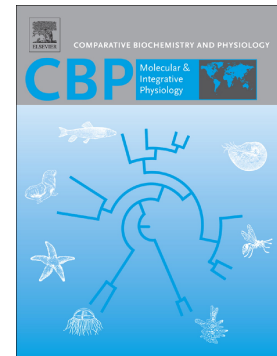# Journal Pre-proof

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

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ABSTRACT

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

Keywords:

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

1. INTRODUCTION

The increase of pollutants in aquatic environments is closely related to the world population growth along with industrial and agricultural activities (Yi et al., 2011; Morais et al., 2012; Zhang et al., 2015). The rapid development and expansion of industry and other anthropogenic activities, such as mining, fuel and energy production, use of pesticides and fertilizers, metallurgy and high tech industries lead to the discharge of effluents containing potentially toxic elements (PTEs) into the aquatic environment, which puts aquatic ecosystems quality at risk (Chowdhury et al., 2016; Buffet et al., 2014; Ventura-Lima et al., 2009, 2011). Among the most common pollutants, mercury (Hg) stands out as frequent environmentally hazardous (ATSDR, 2015) in the field of water policy, whose emissions should be progressively phased out by 2021 (Decision no. 2455/2001/CE). Nevertheless, this metal is still the main component in electronic products, thermometers (for measuring high temperatures) and fluorescent lamps (Dornier et al., 2012; Briant et al., 2016; Pereira et al., 2008; Randall and Chattopadhyay, 2013). As a response to these hazards, throughout the last years a growing interest in the removal of PTEs from effluents has been observed, in particular Hg due to its harmful character to the environment and human health (Xiong et al., 2014; Zhang et al., 2014). Different methodologies to the decontamination of water have been developed, including chemical precipitation using, among others, 2,4,6-trimercapto-1,3,5-triazine, trisodium salt nonahydrate (TMT) ($Na_3S_3C_3N_3 \cdot 9H_2O$) to precipitate metals (Hg, Zn, Pb, Cd) (Matlock et al., 2001; Henke et al., 2001); membrane filtration as ultrafiltration, reverse osmosis, nanofiltration (Aroua et al., 2007; Muthukrishnan and Guha, 2008; Pugazhenthi et al., 2005); coagulation and flocculation, as the use of chitosan, alum or ferric salts (Heredia and Martín, 2009; Renault et al., 2009); electrochemical methods and nanomaterials (Ali et al., 2012; Anjum et al., 2016; Huang et al., 2015; Li et al., 2010). Most of these methodologies are low cost but inefficient, or efficient but very costly (Babel and Kurniawan, 2003; Gehrke et al., 2015; Mohan and Pittman, 2007). For these reasons, it is important to develop alternative methods and new materials, such as nanostructured

materials (NSMs), which have proved good effectiveness to remove metal(loid)s from water (Chen and Mao, 2007; Jackson et al., 2012; Paul et al., 2015; Vilela et al., 2016).

Since the discovery of graphene in 2004, the 2-dimensional hexagonal network of carbon atoms graphitic material has received worldwide attention and research (Adeleye et al., 2015, 2016). Presently, graphene-based materials (GBM) are extensively investigated in electronics, biological engineering, being widely used in a vast range of applications including filtration systems, lightweight/strong composite materials, photovoltaic and energy storage, due to its excellent electrical conductivity, high mechanical strength and thermal conductivity, high impermeability to gases and optical transparency (Henriques et al., 2016; Yang et al., 2018). Due to its multifunctionality, GBM started to be investigated as alternative to conventional water treatment methods, resulting very promising (Ali, et al., 2018; Nupearachchi et al., 2017; Xiong et al., 2014; Zhang et al., 2014). For example, graphene oxide (GO) showed that oxygen functional groups play a key role in the sorption of metals, especially multivalent metal ions (Bian et al., 2015; Feng et al., 2011; Vilela et al., 2016). More recently, studies highlighted the performance of GO-functionalized with polyethyleneimine (PEI), GO-PEI in freshwater, with removals of 35% for As, 96% for Hg, 99% for Cd, 96% for Pb, and 92% for Cr and 98% for Ni, using only 50 mg/L of GO-PEI (all contaminants at equal initial concentration of 2.5 μmol/L) (Henriques et al., 2016; Rute, 2017). This material proved also to be very effective to remove Hg ions from seawater, with 81% of removal after just 6 hours (results under publication).

Despite the high removal efficiencies, it is imperative to assess environmental toxicity risks derived from the application of these materials, especially for coastal aquatic systems, as this information is inexistent. In this context, benthic species are good biological models as they accumulate and reflect the impacts of different substances (Attig et al., 2014; Coppola et al., 2017, 2018; Freitas et al., 2018; Nardi et al., 2017; Velez et al., 2015; Hu et al., 2015; Banni et al., 2014). Studies conducted on oysters (*Saccostrea cucullata, Crassostrea gigas* (Gagnaire et al., 2004; Pan and Wang, 2011)), clams *(Anodonta anatina, Corbicula fluminea, Ruditapes decussatus, Ruditapes philippinarum* (Oliveira et al., 2017;

Pan and Wang, 2011; Velez et al., 2016b)), mussels (*Perna viridis, Septifer virgatus, Mytilus galloprovincialis, Mytilus edulis* (Attig et al., 2014; Mubiana and Blust, 2007; Pan and Wang, 2011)) and cockles (*Cerastoderma edule* (Freitas et al., 2012)) showed the capacity of PTEs to impair organism's redox status, increasing the activity of antioxidant enzymes, and to decrease organisms metabolic capacity, namely when exposed to Hg (Raftopoulou et al., 2011; Azizi et al., 2018; Coppola et al., 2017, 2018; Mubiana and Blust, 2007; Nardi et al., 2017).

The present study evaluated the toxicity of seawater, previously contaminated with Hg and remediated by GO-PEI, using the mussel *M. galloprovincialis*. This species is one of the most popular environmental bioindicators, presenting a wide spatial distribution and economic relevance (Fattorini et al., 2008; Kristan et al., 2014; Velez et al., 2016abc; Coppola et al., 2017; Richir and Gobert, 2014; Majdoub et al., 2017). It is a sedentary filter-feeder organism and possess a large capacity to accumulate pollutants (Coppola et al., 2018; Livingstone et al., 2000; Selvin et al., 2009). The experimental setup consisted of the exposure of mussels, during 28 days, to different treatments: clean seawater (control); remediate seawater; and seawater containing Hg (50 µg/L), GO-PEI (10 mg/L) or the mixture of both. At the end of the exposure period, Hg concentrations in mussel's soft tissues, histopathological alterations, as well as biochemical responses related to mussels' metabolic, oxidative stress and neurotoxic status were evaluated.

