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Will temperature rise change the biochemical alterations induced in *Mytilus galloprovincialis* by Cerium oxide nanoparticles and Mercury?

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Abstract

It is known that, for marine coastal ecosystems, pollution and global warming are among the most threatening factors. Among emerging pollutants, nanoparticles (NPs) deserve particular attention as their possible adverse effects are significantly influenced by environmental factors such as salinity, pH and temperature, as well as by their ability to interact with other contaminants. In this framework, the present study aimed to evaluate the potential interactions between CeO₂ NPs and the toxic classic metal mercury (Hg), under current and warming conditions. The marine bivalve *Mytilus galloprovincialis* was used as biological model and exposed to CeO₂ NPs and Hg, either alone or in combination, for twenty-eight days at 17 °C and 22 °C. A suite of biomarkers related to energetic metabolism, oxidative stress/damage, redox balance, and neurotoxicity was applied in exposed and non-exposed (control) mussels. The Hg and CeO₂ NPs accumulation was also assessed. Results showed that the exposure to CeO₂ NPs alone did not induce toxic effects in *M. galloprovincialis*. On the contrary, Hg exposure determined a significant loss of energetic metabolism and a general decrease in biochemical performances. Hg accumulation in mussels was not modified by the presence of CeO₂ NPs, while the biochemical alterations induced by Hg alone were partially canceled upon co-exposure with CeO₂ NPs. The temperature increase induced loss of metabolic and biochemical functions and the effects of temperature prevailed on mussels exposed to pollutants acting alone or combined.

Keywords

Nanoparticles; metals, ocean warming; mussels; metabolic capacity

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1. INTRODUCTION

Nanotechnology is one of the most rapidly developing research fields of the 21st century (Aithal et al., 2015). It involves the study, production, and manipulation of structures, devices, materials or particles with at least one dimension that falls within the range of 1-100 nm length. The unique physicochemical properties of nanoparticles (NPs), substantially different from those of the same bulk materials, made them attractive for a broad range of technological applications and consumer goods (Kotagiri et al., 2015; Shehada et al., 2015). The massive use of NPs resulted in their release into the environment generating an urgent need to predict their impacts on natural ecosystems. In particular, the marine ecosystem represents the final sink of the released NPs in soils and waterways; moreover, it suffers also for the direct release of NPs developed for marine applications (Corsi et al., 2014).

As a consequence of their high surface-area-to-volume ratio, NPs can adsorb other pollutants, which might affect their transport and bioavailability in natural systems and, ultimately, their ecotoxicity (Navarro et al., 2008). The sorption of pollutants onto nano-sized particles was already explored in various studies and attempts to investigate this interaction recently increased (Canesi et al., 2015; Liu et al., 2018; Naasz et al., 2018 and citations therein). The joint effects of contaminants and NPs on organisms can be categorized as synergistic, antagonistic or independent, depending on the type of NPs and the associated contaminant (Canesi, et al., 2015). Most of the studies investigating the combined effects of NPs and pollutants dealt with carbon-based NPs (nanotubes, fullerenes) and n-TiO₂, whilst fewer studies focused on other metal-based NPs (Canesi et al., 2015; De Marchi et al., 2019). Notwithstanding, assessing this topic is essential to properly predict the risk related to the presence of NPs in the environment, since organisms are exposed to complex mixtures of contaminants in natural conditions.

Among NPs, cerium oxide nanoparticles (CeO₂ NPs) are largely employed in many industrial applications, being used as catalysts in diesel fuel oil production, glass polishers, antioxidant agents in biomedicine or as ultraviolet absorbent (Sun et al., 2012; Truffault et al., 2012). The environmental concentration of CeO₂ NPs is expected to be in the range of ng L⁻¹ in surface water, pg L⁻¹ in seawater and µg L⁻¹ in hotspots as wastewater treatment plants (Giese et al., 2018; Gottschalk et al., 2015; Keller & Lazareva, 2013). The toxic effects of CeO₂ NPs were reported on different marine taxa such as bacteria, algae, bivalves, echinoderms and fish (Sendra et al., 2018; Canesi et al., 2014; García et al., 2012; Fairbairn et al., 2011; Rodea-Palomares et al., 2011; Van Hoecke et al., 2009). It was demonstrated that CeO₂ NPs may target the organism at different biological scales, inducing oxidative stress and immunomodulation, altering organisms behavior, reducing growth or development, and increasing lethality (Auguste et al., 2019; Bour et al., 2015; Conway et al., 2014; Garaud et al., 2015; Koehlé-Divo et al., 2018).

