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How efficient is graphene-based nanocomposite to adsorb Hg from seawater. A laboratory assay to assess the toxicological impacts induced by remediated water towards marine bivalves

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HIGHLIGHTS

- Graphene oxide functionalized with polyethyleneimine (GO-PEI) removed Hg from seawater.
- Low Hg accumulation in clams under remediated seawater, especially at 22 °C.
- Clams under Hg free treatments (CTL, GO-PEI, RSW) presented similar responses.
- Hg treatment was the most deleterious condition, regardless the temperature.
- Clams exposed to different temperatures showed a different behaviour.

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ABSTRACT

Advanced investigations on the use of graphene based nanomaterials have highlighted the capacity of these materials for wastewater treatment. Research on this topic revealed the efficiency of the nanocomposite synthetized by graphene oxide functionalized with polyethyleneimine (GO-PEI) to adsorb mercury (Hg) from contaminated seawater. However, information on the environmental risks associated with these approaches are still lacking. The focus of this study was to evaluate the effects of Hg in contaminated seawater and seawater remediated by GO-PEI, using the species *Ruditapes philippinarum*, maintained at two different warming scenarios: control (17 °C) and increased (22 °C) temperatures. The results obtained showed that organisms exposed to non-contaminated and remediated seawaters at control temperature presented similar biological patterns, with no considerable differences expressed in terms of biochemical and histopathological alterations. Moreover, the present findings revealed increased toxicological effects in clams under remediated seawater at 22 °C in comparison to those subjected to the equivalent treatment at 17 °C. These results confirm the capability of GO-PEI to adsorb Hg from water with no noticeable toxic effects, although temperature could alter the responses of mussels to remediated seawater. These materials seem to be a promise eco-friendly approach to remediate wastewater, with low toxicity evidenced by remediated seawater and high regenerative capacity of this nanomaterial, keeping its high removal performance after successive sorption-desorption cycles.

1. Introduction

Coastal marine ecosystems have been influenced by a vast variety of natural and anthropogenic substances such as metal(loid)s (Izagirre et al., 2014; Lamborg et al., 2014; Maulvault et al., 2017;

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Klaver et al., 2014; Schaller et al., 2011). Increased concentrations of these pollutants it is also associated with world population growth, especially around coastal areas, mainly resulting from industrial and agricultural activities (Fattorini et al., 2008; Marques et al., 2017; Nardi et al., 2018; Randall and Chattopadhyay, 2013; Pereira et al., 2008). Anthropogenic sources of these pollutants include alloy and batteries production, coating, explosive manufacturing, pesticides and phosphate fertilizers (Ayangbenro and Babalola, 2017). As a result, several studies already reported high metal(-loid)s concentrations in marine and estuarine systems worldwide, in water, sediments and inhabiting organisms (Bakary et al., 2015; Randall and Chattopadhyay, 2013; Tchounwou et al., 2012). Amongst the most toxic elements in aquatic systems, mercury (Hg) has been recognized as one of the most highly dangerous substances (ATSDR, 2019), a situation that may continue due to its use in electronic products and fluorescent lamps (among others, Donnici et al., 2012; Briant et al., 2016).

The booming of nanotechnology and outstanding advances in research concerning graphene-based nanomaterials have provided great promise for wastewater treatment (Mokhtar et al., 2019; Nupearachchi et al., 2017; Stobel et al., 2019; Zhang et al., 2010; Zhou et al., 2015). Recently, several methods have been tested as an attempt to remove metals, including Hg, from contaminated water, such as chemical precipitation (Henke et al., 2001; Matlock et al., 2001), ultra and nano-filtration as well reverse osmosis (Aroua et al., 2007; Muthukrishnan and Guha, 2008; Pugazhenthi et al., 2005) and the use nanomaterials as sorbents (Ali et al., 2012, 2019; Anjum et al., 2016; Babel and Kurniawan, 2003; Huang et al., 2015; Li et al., 2010). Among these techniques, Bessa et al. (2020) studied a low-cost nanocomposite, developed with graphene oxide (GO) combined with polyethyleneimine (PEI) that efficiently removed Hg from water (93% from contaminated seawater with 50 μg/L of Hg). Still, scarce information exists regarding the possible toxicity associated with remediated water obtained from such approaches, especially considering predicted climate change scenarios (Coppola et al., 2019, (2020a,b); Falinski et al., 2020).

Besides pollutants, aquatic systems have been also subjected to climate changes, with the intergovernmental panel on climate change (IPCC, 2018) highlighting the increase of global temperature as an imminent climatic problem derived from anthropogenic activities. It was estimated that human activities resulted into a global atmospheric warming close to 1.0 °C above pre-industrial levels, which may reach 1.5 °C between 2030 and 2052 (IPCC, 2018; IPCC, 2019). Although at a slower rate, also seawater temperature is rising in oceans and especially in marine coastal systems (Boyer et al., 2005; Bindoff et al., 2007; Fogarty et al., 2008; Levitus et al., 2009). However, differently from open ocean (with a thermal inertia due to its high heat capacity of water and high total volume), shallow water bodies like coastal areas, lagoons and estuaries are more susceptible to air temperature's influence because of their restricted heat exchange with open waters (Lloret et al., 2008; Newton et al., 2018; Pan and Wang, 2011). Jakimavičius et al. (2018) predicted a temperature rise up to 6 °C by the year 2100 in the Curonian lagoon. Associated with warming of coastal areas several authors have already demonstrated harmful effects in inhabiting organisms (Rosa et al., 2012; Pörtner and Knust, 2007; Boukadida et al., 2016, among others). As an example, Jiang et al. (2016) highlighted the increase of seawater temperature from 15 to 20 °C could affect negatively the physiological as well as the biochemical behaviour of the yesso scallop species, *Patinopecten yessoensis*.

Based on their sessile nature, filter-feeding habit, high tolerance, and tendency to bioconcentrate pollutants, high distribution and economic importance in Europe as well as in the oriental continent (namely China), *Ruditapes philippinarum* is considered a good sentinel for monitoring marine pollution and climate changes (Bebianno et al., 2004; Freitas et al., 2018; Ji et al., 2006; Jiang et al., 2019; Yang et al., 2013; Velez et al., 2015). The present study aimed to understand the possible impacts derived from Hg remediated seawater using GO-PEI, under control temperature (17 °C) and predicted warming scenario (22 °C), in the species *R. philippinarum,* collected from the Red island, a non-contaminated area in the Yellow Sea (Qingdao, China). Clams were subjected, during 28 days, to five treatments: 1) non-contaminated seawater (CTL); 2) seawater with nanocomposite (graphene oxide combined with polyethyleneimine, GO-PEI); 3) seawater contaminated with mercury (Hg); 4) seawater with mixture of nanocomposite and Hg (GO-PEI + Hg) and 5) remediate seawater (RSW). Each treatment was tested under both temperatures. After exposure, clams Hg accumulation levels, histopathological alterations and biochemical responses were evaluated.