2. MATERIALS AND METHODS

2.1 Experimental treatments

The species *Mytilus galloprovincialis* was collected in the Ria de Aveiro, Portugal (40°38'51.1"N 8°44'05.5"W), with a mean body weight of 21.3 ± 6.61 g, mean length of 6.18 ± 0.46 cm and a mean width of 3.52 ± 0.27 cm.

Organisms were transported from the field to the laboratory using plastic containers filled with seawater from the sampling site. A depuration and acclimation period of two weeks was performed placing mussels in 100 L tanks filled with artificial seawater (salinity 30 ± 1) (Tropic Marin® SEA SALT from Tropic Marine Center) at temperature 17.0 ± 1.0 ºC and pH 8.0 ± 0.10. Seawater was renewed daily during the first three days. Then water was renewed every two-three days and animals were fed with Algamac protein plus (150.000 cells/animal). Temperature 17 ºC, pH 8.0 and salinity 30 were selected considering values measured during mussel's collection and mean values observed during the year in the sampling area (IPMA, 2017).

After this period, organisms were maintained under the same temperature, salinity and pH conditions but exposed to five different treatments as described in Table 1. Per treatment three aquaria were used with six individuals each.

The concentration of mercury (Hg) used in the present study, 50 µg/L, was selected taking into consideration that this is the maximum allowable limit in wastewater discharges from industry (Directive, 2013/39/EU, 2013). The graphene as graphene oxide (GO) functionalized with polyethyleneimine (GO-PEI) 10 mg/L was selected according to the capacity of this nanostructured material (NSMs) to remove Hg (preliminary assays). The remediate seawater was prepared by spiking clean seawater with Hg (1000 mg/L of Hg, Sigma Aldrich) first, and then remediating it using GO-PEI (10 mg/L) for 24 hours, after which the material was separated from the seawater by filtration (remediated seawater).

During the experimental period of 28 days, temperature and salinity were daily checked as well as mortality. Animals were fed with Algamac protein plus (150.000 cells/animal) three times per week. During the experiment, seawater from each aquarium was renewed weekly

and treatments reestablished, including temperature, salinity and concentrations of Hg and GO-PEI. Seawater samples from each aquarium were collected immediately after weekly water exchange for Hg quantification aiming to compare real concentrations with nominal ones.

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

2.2 Synthesis and characterization of graphene oxide functionalized with polyethyleneimine

Graphene oxide (GO) water dispersion (0.4 wt% concentration from Graphenea) was directly mixed with ethyleneimine polymer (PEI) solution 50% (w/v) in water with M.W. ~750000 (Sigma Aldrich) with a ratio of 24% v/v (GO/polymer). The pH of both solutions, GO and polymer, was adjusted to 2 before mixing, using 0.1 mol/L NaOH or HCl solutions. After mixing, the solution was rapidly shaken for 10 s to form a hydrogel. The hydrogel was freeze-dried (Telstar LyoQuest HT-40, Beijer Electronics Products AB, Malmoe, Sweden) at -80 ºC obtaining three dimensional (3D) porous structures. The lyophilized samples were then washed in MilliQ water for 12 h to remove acidic residues. Finally, samples were freeze-dried again resulting in a foam (Figure1).

2.3 Mercury quantification

The quantification of Hg in seawater aliquots was performed by cold vapor atomic fluorescence spectroscopy (CV-AFS), using a PSA 10.025 Millennium Merlin Hg analyser and $SnCl_2$ (2% m/v in HCl 10% v/v) as a reducing agent (Henriques et al., 2019). The calibration curve ($r^2 \geqslant 0.999$) was plotted and checked daily, using five standard solutions

(0.0–0.5 µg/L). Detection and quantification limits obtained through blank measurements (n = 15) were 0.007 µg/L and 0.021 µg/L, respectively.Three replicate measurements were carried out for each sample (aceptable relative standard deviation between replicates: <5%).

The concentration of Hg in organism's tissues was quantified by thermal decomposition atomic absorption spectrometry with gold amalgamation (LECO model AMA-254), as described in Costley et al. (2000). Analytical quality control was performed by using Certified Reference Material, TORT 2 (*Lobster hepatopancreas*; 0.27 ± 0.06 mg/kg (total Hg)), which was analysed every day prior to the beginning of the analysis and repeated at the end of the day. All percentages of recovery were within the accepted range of 99-113%. Blanks, with the empty vessel, were analysed between samples to assure that no Hg was remained between analysis.

2.4 Biochemical markers

The selected biomarkers included: i) metabolic capacity (electron transport system activity, ETS); ii) energy-related parameters (glycogen content, GLY; total protein content, PROT); iii) phase I antioxidant enzymes activity (namely, enzymes superoxide dismutase, SOD; catalase, CAT; glutathione peroxidase, GPx; glutathione reductase, GRed); iv) phase II antioxidant isoenzymes activity (glutathione-S-transferases, GSTs); v) extent of cellular damage (lipid peroxidation levels, LPO; protein carbonylation levels, PC); vi) redox balance (assessed by the ratio between reduced (GSH) and oxidized (GSSG) glutathione); and vii) neurotoxicity (acetylcholinesterase activity, AChE).

The biochemical parameters were determined as described in Carregosa et al. (2014) and Coppola et al. (2019). All biochemical parameters were performed in duplicate and measurements made on a microplate reader (Biotek). All extractions for each biomarker were performed with specific buffers: i) samples used for ETS quantification were extracted in homogenizing buffer (0.1 mol/L Tris-HCl, pH 8.5, 15% (w/v) PVP, 153 mmol/L magnesium sulfate ($MgSO_4$) and 0.2% (v/v) Triton X-100); ii) for LPO determination samples were extracted with 20% (v/v) trichloroacetic acid (TCA); iii) for GSH/GSSG quantification

extraction was done using KPE buffer made with 0.1% Triton X-100 and 0.6% sulfosalisylic acid in KPE (0.1 mol/L potassium phosphate buffer, 5 mmol/L EDTA, pH = 7.5); iv) for the quantification of the remaining biomarkers (GLY, PROT, SOD, CAT, GPx, GRed; PC, GSTs, AChE) extraction was done using potassium phosphate buffer (50 mmol/L potassium dihydrogen phosphate; 50 mmol/L potassium phosphate dibasic; 1 mmol/L ethylenediamine tetraacetic acid disodium salt dihydrate (EDTA). These samples were disrupted using the TissueLyser II, which has the capacity of simultaneously disrupts multiple biological samples through high-speed shaking in plastic tubes with stainless steel, tungsten carbide for 60 s at 4 ºC. After this procedure samples were centrifuged for 25 min (or 10 min for GSH/GSSG) at 10000 g (or 3000 g for ETS) and 4ºC, and supernatants collected and stored at -80 ºC or immediately used.

