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The Role of Temperature on the Impact of Remediated Water towards Marine Organisms

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Abstract: Marine organisms are frequently exposed to pollutants, including trace metals, derived from natural and anthropogenic activities. In order to prevent environmental pollution, different approaches have been applied to remove pollutants from waste water and avoid their discharge into aquatic systems. However, organisms in their natural aquatic environments are also exposed to physico-chemical changes derived from climate change-related factors, including temperature increase. According to recent studies, warming has a negative impact on marine wildlife, with known effects on organisms physiological and biochemical performance. Recently, a material based on graphene oxide (GO) functionalized with polyethyleneimine (PEI) proved to be effective in the remediation of mercury (Hg) contaminated water. Nevertheless, no information is available on the toxic impacts of such remediated water towards aquatic systems, neither under actual nor predicted temperature conditions. For this, the present study assessed the toxicity of seawater, previously contaminated with Hg and remediated by GO-PEI, using the clam species *Ruditapes philippinarum* exposed to actual and a predicted temperature conditions. The results obtained demonstrated that seawater contaminated with Hg and/or Hg+GO-PEI induced higher toxicity in clams exposed to 17 and 22 °C compared to organisms exposed to remediated seawater at the same temperatures. Moreover, similar histological and biochemical results were observed between organisms exposed to control and remediated seawater, independently of the temperatures (17 and 21 °C), highlighting the potential use of GO-PEI to remediate Hg from seawater without significant toxicity issues to the selected marine species.

Keywords: biomarkers; toxicity; *Ruditapes philippinarum*; GO-PEI; seawater remediation; mercury; bioaccumulation

1. Introduction

Studies conducted in the last decade have demonstrated that the increase of greenhouse effect gases, such as carbon dioxide, is intrinsically related with global warming [1–5]. Global warming is not only responsible for atmospheric temperature rise but also for the increase in mean water temperature

in aquatic systems. According to the Intergovernmental Panel on Climate Change (IPCC) [3], global warming is likely to reach 1.5 °C between 2030 and 2052 if it continues to increase at the current rate. Temperature increase may be of greater magnitude in estuaries and coastal lagoons due to their physical-chemical characteristics, including low water exchange [6–8]. Associated with temperature increase in aquatic systems, it is predicted that inhabiting organisms may be subjected to deleterious effects as already shown by different authors [9–11]. Studies with bivalves already showed that temperatures exceeding an organism's thermal tolerance range can cause physiological perturbations with consequences on growth and reproduction of mussels [12–15], as well as decrease of metabolic rate and respiratory capacity in clams and mussels [16–18]. Warming conditions can also enhance reactive oxygen species (ROS) production in the cells, leading to oxidative stress in different marine species, including bivalves [19–24]. Particularly, biochemical alterations have been observed in different clam's species in response to temperature rise, including increased antioxidant capacity [18,25] and cellular damage [26,27]. Recent studies further demonstrated that change in temperature negatively impacted bivalve's embryo-larval development [28,29].

In the aquatic environment organisms may not only be subject to climate changes but are also exposed to pollutants, such as metals, derived from natural and anthropogenic activities, associated with world population growth [30–34]. Coastal ecosystems have been particularly affected by metals (e.g., lead (Pb), mercury (Hg), cadmium (Cd) and others) with well-known toxic effects towards aquatic organisms [21,35–38]. Studies conducted with top list hazardous elements [39] as Hg, Cd and arsenic (As) already showed their capacity to interfere on bivalve's biochemical performance [20,21,40–44]. In particular, studies assessing the effects of Hg in bivalves showed that this metal induced histological, physiological and biochemical impairments in oysters (*Saccostrea cucullata*, *Crassostrea gigas*) [8,45], clams (*Anodonta anatina*, *Corbicula fluminea*, *Ruditapes decussatus* and *R. philippinarum*) [8,38,46], mussels (*Perna viridis*, *Septifer virgatus*, *Mytilus galloprovincialis*, and *M. edulis*) [12,14,47,48] and cockles (*Cerastoderma edule*) [49].

Although environmental threats caused by Hg are well-known, the concentration of this metal has increased in the environment due to its use as main component in electronic products, thermometers (for measuring high temperatures) and fluorescent lamps [33,50,51]. In the aquatic environment Hg has been identified in coastal and bay waters in concentrations ranging from 0.10 ng/L (Chesapeake Bay, MD, USA) to 1200 ng/L (Marano and Grado lagoons, Venice, Italy), reaching 2700 ng/L in Bohai Sea coast (China); whereas in open seawater Hg concentrations range from 0.08 ng/L (Pacific Ocean) to 0.20 ng/L (Atlantic Ocean) [52,53]. As an attempt to remove metal (oid)s, in particular Hg, from water and avoid their discharge into aquatic systems, different approaches have been applied such as chemical precipitation [54,55], ultrafiltration [56,57], reverse osmosis [58], nanofiltration [56–58], and sorption on nanomaterials [59–62]. The main concerns regarding these methodologies are the fact that they are low cost but inefficient, or efficient but expensive [63–65]. In order to overcome these issues, Henriques et al. [66] synthesized and characterized new nanostructured materials (NSMs), based on graphene oxide (GO) that proved to be effective to remove Hg from water. The remarkable breakthroughs in research on graphene-based materials (GBM) have revealed its great potential for environmental remediation. GO can be produced by oxidation of graphite in laboratory [67], composed on a substrate or porous material and used as a membrane. Different GBM have been developed for water desalination, sorption processes, degradation of organic contaminants and the removal of potential toxic elements from polluted waters [68–70]. Recently our group developed a material based on GO functionalized with polyethyleneimine (PEI) that proved to be effective in the remediation of Hg 50 μg/L contaminated seawater, with 81% of removal efficiency after just 6 h [71]. Nevertheless, up to now, no information exists on the toxicity of the remediated seawater, i.e., no information is available on possible effects in aquatic organisms exposed to water after the remediation treatment.

Although recent literature has demonstrated the impacts of temperature in bivalves physiological and biochemical performance, the co-occurrence of temperature increase and pollutants is not yet well understood. The simultaneous occurrence of temperature rise and the presence of pollutants

may result in organisms increased sensitivity to each of the stressors but may also alter pollutants' toxicity, leading to additive or antagonist effects as reported in several studies [23,28,29,47,72–74]. According to Coppola et al. [21], oxidative stress was enhanced in *M. galloprovincialis* exposed to Hg under warming conditions.

For the aforementioned, the present study aimed to assess the possible toxicity of seawater, previously contaminated with Hg and remediated by GO-PEI, using the clam species *R. philippinarum* under different temperature scenarios, to assess the effects of temperature rise on the impacts induced by remediated water. Previous studies already demonstrated that this clam species is a good bioindicator, being commonly used in field and laboratory studies to evaluate the effects derived from the exposure to different pollutants, including metals [38,43,75], drugs [76–78], or nanoparticles [79,80]. *R. philippinarum* specimens were exposed for 28 days, at different treatments, including clean seawater (control-CTL); remediate seawater; and seawater containing Hg (50 µg/L), GO-PEI (10 mg/L) or the mixture of both. Each treatment was conducted under control (17 °C) and increased (22 °C) temperatures. At the end, Hg concentrations in clam's soft tissues, histopathological alterations, as well as biochemical responses related to clams' metabolic, cellular damage and oxidative stress status were measured.

2. Materials and Methods

2.1. Laboratory Conditions and Experimental Setup

The species *Ruditapes philippinarum* were collected in the Mira channel (Ria de Aveiro lagoon, Portugal), with a mean total weight of 12.1 ± 2.6 g, mean length of 3.53 ± 0.29 cm and a mean width of 4.53 ± 0.42 cm.

In the laboratory clams were placed under acclimation (one week), with water conditions similar to the sampling site. After this initial period, clams were divided in two groups: one exposed at 17 ± 1 °C and another at 22 ± 1 °C (with a gradual temperature increase), for the acclimation to test conditions during an extra week. Throughout these two weeks all organisms were maintained in artificial seawater (salinity 30 ± 1) at pH 8.0 ± 0.1 and constant aeration. Seawater was renewed every 2–3 days, after which animals were fed with Algamac protein plus. Environmental conditions measured during clam's field sampling (temperature 17 °C, pH 8.0, salinity 30) were considered as control levels that were also in agreement with the mean values observed during the year in the sampling area [81]. The highest tested temperature (22 °C) was selected considering predicted global warming conditions [3].

After this period, organisms were maintained during 28 days in two groups under test temperatures, salinity and pH conditions, with organisms divided in five different treatments as described in Table 1. Per treatment three aquaria were used with six individuals in each aquarium (5 L glass aquaria).

Table 1. Experimental treatments. GO-PEI: Graphene oxide functionalized with polythyleneimine; Hg: mercury.