Cerium-based oxides and their composites were also reported to be reliable adsorbents for metal traces (Duncan & Owens, 2019). Indeed, CeO₂ NPs have been employed for sorption from water media of many different cations, such as As⁵⁺, As³⁺, Cr⁶⁺, Pb²⁺, Cd²⁺, F⁻, Hg⁰, Hg²⁺ and U⁶⁺ (Olivera et al., 2018). Although the potential use of these NPs can be a promising solution for water decontamination, several crucial issues

should be addressed regarding this application, such as the possible occurrence of unexpected effects due to the interactions between NPs, including CeO₂ NPs, and metals.

Among metals, mercury (Hg) ranks in the third position in the priority list of substances that can potentially threaten human health by ATSRD (2017) due to its persistence in the environment and its propensity to bioaccumulate in the organism (Jiang et al., 2006). Mercury has been found in coastal ecosystems since decades ago, derived from many activities, including chloro-alkali industry (among others, Nunes et al., 2008). Although along recent years some of these activities have been limited, Hg has been used in novel applications, including fluorescent lamps, together with rare earth elements as Ce (among others, Tunsu et al., 2014, 2016). In coastal waters, Hg was detected in concentrations up to $\mu\text{g L}^{-1}$ (Gworek et al., 2016), whereas in open sea Hg concentrations were estimated to be in the range of 0.5-3.0 ng L^{-1} (Faganeli et al., 2012). The capacity of Hg to affect biological processes, including biochemical mechanisms involved in organisms' oxidative stress status, was assessed by several authors (Chen et al., 2014; Coppola et al., 2018; Velez et al., 2016). The negative impacts that Hg has on energy metabolism, physiological mechanisms and reproduction were also ascertained (Pytharopoulou et al., 2013; Velez et al., 2016).

Besides pollution, coastal ecosystems are affected by the ever-increasing threat posed by the changing climate. Indeed, the adverse effects of contaminants could be further amplified if combined with other environmental stressors such as global warming (Sokolova & Lanning, 2008). Several studies highlighted that an increase of water temperature is able to affect the bioaccumulation of pollutants, including metals, but also to modify organism's susceptibility to contaminants (Guinot et al., 2012; Banni et al., 2014; Coppola et al., 2017; Nardi et al., 2017). However, the effect of increasing temperature on NPs is still barely investigated (Andrade et al., 2019a), and less is known on the influence of temperature rise on the combined effects between NPs and metals (Freitas et al., 2018).

In this view, the aim of this study was to assess the potential interaction of CeO₂ NPs with the toxic metal Hg²⁺ under the temperature representative of current conditions at the sampling area, and the temperature increase forecasted to occur at the end of the 21st century (IPCC, 2014). The marine bivalve *Mytilus galloprovincialis* was used in the present study since it has been widely used as bioindicator species for a vast variety of pollutants, including metals as Hg and nanoparticles (Canesi et al., 2015; Coppola et al., 2018; Barranger et al., 2019; Freitas et al., 2019; Pinto et al., 2019). Mussels were exposed to CeO₂ NPs and Hg²⁺ individually and in combination at 17 and 22 °C, for twenty-eight days. Several biochemical endpoints were evaluated such as glycogen (GLY) content, protein (PROT) content and electron transport system (ETS) activity, as markers of energy alteration and metabolism; catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR) and glutathione-S-transferases (GSTs) activities, as markers of oxidative stress; lipid peroxidation (LPO) and protein carbonyl (PC) levels, as markers of oxidative damage. Moreover, reduced glutathione (GSH) content was evaluated to measure mussel's redox balance, while acetylcholinesterase (AChE) activity was used as a neurotoxicity endpoint.

2 . MATERIALS AND METHODS

2.1. Nanoparticles synthesis and characterization

CeO₂ NPs were prepared following Plakhova et al. (2016) procedure, with modifications fully described by Villa et al. (2020). The hydrodynamic size and ζ -potential of NPs were characterized through Dynamic Light Scattering (DLS) using a Zetasizer nano ZS instrument (Malvern) equipped with a 633 nm solid state He–Ne laser at a scattering angle of 173°, operating at 25 °C. The size and charge analyses were averaged from at least three repeated measurements.