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

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

Phase I antioxidant enzymes activity - The activity of SOD was quantified by following the method of Beauchamp and Fridovich (1971). The standard curve was obtained using SOD standards (0.25-60 U/mL). Absorbance was measured at 560 nm after 20 min of

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

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

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

coefficient Ɛ=22,308 (mol/L)$^{-1}$ cm^{-1} was used to calculated PC levels, expressed in nmol per g of FW.

Redox balance - GSH and GSSG were used as standards (0–60 μmol/L) and absorbance was measured at 412 nm (Rahman et al., 2014). The calculation of the ratio between oxidized and reduced glutathione was done in account the number of thiol equivalents (GSH/2 $*$ GSSG).

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

2.5 Histopathological markers

After the experimental period, mussels used for histopathological analyses were fixed in Bouin's fluid for 24 hours at room temperature. Then, each specimen was placed in ethanol 70% which was changed daily to wash out the fixative. Afterwards, organisms were gradually dehydrated from ethanol 70% to absolute alcohol in graded alcohols, cleared in xylene and embedded in paraffin (56–58°C). Serial sections (10 μm thick) obtained at microtome were mounted in albumin-coated slides and stained with haematoxylin and toluidine blue as described in Pinto et al. (2019). At the end of this process, all specimens were analysed to identify histopathological alterations in gonads (female and males), gills and digestive tubules. For each individual and treatment, the histopathological index (I_h) in gills and digestive tubules was obtained based on Costa et al. (2013).

2.6 Integrated biomarker response

To integrate results obtained from biochemical markers and to try to understand the general mussel's biochemical response, the integrated biomarker response (IBR) was used, which was calculated according to Beliaeff and Burgeot (2002). Thus, results obtained from all studied biomarkers were used in order to evaluate the general response of *M.*

galloprovincialis to different treatments. IBR index was calculated as described by Pinto et al. (2019). Biomarkers were arranged in the following order: SOD, CAT, LPO, GLY, GSTs, GPx, AChE, GRed, PROT, PC, ETS and GSH/GSSG. Values were discussed in terms of a general response given by the final IBR value, where higher values correspond to higher mussels' response.

2.7 Statistical analyses

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

3. RESULTS

3.1 Mortality

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

3.2 Mercury concentration in mussels and seawater

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

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

3.3 Biochemical markers

Metabolic capacity

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

differences on ETS activity were observed among treatments A, C and E (Figure 2A, Table 4).

Energy related parameters

Significantly lower GLY content was observed in organisms exposed to control (A) in comparison to organisms under the remaining treatments (Figure 2B, Table 4). Organisms exposed to remediated seawater (E) as well as organisms exposed to Hg (treatment D) showed significantly higher GLY content than organisms under control (A) and GO-PEI (B) (Figure 2B, Table 4). No significant difference was detected in organisms exposed to Hg+GO-PEI in comparison to the other ones with exception to the control (Figure 2B, Table 4).

Significantly higher PROT content was observed in organisms exposed to GO-PEI contaminated seawater (B) in comparison to the remaining treatments (Figure 2C, Table 4). No significant differences in terms of PROT content were detected in organisms exposed to Hg between A, C and E treatments. Moreover, no significant differences were found between C and E treatments (Figure 2C, Table 4). On the other hand, the PROT content in organisms exposed to control (A) was significantly lower than the values observed in mussels exposed to B, C and E treatments (Figure 2C, Table 4).

Phase I antioxidant enzymes activity

Significantly lower SOD activity was observed in organisms exposed to control (A) and organisms exposed to remediated seawater (E) in comparison to organisms exposed to contaminated treatments (B, C and D) (Figure 3A, Table 4). No significant differences on SOD activity were observed among B, C and D treatments (Figure 3A, Table 4).

Significantly higher CAT activity was observed in organisms exposed to treatments D and C, with the highest values were in organisms exposed to Hg (treatment D) (Figure 3B, Table 4). No significant differences in terms of CAT activity were observed in organisms exposed to remediated seawater in comparison to organisms exposed to A and B treatments (Figure 3B, Table 4). Moreover, significantly lower antioxidant enzymes activity was

observed in organisms exposed to control in comparison to B, C and D treatments (Figure 3B, Table 4).

Significantly lower GPx activity was observed in organisms exposed to control (A) in comparison to the remaining treatments (Figure 3C, Table 4). No significant difference was observed in mussels exposed to Hg+GO-PEI and B treatment as well as between Hg+GO-PEI and D treatment. Mussels exposed to remediated seawater presented antioxidant activity similar to organisms exposed to GO-PEI (treatment B) (Figure 3C, Table 4).

Significantly lower GRed activity was observed in organisms exposed to control (A) and to remediated seawater (E) in comparison to contaminated treatments (B, C and D) (Figure 3D, Table 4). No significant differences in terms of GRed activity were observed among treatments B, C and D (Figure 3D, Table 4).

Phase II antioxidant isoenzymes activity

Significantly lower GSTs activity was observed in organisms exposed to control (A) in comparison to organisms exposed to contaminated treatments (B, C and D) (Figure 4, Table 4). Significantly higher GSTs activity was observed in organisms exposed to Hg (C and D) with the highest values in organisms exposed to Hg alone (treatment D) in comparison to the remaining treatments (Figure 4, Table 4). Mussels exposed under remediated seawater showed no significant differences in comparison to organisms at control and GO-PEI treatments (Figure 4, Table 4).

Cellular damage

Significantly lower LPO levels were observed in organisms exposed to control (A) and to GO-PEI (B) in comparison to remaining treatments (Figure 5A, Table 4). Significantly higher LPO levels were observed in organisms exposed to Hg treatments (C and D), with the highest values in organisms exposed to Hg alone (treatment D) (Figure 5A, Table 4). Mussels exposed to remediated seawater presented higher LPO than values recorded at

control and GO-PEI treatments but significantly lower than values observed in organisms exposed to C and D treatments (Figure 5A, Table 4).

Significantly lower PC content was observed in organisms exposed to control (treatment A) in comparison to organisms exposed to the remaining treatments (Figure 5B, Table 4). No significant differences in terms of PC values were observed in organisms exposed to contaminated seawater (B, C and D) (Figure 5B, Table 4). Mussels exposed to remediated seawater presented an intermediate PC value between control and contaminated treatments (Figure 5B, Table 4).

Redox balance

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

Neurotoxicity

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

3.4. Histopathological markers

Gonads

Organisms exposed to control (A) and seawater with GO-PEI (B) showed abundant vitellogenic oocytes in gonads. Mussels exposed to Hg+GO-PEI and Hg (treatments C and D) showed a reduction in the number of the oocytes and the presence of necrotic forms (Figure 7). In females, no oocytes were detected in remediated seawater (E) (Figure 7). In

males, no differences between control and treated mussels were observed (data not shown). No enough morphological effects were detectable to calculate the I_h in gonads.