2. Materials and methods

2.1. Laboratory conditions and experimental setup

Clams were collected in the Red island (Yellow Sea, Qingdao, China), considered as a non-contaminated area (Yang et al., 2013; Jiang et al., 2019), in October 2019. Specimens with similar size were selected: length of 29.2 ± 1.9 mm, width of 21.2 ± 1.5 mm and height of 13.8 ± 1.0 mm.

In the laboratory, clams were placed in a 100 L glass aquarium with sand and seawater from the sampling site (salinity 30 ± 1; pH 8.0 ± 0.1; temperature 17.0 ± 1 °C; dissolved oxygen 7.7 ± 0.2 mg/L) with constant aeration for two weeks. During the first week all organisms were maintained under the same temperature 17 °C (depuration period). In the second week (acclimation period), half of the clams was kept at 17 ± 1 °C (control temperature) and another half was subjected to a gradual temperature increase up to 22 ± 1 °C (warming scenario). During depuration and acclimation periods both groups were fed with a solution of algae (1 g of Algamac protein plus powder per 1.5 L of distilled water) every 3–4 days.

The control temperature (17 °C) was selected based on the mean values recorded along the year in the sampling area (Wang, 2014). The warming scenario (22 °C) was selected considering predicted increased temperatures in coastal systems (IPCC, 2019; Sun et al., 2011; Zhu et al., 1991).

The exposure assay (28 days) was carried out for the two groups (17 and 22 °C) at the same salinity and pH conditions (salinity 30 and pH 8.0). Organisms at the two temperatures were subjected to five treatments as described in Table 1: control (CTL); GO-PEI; Hg; GO-PEI + Hg; RSW. Per treatment three glass aquaria of 3 L were used, with 12 clams in each replicate.

The remediated seawater was obtained after a treatment of 24 h where the seawater was previously contaminated with 50 μg/L of Hg stock solution (Hg 1000 mg/L in 1 mol/L HNO_3, 99.9999% trace metals basis) and remediated with GO-PEI as referred in (Coppola et al. (2020a,b)). The Hg concentration of 50 μg/L used in this study was selected taking into consideration that this is the maximum allowable limit in wastewater discharges from industry, in the European Union (Directive, 2013/39/EU, 2013). The GO-PEI concentration (10 mg/L) was selected according to the capacity of this material to remove Hg (Bessa et al., 2020).

During the experiment clams' mortality was checked and one day per week seawater from each aquarium was renewed with the re-establishment of all initial conditions. To feed the animals a commercial Algamac protein plus solution was prepared and 1 mL/L was added to each aquarium three times per week.

Before spiking, water samples were collected from all aquaria to

Table 1
Treatments evaluated (conditions in aquariums).

TREATMENTS	DESCRIPTION
CTL	Hg 0 μg/L + GO-PEI 0 mg/L
GO-PEI	GO-PEI 10 mg/L
Hg	Hg 50 μg/L
GO-PEI + Hg	GO-PEI 10 mg/L + Hg 50 μg/L
Remediated seawater (RSW)	Seawater remediated with GO-PEI (10 mg/L) for 24 h after Hg contamination (50 μg/L)

assess Hg background levels in seawater medium. To determine the real concentrations of Hg in water medium, every week water samples were collected (from control and contaminated treatments) immediately after water renewal and spiking.

At the 28th day, organisms from each aquarium were sacrificed thought the separation of the shell from soft tissue. Three clams per treatment were used for the histological measurements (one from each aquarium); gills and digestive tubules were vivisected and immediately fixed in Davidson's fluid for 24 h at room temperature. The remaining clams were frozen with liquid nitrogen and maintained at −80 °C. From each clam, soft tissues were manually homogenized (using pestle and mortar) and separated in five different microtubes with aliquots of 0.3 g fresh weight (FW). Four of them were used for biochemical analyses and the remaining one was lyophilized during 1 week for the Hg quantification.

2.2. Mercury quantification

The Hg cquantification in water samples and clams' tissues were conducted by the Societe Generale de Surveillance − China Standard Technology Development Corporation, SGS − CSTC Standards Technical Services CO., Ltd. (Qingdao, Shandong, China). The concentration of Hg in seawater and organisims were expressed in μg/L and μg/g, respectively, as reported by Coppola et al. (2020b) and Jiang et al. (2019). For each aquarium three replicates were measured. The quantification limits of Hg in seawater and tissue were 5.0 μg/L and 0.005 μg/g, respectively.

2.3. Quality assurance and quality control

All samples were evaluated in duplicates to obtain parallel results and reduce uncertainties. The average data obtained from tests under the same conditions were used to obtain the final result. All samples including blanks and standard calibration curve were assessed by using the same procedures at the same conditions. The recovery of Hg was 97% in relation to the standard (0,0 -2.0 μg/L) and the relative percentage differences were all within 10%.

2.4. Biochemical markers

The electron transport system (ETS) activity is a proxy of the metabolic capacity of an organisms and determined as reported by Andrade et al. (2018) and Coppola et al. (2019). It was read in a microplate at 490 nm and expressed in nmol/min/g FW. All the remaining parameters were determined using commercial kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) already used in previous studies with the same species or other bivalves (Jiang et al, 2016, 2019), including: i) energy reserves, namely glycogen (GLY) content (CAS A045-2-2), and protein total (PROT) content (CAS A043-1-1); ii) enzymatic scavengers as total superoxide dismutase (T-SOD) activity (CAS A001-1-2), catalase (CAT) activity (CAS A007-1-1), and glutathione peroxidase (GSH-PX) activity (CAS A005-1-2); iii) cellular damage as malondialdehyde (MDA) levels (CAS A003-1-2); iv) neurotoxicity as true choline esterase (T-CHE) activity (CAS A024-1-1). All biomarkers were carried out in duplicate and measured using a microplate reader (Multiskan FC, Thermo Fisher Scientific, China).

2.5. Histopathological measurements

After 24 h in Davidson fluid, the clams' gills (G) and digestive tubules (D.T.) were placed in ethanol 70%. Afterwards, organisms were sent to the Service bio-Technology Co., Ltd. Laboratory (Wuhan, Hubei, China) for histopathological analyses. Tissue sections were acquired following the method reported by Coppola et al. (2018). The histopathological index (*ih*) was evaluated as described in Leite et al. (2020) where for each slide (6 per tissue) 6 pictures at 50× and 100× magnification were taken for a total of n = 36 per organ. The *ih* values were calculated based on biological differences of each surveyed alteration with a value ranging between minimum and maximum severity (1−3) and its degree of dissemination with the score ranging between 0 (feature/alteration not observed in any of the 6 pictures made) and 6 (maximal diffusion where the alteration was detectable in each picture) as reported by Coppola et al. (2020a,b) and Costa et al. (2013).