Treatments	Description
CTL	Artificial seawater (Hg 0.0 µg/L + GO-PEI 0.0 mg/L)
GO-PEI	Artificial seawater with GO-PEI 10 mg/L
Hg+ GO-PEI	Artificial seawater with Hg 50 µg/L and GO-PEI 10 mg/L
Hg	Artificial seawater with Hg 50 µg/L
Remediated seawater	Artificial seawater previously contaminated with Hg (50 µg/L), and remediated by GO-PEI (10 mg/L) during 24 h.

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 [82]. A concentration of 10 mg/L of graphene oxide (GO) functionalized with polyethyleneimine (GO-PEI)

was selected according to the capacity of this nanostructured material (NSMs) to remove Hg from seawater (preliminary assays). The remediated seawater was prepared by the contamination of clean seawater with Hg using a defined volume of a stock solution (1000 mg/L of Hg, Sigma Aldrich) followed by remediation with GO-PEI (10 mg/L) during 24 h, after which the material was separated from the seawater by filtration.

Throughout the experimental period, water conditions were checked daily as well as clams' mortality. Animals were fed with Algamac protein three times per week. During this period, seawater from each aquarium was renewed weekly and treatments reestablished, including temperatures, salinity and concentrations of Hg and GO-PEI. Seawater samples from each aquarium were collected immediately after weekly water exchange for Hg quantification, to compare real concentrations with nominal ones.

At the end of the exposure clams were meticulously opened to separate the shell from soft tissue. One clam (soft tissue) per aquarium (three per treatment) was fixed in Bouin's fluid for 24 h at room temperature for the histological evaluation. For biochemical analyses and Hg quantification six organisms per treatment (two per aquarium) were frozen in liquid nitrogen and manually homogenized with a mortar and a pestle. Each organism' soft tissue was divided into aliquots of 0.3 g fresh weight (FW) and stored at −80 °C.

2.2. Synthesis and Characterization of Graphene Oxide Functionalized with Polyethyleneimine

Graphene oxide water dispersion (0.4 wt % concentration from Graphenea) was directly mixed with ethyleneimine polymer (PEI) solution at 50% (*w*/*v*) in water, with molecular weight (M.W.) ~750,000 and a ratio GO/polymer of 24% *v*/*v*. 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 frozen at −80 °C 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-like macrostructure (Figure 1).

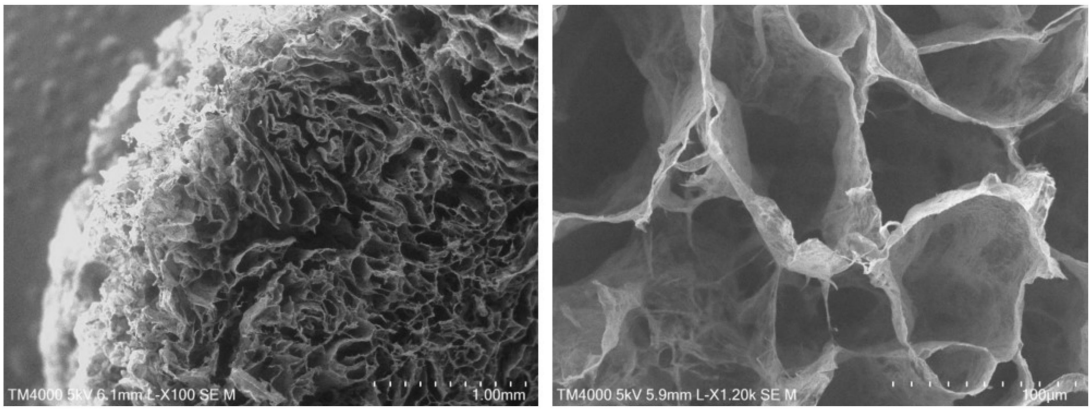

Figure 1. SEM (scanning electron microscopy) images of GO-PEI macrostructure obtained after lyophilization evidencing the porous nature of this materials.

2.3. Mercury Quantification

The quantification of Hg in seawater aliquots was performed following Henriques et al. [83] methods using cold vapor atomic fluorescence spectroscopy (CV-AFS).

The concentration of Hg in the organism's tissues was quantified by thermal decomposition atomic absorption spectrometry with gold amalgamation, as described in Costley et al. [84].

2.4. Biochemical Markers

The selected biomarkers included: (i) metabolic capacity (electron transport system, ETS); (ii) antioxidant enzymes activity (superoxide dismutase, SOD; glutathione peroxidase, GPx; glutathione

reductase, GRed); (iii) extent of cellular damage levels (lipid peroxidation, LPO; protein carbonylation, PC); (iv) redox balance (ratio between reduced (GSH) and oxidized (GSSG) glutathione content). The biochemical parameters were determined as described in Coppola et al. [79]. All biochemical parameters were performed in duplicate and measurements made on a microplate reader (BioTek Synergy HT).

2.5. Histopathological Measurements

After the experimental period, the clams fixed in Bouin's fluid for histopathological analyses were placed in ethanol 70% which was changed daily to wash out the fixative left over. Afterwards, organisms were gradually dehydrated from ethanol 70% to absolute alcohol in graded alcohols, cleared in xylene, embedded in paraffin (56–58 °C), and serial sections (7 µm thick) were obtained using a microtome as described in Pinto et al. [85]. Histopathological alterations in gills and digestive tubules were identified as described previously [86,87].

2.6. Integrated Biomarker Response

The integrated biomarker response (IBR) index was calculated according to Beliaeff and Burgeot [88] and detailed in Coppola et al. [79]. Biomarkers were arranged in the following order: ETS, SOD, GPx, GRed, LPO, PC, and GSH/GSSG. Values were discussed in terms of a general response given by the final IBR value, where higher values correspond to higher clams' response.

2.7. Statistical Analyses

Mercury concentration in seawater and the clam's soft tissues, biochemical markers and histopathological indices, obtained for each tested treatment, were submitted to a statistical hypothesis testing using permutational analysis of variance [89]. The null hypotheses tested were: (i) for each response (Hg concentration in seawater and clams, biomarkers and histopathological indices), no significant differences were observed among treatments (CTL, GO-PEI, Hg+GO-PEI, Hg and remediated seawater) at 17 °C (uppercase letters in Table 2) and 22 °C (lowercase letters in Table 2); (ii) for each response and for each treatment no significant differences existed between temperatures (17 and 22 °C), represented in Table 2 by an asterisk.

The matrix expressing histopathological and biochemical markers as well as Hg concentrations per treatment under both temperatures were normalized and the Euclidean distance calculated among centroids was visualized in principal coordinates ordination (PCO) analysis. In the PCO graph, the variables presenting a correlation higher than 75% with treatments spatial distribution were represented as superimposed vectors.

3. Results

3.1. Mortality

At the end of the experimental period the highest mortality (44%) was recorded in clams submitted to Hg at both temperatures (17 and 22 °C) and in organisms exposed to GO-PEI at 17 °C and Hg+GO-PEI at 22 °C (44%). Lower mortality was observed in GO-PEI at 22 °C and Hg+GO-PEI treatments at 17 °C (11% and 33%, respectively). The organisms exposed to remediated seawater at both temperatures presented the same mortality (33%). A mortality rate of 22% was recorded in CTL treatment at 17 and 22 °C. Due to high mortality rates observed in all tested conditions, including control, the results achieved must be considered with precaution.

3.2. Mercury Concentration in Seawater and Clams

Mercury concentration in seawater samples collected weekly in aquaria (immediately after water renewal and spiking) from Hg+GO-PEI and Hg treatments at 17 and 22 °C were very close to the nominal concentration (50 µg/L), while Hg levels in CTL and GO-PEI treatments at both

temperatures were below the limit of quantification (<LOQ) (Table 2). Under 17 °C, significantly lower Hg concentrations were observed in seawater samples collected from remediated seawater comparing with those contaminated by Hg+GO-PEI and Hg treatments. Similar results were observed under 22 °C. Between temperatures no significant differences were found regardless the treatment (Table 2).

At 17 °C, significantly lower Hg concentration was found in clams exposed to remediated seawater compared with Hg values measured in clams subjected to Hg+GO-PEI and Hg treatments. Under 22 °C, clams showed significantly lower Hg concentrations when exposed to remediated seawater and to Hg+GO-PEI in comparison to organisms under Hg treatment. Between temperatures, significant differences were found in all treatments except for GO-PEI, with clams exposed to 17 °C presenting significantly lower Hg concentrations in specimens under CTL and Hg treatments while significantly higher values were observed at 17 °C in clams exposed to Hg+GO-PEI and remediated seawater.

3.3. Biochemical Markers

All results obtained from biochemical markers were expressed as mean ± standard deviation and values are shown in Table 2.

3.3.1. Metabolic Capacity

At 17 °C significantly lower ETS activity was detected in organisms exposed to Hg+GO-PEI and remediated seawater compared to the remaining treatments. At 22 °C, significant differences were observed among treatments with exception to clams exposed to Hg+GO-PEI and CTL. Organisms exposed to Hg (22 °C) and remediated seawater (17 °C) showed the highest and the lowest ETS activity, respectively. Between temperatures, significant differences were observed at CTL and GO-PEI treatments, with higher metabolic capacity under 17 °C compared to 22 °C.