Gills

Gills of mussels kept in control condition (A) displayed epithelial cells with a regular distribution of lateral frontal cilia and no morphological abnormalities. The central vessel resulted tight. A very low number of lipofuscin aggregates and infiltrated haemocytes were observed (Figure 7). Exposure to Hg (D) leads to several histopathological alterations including cilia loss, haemocytes infiltration and enlargement of the central vessel (Figure 7). Mussels exposed to remediated seawater (E) showed less histopathological alterations but still enlargement of the gill vessel and lipofuscin aggregates were observed (Figure 7).

For gills, significantly lower I_h was obtained for organisms exposed to treatment A in comparison to treatments D and E (Figure 8A, Table 4). No significant differences were observed between I_h of mussels exposed to A, B and C treatments (Figure 8A, Table 4). Significantly higher I_h values were found in organisms exposed to Hg alone (treatment D) in comparison with organisms exposed to GO-PEI and combination of both (treatments B and C, respectively) (Figure 8A, Table 4). No significant differences in I_h were observed among B, C and E treatments (Figure 8A, Table 4).

Digestive tubules

Mussels' digestive tubules under control treatment (A) (Figure 7) result constituted by a single layer of cells surrounding a narrow or occluded tubular lumen. Atrophy increment was observed in organisms exposed to GO-PEI and Hg (treatments B and D, respectively), but especially in the treatment D (Figure 7). Lipofuscin aggregates were detected in organisms exposed to Hg+GO-PEI (C), while under remediated seawater (E) digestive tubules showed an increase in haemocyte infiltration. In all analysed organisms' necrosis was never detected.

Significantly lower I_h was obtained in organisms exposed to treatment A in comparison to the remaining treatments (Figure 8B, Table 4). Comparing I_h values significantly higher

values were observed in organisms exposed to Hg (treatment D) in comparison to the other ones (Figure 8B, Table 4). No significant differences were found between mussels exposed to C and E (Figure 8B, Table 4).

3.5. Integrated Biomarker Response (IBR)

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

4. DISCUSSION

In the last few years, a series of investigations involving the use of different synthetic materials (e.g. nanoparticles (NPs)) for water remediation (namely from metal(loid)s), have been conducted (Nupearachchi et al., 2017; Zhang et al., 2010; Coppola et al., 2019, Gehrke et al., 2015; Mohmood et al., 2016; Davidescu et al., 2015). However, the combination between nanoparticles (NPs) and metal(loid)s (for example Hg and As) can generate an interactive toxic influence in the environment, especially in aquatic systems (Coppola et al., 2019; Moore et al., 2006; Fabrega et al., 2011). Previous studies based on the NPs such as zinc oxide (ZnO_2-NPs), titanium dioxide (TiO_2-NPs), gold nanoparticles (AuNPs) and carbon nanotubes (CNTs) were focused only on NPs toxicity to marine organisms as mussels (*Mytilus edulis* and *M. galloporivincialis*), oysters (*Crassostrea gigas*) and clams (*Ruditapes philippinarum*) (Sun et al. 2016; Volland et al., 2015; Freitas et al., 2018; De Marchi et al., 2017a; Pan et al., 2012; Canesi et al., 2015; Trevisan et al., 2014). These studies reported alterations on organism's metabolic capacity and energy reserve contents as well as increase of oxidative stress and cellular damage when the organisms were in contact with NPs and metal(loid)s. However, scarce information is available on the use of NPs to remove contaminants from water and the possible toxic impacts of this remediated water towards marine organisms. Recently, Coppola et al. (2019) observed lower cellular damage and oxidative stress but higher metabolism in *M. galloprovincialis* after 28 days of exposure to remediated seawater (previously contaminated with As (1000 µg/L) and decontaminated by $MnFe_2O_4$, NPs (50 mg/L)) in comparison to contaminated seawater. Still, to the best of our knowledge, no studies evaluated the potential toxic effects, in marine organisms (e.g. bivalve), resulting from seawater decontaminated from metal(loid)s using graphene oxide functionalized with polyethyleneimine (GO-PEI).

The present study demonstrated that when under Hg contaminated seawater mussels *Mytilus galloprovincialis* presented higher concentration of mercury (Hg) in their tissues in comparison to organisms exposed to remediated seawater. These results are in line with

previous studies that showed the capacity of bivalves (e.g. oysters, clams and mussels) to accumulate metal(loid)s (Hg, lead (Pb) and As) when exposed to high concentrations of these contaminants (Coppola et al., 2019, 2018; Pirone et al., 2019; Freitas et al., 2019; Moreira et al., 2017).

Previous studies also demonstrated that the accumulation of metal(loid)s by bivalves may lead to changes on their metabolic capacity (assessed by measuring the activity of ETS) and the occurrence of cellular damages, including peroxidation of the lipid membrane and carbonylation of proteins. Furthermore, as already demonstrated from the literature, under elevated concentrations of metal(oid)s bivalves are also able to activate their antioxidant defences in order to eliminate reactive oxygen species (ROS), and avoid or limit cell damages.

The present study showed that mussel exposed to Hg (50 µg/L, corresponding to the maximum permissible concentration of Hg in wastewater discharges (treatment D)), had a decrease on their metabolic capacity in comparison with the control (treatment A). This decrease in mussel's metabolism was also observed in previous studies that assessed bivalve's metabolism after metal(loid)s exposure (Coppola et al., 2017, 2018; Bielen et al., 2016, Freitas et al., 2017). A similar pattern was observed in mussels exposed to GO-PEI (treatment B). A decrease on organism's metabolic capacity was already observed in other bivalve species (mussels, *Mytiuls coruscus,* and clams, *Ruditapes philippinarum*) exposed to NPs of carbon and ZnO_2 (De Marchi et al., 2017a; Huang et al., 2018). This fact may indicate that mussels exposed to potentially toxic substances can prevent their impacts by reducing their metabolic capacity through the closure of their valves as identified by Gosling (2003). When analysing mussels exposed to the combination of Hg and GO-PEI, results revealed an ETS capacity similar to control and remediated seawater. These findings contrast with a study conduct by Freitas et al. (2018) that showed a decrease of *R. philippinarum* metabolic capacity exposed to As and carbon nanotubes treatment. Probably the binding of Hg by GO-PEI led to a decrease in water toxicity, either because a) the GO-PEI+Hg complex remained

in solution but is less toxic to organisms than free Hg, or b) the GO-PEI+Hg complex settled, being unavailable for uptake by the mussels. Moreover, a similar metabolic capacity was observed when comparing mussels exposed to remediated seawater (treatment E) with control, which may result from lower Hg concentration observed in mussel's tissues under this condition. Similarly, Coppola et al. (2019) demonstrated that the ETS activity was significantly higher in organisms exposed to decontaminated seawater in comparison to organisms exposed to As contaminated seawater, with values obtained in mussels under decontaminated treatments closer to control than to contaminated ones.

