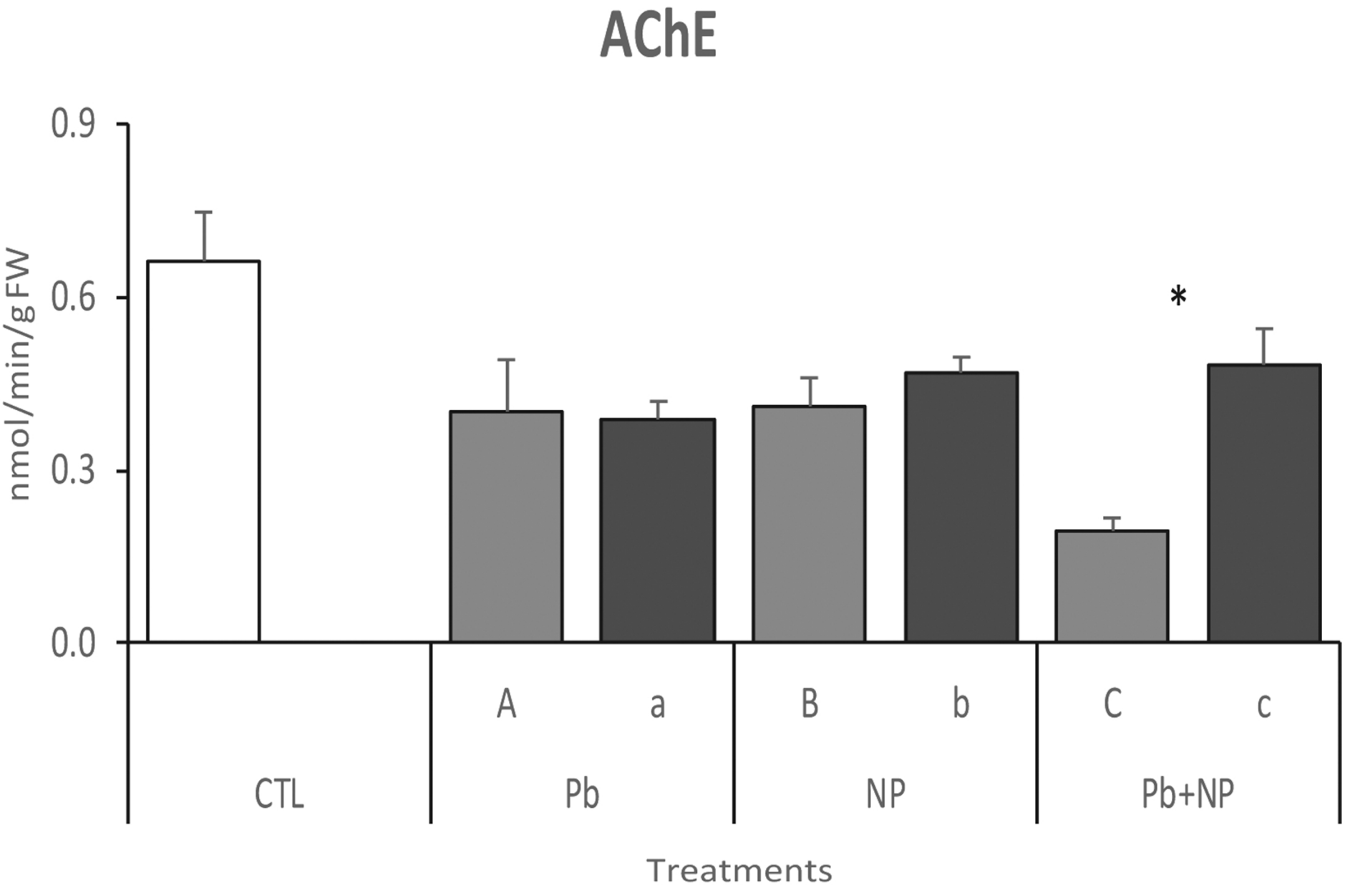

Figure 5

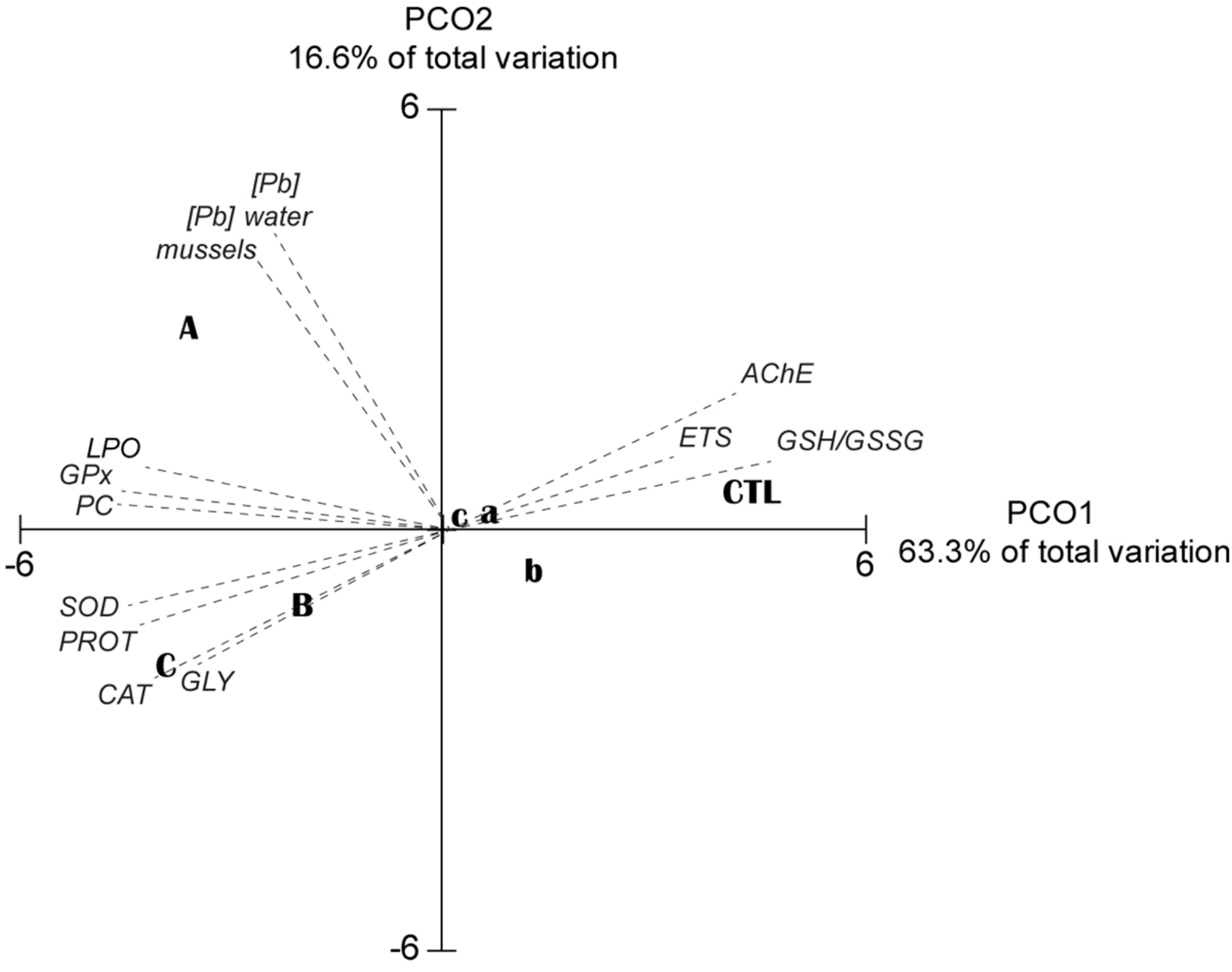
PCO2
16.6% of total variation
6
-6
[Pb]
[Pb] water
mussels
A
AChE
ETS
GSH/GSSG
LPO
GPx
PC
CTL
c
a
PCO1
63.3% of total variation
-6
6
b
SOD
PROT
B
C
CAT
GLY

Figure 6