3.3.2. Antioxidant Enzymes Activity

At 17 and 22 °C, significantly higher SOD activity was observed in clams exposed to Hg in comparison to organisms under the remaining treatments. Between temperatures, significant differences were observed at CTL and Hg treatments, with higher SOD activity at 17 °C compared to 22 °C.

At 17 °C, significant differences in GPx activity were observed between CTL, GO-PEI and Hg+GO-PEI, with the highest values in *R. philippinarum* under Hg+GO-PEI treatment. No significant differences were observed between clams exposed to CTL and remediated seawater, as well as among GO-PEI, Hg and remediated seawater. At 22 °C, significantly lower antioxidant defence was observed at CTL compared to GO-PEI and Hg treatments. Between temperatures, significantly higher GPx activity was only recorded in clams exposed to Hg+GO-PEI at 17 °C compared to treatment at 22 °C.

At 17 °C, significant differences in terms of GRed activity were observed between GO-PEI, Hg and remediated seawater showing the highest values in organisms under GO-PEI treatment. Specimens exposed to remediated seawater did not present significant differences between CTL and Hg+GO-PEI. In addition, Hg+GO-PEI condition did not evidence difference with Hg exposed clams. At 22 °C, significant differences were observed among all treatments except between clams exposed to Hg and remediated seawater. Under this temperature (22 °C), organisms exposed to GO-PEI and Hg treatments showed the highest and the lowest GRed activity, respectively. Between temperatures, significantly lower enzymatic activity was measured in clams under CTL at 17 °C compared to 22 °C, while bivalves exposed to Hg treatments presented significantly higher enzymatic activity at 17 °C.

Table 2. (i) Hg quantification: water samples ([Hg] W) µg/L collected immediately after the weekly water renewal for each treatment; clams ([Hg] C) mg/Kg collected at the end of the experiment. Levels not detectable (below the limit of quantification, <LOQ). (ii) Biochemical markers in *Ruditapes philippinarum* collected 28 days after the beginning of the experiment: electron transport system (ETS) activity nmol/min/g fresh weight (FW); superoxide dismutase activity (SOD) U/g FW; glutathione peroxidase (GPx) activity U/g FW; glutathione reductase (GRed) activity U/g FW; lipid peroxidation levels (LPO) nmol MDA/g FW; protein carbonyl levels (PC) nmol/g FW; ratio between reduced and oxidized glutathione (GSH/GSSG). (iii) Histopathological markers collected 28 days after the beginning of the experiment: Gills (*Ih* G) *ih*; digestive tubules (*Ih* DT) *ih*. Results are mean + standard deviation. Statistical differences among the treatments at 17 and 22 °C (The meaning of the letters is in the legend. Uppercase letter are used to identify statistical differences among treatments at 17 °C. While lowercase letter are used to identify statistical differences among treatments at 22 °C were presented with different uppercase letters and lowercase letters, respectively. Significant differences between treatments 17 °C vs. 22 °C (*) are presented with asterisks. The highest values for each biomarker were highlighted in bold, while the lowest values were underlined.

		CTL			GO-PEI			Hg+GO-PEI			Hg			Remediated Seawater		
		17 °C		22 °C	17 °C		22 °C	17 °C		22 °C	17 °C		22 °C	17 °C		22 °C
Hg Quantification	*[Hg]W*	<LOQ		<LOQ	<LOQ		<LOQ	50.0 ± 3.90 ^{A}		49.6 ± 3.26 ^{a}	50.4 ± 2.95 ^{A}		49.4 ± 5.09 ^{a}	11.5 ± 3.71 ^{B}		11.5 ± 3.71 ^{b}
	[Hg]C	0.18 ± 0.02 ^{A}	*	0.29 ± 0.01 ^{a}	0.14 ± 0.03 ^{B}		0.16 ± 0.0056 ^{b}	7.3 ± 0.63 ^{C}	*	3.6 ± 0.29 ^{c}	9.1 ± 1.9 ^{C}	*	12 ± 2.5 ^{d}	4.7 ± 0.34 ^{D}	*	2.9 ± 0.98 ^{c}
Biochemical Markers	*ETS*	31.7 ± 4.86 ^{A}	*	25.5 ± 5.44 ^{a}	35.5 ± 5.99 ^{A}	*	15.5 ± 3.42 ^{b}	13.2 ± 0.76 ^{B}		20.2 ± 7.09 ^{a,b}	30.1 ± 4.65 ^{A}		**39.0 ± 2.82 ^{c}**	11.1 ± 0.60 ^{C}		11.1 ± 1.34 ^{d}
	SOD	0.43 ± 0.030 ^{A}	*	0.24 ± 0.040 ^{a}	0.46 ± 0.050 ^{A}		0.34 ± 0.03 ^{a}	0.41 ± 0.05 ^{A}		0.33 ± 0.02 ^{a}	**0.77 ± 0.07 ^{B}**	*	0.54 ± 0.13 ^{b}	0.28 ± 0.03 ^{A}		0.29 ± 0.01 ^{a}
	GPx	0.03 ±0.005 ^{A}		0.03 ± 0.004 ^{a}	0.04 ± 0.004 ^{B}		**0.05 ± 0.01 ^{b}**	0.06 ± 0.008 ^{C}	*	0.04 ± 0.004 ^{a,b}	0.04 ± 0.009 ^{B,C}		0.04 ± 0.006 ^{b,c}	0.04 ± 0.01 ^{A,B}		0.04 ± 0.006 ^{a,b}
	GRed	0.030 ± 0.0040 ^{A,D}	*	0.060 ± 0.010 ^{a}	0.14 ± 0.020 ^{B}		**0.12 ± 0.010 ^{b}**	0.060 ± 0.010 ^{C,D}		0.070 ± 0.0070 ^{c}	0.060 ± 0.010 ^{C}	*	0.030 ± 0.0070 ^{d}	0.035 ± 0.014 ^{D}		0.041 ± 0.013 ^{d}
	LPO	15.4 ± 0.75 ^{A}		14.9 ± 1.09 ^{a,d}	16.2 ± 0.64 ^{A}	*	13.5 ± 0.48 ^{a}	20.6 ± 0.58 ^{B,C}		22.6 ± 3.67 ^{b}	22.0 ± 0.25 ^{B}	*	**28.2 ± 0.384 ^{c}**	17.3 ± 3.049 ^{A,C}	*	14.9 ± 0.645 ^{d}
	PC	0.90 ± 0.13 ^{A}		0.89 ± 0.06 ^{a}	0.99 ± 0.19 ^{A}		0.97 ± 0.11 ^{a}	0.95 ± 0.08 ^{A}		0.88 ± 0.04 ^{a}	**1.03 ± 0.13 ^{A}**		*0.87 ± 0.06 ^{a}*	0.89 ± 0.007 ^{A}		0.95 ± 0.12 ^{a}
	GSH/GSSG	0.49±0.05 ^{A}	*	**0.75 ± 0.04 ^{a}**	0.13 ± 0.02 ^{B}	*	0.23 ± 0.04 ^{b}	0.13 ± 0.01 ^{B}	*	0.21 ± 0.03 ^{b}	0.12 ± 0.008 ^{B}	*	0.22 ± 0.04 ^{b}	0.11 ± 0.02 ^{B}	*	0.23 ± 0.04 ^{b}
Histopathological Index	*Ih G*	0.05 ± 0.02 ^{A}	*	0.08 ± 0.03 ^{a}	0.15 ± 0.02 ^{B}		0.13 ± 0.05 ^{b}	0.17 ± 0.08 ^{B}		0.18 ± 0.07 ^{c}	0.27 ± 0.05 ^{C}	*	**0.33 ± 0.05 ^{d}**	0.12 ± 0.06 ^{B}		0.16 ± 0.06 ^{c,b}
	Ih DT	0.23 ± 0.09 ^{A}		0.16 ± 0.07 ^{a,b}	**0.38 ± 0.001 ^{B}**	*	0.21 ± 0.19 ^{a}	0.23 ± 0.07 ^{A}	*	0.09 ± 0.001 ^{b}	0.31 ± 0.001 ^{C}	*	0.37 ± 0.05 ^{c}	0.21 ± 0.11 ^{A}		0.19± 0.13 ^{a}
IBR				2.81	2.01		4.16	3.44		2.39	3.38		**4.27**	2.20		2.09

3.3.3. Cellular Damage

Under 17 °C, significantly higher LPO levels were observed only in organisms exposed to Hg compared to the remaining treatments with exception to Hg+GO-PEI. Clams under CTL conditions showed significantly lower cellular damage when compared with those at Hg+GO-PEI and Hg treatments. At 22 °C, significant differences were observed among all treatments with exception to CTL, GO-PEI and remediated seawater. Between temperatures, significantly higher cellular damage was shown at GO-PEI and remediated seawater treatments at 17 °C compared to 22 °C, while significantly lower LPO levels were found in organisms exposed to Hg at 17 °C.