2.2. Experimental conditions

Specimens of the Mediterranean mussel *Mytilus galloprovincialis* (9.09 ± 3.3 g without shell) were collected from the Mira channel at the Ria de Aveiro lagoon (northwest coast of Portugal), considered a non-contaminated area, namely in terms of metals (among others, Freitas et al., 2014). After sampling, mussels were acclimated and depurated for one week in the laboratory, under the following controlled conditions: temperature 17.0 ± 1.0 °C; pH 8.0 ± 0.1 , photoperiod 12 h light and 12 h dark in continuous aeration and artificial seawater (salinity 30 ± 1). Artificial seawater was prepared by mixing a commercially available salt mixture (Tropic Marin Pro Reef salt; Tropic Marine, Germany) with freshwater obtained by reverse osmosis. A temperature of 17 °C and a pH value of 8.0 were selected as representative of mean values measured at the sampling site during mussels field sampling.

After the acclimation period, organisms were exposed in triplicates (three mussels per aquarium, three aquaria per treatment, nine individuals per treatment), in 3 L aquaria, considering the following treatments: A) CTL-17, seawater at 17 °C; B) CeO₂ NPs-17, 1 μ g/L of CeO₂ NPs at 17 °C; C) Hg-17, 10 μ g/L of Hg²⁺ at 17 °C; D) Hg+Ce NPs-17, 10 μ g/L of Hg²⁺ and 1 μ g/L of CeO₂ NPs at 17 °C; E) CTL-22, seawater at 22 °C; F) CeO₂ NPs-22, 1 μ g/L of CeO₂ NPs at 22 °C; G) Hg-22, 10 μ g/L of Hg²⁺ at 22 °C; H) Hg+Ce NPs-22, 10 μ g/L of Hg²⁺ and 1 μ g/L of CeO₂ NPs at 22 °C. The temperature of 22 °C was selected as representative of the predicted global mean surface temperature increase by the end of the 21st century (2.6 °C to 4.8 °C under RCP8.59) (IPCC, 2014). Different temperatures (17 ± 1 and 22 ± 1 °C) were achieved by maintaining each group of aquaria in different climatic rooms. The water temperature of 22 °C was gradually obtained before the experiment beginning. The temperature in each aquarium was daily monitored with a thermometer and adjusted if necessary.

Mussels were exposed to the above-mentioned conditions for twenty-eight days, during which water was changed weekly and exposure conditions re-established immediately. Every week, after spiking, a sample of water was collected from each aquarium to evaluate the real concentration of pollutants. Every two days animals were fed with Algamac protein Plus. Also, during the entire experimental period, aquarium seawater pH, temperature, and salinity were monitored to avoid any changes that could influence the obtained results.

At the end of the exposure period, animals were collected, individually frozen, and stored at -80 °C until both biochemical analyses and pollutant measurements were carried out. Whole soft tissues of each mussel (three animals per replicate, nine individuals per treatment) were individually and manually homogenized with a mortar and a pestle in liquid nitrogen. Each homogenized sample was divided in different aliquots of 0.5 g fresh weight (FW) each for biochemical assays and quantification of pollutants concentrations.

2.3 Mercury and Cerium quantification in seawater and mussels' soft tissues

The quantification of Hg in seawater aliquots was performed by cold vapour atomic fluorescence spectroscopy, while total Hg in biological material was quantified through Atomic Absorption Spectroscopy. In what regards to Ce, concentrations were quantified in seawater and in mussel's samples using inductively coupled plasma - mass spectrometry. Analytical procedures are fully described in the Supplementary Material (SM).

2.4 Biochemical parameters

Mussels soft tissues were used to carry out the biomarkers analyses following the procedures described in Pirone et al. (2019). For each experimental condition, a suite of biomarkers was analyzed including: metabolic capacity (electron transport system activity, ETS), energy reserves (glycogen content, GLY; protein content, PROT), oxidative stress (catalase activity, CAT; glutathione peroxidase activity, GPx, glutathione S-transferases activity, GSTs; glutathione reductase, GR), oxidative damage and redox status (lipid peroxidation levels, LPO; protein carbonylation levels, PC; reduced glutathione content, GSH) and neurotoxicity (Acetylcholinesterase activity, ACHE). A detailed description of each method is reported in the SM.

2.5 Statistical analysis

All data obtained from the biochemical analysis and pollutants quantification were analyzed employing the PERMANOVA+ add-on in PRIMER v6 (Anderson et al. 2008). The permutation method used was the unrestricted permutation of raw data, testing the maximum number of permutations (9999), while the Monte Carlo test was selected to obtain numerical results for each pair of comparisons. Values lower than 0.05 ($p \leq 0.05$) were considered as significantly different and are reported in Table S2. Degrees of freedom = 4. The null hypothesis tested was: for Hg accumulation and each biomarker response, no significant differences existed among different exposure treatments.