2.6. Statistical analyses

The statistical permutational analysis of variance was conducted for all the obtained results (Hg concentration in clam's tissues, histological alterations and biomarkers) using the software PERMANOVA + add-on in PRIMER v6 (Anderson et al., 2008). Pairwise comparisons were performed and the significant differences were accepted when $p < 0.05$. The null hypotheses tested were: a) for each response (Hg concentration, histological and biochemical markers), no significant differences were detected among treatments (CTL, GO-PEI, Hg, GO-PEI + Hg and RSW) at 17 °C (uppercase letters in Tables 2 and 3, Figs. 1) and 22 °C (lowercase

Table 2
Mercury concentration [Hg] in clams' soft tissues (μg/g) at the end of the experiment. Results are mean ± standard deviation. Significant differences among treatments are represented with different letters: uppercase letters for 17 °C and lowercase letters for 22 °C. Differences between both temperatures for each treatment are represented with an asterisk.

[Hg] μg/g	CTL	GO-PEI	Hg	GO-PEI + Hg	RSW
17 °C	0.021 ± 0.001^{A}	0.024 ± 0.001^{A}	3.2 ± 0.8^{B}	3.9 ± 1.2^{B} *	1.8 ± 0.18^{C} *
22 °C	0.019 ± 0.002^{a}	0.023 ± 0.003^{a}	3.0 ± 0.04^{b}	1.2 ± 0.08^{c}	0.19 ± 0.009^{d}

Mean values were obtained considering three true replicates per treatment (three different aquaria per treatment; n = 3), and from each aquarium one replicate was used.

Table 3
Biochemical markers in *Ruditapes philippinarum* at the end of the experiment: Electron transport system (ETS) activity nmol/min/g FW; glycogen (GLY) content mg/g FW; protein total (PROT) content mg/g FW; total superoxide dismutase (T-SOD) activity U/g FW; catalase (CAT) activity U/g FW; glutathione peroxidase (GSH-PX) activity U/g FW; malondialdehyde (MDA) levels nmol/g FW; neurotoxicity as true choline esterase (T-CHE) activity U/g FW. Results are mean ± standard deviation. Differences among the treatments at 17 and 22 °C were presented in uppercase letter and lowercase letter, respectively. Significant differences between 17 and 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			RSW		
		17°C		*22°C*	*17°C*		*22°C*	*17°C*		*22°C*	*17°C*		*22°C*	*17°C*		*22°C*
Biochemical markers	*ETS*	27.43 ± 3.54^{A}	*	54.68 ± 7.63^{a}	31.92 ± 3.95^{A}	*	57.21 ± 4.69^{a}	31.25 ± 1.90^{A}		37.09 ± 9.65^{b}	29.16 ± 2.23^{A}	*	**58.89 ± 2.94^{a}**	23.62 ± 1.40^{B}	*	52.20 ± 9.35^{a}
	GLY	8.03 ± 0.35^{A}		7.41 ± 1.13^{a}	11.30 ± 1.63^{B}	*	7.53 ± 0.30^{a}	17.29 ± 0.44^{C}	*	**20.61 ± 2.33^{b}**	14.01 ± 0.62^{D}		17.00 ± 2.26^{b}	8.47 ± 0.57^{A}	*	12.00 ± 0.38^{c}
	PROT	2.66 ± 0.17^{A}	*	3.31 ± 0.73^{a,c}	2.67 ± 0.30^{A}	*	**3.69 ± 0.23^{a}**	2.38 ± 0.37^{A}		2.44 ± 056^{b}	3.64 ± 0.34^{B}	*	1.99 ± 0.85^{b}	3.44 ± 0.67^{B}	*	2.51 ± 0.69^{b,c}
	T-SOD	6.94 ± 0.08^{A}	*	8.82 ± 0.11^{a}	7.06 ± 0.01^{A}	*	8.8 ± 0.1^{a}	7.59 ± 0.54^{A,B}	*	8.88 ± 0.12^{a}	7.89 ± 0.15^{B}	*	**9.97 ± 0.2^{b}**	7.94 ± 0.46^{B}	*	9.89 ± 0.22^{b}
	CAT	7.0 ± 0.28^{A,B}	*	3.57 ± 0.13^{a,c}	**7.34 ± 0.22^{A}**	*	3.6 ± 0.1^{a}	6.54 ± 0.31^{B,C}	*	3.65 ± 0.08^{a}	6.67 ± 0.08^{B,C}	*	3.21 ± 0.24^{b}	5.97 ± 0.32^{C}	*	3.18 ± 0.41^{b,c}
	GSH-PX	2.63 ± 0.73^{A}		3.46 ± 0.85^{a}	2.49 ± 0.48^{A}	*	**5.8 ± 0.8^{b}**	2.07 ± 0.78^{A}	*	5.35 ± 0.84^{b}	1.79 ± 0.94^{A}	*	4.91 ± 1.09^{b}	2.66 ± 0.48^{A}	*	5.24 ± 0.58^{b}
	MDA	124.86 ± 18.86^{A}		129.37 ± 25.44^{a}	234.00 ± 39.71^{B}	*	158.30 ± 56.51^{a}	97.23 ± 9.04^{A}		145.57 ± 21.23^{a}	98.44 ± 17.81^{A}	*	295.89 ± 27.42^{b}	115.13 ± 12.38^{A}	*	**348.21 ± 78.53^{b}**
	T-CHE	3.60 ± 0.38^{A}		4.64 ± 0.69^{a,b}	3.74 ± 0.34^{A}		3.0 ± 1.0^{b,c}	6.78 ± 1.14^{B}	*	2.41 ± 0.53^{c}	5.96 ± 0.65^{B}	*	4.58 ± 0.82^{a}	6.49 ± 0.75^{B}		**7.17 ± 1.21^{d}**

Mean values were obtained considering three true replicates per treatment (three different aquaria per treatment; n = 3), and from each aquarium three replicates were used.

letters in Tables 2 and 3, Fig. 1); b) for each response and for each treatment, no significant differences existed between temperatures (17 and 22 °C), highlighted with asterisks in Tables 2 and 3, Fig. 1.