No significant PC levels at 17 °C and/or 22 °C differences were observed among treatments. No significant differences were observed between temperatures regardless of the treatment tested.

3.3.4. Redox Balance

At 17 °C, significantly higher GSH/GSSG ratio were observed in *R. philippinarum* exposed to CTL when compared to all the other treatments. Similar results were obtained in organisms under 22 °C. Between temperatures, significant differences were observed among all treatments, with higher GSH/GSSG ratio under 22 °C compared to 17 °C.

3.4. Histopathological Measurements

All results obtained from histopathological measurements were expressed as mean ± standard deviation for seawater and clams (Table 2).

3.4.1. Gills

Figure 2 shows the haemocytes infiltration (arrows), evident enlargement of the central vessel (long arrows), abundance of lipofuscin aggregates (*) in gills for each treatment at 17 °C and 22 °C. At 17 °C significant gills histopathological (*ih*) differences were presented between all treatments with the exception among GO-PEI, Hg+GO-PEI and remediated seawater. Moreover, organisms exposed to CTL and Hg showed the lowest and the highest histopathological alterations, respectively. At 22 °C significant *Ih* differences were identified among all treatments in comparison to CTL. No significant differences were observed among clams exposed to remediated seawater compared to GO-PEI and Hg+GO-PEI. Organisms exposed to CTL and Hg showed the lowest and the highest gill alterations, respectively. Between the temperatures, significant differences were identified at CTL and Hg, with higher histopathological alterations under 22 °C compared to 17 °C.

3.4.2. Digestive Tubules

The haemocytes infiltration (arrows), abundance of lipofuscin aggregates (*) and atrophied (at) in digestive tubules of each treatment at both temperatures are shown in Figure 2. At 17 °C significant differences were shown among the treatments with the exception between CTL, Hg+GO-PEI and remediated seawater. At this temperature (17 °C), organisms exposed to remediated seawater showed the lowest *Ih* values, while the highest values were found at GO-PEI treatment. Organisms under 22 °C showed no significant *Ih* differences among CTL, GO-PEI, Hg+GO-PEI and remediated seawater. Organisms exposed to Hg showed the highest *Ih* values. Between the temperatures, significantly higher *Ih* values were found at 17 °C for GO-PEI and Hg+GO-PEI treatments, while in clams exposed to Hg higher values were obtained at 22 °C.

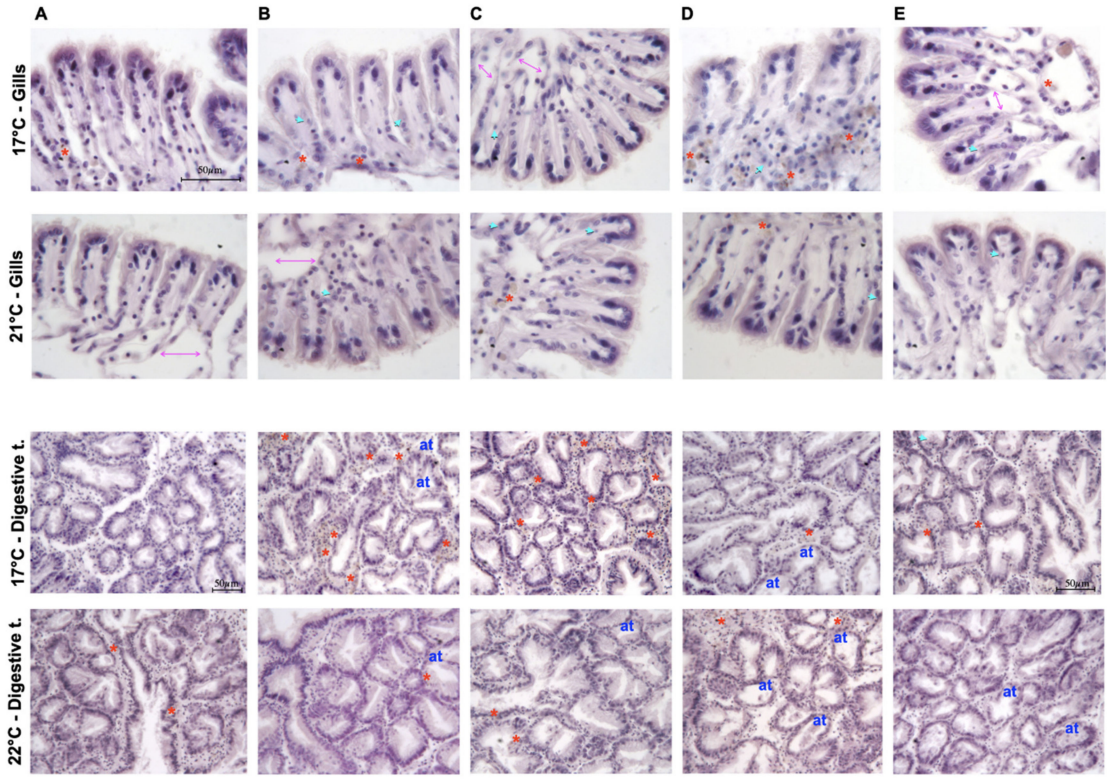

Figure 2. Micrographs of different tissues in *Ruditapes philippinarum* exposed to different treatments stained with haematoxylin. (i) Gills: haemocytes infiltration (light blue arrows), evident enlargement of the central vessel (pink long arrows), abundance of lipofuscin aggregates (red asterisks); (ii) digestive tubules: haemocytes infiltration (light blue arrows), abundance of lipofuscin aggregates (red asterisks) atrophied digestive tubules (blue *at*). Scale bar = 50 µm.

3.5. Integrated Biomarker Response (IBR)

The highest IBR value (4.27) was found for the clams exposed to Hg at 22 °C, which indicates higher impacts in Hg contaminated organisms under warming conditions. By contrast, lower IBR values were observed in organisms exposed to GO-PEI at 17 °C (2.01) and remediated seawater (2.20 and 2.09 at 17 and 22 °C, respectively). The results obtained for organisms exposed to the remaining treatments were showed in Table 2.

3.6. Multivariate Analysis

The principal coordinates ordination analysis (PCO) obtained for Hg in clams and water, biochemical and histopathological alterations is shown in Figure 3, with the PCO axis 1 explaining 45.3% of the total variation and PCO axis 2 21.6%. PCO1 separated organisms exposed to CTL (17 and 22 °C), remediated seawater (17 and 22 °C) and GO-PEI (22 °C) in the positive side from the remaining treatments in the negative side. PCO2 separated organisms exposed to GO-PEI (17 °C) and Hg (17 °C) in the negative side from the remaining treatments in the positive side. Remediated seawater (17 and 22 °C) as well as CTL (17 and 22 °C) clams were associated with GSH/GSSG values; clams exposed to GO-PEI (17 °C) were close related with the highest GRed and PC values; Hg contaminated clams at 17 °C were associated to the highest values of SOD; while Hg-contaminated clams at 22 °C were associated with the highest values of LPO and Hg concentrations in water and tissues.