3. RESULTS

3.1. *CeO₂ nanoparticles characterization*

Ad hoc synthesized CeO₂ NPs were homogeneous in size, with a mean diameter centered at ~ 5 nm (transmission electron microscopy micrograph SI1). DLS measurements were carried out in MilliQ water, and the fitting of the correlation function by a bi-exponential function showed the presence of two populations centered at 146 ± 152 and 400 ± 75 , the latter being the most relevant, as stated by the number-weighted size distribution graph (Fig. S1). The ζ -potential in MilliQ water indicated a strongly positive surface charge ($+36.7 \pm 2.3$ mV). In seawater higher aggregation occurred, leading to the formation of larger CeO₂ NPs aggregates (1760 ± 270 nm). In this medium the measurement of ζ -potential was hampered by the too high value of the ionic strength.

3.2. *Mercury and Cerium quantification in seawater and mussels' soft tissues*

Concerning the Hg concentration in water, no differences were observed between Hg levels measured in the aquaria contaminated with the metal alone ($4.12 \pm 0.51 \mu\text{g L}^{-1}$) with respect to Hg + Ce ($2.95 \pm 1.38 \mu\text{g L}^{-1}$) at 17 °C. Similarly, no differences were observed at 22 °C ($2.1 \pm 0.4 \mu\text{g L}^{-1}$ for Hg vs $4.6 \pm 1.4 \mu\text{g L}^{-1}$ for Hg + Ce) (Table S1). The measurement of Ce levels in water confirmed a fast sedimentation of the NPs since no differences were observed between the concentration of the CTL and the NP contaminated waters at both temperatures (Table S1). However, given the low concentration of NPs used in this study, this data should be considered with caution.

The amount of Hg²⁺ in soft tissue confirmed the uptake and bioaccumulation of the metal by mussels (Table 1). The concentration of Hg²⁺ was significantly higher in the group exposed to Hg²⁺ alone and combined with CeO₂ NPs than to CTL, both at 17 and 22 °C. The co-exposure to Hg²⁺ and NPs did not affect metal accumulation at 17 °C, while a slight increase of metal content was observed in mussels exposed to Hg + Ce NPs at 22 °C compared to the exposure to the metal alone as well as to the metal NP combination at 17 °C. The tissue level of CeO₂ NPs did not show any significant differences among exposure conditions.

3.3 *Biochemical assays*

3.3.1 *Metabolic capacity and energy reserves*

At 17 °C the exposure to CeO₂ NPs did not affect ETS activity compared to the CTL. On the contrary, a significantly lower ETS activity was observed in mussels exposed to Hg²⁺ alone and combined with NPs with respect to the CTL and CeO₂ NPs. At 22 °C no significant differences in ETS activity were observed among all treatments. Comparing the ETS activity measured at 17 °C and 22 °C it was possible to observe significantly lower values at higher temperature in CTL and in Ce groups, and an opposite pattern at Hg²⁺ treatment (Fig 1A).

At 17 °C, the GLY level was significantly lower in all exposed groups in comparison to CTL. On the contrary, at 22 °C a significant increase of GLY was observed in mussels exposed to CeO₂ NPs and Hg²⁺ alone compared to the CTL. Comparing the data at the two different temperatures, significant differences were observed in non-contaminated mussels, with higher values at 17 °C (Fig. 1B).

Concerning the PROT content, at 17 °C significantly lower values were found in Hg²⁺ and Hg+Ce exposed mussels in comparison to CTL and Ce treatments. At 22 °C no significant differences were observed among treatments. Between both temperatures, significant differences were observed at CTL and CeO₂ NPs exposed mussels, with significantly higher PROT content at 17 °C (Fig 1.C).

3.3.2 Antioxidant and biotransformation defenses

At 17 °C mussels exposed to Hg²⁺ and Hg+Ce NPs showed a significant decrease in CAT activity in comparison to the CTL, while no significant differences were observed among treatments at 22 °C. Significant differences between temperatures were observed in the CTL groups, with higher CAT activity at 17 °C (Fig 2A).

The activity of GPx showed no significant differences among treatments, at both 17 °C and 22 °C. Significantly higher values were measured at 22 °C in comparison to 17 °C when organisms were exposed to Hg²⁺ and Hg+Ce (Fig 2B).