As a response to metabolic decrease, the results obtained revealed higher PROT and especially GLY content in mussels under the Hg (treatment D) when compared to the control, demonstrating the capacity of organisms to save their energy reserves under reduced metabolic activity. In accordance to these findings, other studies have also showed lower metabolic activity associated with an increase of energy reserves content in marine organisms exposed to metal(loid)s, namely in *M. galloprovincialis* exposed to Ti (titanium), Hg and As (Coppola et al., 2017, 2018; Freitas et al., 2017; Monteiro et al., 2019). A similar pattern was observed when mussels exposed to GO-PEI (treatment B) which is also in accordance with previous studies by De Marchi et al. (2017a, b) that showed an increase of GLY and PROT content associated with lower ETS when the polychaete *Diopatra neapolitana* was exposed to graphene oxide (GO). Moreover, the increase of energy reserves was observed in mussels exposed to combination of GO-PEI and Hg (treatment C) in comparison to control. In accordance to these results, Freitas et al. (2018) highlighted the increase of energy reserves in clams *R. philippinarum* exposed to combination of As and functionalized multi-walled carbon nanotubes (f-MWCNTs). The present findings further revealed an increase of energy reserve content in organisms exposed to remediated seawater (treatment E) compared to control organisms. These findings may indicate that mussels under this treatment were able to avoid the expenditure of energy reserves, although their metabolic capacity was similar to control values.

Although mussels tend to decrease their metabolic capacity when exposed to any of the contamination treatments (B, C and D), an increase of antioxidant enzymes activities was observed in mussels exposed to these treatments, demonstrating the capacity of bivalves to activate their antioxidant defences to fight against the excess of ROS produced by the presence of Hg, GO-PEI or both contaminants acting in combination. In particular, mussels exposed to Hg (treatment D) increased the activity of antioxidant enzymes in comparison to the control and remediated seawater (treatment E). A similar pattern was observed when mussels exposed to GO-PEI (treatment B). Also, mussels exposed to the combination of Hg and GO-PEI showed an increase of SOD, CAT, GRed, GPx activities in comparison to organisms exposed to control and remediated seawater (E). These results are in line with analogues studies based on marine organisms (mussels, *M. galloprovincialis* and clams, *R. philippinarum*) exposed to metal(loid)s (Pb, Hg and As) (Pirone et al., 2019; Velez et al., 2016ab; Coppola et al., 2017, 2018), NPs (De Marchi et al., 2017ab; Xia et al., 2017; Cid et al., 2015; Gomes et al., 2012), or the combination of NPs and metal(oid)s (Coppola et al., 2019; Freitas et al., 2018). Moreover, similar antioxidant activity was observed comparing mussels exposed to control and remediated seawater (treatment E) which indicate that remediated seawater induced lower toxicity than seawater with Hg, GO-PEI or GO-PEI with Hg (treatments D, B and C, respectively) and the low Hg accumulated in mussels resulted in a similar defence activity. Similarly, a study conducted by Coppola et al. (2019) demonstrated that mussels exposed to control and As remediated seawater presented similar antioxidant enzymes activity while mussels exposed to contaminated treatments showed activation of their antioxidant defences.

Regarding the antioxidant phase II defence mechanisms, GSTs enzymes are known for their ability to catalyse the conjugation of the reduced form of glutathione (GSH) to xenobiotic substrates (Regoli and Giuliani, 2014). In the present study, comparing to control, GSTs clearly increased their activity on mussels exposed to Hg (treatment D), the combination of both contaminants (treatment C) and at a lower extent in mussels exposed to GO-PEI acting

alone. These results are in line with previous studies that revealed that GSTs are activated by the presence of contaminants (including As, Pb and Hg) in bivalves including clams and mussels (Freitas et al., 2016, 2017, Velez et al, 2016abc, Coppola et al., 2017, 2018; Jaishankar et al., 2014). Previous studies also showed an increase in bivalves' biotransformation activity related to combination of NPs and metal(loid)s exposure compared to the control (Freitas et al., 2018, Coppola et al., 2019). Lower GSTs activity in mussels exposed to treatment C and especially treatment B may indicate that GSTs are not involved in the biotransformation of NPs of carbon, namely in the biotransformation of GO-PEI into less toxic excreted substances. Similarly, De Marchi et al. (2017) showed that clams exposed to f-MWCNTs presented lower GSTs activity compared to non-contaminated clams. In this study, organisms at control treatment had a similar GSTs activity of mussels exposed to remediated seawater. Lower GSTs activity in mussels exposed to remediated seawater may indicate that the concentration of Hg in this treatment was not enough to activate these enzymes, which agrees with the recent work by Coppola et al. (2019) that reported similar GSTs activity in mussels exposed to As seawater decontaminated and control organisms.

Although antioxidant and biotransformation enzymes were activated in contaminated mussels (treatments C and D), these defence mechanisms were not enough to prevent cellular damages in mussels exposed to these treatments (identified by higher LPO and PC levels). The increase of antioxidant and biotransformation defences may prevent cellular damages, avoiding the occurrence of LPO and PC (Li et al., 2008; Yang et al., 2008; Chang et al., 2011; Coppola et al., 2017, 2018; Freitas et al., 2019). In the present study Hg, GO-PEI, and the combination of both showed cellular damages and oxidative stress (shown by lower GSH/GSSH ratio), demonstrating the inefficient capacity of antioxidant and biotransformation enzymes to eliminate the excess of ROS and avoid LPO and PC, as well as the loss of redox balance. Similar results were observed by Coppola et al. (2018) in mussels exposed to Hg concentration, by De Marchi et al. (2017a) in *D. neapolitana* under the presence of high GO concentration reduced the GHS/GSSG ratio and increased the LPO

value, and by Freitas et al. (2018) that showed the induction of cellular damages on mussels exposed to f-MWCNTs and As. Furthermore, although at a limited extent, mussels exposed to remediated seawater showed higher LPO and PC levels than control organisms and lower GSH/GSSG values. These findings may indicate that although less Hg was accumulated, lower metabolic changes were observed, and limited activation of antioxidant and biotransformation enzymes was observed in mussels exposed to this treatment that still presented oxidative stress and cellular damages. Opposite results were found by Coppola et al. (2019) that reported a decrease of LPO and PC values following by increase of ratio GSH/GSSG in mussels under seawater decontaminated from As by $MnFe_2O_4$, NPs in comparison to non-remediated conditions. Such findings may indicate higher toxicity of Hg remediated seawater in comparison to As remediated seawater.