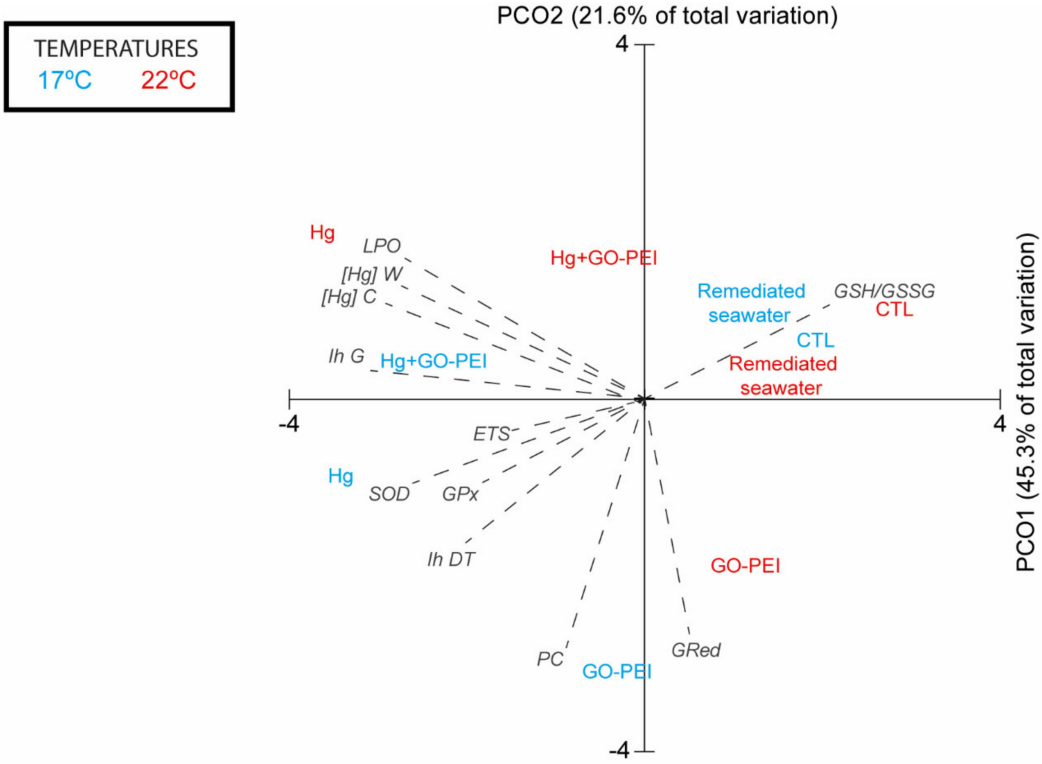

Figure 3. Principal coordinated ordination (PCO) analyses based on biochemical parameters, measured in *Ruditapes philippinarum* exposed to different conditions (CTL, GO-PEI, Hg+GO-PEI, Hg and Remediated seawater). Pearson correlation vectors are superimposed as supplementary variables, namely biochemical data (r > 0.75): ETS, PC, SOD, LPO, GPx, GRed, GSH/GSSG, [Hg] C and [Hg] W, *Ih* DT and *Ih* G.

4. Discussion

In the present study the increase of seawater temperature influenced the accumulation of Hg, with higher concentration measured in *Ruditapes philippinarum* exposed to warming conditions (22 °C) compared to organisms at 17 °C. Higher Hg concentration in clams exposed to 22 °C may be related to increased metabolic capacity (measured by the electron transport system activity) observed at 22 °C. The present findings further revealed that clams under Hg+GO-PEI and remediated seawater treatments presented higher Hg concentration at control temperature (17 °C) than in warming conditions (22 °C), and in this case no differences were observed in terms of clam's metabolic capacity at both temperature regimes. These results can indicate that in the presence of GO-PEI (Hg+GO-PEI and remediated seawater treatments) Hg is not as easily accumulated at 22 °C as it is at 17 °C, a result that will need further investigation. Similarly, Leite et al. [86] demonstrated that *Mytilus galloprovincialis* presented the highest accumulation of rutile at 18 °C compared to 22 °C. The authors explained that at higher temperature the lowest accumulation could be explained by higher precipitation of larger aggregates limiting the availability and accumulation of the contaminated particles. Nevertheless, higher accumulation of metals in bivalves under temperature rise in comparison to control temperature was previously observed in mussels (*M. galloprovincialis*) exposed to As [20], which was also associated with increased metabolic activity in these species. Nevertheless, Sanni et al. [90] demonstrated that temperature (12, 20 and 28 °C) did not influence the accumulation of Cd in the oyster *Crassostrea virginica*; and Izagirre et al. [74] showed similar results for the species *M. galloprovincialis* exposed to Cd at 18 and 26 °C. Therefore, the present and previous studies indicate that bioconcentration may not only depend on the exposure concentration levels, exposure time and temperature conditions but also the element and its behaviour.

The present findings clearly demonstrated that biochemical responses and histopathological alterations were close related to stress induced by Hg bioaccumulation, with a lower influence of the temperature on a clam's performance. These findings are evidenced by the PCO analysis, where clams exposed to control (CTL) and remediated seawater at both temperatures were grouped together in terms of biochemical and histopathological responses; clams exposed to GO-PEI at 17 and 22 °C were graphically close indicating similar biochemical performance of organisms under these conditions; while clams exposed to Hg and Hg+GO-PEI were apart from all the other treatments, indicating similar effects induced by the presence of Hg. These findings suggest that: (i) regardless of the temperature, clams exposed to remediated seawater were exposed to low stress conditions due to lower Hg exposure concentration and accumulation, with biochemical and histopathological responses similar to CTL organisms; (ii) clams exposed to Hg at both temperatures and Hg+GO-PEI at 17 °C presented the highest Hg concentrations in their tissues and showed similar biochemical performance and histopathological alterations.

Similar biochemical and histopathological alterations induced in clams exposed to remediated seawater and CTL conditions at both temperatures revealed a low effect of temperature and indicate the low toxicity of remediated seawater as a consequence of low Hg concentration in this water. Higher GSH/GSSG values observed in clams under control conditions (17 and 22 °C) evidence the maintenance of the redox balance under these conditions, regardless of the temperature of exposure. It is well known that under non-stressful conditions organisms tend to have higher reduced glutathione (GSH) in comparison to oxidized glutathione (GSSG) content, with higher GSH/GSSG values at non-stressful conditions [20,21,75,80]. The results obtained indicate that the concentrations of Hg in remediated water were not high enough to induce significant alterations in clams compared to organisms in control conditions. Previous studies exposing bivalves to similar Hg concentrations also demonstrated limited biochemical impacts [38,44]. Furthermore, similar findings were already revealed by Coppola et al. [79] when exposing bivalves to seawater previously contaminated by As and remediated by manganese-ferrite ($MnFe_2O_4$) nanoparticles.

Clams exposed to GO-PEI, both under control and increased temperatures, evidenced limited biochemical and histopathological alterations, although higher than alterations observed in organisms under remediated seawater and CTL treatments but lower than clams exposed to Hg and Hg+GO-PEI treatments. The present results also demonstrated no interactive effects between the presence of GO-PEI and temperature rise, with no clear separation on a clam's responses exposed to GO-PEI at control and increased temperature. Previous studies investigating the impacts of similar nanoparticles evidenced limited biochemical alterations in bivalves [40,79,80].

In the presence of Hg, both with or without GO-PEI, clams evidenced higher Hg concentrations resulting in greater biochemical and histopathological alterations. This is due to the activation of antioxidant mechanisms in clams exposed to these conditions, which were inefficient to avoid cellular damage, especially under warming conditions. Considering that clams exposed to Hg and Hg+GO-PEI under warming conditions presented higher cellular damage than at 17 °C, and since Hg concentrations were lower in clams exposed to Hg+GO-PEI at 22 °C than at 17 °C, these results indicate that temperature rise will enhance the impacts caused by Hg and/or the sensitivity of clams towards this metal. Data on the IBR index also corroborate these results, with the highest values in clams exposed to Hg at 22 °C. Studies conducted by Coppola et al. [28] also showed the increase of oxidative stress in *M. galloprovincialis* when exposed to a combination of Hg and warming scenario. In the same species, Coppola et al. [87] further demonstrated that the combination between GO-PEI and Hg caused lower oxidative stress and cellular damage than organisms only exposed to treatments with Hg.

5. Conclusions

Overall, the present study clearly showed that remedied seawater induces less biochemical and histopathological alterations than Hg and GO-PEI treatments. Furthermore, the temperature rise seemed to enhance the impacts cause by Hg (both acting alone or combined with GO-PEI), which can negatively impact the clam's population growth and reproduction in future warming conditions and in the presence of Hg.

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References

1. Dahlke, F.; Butzin, M.; Nahrgang, J.; Puvanendran, V.; Mortensen, A.; Pörtner, H.-O.; Storch, D. Northern cod species face spawning habitat losses if global warming exceeds 1.5 °C. *Sci. Adv.* **2018**, *4*, eaas8821. [CrossRef]
2. IPCC. *Climate Change 2007: The Physical Science Basis. Contribution of Work Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate*; Cambridge University Press: Cambridge, UK, 2007.
3. IPCC. *Global Warming of 1.5 °C. An IPCC Special Report on the Impacts of Global Warming of 1.5°C above Pre-Industrial Levels and Related Global Greenhouse Gas Emission Pathways, in the Context of Strengthening the Global Response to the Threat of Climate Change, Sustainable Development, and Efforts to Eradicate Poverty*; Masson-Delmotte, V., Zhai, P., Pörtner, H.-O., Roberts, D., Skea, J., Shukla, P.R., Pirani, A., Moufouma-Okia, W., Péan, C., Pidcock, R., Eds.; Springer: Geneva, Switzerland, 2018.
4. Manciocco, A.; Calamandrei, G.; Alleva, E. Global warming and environmental contaminants in aquatic organisms: The need of the etho-toxicology approach. *Chemosphere* **2014**, *100*, 1–7. [CrossRef]
5. Sokolova, I.M.; Lannig, G. Interactive effects of metal pollution and temperature on metabolism in aquatic ectotherms: Implications of global climate change. *Clim. Res.* **2008**, *37*, 181–201. [CrossRef]
6. Newton, A.; Brito, A.C.; Icely, J.D.; Derolez, V.; Clara, I.; Angus, S.; Schernewski, G.; Inácio, M.; Lillebø, A.I.; Sousa, A.I.; et al. Assessing, quantifying and valuing the ecosystem services of coastal lagoons. *J. Nat. Conserv.* **2018**, *44*, 50–65. [CrossRef]
7. Earp, H.S.; Prinz, N.; Cziesielski, M.J.; Andskog, M. For a World without Boundaries: Connectivity between Marine Tropical Ecosystems in Times of Change. In *YOUMARES 8—Oceans across Boundaries: Learning from Each Other*; Jungblut, S., Liebich, V., Bode, M., Eds.; Springer: Cham, Switzerland, 2018; pp. 125–144.
8. Pan, K.; Wang, W.-X. Mercury accumulation in marine bivalves: Influences of biodynamics and feeding niche. *Environ. Pollut.* **2011**, *159*, 2500–2506. [CrossRef] [PubMed]