Concerning the GR, at 17 °C significantly lower activity was observed in mussels exposed to Hg²⁺ and Hg+Ce in comparison to the CTL and CeO₂ NPs alone. At 22 °C no significant differences were observed among treatments. On the contrary, significant differences between temperatures were observed at the CTL and CeO₂ NPs exposed mussels, with higher GR activity at 17 °C (Fig. 2C).

At 17°C the GSTs activity decreased significantly in mussels exposed to Hg²⁺ and Hg+Ce NPs in comparison to CTL, while was significantly increased in mussels exposed to Hg+Ce NPs compared to Hg²⁺ alone. At 22 °C no significant differences were found among treatments. Between temperatures, the GSTs activity significantly decreased in CTL, CeO₂ NPs and Hg+Ce NPs mussels at 22 °C in comparison with the same groups at 17°C (Fig. 2D).

3.3.3 Oxidative damage

At 17 °C LPO levels were significantly lower in mussels exposed to Hg²⁺ and Hg+Ce NPs compared to the CTL and CeO₂ NPs. At 22 °C LPO showed significant differences between Hg+Ce and CeO₂ NPs mussels, as well as between Hg+Ce and Hg²⁺ mussels, with lower values in mussels exposed to Hg+Ce NPs. Significant differences between temperatures were observed at CTL, with higher LPO levels at 17 °C (Fig. 3A).

The PC levels were significantly lower only in Hg^{2+} exposed mussels in comparison to CTL and CeO_2 NPs treatments at 17 °C. At 22 °C no significant differences were observed among treatments. Between temperatures no significant difference was observed at each of the tested conditions (Fig. 3B).

3.3.4 Redox balance

At 17 °C, GSH showed significantly lower levels at Hg^{2+} and $\text{Hg}+\text{Ce}$ NPs mussels in comparison to the CTL ones. At 22 °C no significant differences were observed among conditions. Significant differences between temperatures were observed for the CTL, Hg^{2+} and $\text{Hg}+\text{Ce}$ NPs groups, with higher values at 17 °C for the CTL mussels and an opposite trend for Hg^{2+} and $\text{Hg}+\text{Ce}$ NPs mussels (Fig. 4).

3.3.5 Neurotoxicity

At 17 °C, significantly lower values of AChE activity were observed for Hg^{2+} exposed mussels compared to the CTL and CeO_2 NPs exposed mussels, resulting in 56 % inhibition of AChE activity. The mussels exposed to the combination of Hg^{2+} and CeO_2 NPs groups showed a 50 % of inhibition higher than controls. In this group, a significant increase in AChE activity was also observed in comparison to the group exposed to Hg^{2+} alone. At 22 °C higher AChE activity was observed for mussels exposed to Hg^{2+} and $\text{Hg}+\text{Ce}$ NPs compared to the CTL (60% enhancement in both groups). The increased temperature at 22 °C led to significant inhibition of AChE up to 53% in the CTL and 38% for CeO_2 NPs exposed mussels, compared to 17 °C. An opposite response was observed for Hg^{2+} and $\text{Hg}+\text{Ce}$ NPs contaminated mussels, where an increase of 75% and 63% activity, respectively, was measured (Fig. 5).

4. DISCUSSION

This study aimed to evaluate the potential effects arising from the interactions between CeO₂ NPs and the toxic metal Hg²⁺ on the bivalve *M. galloprovincialis*. The combined effects of NPs and pollutants represent a very important but still controversial aspect of NPs ecotoxicity, as several research studies pointed out that not only the intrinsic properties of NPs, but also their ability to interact and often behave as pollutant carriers, could amplify the adverse impacts of NPs for aquatic ecosystems. Although the interactions of CeO₂ NPs with metals in the aquatic environment are likely to occur, studies on their combined effects on organisms are lacking. Therefore, our first aim was to fill this gap of knowledge. The influence of sea warming on this interaction was also evaluated. Indeed, several studies highlighted that the increase in temperature amplifies the toxicity of different classes of environmental pollutants (Sokolova & Lannig, 2008; Coppola et al., 2018; Andrade et al., 2019a). It is well known that changes in seawater parameters such as temperature would affect significantly NP fate and, as a consequence, the bioavailability and toxicity to organisms (Corsi et al., 2014). Nevertheless, very few studies investigated the consequences of seawater warming on NP ecotoxicity and none on ceria NPs. Moreover, up to now no studies assessed the influence of warming on the ecotoxicity of NPs combined with metals. Therefore, a further novelty in our study is represented by the attempt to show the effects resulting from the exposure to such combination of stressors.