In marine bivalves the neurotoxic impacts of different pollutants were evidenced by the decrease on AChE activity due to its high affinity for many neurotoxic compounds such as metal(loid)s and NPs (Wang et al., 2009; Maisano et al., 2017; Coppola et al 2019; Freitas et al., 2018). The present results evidenced that mussels exposed to contaminated and remediated seawater showed neurotoxicity effects, with lower AChE activity under these treatments in comparison to control organisms. In accordance, several other studies revealed similar impacts with inhibition of AChE activity when organisms are under the presence of metal(loid)s (Coppola et al. 2017, 2018; Freitas et al., 2017; Pirone et al., 2018), f-MWCNTs (De Marchi et al., 2018) or the combinations of f-MWCNTs and As (Freitas et al., 2018). Also Coppola et al. (2019) showed similar results in mussels *M. galloprovincialis* exposed to seawater previously contaminated with As and remediated by $MnFe_2O_4$, NPs.

The results obtained with IBR index showed higher values in mussels exposed to Hg contaminated seawater. These results are in agreement with several studies which showed higher IBR values in bivalves exposed to pollutants, including Hg (Bigot et al., 2011; Pinto et al., 2019; Yuan et al., 2017; Marigómez et al., 2016; Serafim et al., 2012). On the other hand,

the lowest IBR index was observed in *M. galloprovincialis* exposed to GO-PEI. This result disagreed with previously similar study by Coppola et al. 2019 which showed an increase of IBR index when mussels were exposed to NPs. Also, Xia et al. (2017) detected the highest oxidative stress and histopathological alteration in marine scallop *Chlamys farreri* under higher concentration of TiO2 NPs correlated to increase of IBR values. Still, to our knowledge, no studies evaluated the potential toxic effects, in marine organisms (e.g. bivalve), resulting from seawater decontaminated from metal(loid)s using GO-PEI or the combination of Hg and GO-PEI.

In what regards to histopathological alterations, the present study also demonstrated the toxic action of Hg in mussels' tissues. In particular, obtained results showed that female gonads exposed to Hg had semi-empty follicles with the few damaged oocytes, while loss of cilia, haemocytes infiltration and enlargement of the central vessel were observed in gills. Similar histopathological alterations were observed by other authors when exposing mussels to contaminants (Amachree et al. 2014; Cuevas et al., 2015; Sonawane, 2015; Sunila, 1988), including the study by Maina et al. (2017) that showed several histopathological changes on mussels' gills collected from a heavily Hg polluted area. The lack of cilia may lead to difficulties in filtering food and breathing problems, compromising the survival of the animals (Pagano et al., 2016). Haemocyte infiltration is often observed in organisms exposed to xenobiotics and it is sign of inflammation. Also, the digestive tubules of mussels exposed to Hg resulted seriously damaged showing an increase of atrophy with a reduction in thickness of epithelia accompanied by the enlargement of the tubules lumen and haemocytes infiltration. The digestive tubules of molluscs is the main organ for detoxification of xenobiotic compounds, and it has therefore been extensively used for toxicity assessment (Moore and Allen, 2002, Livingstone et al., 1992). Therefore, severe alterations in his organ could seriously affect survival of the organisms.

The present study further showed that while in female gonads GO-PEI induces necrosis and apoptotic events in mature oocytes, in gills the nanostructured materials did not

have any significant effect. The I_h for gills resulted quite similar to control, suggesting no uptake of the GO-PEI at gills level. At digestive tubules level, instead, exposure to GO-PEI led an increase of histopathological damages resulting in a higher I_h not only than control, but also than C (GO-PEI+Hg) and E (remediated seawater) treatments while lower than treatment D (Hg exposure). The main alterations encountered on digestive tubules were the presence of cellular atrophy with changes in the morphology of the epithelial cells, that became thinner. Although these results have no comparison in the literature due to the lack of studies performed with GO-PEI, a study on zebrafish exposed to GO for 14 days revealed several cellular alterations of the liver tissue (Chen et al., 2016). Furthermore, another study, using GO, showed numerous degenerative changes in gut and testis cells of the cricket *Acheta domesticus* (Dziewięcka et al., 2017).

Up today no studies are available on histopathological alterations induced by the combination of Hg and GO-PEI. However in this work the gills tissue did not appear to be affected. In fact, the gills' I_h results were similar to treatments A and C. Also, for the digestive tubules the histopathological impact of the Hg combined to GO-PEI resulted lower than Hg and GO-PEI by them self, even though the I_h calculated was slightly higher than control. These findings suggest that the combination of Hg and GO-PEI may prevent damages induced by Hg and hypothetically other metals too.

Regarding the results of organisms exposed to remediated seawater, the gills showed higher I_h at treatment E in comparison to control but lower values in comparison to the treatments B and D. The most common histopathological alteration observed in gills on treatment E was the presence of lipofuscin aggregates. Lipofuscin results from the oxidation of membrane lipids and proteins by free radicals. The presence of lipofuscin aggregates may indicate oxidative stress or defective detoxification of free radicals in the affected cells (Höhn and Grune 2013).

CONCLUSION

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

Acknowledgments

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

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

Figure 3. A: Superoxide dismutase activity (SOD); B: Catalase activity (CAT); C: Glutathione peroxidase activity (GPx); D: glutathione reductase (GRed), in *Mytilus galloprovincialis* exposed to different treatments (A, B, C, D and E) at the end of the experiment. Results are mean (n=9) + standard deviation Significant differences among the treatments are presented with uppercase letters.

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

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

Figure 6. Acetylcholinesterase activity (AChE), in *Mytilus galloprovincialis* exposed to different treatments (A, B, C, D and E) at the end of the experiment. Results are mean (n=9)

+ standard deviation. Significant differences among the treatments are presented with uppercase letters. n=9.

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

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

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

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

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

Hg water concentration (µg/L)

A	$\leq 0.01^a$
B	$\leq 0.01^a$
C	35 ± 3.8^b
D	49 ± 3.7^c
E	12 ± 1.9^d

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

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

Table 4. *p-values, F- values, and DFn, DFd* (Degrees of freedom numerator and denominator, respectively) obtained by pair-wise comparisons between treatments (A, B, C, D and E) for each biomarker: Electron transport system activity (ETS); Glycogen content (GLY); Total protein content (PROT); Superoxide dismutase activity (SOD); Catalase activity (CAT); Glutathione peroxidase activity (GPx); Glutathione reductase (GRed); Glutathione-S-