9. Boukadida, K.; Banni, M.; Gourves, P.-Y.; Cachot, J. High sensitivity of embryo-larval stage of the Mediterranean mussel, *Mytilus galloprovincialis* to metal pollution in combination with temperature increase. *Mar. Environ. Res.* **2016**, *122*, 59–66. [CrossRef]
10. Pörtner, H.-O.; Knust, R. Climate Change affects marine fishes through the oxygen limitation of thermal tolerance. *Science* **2007**, *315*, 95–97. [CrossRef] [PubMed]
11. Rosa, R.; Pimentel, M.; Boavida-Portugal, J.; Teixeira, T.; Trübenbach, K.; Diniz, M.S. Ocean Warming enhances malformations, premature hatching, metabolic suppression and oxidative stress in the early life stages of a keystone squid. *PLoS ONE* **2012**, *7*, e38282. [CrossRef]
12. Bodin, N.; Burgeot, T.; Stanisière, J.; Bocquené, G.; Menard, D.; Minier, C.; Boutet, I.; Amat, A.; Cherel, Y.; Budzinski, H. Seasonal variations of a battery of biomarkers and physiological indices for the mussel *Mytilus galloprovincialis* transplanted into the northwest Mediterranean Sea. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2004**, *138*, 411–427. [CrossRef]
13. Hiebenthal, C.; Philipp, E.E.R.; Eisenhauer, A.; Wahl, M. Effects of seawater pCO_2 and temperature on shell growth, shell stability, condition and cellular stress of Western Baltic Sea *Mytilus edulis* (L.) and *Arctica islandica* (L.). *Mar. Biol.* **2012**, *160*, 2073–2087. [CrossRef]
14. MacKenzie, C.L.; Lynch, S.A.; Culloty, S.C.; Malham, S.K. Future Oceanic Warming and Acidification alter immune response and disease status in a commercial shellfish species, *Mytilus edulis* L. *PLoS ONE* **2014**, *9*, e99712. [CrossRef] [PubMed]
15. Petes, L.E.; Menge, B.A.; Harris, A.L. Intertidal mussels exhibit energetic trade-offs between reproduction and stress resistance. *Ecol. Monogr.* **2008**, *78*, 387–402. [CrossRef]
16. Jansen, J.M.; Hummel, H.; Bonga, S.W. The respiratory capacity of marine mussels (*Mytilus galloprovincialis*) in relation to the high temperature threshold. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2009**, *153*, 399–402. [CrossRef] [PubMed]
17. Pörtner, H.-O. Oxygen- and capacity-limitation of thermal tolerance: A matrix for integrating climate-related stressor effects in marine ecosystems. *J. Exp. Biol.* **2010**, *213*, 881–893. [CrossRef] [PubMed]
18. Velez, C.; Figueira, E.; Soares, A.M.; Freitas, R. Effects of seawater temperature increase on economically relevant native and introduced clam species. *Mar. Environ. Res.* **2017**, *123*, 62–70. [CrossRef] [PubMed]
19. Freitas, R.; Silvestro, S.; Pagano, M.; Coppola, F.; Meucci, V.; Battaglia, F.; Intorre, L.; Soares, A.M.V.M.; Pretti, C.; Faggio, C. Impacts of salicylic acid in *Mytilus galloprovincialis* exposed to warming conditions. *Environ. Toxicol. Pharmacol.* **2020**, *252*, 103448. [CrossRef]
20. Coppola, F.; Almeida, Â.; Henriques, B.; Soares, A.M.; Figueira, E.; Pereira, E.; Freitas, R. Biochemical responses and accumulation patterns of *Mytilus galloprovincialis* exposed to thermal stress and Arsenic contamination. *Ecotoxicol. Environ. Saf.* **2018**, *147*, 954–962. [CrossRef]
21. Coppola, F.; Henriques, B.; Soares, A.M.; Figueira, E.; Pereira, E.; Freitas, R. Influence of temperature rise on the recovery capacity of *Mytilus galloprovincialis* exposed to mercury pollution. *Ecol. Indic.* **2018**, *93*, 1060–1069. [CrossRef]
22. Kefaloyianni, E.; Gourgou, E.; Ferle, V.; Kotsakis, E.; Gaitanaki, C.; Beis, I. Acute thermal stress and various heavy metals induce tissue-specific pro- or anti-apoptotic events via the p38-MAPK signal transduction pathway in *Mytilus galloprovincialis* (Lam.). *J. Exp. Biol.* **2005**, *208*, 4427–4436. [CrossRef]
23. Coppola, F.; Almeida, Â.; Henriques, B.; Soares, A.M.; Figueira, E.; Pereira, E.; Freitas, R. Biochemical impacts of Hg in *Mytilus galloprovincialis* under present and predicted warming scenarios. *Sci. Total Environ.* **2017**, *601*, 1129–1138. [CrossRef]
24. Verlecar, X.; Jena, K.; Chainy, G. Biochemical markers of oxidative stress in *Perna viridis* exposed to mercury and temperature. *Chem. Interact.* **2007**, *167*, 219–226. [CrossRef]
25. Greco, L.; Pellerin, J.; Capri, E.; Garnerot, F.; Louis, S.; Fournier, M.; Sacchi, A.; Fusi, M.; Lapointe, D.; Couture, P. Physiological effects of temperature and a herbicide mixture on the soft-shell clam *Mya arenaria* (Mollusca, Bivalvia). *Environ. Toxicol. Chem.* **2010**, *30*, 132–141. [CrossRef] [PubMed]
26. Maulvault, A.L.; Camacho, C.; Barbosa, V.; Alves, R.; Anacleto, P.; Fogaça, F.H.D.S.; Kwadijk, C.; Kotterman, M.; Cunha, S.C.; Fernandes, J.; et al. Assessing the effects of seawater temperature and pH on the bioaccumulation of emerging chemical contaminants in marine bivalves. *Environ. Res.* **2018**, *161*, 236–247. [CrossRef] [PubMed]