4.1 Effects of CeO₂ NPs and Hg²⁺

Bivalve mollusks are identified as ideal sentinel species for the assessment of ecotoxicological impacts of NPs due to their eco-physiological features (Canesi et al., 2012). Among them, the high filter-feeding capacity shown by *M. galloprovincialis* (about 50 mL of seawater per min (Famme et al., 1986)) makes this species an efficient accumulator of pollutants from the environment, leading to the opportunity to evaluate the concentration and bioavailability of toxic agents *in situ*. Moreover, *M. galloprovincialis* constitutes a source of food and habitat for many other marine species (Beyer et al., 2017; Świacka, et al., 2019), therefore contributing to trophic transfer of pollutants (Farrell and Nelson 2013; Larsen et al., 2016). Focusing on CeO₂ NPs, a previous study on *M. galloprovincialis* reported that most of the CeO₂ NPs filtered from the water column were ejected through pseudo-feces, but a non-negligible fraction could also bioaccumulate in tissues upon long-term exposure to CeO₂ NPs in the mg L⁻¹ range (Conway et al., 2014). In agreement with this observation, the study by Montes and co-authors (2012) showed that in mussels exposed for four days to increasing doses of CeO₂ NPs (1-10 mg L⁻¹), a significant bioaccumulation was measured only at the highest concentration. Some studies showed also the ability of CeO₂ NPs to induce toxic effects in *M. galloprovincialis* either *in vitro* (Sendra et al., 2017) and *in vivo* (Auguste et al., 2019). In this latter study, the exposure for 96 h at 100 µg L⁻¹ triggered oxidative stress and modulated these genes involved in detoxification, immune response and neuroendocrine signaling.

Conversely to these findings, in the present study a negligible accumulation of Ce was measured in mussels exposed to the CeO₂ NPs acting individually as well as in combination with Hg²⁺. The low accumulation levels were also reflected in the absence of biological effects observed upon exposure to NPs alone. This result is likely due to the low concentration of NPs tested in this study (which are approaching the environmental ones), combined with the fast aggregation and sedimentation of the NPs occurring in the exposure media, which reduced further the NP bioavailability for mussels.

On the contrary, the present study revealed that Hg²⁺ was able to affect significantly the majority of the investigated biomarkers. Overall, the present results pointed out that Hg²⁺ tends to inhibit the metabolic capacity of the organisms, as the activity of electron transport chain was decreased. Lower PROT and GLY concentrations observed in contaminated mussels suggested a decrease of energy supplies. Previous studies reported a down-regulation of protein synthesis in mussels exposed to metals such as Cr, Cu and Mn (Pytharopoulou et al., 2008; Kalpaxis et al., 2004). Besides, the observed lowering of LPO might suggest a potential decrease of the total lipid content, but may also result from the decrease of the ETS activity associated with lower reactive oxygen (ROS) generation.

Concerning the effects on the antioxidant machinery, almost all enzymes were inhibited in organisms exposed to Hg²⁺ and to the combination of Hg²⁺ and CeO₂ NPs, regardless the temperature tested. Singaram and co-authors (2013) reported similar results in the crab *Scylla serrata* exposed to 1 and 10 µg L⁻¹ of Hg for 14 days, showing inhibition of CAT and GPx enzymes and detecting a lower content of GSH, but unlike the present study authors reported a parallel increase of GSTs activity and LPO levels. Similarly, Coppola and co-workers (2017) reported an increase of GSTs, CAT and SOD activities in *M. galloprovincialis* exposed to 0.25 mg L⁻¹ Hg²⁺ for twenty-eight days.

The observed reduced antioxidant activity could be associated with an imbalance of the antioxidant enzymes due to ROS overproduction, a mechanism already suggested for metals, including Hg²⁺ (Franco et al., 2009; Pytharopoulou et al., 2011). Nevertheless, since higher ETS activity was observed at control organisms (i.e., uncontaminated mussels at control temperature - 17 °C) this could result into higher ROS content at this condition and higher antioxidant enzymes activity. On the other end, lower ETS activity observed in contaminated organisms resulted into less ROS generation with no need for antioxidant defenses activation.

As far as the redox balance is concerned, the GSH is a key molecule involved in the excretion/detoxification of inorganic Hg, through the conjugation mediated by GSTs (Long et al., 2011). Therefore, the decrease of GSH could be due to its binding with the metal followed by excretion of the GSH-Hg complex, in agreement with the hypothesis already suggested by Franco and coauthors (2009) for this metal. The present findings further revealed that such depletion could also have resulted from the lack of GR activation that decreased its activity.