Using the same software (PRIMER v6), Principal Coordinates Analysis (PCoA) was calculated by the Euclidean distance similarity matrix considering Hg concentrations, histopathological and biochemical markers for each treatment and temperature. On top of the PCoA graph, tissues Hg concentration, histopathological and biochemical descriptors were provided as Pearson correlation vectors.

3. Results

3.1. Mortality

During the experimental exposure, the highest mortality (50%) was recorded in clams submitted to Hg and GO-PEI + Hg at 22 °C. Moreover, high mortality was also detected in organisms under Hg and GO-PEI + Hg at 17 °C (47 and 39%, respectively). Mortality was also observed at 22 °C in non-contaminated (CTL) and GO-PEI exposed clams (both 36%) as well as in clams subjected to remediated seawater (RSW) (33%). The organisms under CTL, GO-PEI and RSW at 17 °C presented the mortality lower than 19%.

3.2. Mercury quantification

The background concentration of Hg in seawater was below the quantification limit (5.0 μg/L). Moreover, the Hg concentration in water samples collected from all aquaria after spiking showed the mean ± standard values close to the nominal concentration (64.1± 8.6 μg/L compared with 50 μg/L). The treatments CTL, GO-PEI and RSW at both temperatures presented a Hg level below the quantification limit (5.0 μg/L).

The Hg quantification in clams' tissues from CTL and GO-PEI treatments at 17 °C showed significantly lower Hg concentrations in comparison to the remaining treatments (Hg, GO-PEI + Hg, RSW), with significantly higher levels in organisms exposed to Hg and GO-PEI + Hg (Table 2). At 22 °C significantly lower Hg values were observed at CTL and GO-PEI treatments, while significantly higher Hg concentration was found in clams subjected to Hg (Table 2). Between temperatures, significant differences were observed in GO-PEI + Hg and RSW treatments, with higher values at 17 °C (Table 2).

3.3. Biochemical markers

All biochemical results are showed in Table 3.

3.3.1. Metabolic capacity

At 17 °C significantly lower ETS activity was identified in organisms from RSW compared to the remaining treatments. At 22 °C the lowest values were observed at Hg treatment, with significant differences to the remaining treatments. Between temperatures, significantly higher values were found at 22 °C except for Hg treatment.

3.3.2. Energy reserves

At 17 °C significantly higher GLY content was detected in clams under GO-PEI, Hg and GO-PEI + Hg treatments in comparison to the remaining ones, with the highest value at Hg treatment and no significant differences between CTL and RSW. At 22 °C the highest GLY content was found in clams exposed to Hg, followed by clams under GO-PEI + Hg, with significant differences to the remaining treatments. Between temperatures, significantly higher values were found at 17 °C for GO-PEI treatment, while an opposite

response was observed at Hg and RSW treatments.

At 17 °C significantly higher PROT content was obtained in clams subjected to GO-PEI + Hg and RSW treatments in comparison to the remaining ones. At 22 °C significantly lower PROT values were observed in clams exposed to Hg and GO-PEI + Hg with significant differences to control. Between temperatures, significantly higher values were found at 22 °C for CTL and GO-PEI treatments, while an opposite response was observed at GO-PEI + Hg and RSW treatments.

3.3.3. Enzymatic scavengers

At 17 and 22 °C significantly higher T-SOD activity was detected at GO-PEI + Hg and RSW treatments in comparison to organisms exposed to CTL and GO-PEI. Between temperatures, significant differences were observed at all treatments, with higher T-SOD activity at 22 °C.

At 17 °C significantly lower CAT activity was observed in organisms subjected to Hg, GO-PEI + Hg and RSW in comparison to CTL and GO-PEI treatments. The lowest values were found in clams under RSW, and no significant differences between CTL and GO-PEI as well as between Hg and GO-PEI + Hg. At 22 °C significantly lower values were observed at GO-PEI + Hg and RSW treatments, with no significant differences between CTL, GO-PEI and Hg treatments. Comparing both temperatures, significantly higher CAT activity was recorded at 17 °C regardless the treatment tested.

At 17 °C GSH-PX activity showed no significant difference among all treatments. At 22 °C significantly lower values were obtained at CTL treatment compared to the other ones, with no significant differences among GO-PEI, Hg, GO-PEI + Hg and RSW treatments. Between temperatures, significantly higher GSH-PX activity was recorded at 22 °C regardless the treatment tested, with exception for clams exposed to CTL.

3.3.4. Cellular damage

At 17 °C significantly higher MDA levels were detected in organisms exposed to GO-PEI compared to the remaining treatments. At 22 °C significantly higher values were observed in clams exposed to GO-PEI + Hg and RSW treatments. Between temperatures, significantly higher values were observed at GO-PEI for 17 °C, while higher values were detected at 22 °C in clams subjected to GO-PEI + Hg and RSW treatments.

3.3.5. Neurotoxicity

At 17 °C significantly higher T-CHE activity was observed under Hg, GO-PEI + Hg and RSW compared to other treatments, with no significant differences between CTL and GO-PEI as well as between Hg, GO-PEI + Hg and RSW treatments. At 22 °C significantly higher activity was found in *R. philippinarum* under RSW in comparison to the remaining treatments, while significantly lower values were found in clams subjected to GO-PEI and Hg compared to the remaining treatments. Between temperatures, significant differences were observed at Hg and GO-PEI + Hg treatments, with higher T-CHE activity at 17 °C.

3.4. Histopathological measurements

3.4.1. Digestive tubules

At 17 and 22 °C the highest digestive tubules histopathological index (*ih*) was observed in organisms exposed to Hg, with significant differences to the rest of treatments (except with RSW at 22 °C) and no significant differences were detected between CTL and GO-PEI treatments (Fig. 1A). Between temperatures, significantly higher values were found at 22 °C for CTL and RSW treatments (Fig. 1A).

Fig. 2 shows the haemocytes infiltration (arrows), high evidence of lipofuscin aggregates (*) and atrophied (a) in digestive tubules for each treatment at 17 and 22 °C.

3.4.2. Gills

At 17 °C significantly higher *ih* values were found in gills of clams subjected to Hg and GO-PEI + Hg as compared to the rest of treatments, with no significant differences between CTL and GO-PEI as well as between CTL and remediated seawater treatments (Fig. 1B). At 22 °C the highest *ih* values were observed at GO-PEI + Hg treatment, with no significant differences among GO-PEI, Hg and RSW (Fig. 1B). Between the temperatures, significantly higher values were detected at 22 °C for all treatments except for CTL and Hg (Fig. 1B).

The haemocytes infiltration (arrows), huge enlargement of the central vessel (long arrows), high evidence of lipofuscin aggregates (*) in gills for each treatment under both temperatures were showed in Fig. 2.