$\rho< 0.05$	ETS	GLY	PROT	SOD	CAT	GPx	GRed	GSTs	LPO	PC	GSH/G
A vs B	**0.0005**	**0.0003**	**0.0001**	**0.0031**	**0.0068**	**0.000**	**0.0002**	**0.0051**	0.6755	**0.0001**	**0.00**
A vs C	0.0767	**0.0001**	**0.0020**	**0.0025**	**0.0001**	**0.0001**	**0.0001**	**0.0004**	**0.0002**	**0.0001**	**0.00**
A vs D	**0.0006**	**0.0001**	0.1670	**0.0021**	**0.0001**	**0.0001**	**0.0011**	**0.0001**	**0.0001**	**0.0001**	**0.00**
A vs E	0.4620	**0.0001**	**0.0020**	0.5634	0.1772	**0.0001**	0.0920	0.0451	**0.0002**	**0.0001**	**0.00**
B vs C	0.2676	0.1255	**0.0031**	0.6695	**0.0004**	0.0591	0.1476	**0.0115**	**0.0001**	0.7717	**0.00**
B vs D	0.7690	**0.0094**	**0.0005**	0.8035	**0.0001**	**0.0262**	0.8466	**0.0001**	**0.0001**	0.5395	**0.00**
B vs E	**0.0100**	**0.0028**	**0.0059**	**0.0070**	0.4533	0.0562	**0.0240**	0.7822	**0.0001**	**0.0001**	**0.00**
C vs D	0.2126	0.4900	0.0873	0.8063	**0.0027**	0.7566	0.2952	**0.0097**	**0.0005**	0.6223	**0.00**
C vs E	0.2609	0.0750	0.8074	**0.0027**	**0.0003**	**0.0002**	**0.0008**	0.0464	0.0405	**0.0001**	**0.00**
D vs E	**0.0078**	0.1176	0.0722	**0.003**	**0.0001**	**0.0002**	0.0389	**0.0001**	**0.0001**	**0.0001**	**0.00**
F-value	*5.053*	*15.423*	*13.672*	*8.1 1*	*45.950*	*43.947*	*9.683*	*20.672*	*63.450*	*107.850*	*294.*
DFn, DFd						**4, 40**					

transferases (GSTs); Lipid peroxidation levels (LPO); protein carbonyl levels (PC); ratio between reduced and oxidized glutathione (GSH/GSSG); Acetylcholinesterase activity (AChE) and histopathological index: Gills; Digestive Tubules; Significant differences ($p \leq$ 0.05) are highlighted in bold.

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.

- Mussels bioaccumulated Hg, with lower concentration in remediated seawater

- Antioxidant defences were activated in mussels exposed to Hg, GO-PEI and Hg+GO-PEI

- Contaminated mussels showed lower metabolism, higher cellular damage and loss of redox balance

- Neurotoxicity was induced in contaminated mussels

- Histopathological alterations were limited in mussels under remediated seawater
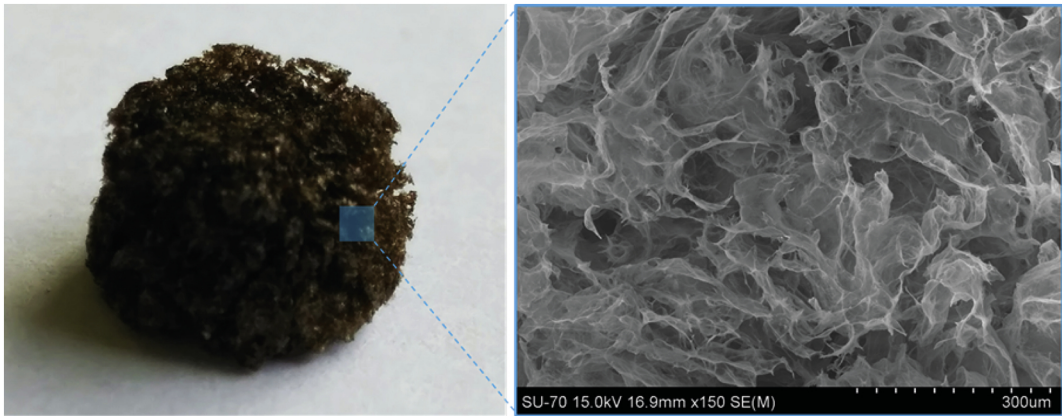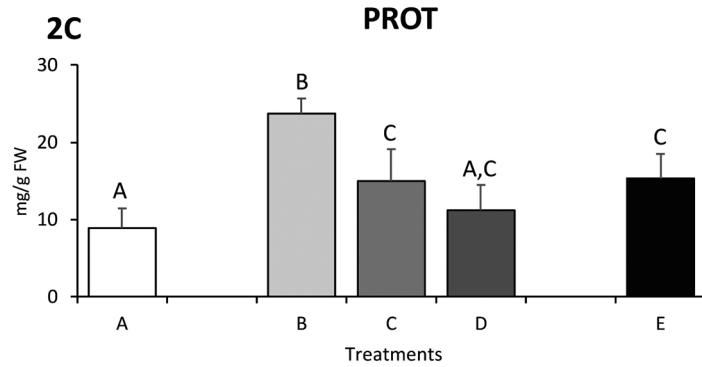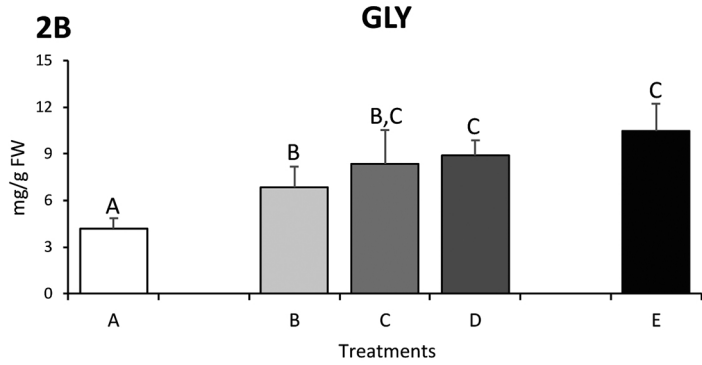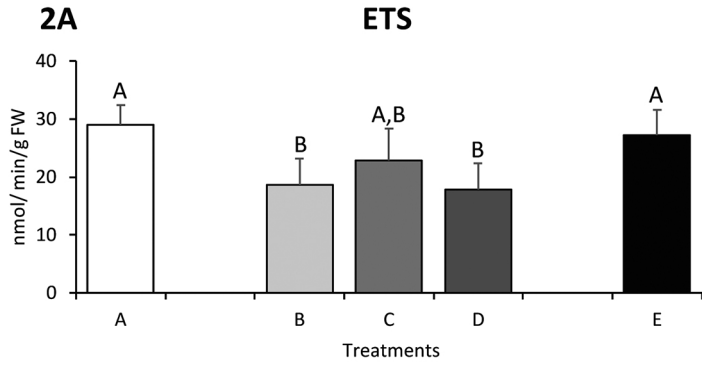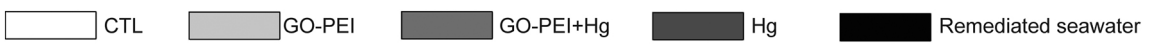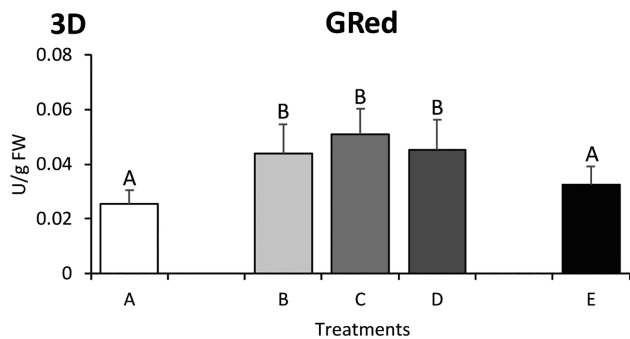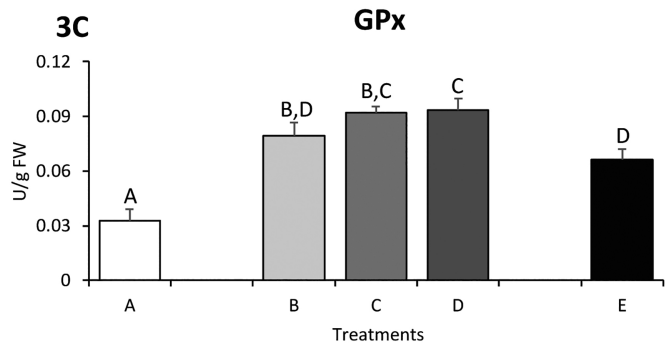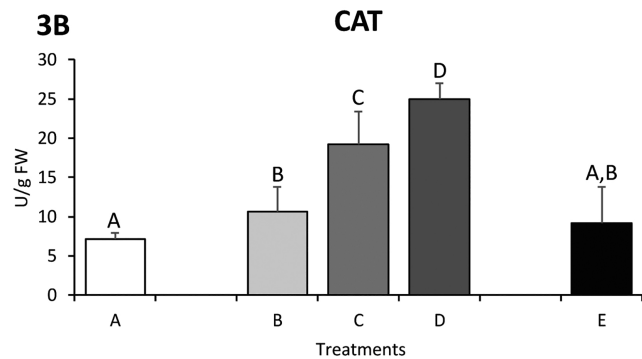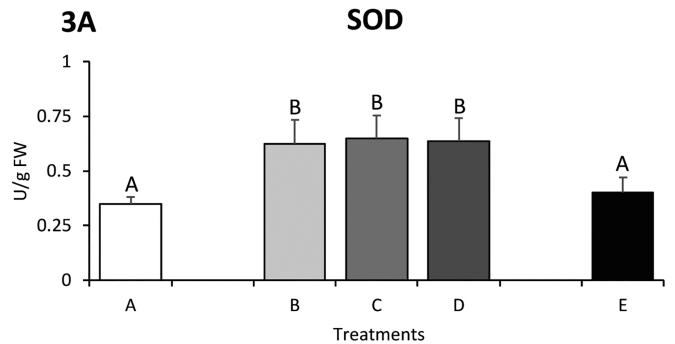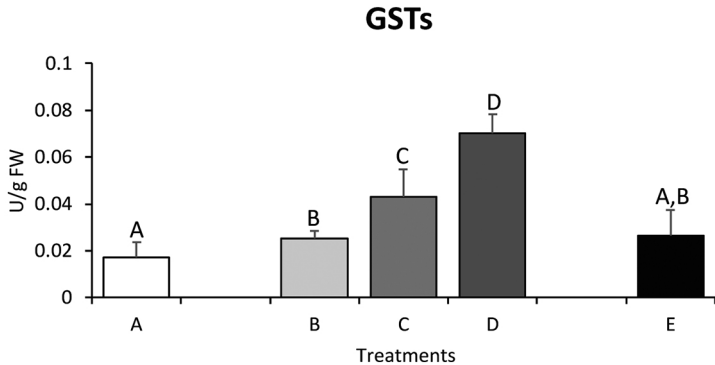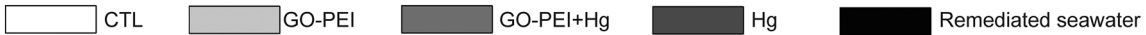
Figure 1