The decreased activity of ETS, one of the cellular sources of ROS, and the absence of protein damage suggested that the organisms exposed to Hg^{2+} were not undergoing an overproduction of ROS, but rather a decrease of filtrating rate due to valve closure, still using energy reserves. Indeed, the closure of valves is a protective strategy that mussel activates to cope with stressful conditions and avoid accumulation of pollutants, some evidence indeed demonstrated that lower ETS activity resulted in a lower accumulation in bivalves (Gosling, 2003; Coppola et al., 2018; Freitas et al., 2018). Nevertheless, this last hypothesis should be further confirmed.

Concerning AChE activity, the present findings are in line with results from other studies in which Hg^{2+} determined an inhibition of this enzyme in different species, as in the freshwater fish *Cyprinus carpio* (Suresh et al., 1992) and in the crab *Carcinus maenas* (Elumalai et al., 2007), suggesting an interference of this metal with the cholinergic neurotransmission.

A different pattern of biomarker modulation was observed upon the co-exposure of Hg^{2+} and CeO_2 NPs. The combination of both contaminants seems to partially recover the toxic effect induced by Hg^{2+} alone on GSTs, GR and AChE activities. The same was observed for GSH content that increased in $\text{Hg} + \text{Ce}$ NPs group with respect to the group exposed to Hg^{2+} alone. Thus, these results seem to suggest an antagonistic interaction between the two contaminants, even though the biological effect is given mostly by the chemical pollutant, which always prevails on the physical one. A similar antagonistic behavior was reported from Anjum and co-workers (2014) between iron oxide NPs functionalized with dithiocarbamate ($\text{Fe}_3\text{O}_4@\text{SiO}_2/\text{SiDTC}$ also called IONP) and Hg^{2+} in the brain of the European eel *Anguilla anguilla*. Authors showed that the extent of LPO and the activity of GR and GSTs decreased with increasing exposure time to the two contaminants, suggesting a recovery against the negative effects triggered by the exposure to Hg^{2+} alone upon co-exposure conditions. On the other hand, a study conducted with *A. anguilla* blood cells highlighted the occurrence of this antagonism only at the initial period, whereas at late hours of exposure, IONP were unable to mitigate the negative effects triggered by Hg^{2+} (Mohmood et al., 2015). Another study showed also that Hg^{2+} can bind to the surface of Ag NPs (Katok et al., 2012) decreasing its bioavailability for the organism.

The observed decrease of Hg^{2+} toxicity in the presence of CeO_2 NPs could be due to the adsorption of the metal on NPs that undergoing fast agglomeration and sedimentation might reduce the bioavailability for the organisms. Some studies in fact reported the ability of Cerium-based nanomaterials to adsorb and sequester other metals such as As and Cr (Recillas et al., 2010; Dados et al., 2014; Olivera et al., 2018). As an example of successful use for the removal of Hg^{2+} from water medium we can cite a work employing a nano ceria-impregnated silica-iron oxide material (Dados et al., 2014) and a composite of ceria-zirconia-titania for the removal of metallic Hg^0 exploiting a catalytic oxidation method (Li et al., 2016). With the aim to test this hypothesis, the influence of nano ceria on Hg^{2+} bioavailability and bioaccumulation was assessed. Results showed that the Hg^{2+} levels were only slightly reduced in water in the presence of CeO_2 NPs and the bioaccumulation was similar in the organisms exposed to the metal alone and in combination with NPs.

Therefore, a physico-chemical interaction in water medium is unlikely to occur, but rather an antagonistic effect could take place on biological target inside the organism, potentially affecting the metabolism of the metal. In support to this hypothesis, the most marked differences between Hg^{2+} alone and $\text{Hg}+\text{NPs}$ were observed on GSH and enzymes involved in its pathway, such as GR and GSTs. Due to the crucial role of GSH in Hg detoxification, it is possible that the combination with CeO_2 NPs allows Hg to bypass the GSH response mechanism. Nevertheless, this hypothesis needs further confirmations. The presence of the NPs could also alter the biodistribution of the metal in the organism. In particular, biodistribution is essential for determining the adverse effects triggered by pollutants, as different compartments in the organism could show different sensitivity to contaminants. For instance, nTiO_2 not only adsorbed and enhanced the accumulation of As in *Artemia salina*, but also modified the subcellular allocation of As in the organism (Liu et al., 2018).