27. Matozzo, V.; Chinellato, A.; Munari, M.; Bressan, M.; Marin, M.G. Can the combination of decreased pH and increased temperature values induce oxidative stress in the clam *Chamelea gallina* and the mussel Mytilus galloprovincialis? *Mar. Pollut. Bull.* **2013**, *72*, 34–40. [CrossRef] [PubMed]
28. Moreira, A.; Freitas, R.; Figueira, E.; Ghirardini, A.V.; Soares, A.M.; Radaelli, M.; Guida, M.; Libralato, G. Combined effects of arsenic, salinity and temperature on *Crassostrea gigas* embryotoxicity. *Ecotoxicol. Environ. Saf.* **2018**, *147*, 251–259. [CrossRef] [PubMed]
29. Van Colen, C.; Jansson, A.; Saunier, A.; Lacoue-Labathe, T.; Vincx, M. Biogeographic vulnerability to ocean acidification and warming in a marine bivalve. *Mar. Pollut. Bull.* **2018**, *126*, 308–311. [CrossRef] [PubMed]
30. Tankoua, O.F.; Buffet, P.; Amiard-Triquet, C.; Amiard-Triquet, C.; Méléder, V.; Gillet, P.; Mouneyrac, C.; Berthet, B. Intersite variations of a battery of biomarkers at different levels of biological organisation in the estuarine endobenthic worm *Nereis diversicolor* (Polychaeta, Nereididae). *Aquat. Toxicol.* **2012**, *114*, 96–103. [CrossRef]
31. Giani, M.; Djakovac, T.; Degobbis, D.; Cozzi, S.; Solidoro, C.; Umani, S.F. Recent changes in the marine ecosystems of the northern Adriatic Sea. *Estuarine Coast. Shelf Sci.* **2012**, *115*, 1–13. [CrossRef]
32. Pereira, E.; Abreu, S.; Coelho, J.P.; Pato, P.; Pardal, M.; Duarte, A.C. Influence of tidal resuspension on seston lithogenic and biogenic partitioning in shallow estuarine systems: Implications for sampling. *Mar. Pollut. Bull.* **2008**, *56*, 348–354. [CrossRef]
33. Randall, P.M.; Chattopadhyay, S. Mercury contaminated sediment sites—An evaluation of remedial options. *Environ. Res.* **2013**, *125*, 131–149. [CrossRef]
34. Suriya, J.; Bharathiraja, S.; Sekar, V.; Rajasekaran, R. Metallothionein induction and antioxidative responses in the estuarine polychaeta *Capitella capitata* (Capitellidae). *Asian Pac. J. Trop. Biomed.* **2012**, *2*, S1052–S1059. [CrossRef]
35. Al Naggar, Y.; Khalil, M.S.; Ghorab, M.A. Environmental Pollution by Heavy Metals in the Aquatic Ecosystems of Egypt. *Open Access J. Toxicol.* **2018**, *3*, 555603. [CrossRef]
36. Bielen, A.; Bošnjak, I.; Sepčić, K.; Jaklič, M.; Cvitanić, M.; Lušić, J.; Lajtner, J.; Simčič, T.; Hudina, S. Differences in tolerance to anthropogenic stress between invasive and native bivalves. *Sci. Total Environ.* **2016**, *543*, 449–459. [CrossRef] [PubMed]
37. Freitas, R.; Leite, C.; Pinto, J.; Costa, M.; Monteiro, R.; Henriques, B.; Di Martino, F.; Coppola, F.; Soares, A.M.; Solé, M.; et al. The influence of temperature and salinity on the impacts of lead in *Mytilus galloprovincialis*. *Chemosphere* **2019**, *235*, 403–412. [CrossRef] [PubMed]
38. Velez, C.; Freitas, R.; Antunes, S.C.; Soares, A.M.; Figueira, E. Clams sensitivity towards As and Hg: A comprehensive assessment of native and exotic species. *Ecotoxicol. Environ. Saf.* **2016**, *125*, 43–54. [CrossRef] [PubMed]
39. ATSDR. Priority List of Hazardous Substances. 2015. Available online: https://www.atsdr.cdc.gov/spl/index.html (accessed on 16 January 2007).
40. Freitas, R.; Coppola, F.; De Marchi, L.; Codella, V.; Pretti, C.; Chiellini, F.; Morelli, A.; Polese, G.; Soares, A.M.; Figueira, E.; et al. The influence of Arsenic on the toxicity of carbon nanoparticles in bivalves. *J. Hazard. Mater.* **2018**, *358*, 484–493. [CrossRef]
41. Nardi, A.; Mincarelli, L.F.; Benedetti, M.; Fattorini, D.; D'Errico, G.; Regoli, F. Indirect effects of climate changes on cadmium bioavailability and biological effects in the Mediterranean mussel *Mytilus galloprovincialis*. *Chemosphere* **2017**, *169*, 493–502. [CrossRef]
42. Samuel, S.; Kathirvel, R.; Jayavelu, T.; Chinnakkannu, P. Protein oxidative damage in arsenic induced rat brain: Influence of dl-α-lipoic acid. *Toxicol. Lett.* **2005**, *155*, 27–34. [CrossRef]
43. Zhang, Y.; Song, J.; Yuan, H.; Xu, Y.; He, Z.; Duan, L. Biomarker responses in the bivalve (*Chlamys farreri*) to exposure of the environmentally relevant concentrations of lead, mercury, copper. *Environ. Toxicol. Pharmacol.* **2010**, *30*, 19–25. [CrossRef]
44. Velez, C.; Galvao, P.; Longo, R.; Malm, O.; Soares, A.M.; Figueira, E.; Freitas, R. *Ruditapes philippinarum* and *Ruditapes decussatus* under Hg environmental contamination. *Environ. Sci. Pollut. Res.* **2015**, *22*, 11890–11904. [CrossRef]
45. Gagnaire, B.; Thomas-Guyon, H.; Renault, T. In vitro effects of cadmium and mercury on Pacific oyster, *Crassostrea gigas* (Thunberg), haemocytes. *Fish Shellfish Immunol.* **2004**, *16*, 501–512. [CrossRef] [PubMed]

46. Oliveira, P.; Almeida, Â.; Calisto, V.; Esteves, V.I.; Schneider, R.J.; Wrona, F.; Soares, A.M.; Figueira, E.; Freitas, R. Physiological and biochemical alterations induced in the mussel *Mytilus galloprovincialis* after short and long-term exposure to carbamazepine. *Water Res.* **2017**, *117*, 102–114. [CrossRef] [PubMed]
47. Attig, H.; Kamel, N.; Sforzini, S.; Dagnino, A.; Jamel, J.; Boussetta, H.; Viarengo, A.; Banni, M. Effects of thermal stress and nickel exposure on biomarkers responses in *Mytilus galloprovincialis* (Lam). *Mar. Environ. Res.* **2014**, *94*, 65–71. [CrossRef] [PubMed]
48. Mubiana, V.K.; Blust, R. Effects of temperature on scope for growth and accumulation of Cd, Co, Cu and Pb by the marine bivalve *Mytilus edulis*. *Mar. Environ. Res.* **2007**, *63*, 219–235. [CrossRef] [PubMed]
49. Freitas, R.; Pinto, L.R.; Sampaio, M.; Costa, A.; Silva, M.; Rodrigues, A.M.; Quintino, V.; Figueira, E. Effects of depuration on the element concentration in bivalves: Comparison between sympatric *Ruditapes decussatus* and *Ruditapes philippinarum*. *Estuarine Coast. Shelf Sci.* **2012**, *110*, 43–53. [CrossRef]
50. Donnici, S.; Serandrei-Barbero, R.; Canali, G. Evidence of climatic changes in the Venetian Coastal Plain (Northern Italy) during the last 40,000 years. *Sediment. Geol.* **2012**, *281*, 139–150. [CrossRef]
51. Briant, L.J.; Salehi, A.A.; Vergari, E.; Zhang, Q.; Rorsman, P. Glucagon secretion from pancreatic α-cells. *Upsala J. Med. Sci.* **2016**, *121*, 113–119. [CrossRef]
52. Faganeli, J.; Hines, M.E.; Covelli, S.; Emili, A.; Giani, M. Mercury in lagoons: An overview of the importance of the link between geochemistry and biology. *Estuarine Coast. Shelf Sci.* **2012**, *113*, 126–132. [CrossRef]
53. Pereira, P.; Korbas, M.; Pereira, V.; Cappello, T.; Maisano, M.; Canário, J.; Almeida, A.; Pacheco, M. A multidimensional concept for Mercury neuronal and sensory toxicity in fish—From toxicokinetics and biochemistry to morphometry and behavior. *BBA Gen. Subj.* **2019**, *1863*, 129298. [CrossRef]
54. Matlock, M.M.; Henke, K.R.; Atwood, D.A.; Robertson, D. Aqueous leaching properties and environmental implications of cadmium, lead and zinc trimercaptotriazine (TMT) compounds. *Water Res.* **2001**, *35*, 3649–3655. [CrossRef]
55. Henke, K.R.; Hutchison, A.R.; Krepps, M.K.; Parkin, S.; Atwood, D.A. Chemistry of 2,4,6-trimercapto-1,3,5-triazine (TMT): Acid dissociation constants and group 2 complexes. *Inorg. Chem.* **2001**, *40*, 4443–4447. [CrossRef] [PubMed]
56. Aroua, M.K.; Zuki, F.M.; Sulaiman, N.M. Removal of chromium ions from aqueous solutions by polymer-enhanced ultrafiltration. *J. Hazard. Mater.* **2007**, *147*, 752–758. [CrossRef] [PubMed]
57. Muthukrishnan, M.; Guha, B. Effect of pH on rejection of hexavalent chromium by nanofiltration. *Desalination* **2008**, *219*, 171–178. [CrossRef]
58. Pugazhenthi, G.; Sachan, S.; Kishore, N.; Kumar, A. Separation of chromium (VI) using modified ultrafiltration charged carbon membrane and its mathematical modeling. *J. Membr. Sci.* **2005**, *254*, 229–239. [CrossRef]
59. Ali, I.; Khan, T.A.; Asim, M. Removal of arsenate from groundwater by electrocoagulation method. *Environ. Sci. Pollut. Res.* **2011**, *19*, 1668–1676. [CrossRef]
60. Anjum, M.; Miandad, R.; Waqas, M.; Gehany, F.; Barakat, M. Remediation of wastewater using various nano-materials. *Arab. J. Chem.* **2019**, *12*, 4897–4919. [CrossRef]
61. Huang, Y.; Su, Y.; Conway, J.R.; Keller, A.A.; Adeleye, A.S.; Garner, K. Engineered nanomaterials for water treatment and remediation: Costs, benefits, and applicability. *Chem. Eng. J.* **2015**, *286*, 640–662. [CrossRef]
62. Li, M.; Feng, C.; Zhang, Z.; Yang, S.; Sugiura, N. Treatment of nitrate contaminated water using an electrochemical method. *Bioresour. Technol.* **2010**, *101*, 6553–6557. [CrossRef]
63. Babel, S.; Kurniawan, T.A. Low-cost adsorbents for heavy metals uptake from contaminated water: A review. *J. Hazard. Mater.* **2003**, *97*, 219–243. [CrossRef]
64. Gehrke, I.; Geiser, A.; Somborn-Schulz, A. Innovations in nanotechnology for water treatment. *Nanotechnol. Sci. Appl.* **2015**, *8*, 1–17. [CrossRef]
65. Mohan, D.J.; Pittman, C.U., Jr. Arsenic removal from water/wastewater using adsorbents—A critical review. *J. Hazard. Mater.* **2007**, *142*, 1–53. [CrossRef] [PubMed]
66. Henriques, B.; Gonçalves, G.; Emami, N.; Pereira, E.; Vila, M.; Marques, P.A. Optimized graphene oxide foam with enhanced performance and high selectivity for mercury removal from water. *J. Hazard. Mater.* **2016**, *301*, 453–461. [CrossRef] [PubMed]
67. Gonçalves, G.; Marques, P.A.; Granadeiro, C.M.; Nogueira, H.I.; Singh, M.K.; Grácio, J. Surface modification of graphene nanosheets with gold nanoparticles: The role of oxygen moieties at graphene surface on gold nucleation and growth. *Chem. Mater.* **2009**, *21*, 4796–4802. [CrossRef]