Figure 2
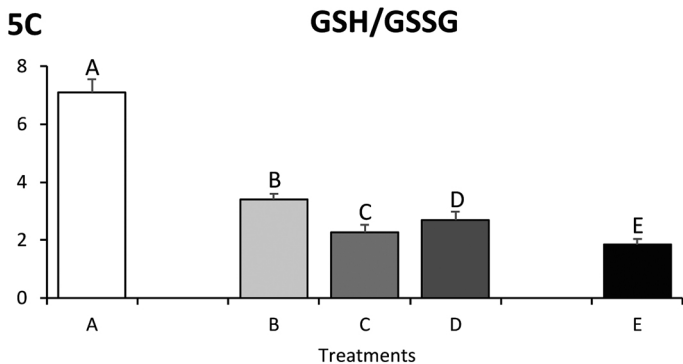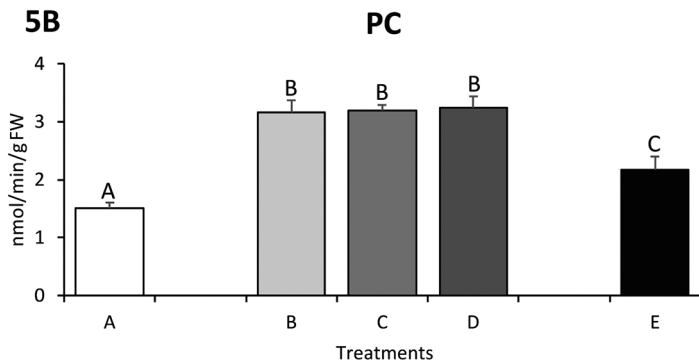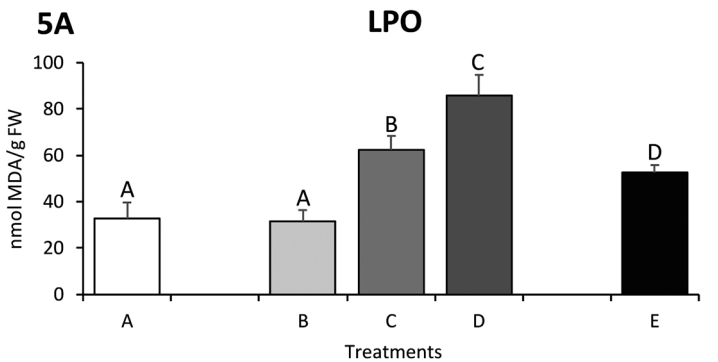
Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8