3.5. Multivariate analysis

The Principal Coordinates Analysis (PCoA) calculated for Hg concentration in clams' soft tissue ([Hg]clams), histopathological index in gills (G (*ih*)) and digestive tubules (D.T. (*ih*)) as well as all biochemical markers (ETS, PROT, GLY, T-SOD, GSH-PX, CAT, MDA, T-CHE) is shown in Fig. 3. The PCoA axis 1 explained 42.1% of the total variation, separating organisms under treatments at 17 °C in the positive side from others at 22 °C in the negative side. The PCoA2, with 27.9% of the total variation, separating organisms under CTL, GO-PEI and RSW under both temperatures in the positive side form the remaining treatments in the negative side. PCoA1 positive side was highly correlated with CAT ($p > 0.89$), while PCoA1 negative side was highly correlated with T-SOD ($p > 0.95$). PCoA2 negative side was correlated with Hg concentration in tissues, D.T. (*ih*) and GLY ($p > 0.71$).

4. Discussion

Several studies have been assessing the use, efficiency and ecological safety of different synthetic materials for remediation of contaminated waters (namely from metal(loid)s) (Coppola et al., 2019; Mohmood et al., 2016; Nupearachchi et al., 2017; Zhang et al., 2010). Up today, limited information exists on the use of these materials and their potential effects towards organisms (in particular marine species), especially when under predicted climate changes (Andrade et al., 2019; Chen et al., 2016; Morosetti et al., 2020). Also, scarce information is available regarding the possible environmental risks of remediated seawater (Coppola et al., 2019, 2020a,b). Therefore, this study aimed to evaluate the impacts on clams *Ruditapes philippinarum* after chronic exposure to remediated seawater from mercury (Hg), using graphene oxide functionalized with polyethyleneimine (GO-PEI) nanocomposite as adsorbent, under actual and predict temperature increase.

In general, the present findings emphasized the efficiency of GO-PEI in removing Hg from seawater, with low Hg accumulation in clams subjected to remediated seawater, especially at 22 °C. *R. philippinarum* exposed to Hg free treatments (CTL, GO-PEI, RSW) presented similar responses, with clams exposed to Hg treatment presenting greater alterations, regardless the temperature tested. Furthermore, clams exposed to different temperatures showed a different behaviour.

The results of this study showed higher mortality in *R. philippinarum* clams maintained at 22 °C in comparison to clams under control temperature (17 °C), in particular when exposed to Hg treatment. Previous studies already proved that temperature can influence marine organisms' metabolic capacity and oxidative

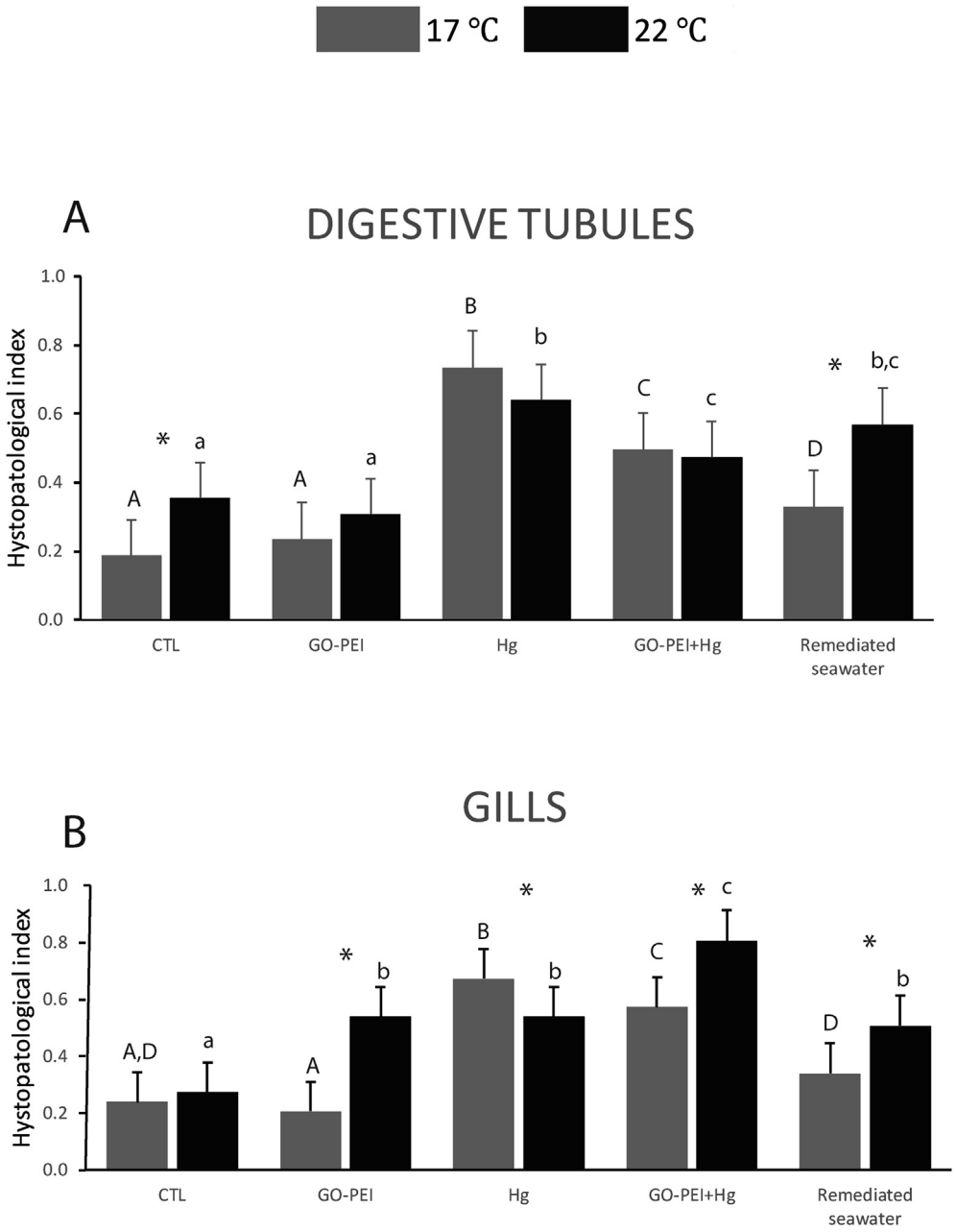

Fig. 1. A: Histopathological index in digestive tubule; **B:** Histopathological index in gills, in *Ruditapes philippinarum* after 28 days-exposure. At each temperature (17 and 22 °C) the tested treatments were: CTL, GO-PEI, Hg, GO-PEI + Hg and Remediated seawater (RSW). Results are mean + standard deviation. Significant differences among treatments are represented with different letters: uppercase letters for 17 °C and lowercase letters for 22 °C. Differences between both temperatures at each treatment are represented with an asterisk.

status (Han et al., 2008; Le Moullac et al., 2007; Velez et al., 2017). Furthermore, it was already demonstrated that warming was responsible for higher sensitivity of organisms to other environmental factors, such as pollutants (Attig et al., 2014; Bat et al., 2000; Coppola et al., 2017, Coppola et al., 2018; Khan et al., 2007; Lannig et al., 2006; Mubiana and Blust, 2007; Sokolova and Lannig, 2008). The present findings further revealed high mortality rate in clams exposed to Hg at control temperature (17 °C), which is in agreement with other works that demonstrated the high toxicity of Hg in marine organisms (Amachree et al., 2014; Chen et al., 2014; Coppola et al., 2018; Pan and Wang, 2011).