68. Cohen-Tanugi, D.; Grossman, J.C. Water Desalination across Nanoporous Graphene. *Nano Lett.* **2012**, *12*, 3602–3608. [CrossRef]
69. Fang, Q.; Zhou, X.; Deng, W.; Liu, Z. Hydroxyl-containing organic molecule induced self-assembly of porous graphene monoliths with high structural stability and recycle performance for heavy metal removal. *Chem. Eng. J.* **2017**, *308*, 1001–1009. [CrossRef]
70. Sahraei, R.; Ghaemy, M. Synthesis of modified gum tragacanth/graphene oxide composite hydrogel for heavy metal ions removal and preparation of silver nanocomposite for antibacterial activity. *Carbohydr. Polym.* **2017**, *157*, 823–833. [CrossRef]
71. Bessa, A.; Henriques, B.; Gonçalves, G.; Irurueta, G.; Pereira, E.; Marques, P.A.A.P. Graphene oxide/polyethyleneimine aerogel for high-performance mercury sorption from natural waters. *Chem. Eng. J.* **2020**, *398*, 125587. [CrossRef]
72. Banni, M.; Hajer, A.; Sforzini, S.; Oliveri, C.; Boussetta, H.; Viarengo, A. Transcriptional expression levels and biochemical markers of oxidative stress in *Mytilus galloprovincialis* exposed to nickel and heat stress. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2014**, *160*, 23–29. [CrossRef]
73. Banni, M.; Hajer, A.; Sforzini, S.; Oliveri, C.; Mignone, F.; Boussetta, H.; Viarengo, A. Transcriptomic responses to heat stress and nickel in the mussel *Mytilus galloprovincialis*. *Aquat. Toxicol.* **2014**, *148*, 104–112. [CrossRef]
74. Izagirre, U.; Errasti, A.; Bilbao, E.; Múgica, M.; Marigómez, I. Combined effects of thermal stress and Cd on lysosomal biomarkers and transcription of genes encoding lysosomal enzymes and HSP70 in mussels, Mytilus galloprovincialis. *Aquat. Toxicol.* **2014**, *149*, 145–156. [CrossRef]
75. Marques, A.; Piló, D.; Carvalho, S.; Araújo, O.; Guilherme, S.; Santos, M.A.; Vale, C.; Pereira, F.; Pacheco, M.; Pereira, P. Metal bioaccumulation and oxidative stress profiles in *Ruditapes philippinarum*—Insights towards its suitability as bioindicator of estuarine metal contamination. *Ecol. Indic.* **2018**, *95*, 1087–1099. [CrossRef]
76. Almeida, A.; Freitas, R.; Calisto, V.; Esteves, V.I.; Schneider, R.J.; Soares, A.M.; Figueira, E.M.D.A.P. Chronic toxicity of the antiepileptic carbamazepine on the clam *Ruditapes philippinarum*. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2015**, *172–173*, 26–35. [CrossRef] [PubMed]
77. Costa, S.; Coppola, F.; Pretti, C.; Intorre, L.; Meucci, V.; Soares, A.M.; Freitas, R.; Solé, M. The influence of climate change related factors on the response of two clam species to diclofenac. *Ecotoxicol. Environ. Saf.* **2019**, *189*, 109899. [CrossRef] [PubMed]
78. Cruz, J.D.; Almeida, Â.; Calisto, V.; Esteves, V.I.; Schneider, R.J.; Wrona, F.; Soares, A.M.; Figueira, E.; Freitas, R. Caffeine impacts in the clam *Ruditapes philippinarum*: Alterations on energy reserves, metabolic activity and oxidative stress biomarkers. *Chemosphere* **2016**, *160*, 95–103. [CrossRef]
79. Coppola, F.; Tavares, D.; Henriques, B.; Monteiro, R.; Trindade, T.; Soares, A.M.; Figueira, E.; Polese, G.; Pereira, E.; Freitas, R. Remediation of arsenic from contaminated seawater using manganese spinel ferrite nanoparticles: Ecotoxicological evaluation in *Mytilus galloprovincialis*. *Environ. Res.* **2019**, *175*, 200–212. [CrossRef]
80. De Marchi, L.; Neto, V.; Pretti, C.; Figueira, E.; Chiellini, F.; Morelli, A.; Soares, A.M.; Freitas, R. The impacts of seawater acidification on *Ruditapes philippinarum* sensitivity to carbon nanoparticles. *Environ. Sci. Nano* **2017**, *4*, 1692–1704. [CrossRef]
81. IPMA. Portuguese Institute for Sea and Atmosphere (IPMA). Available online: http://www.ipma.pt/pt/maritima/sst/ (accessed on 20 August 2019).
82. European Parliament; Council of the European Union. *Directive 2013/39/EU of the European Parliament and of the Council of 12 August 2013 Amending Directives 2000/60/EC and 2008/105/EC as Regards Priority Substances in the Field of Water Policy*; Publications Office of the EU: Brussels, Belgium, 2013.
83. Henriques, B.; Teixeira, A.; Figueira, P.; Reis, A.T.; Almeida, J.; Vale, C.; Pereira, E. Simultaneous removal of trace elements from contaminated waters by living *Ulva lactuca*. *Sci. Total Environ.* **2019**, *652*, 880–888. [CrossRef]
84. Costley, C.T.; Mossop, K.F.; Dean, J.R.; Garden, L.M.; Marshall, J.; Carroll, J. Determination of mercury in environmental and biological samples using pyrolysis atomic absorption spectrometry with gold amalgamation. *Anal. Chim. Acta* **2000**, *405*, 179–183. [CrossRef]
85. Pinto, J.; Costa, M.; Leite, C.; Borges, C.; Coppola, F.; Henriques, B.; Monteiro, R.; Russo, T.; Di Cosmo, A.; Soares, A.M.; et al. Ecotoxicological effects of lanthanum in *Mytilus galloprovincialis*: Biochemical and histopathological impacts. *Aquat. Toxicol.* **2019**, *211*, 181–192. [CrossRef]

86. Leite, C.; Coppola, F.; Monteiro, R.; Russo, T.; Polese, G.; Lourenço, M.A.O.; Ferreira, P.; Soares, A.M.; Pereira, E.; Freitas, R. Toxic impacts of Rutile (TiO_2 nanoparticles) in *Mytilus galloprovincialis* exposed to warming conditions. *Chemosphere* **2020**, in press. [CrossRef]
87. Coppola, F.; Bessa, A.; Henriques, B.; Russo, T.; Soares, A.M.; Figueira, E.; Marques, P.A.; Polese, G.; Di Cosmo, A.; Pereira, E.; et al. Oxidative stress, metabolic and histopathological alterations in mussels exposed to remediated seawater by GO-PEI after contamination with mercury. *Comp. Biochem. Physiol. Part A Mol. Integr. Physiol.* **2020**, *243*, 110674. [CrossRef] [PubMed]
88. Beliaeff, B.; Burgeot, T. Integrated biomarker response: A useful tool for ecological risk assessment. *Environ. Toxicol. Chem.* **2020**, *21*, 1316–1322. [CrossRef]
89. Anderson, M.J.; Gorley, R.N.; Clarke, K.R. PERMANOVAþ for PRIMER. In *Guide to Software and Statistical Methods*; University of Auckland and PRIMER-E: Plymouth, UK, 2008.
90. Sanni, B.; Williams, K.; Sokolov, E.P.; Sokolova, I. Effects of acclimation temperature and cadmium exposure on mitochondrial aconitase and LON protease from a model marine ectotherm, *Crassostrea virginica*. *Comp. Biochem. Physiol. Part C Toxicol. Pharmacol.* **2008**, *147*, 101–112. [CrossRef] [PubMed]