Higher mortality rate found in clams exposed to Hg and GO-PEI + Hg at 22 °C in comparison to organisms exposed to the same treatments but at control temperature were not explained by Hg accumulation in clams' tissues since higher metal concentration was observed in clams under 17 °C. This response may thus corroborate the hypothesis that warming greatly alter the sensitivity of organisms to pollutants, affecting their biochemical performance and, thus, influencing organisms' general health status. Our findings also revealed that lower Hg levels in organisms at 22 °C was not associated with strategies to avoid accumulation, namely filtration and respiration depression, since higher metabolic capacity (assessed by ETS activity) was observed in clams under warming conditions. Therefore, it seems that lower Hg accumulation did not result from clams' decreased filtration capacity associated with lower metabolic capacity but may thus result

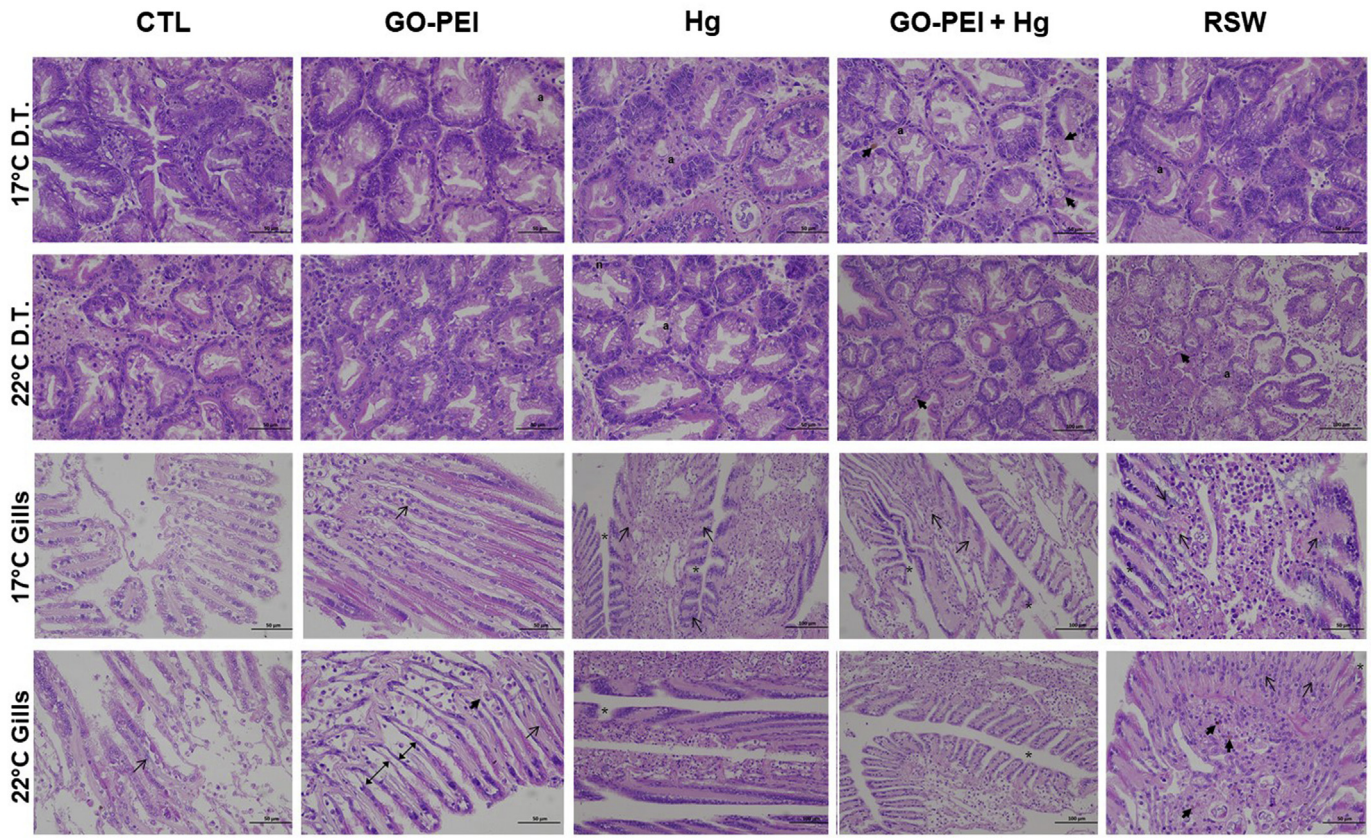

Fig. 2. Micrographs of different tissues in *Ruditapes philippinarum* after 28 days-exposure. At each temperature (17 and 22 °C) the tested treatments were: CTL, GO-PEI, Hg, GO-PEI + Hg and Remediated seawater (RSW). Digestive tubule (D.T.): haemocytes infiltration (arrows), atrophied (a) and necrose (n). Gills: haemocytes infiltration (arrows), evident enlargement of the central vessel (long arrows), abundance of lipofuscin aggregates (*). Scale bar = 50 and 100 μm.

from clams' higher detoxification capacity, which was not evaluated in the present study. Nevertheless, several researches demonstrated that bivalves (clams and mussels) exposed to metals (Hg and Pb) reduce the bioaccumulation by decreasing their metabolic capacity (Freitas et al., 2017b; Casas and Bacher, 2006; Coppola et al., 2018; Velez et al., 2016; Verlecar et al., 2007).

Regardless the accumulation levels, the present findings clearly demonstrated that temperature was the main factor differentiating treatments, with clams' responses clearly distinct between 22 and 17 °C (see Fig. 3). Different studies demonstrated that when bivalves are subjected to temperatures exceeding their thermal tolerance they can experience physiological disturbances (Baek et al., 2014; Han et al., 2008; Marigómez et al., 2017; Moreira et al., 2017; Paillard et al., 2004) and biochemical alterations including cellular damage (Maulvault et al., 2018; Matozzo et al., 2013), increase of oxidative stress (Velez et al., 2017; Greco et al., 2011) as well as metabolic alterations associated with respiratory capacities (Tamayo et al., 2013; Pörtner, 2010; Velez et al., 2017). The results here presented showed that among the most noticeable differences is the metabolic activity, with organisms exposed to warming conditions presenting the highest ETS activity in comparison to clams under control temperature. Results reported by Velez et al. (2017) also showed higher ETS activity in *R. philippinarum* exposed to rise of temperature. Coppola et al. (2018) showed that *Mytilus galloprovincialis* subjected to the combination of temperature rise and Hg presented higher accumulation of this metal in their tissues, with a close relationship with an increase on mussels ETS activity. In the present study, higher metabolic capacity observed in clams under 22 °C was not associated with higher energy reserves expenditure, namely in terms of GLY, especially noticed at Hg, GO-PEI + Hg and RSW treatments. These results may indicate that in the presence of the pollutants, even at lower concentration levels, clams were able to prevent the loss of their energy reserves. Previous studies also demonstrated that in the presence of high temperature bivalves (clam *Macoma balthica* and mussels *M. galloprovincialis*) were also able to preserve their energy reserves content under Cd contamination (Della Torre et al., 2015; Duquesne et al., 2004; Nardi et al., 2017). Nevertheless, the present results revealed that higher metabolism in clams exposed to 22 °C was associated with a general higher antioxidant capacity. In particular, the results here presented revealed that clams under warming conditions presented, in general, higher enzymatic activity (T-SOD and GSH-PX) than clams exposed to 17 °C, a defence strategy already demonstrated by Freitas et al. (2017) who observed the increase of enzymatic scavengers activities (CAT and SOD) in *M. galloprovincialis* under a warming scenario compared to mussels under control temperature. Although higher antioxidant capacity was observed in clams exposed to 22 °C, higher MDA content was generally observed under warming conditions, evidencing that the defence mechanisms were not sufficient to avoid cellular damage. These results agreed with several works that showed an increase of cellular damage in bivalves under warming scenario and/or metals contamination (Coppola et al., 2018; Freitas et al., 2018; Velez et al., 2016), even after increase in antioxidant enzymes activity (Attig et al., 2014; Nardi et al., 2017; Pirone et al., 2019). As an example, Matozzo et al. (2013) showed that in the clam *Chamelea gallina* and in the mussel *M. galloprovincialis* antioxidant enzymes were activated but still MDA levels increased after one week of exposure to

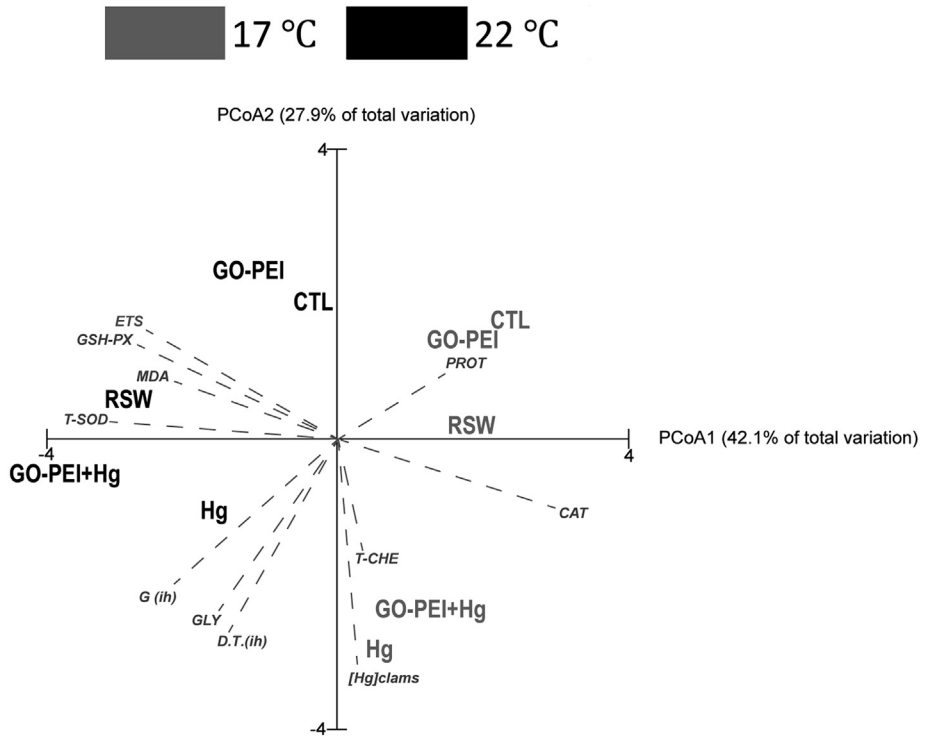

Fig. 3. Principal Coordinated Analyses (PCoA) based on Hg quantification, biochemical parameters and histological alteration measured in *Ruditapes philippinarum* after 28 days-exposure. At each temperature (17 and 22 °C) the tested treatments were: CTL, GO-PEI, Hg, GO-PEI + Hg and Remediated seawater (RSW). Pearson correlation vectors are superimposed as supplementary variables (r > 0.75): ETS, GLY, PROT, MDA, T-SOD, CAT, GSH-PX, T-CHE, [Hg]clams, G *(ih)* and *D.T. (ih)*.

warming scenario (28 °C).

In terms of neurotoxicity, the results here presented showed that increased temperature was responsible for neurotoxic impacts, namely in Hg treatments. Due to the thermo-modulatory function of this enzyme, the thermal stress can be correlated to T-CHE inhibition (Kim et al., 2019), which was already demonstrated by previous researches reporting a significant inhibition of T-CHE activity after exposing the organisms *Pangasianodon hypophthalmus* and *M. galloprovincialis* to metals (zinc (Zn) and Hg) alone and with a simultaneous increase of temperature (Kumar et al., 2020; Morosetti et al., 2020). The obtained results are also in agreement with a study by Costa et al. (2020) which showed increased neurotoxicity in two species of clams (*R. decussadus* and *philippinarum*) in response to increased temperature.

In terms of histopathological alterations, differences between temperatures were also observed in clams under warming scenario and at control treatment temperature, with histopathological impairments including atrophied, haemocytes infiltration and necrose in digestive glands evident at 22 °C. The study conducted by Leite et al. (2020) also showed that temperature rise caused histopathological alterations in gills as increase of haemocytes infiltration, enlargement of the central vessel and abundant lipofuscin aggregates in *M. galloprovincialis* mussels.

Regardless of the temperature tested, the present findings also demonstrated that clams' responses were closely associated with the presence of Hg (Hg and GO-PEI + Hg treatments), with greater alterations in clams exposed to Hg alone than in the presence of GO-PEI. In particular, the results here presented evidence the low toxicity of remediated seawater, with biological responses observed in clams exposed to this treatment similar to the ones observed in clams exposed to CTL and GO-PEI acting alone, regardless the tested temperature (see PCoA graph). Under higher stressful conditions, namely the presence of Hg (Hg and GO-PEI + Hg treatments), the results obtained showed that Hg contaminated clams were not able to present significantly higher antioxidant capacity in comparison to CTL, remediated seawater or GO-PEI exposed clams. Such results may explain high cellular damages in clams exposed to Hg (especially GO-PEI + Hg) at 22 °C, while lower LPO levels in clams subjected to Hg at 17 °C may result from lower ETS activity at this temperature, a mechanism that generates reactive oxygen species. A recent study conducted by Coppola et al. (2019) observed lower oxidative stress and cellular damage in mussels *M. galloprovincialis* when exposed to seawater with low arsenic (As) concentration due to the decontamination of the water by manganese-ferrite ($MnFe_2O_4$) nanoparticles. However, recent literature has demonstrated an increase of non- and enzymatic defences (e.g. lipid peroxidation; superoxidase dismutase; catalase) in clams *R. philippinarum* exposed to different pollutants, mainly metal(loid)s (in specific 100 μg/L of As, 200 μg/L of cadmium (Cd), 1000 μg/L of lead (Pb) and 50 μg/L of Hg) and nanomaterials (Freitas et al., 2018; Marques et al., 2017; Velez et al., 2015). Jiang et al. (2019) showed a rise of antioxidant activity and cellular damage in the same species exposed at low Hg concentration (10 μg/L).

The present study further demonstrated that Hg strongly induced the increase of the T-CHE enzyme at 17 °C. Acetylcholinesterase degrades acetylcholine, a neural transmitter, in choline in cholinergic synapses and neuromuscular junctions (Matozzo et al., 2005). For this reason, the activity of acetylcholinesterase has been used as a biomarker of neurotoxicity. Among metals, Hg is known as a neurotoxic substance, namely to bivalves, by interrupting the nervous transmission. In fact, previous studies demonstrated the inhibition of acetylcholinesterase in bivalves, including in mussels and in clams, can occur due to the presence of metals (Attig et al., 2010; Cajaraville et al., 2000; Chalkiadaki et al., 2014; Matozzo et al., 2005). Nevertheless, since the inhibition of acetylcholinesterase is followed by accumulation of acetylcholine, the elevation of

this compound can also indicate neurotoxicity. This was previously demonstrated by Liu et al. (2011) exposing the clams *R. philippinarum* to Hg. Such findings may explain the results obtained in the present study.

Regarding histopathological impacts, our finding clearly demonstrated greater alterations in clams in the presence of Hg, especially when acting alone, with increase of haemocytes infiltration, atrophied and necrose in digestive tubule tissue. These results are in according with previous studies conducted by Leite et al. (2020) and Cuevas et al. (2015), which showed atrophy in digestive tubules, following a reduction in the thickness of epithelia followed by the expansion of lumen in mussels *M. galloprovincialis* exposed to metals (copper (Cu), Hg, Pb, Zn and titanium (Ti)). Other works also demonstrated that the presence of metals lead to histopathological alterations (haemocytes infiltration and necrose) in gills and digestive tubules of bivalves exposed to Pb (Hariharan et al., 2014) and Cu (Sabatini et al., 2011).

Overall, the present findings demonstrated low impact of remediated seawater towards *R. philippinarum* clams. Such findings are related with the capacity of GO which allows the removal of Hg, highlighting the potential use of this nanomaterial to obtain water quality intended for human consume (Abraham et al., 2017; Kovtun et al., 2019; Tung et al., 2017). Bessa et al. (2020) had already demonstrated the efficiency of GO-PEI (10 mg/L) to remove Hg (50 μg/L) from different water type. While evidencing the capacity to remove Hg from seawater, the present study also demonstrated the low toxicity of the nanomaterial used, with sub-cellular alterations observed in clams exposed to CTL, GO-PEI and remediated seawater being similar, and differing from clams exposed to GO-PEI + Hg and especially Hg. Recent studies conducted with bivalves (oysters, *Crassostrea virginica*; mussels, *M. galloprovincialis*; clams, *R. philippinarum*) exposed to different concentrations of GO (from 1 to 25 mg/L) evaluated possible toxic effects of this nanomaterial (Britto et al., 2020, 2021; Meng et al., 2020; Katsumiti et al., 2017; Khan et al., 2019a,b), showing limited toxic effects of the nanomaterial towards the exposed bivalves. Similarly, the present findings evidence that GO-PEI could be implemented as environment friendly sorbent in industries effluents for water purification before being discharged in aquatic systems. Bessa et al. (2020) further demonstrated the ability of GO-PEI to be regenerated, keeping its high removal performance after successive sorption-desorption cycles allowing its reuse, therefore reducing the environmental impact of its utilization. Although previous studies with graphene nanomaterials always evidenced low toxicity, further studies may be relevant to evaluate the potential use of GO-PEI to remove other pollutants from seawater and the toxicity of resulting remediated water. Recovery of pollutants, although not addressed in this study, should be advised towards a circular and sustainable economy. Commonly, residuals contaminated with Hg are explored by specific industries, that successfully recover Hg from different waste sources (including GO-PEI nanomaterials). However, the recovery of elements from remediated water is still scarcely explored.

5. Conclusion

In general, less biochemical physiological and histopathological alterations were detected in *R. philippinarum* exposed to remediated seawater in comparison to clams subjected to Hg and/or GO-PEI treatments, at control temperature. Furthermore, higher alterations were observed at 22 °C compared to control temperature. This study emphasizes the capability of GO-PEI nanocomposite as a new technology to remove the metal Hg from seawater with low toxic effects in *R. philippinarum* species, although temperature may increase the sensitivity of clams.

Credit author statement

Francesca Coppola: Conceptualization, Formal analysis, Writing – original draft, Weiwei Jiang:, Amadeu M.V.M. Soares: Resources, Paula A.A.P. Marques: Supervision, Resources, Writing – review & editing, Gianluca Polese: Supervision, Resources, Writing – review & editing, Maria Eduarda Pereira: Supervision, Resources, Writing – review & editing, Zengjie Jiang: Supervision, Resources, Writing – review & editing, Rosa Freitas: Supervision, Conceptualization, Resources, Writing – review & editing, Supervision, Resources, Writing – review & editing

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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