4.2 Effects of temperature increase

Due to global warming occurring in coastal marine areas, it is important to understand how the increase in temperature can influence the effects of contaminants for benthic marine species. Several studies highlighted that thermal stress induces a broad range of adverse effects in bivalve mussels such as imbalance of oxidative status, cellular damages, impairment of physiological functions and reduced survival (Munari et al., 2011; Matozzo et al., 2013; Velez et al., 2017). Besides direct impacts on organisms, the rise in temperature could influence the susceptibility of organisms to contaminants including trace metals as Hg, either modifying the bioaccumulation or affecting the organism biochemical performances (Sokolova & Lannig, 2008). For instance, Verlecar and coauthors (2007) showed that the increase of temperature induced oxidative stress in *Perna viridis* and was also able to amplify the oxidative damage triggered by Hg, measured as level of thiobarbituric acid reactive substances. Concerning the effects of the rise in temperature on NPs, Chen and co-authors (2012) demonstrated an enhanced aggregation of CeO_2 NPs in KCl and CaCl_2 solutions at increased temperatures. This effect is probably due to the lower solution viscosity and interfacial energy barrier at higher temperature. These findings are also supported by Yung and co-workers (2017), which detected a higher aggregation and sedimentation of Zn NPs at higher temperature and salinity. Therefore, an increased temperature could affect the bioavailability of the NPs that become less mobile in the water column and tend to easily settle down (Petosa et al., 2010).

On the contrary, in the present study, the effects caused by temperature increase override those caused by pollutants (both acting individually or in combination). In fact, control organisms showed a significant loss of metabolic performance, shifting from 17 °C to 22 °C. As a consequence of this metabolic depression also the activity of antioxidant capacity was lowered. Similarly to the present study, Coppola and co-workers (2018) exposed *M. galloprovincialis* to Hg^{2+} under warming conditions (21°C) and detected a limited capacity to activate the antioxidant defenses due to the reduced metabolic capacity. Also, the study by Coppola et al. (2017) showed that the rise of temperature from 17 °C to 22 °C reduced the ETS activity

preventing the bioaccumulation of Hg in *M. galloprovincialis*. In line with the present results, those authors pointed out that the adverse outcomes induced by temperature overcame the metal effects. Moreover, Andrade et al. (2019b) evidenced that the exposure of *M. galloprovincialis* to tidal regime and to an increased temperature (21°C) resulted in lower metabolic capacity. This may be due to a closure of the valves and metabolic depression put in place as the organism defense response under heat stress. An increase of the frequency and duration of valve closure in response to thermal stress was already documented in the freshwater mussel *Unio tumidus* (Lurman et al., 2014). A similar behavior was observed also in *M. galloprovincialis*, which under heat stress exhibited valve closure and metabolic depression (Anestis et al., 2007).

Finally, neurotoxicity was observed in non-contaminated organisms exposed to increased temperature, with a decrease in AChE activity. Similarly, Kumar et al., (2020) reported a significant inhibition of AChE activity after exposing the fish *Pangasianodon hypophthalmus* to Zn-NPs alone and with concurrent increased temperature. The enzyme has, indeed, a thermo- modulatory function and thermal stress can lead to AChE inhibition (Kim et al., 2019). Notwithstanding, a clear correlation between cholinesterase loss and increasing temperature was poorly described in bivalve species (Kamel et al., 2014). AChE is crucial for cell proliferation, migration and differentiation through signaling process (Layer et al., 2013; Falugi and Aluigi, 2012). Therefore, the loss of AChE activity might lead to neurological disorders and ultimately might affect organism survival (Shinotoh et al., 2000).

5. CONCLUSIONS

The present study represents the first attempt to assess the combined effects of CeO₂ NPs and Hg²⁺ using as biological model an economically and ecologically valuable marine species such as *M. galloprovincialis*. Furthermore, the potential influence of sea warming on this interaction was evaluated. The CeO₂ NPs did not affect significantly the mussel metabolic metabolism and oxidative status. On the contrary, Hg²⁺ induced a general metabolic depression and inhibition of antioxidant enzymes in organisms. A slight antagonistic effect of the two pollutants was observed, even if the effects of the metal prevailed on the NPs. The present results highlighted also that warming conditions do affect metabolic performance and reduce neurological functions of mussels. These effects might impact on the resilience of organisms towards chemical stress and lead to negative repercussion on key physiological functions such as feeding, growth and reproduction, ultimately affecting mussel population health.

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Figure legends

Fig. 1. A: Electron transport system (ETS) activity; B: Glycogen (GLY) content; C: Protein (PROT) content (mean + standard deviation) (n=9), in *M. galloprovincialis* exposed to CTL, as well as CeO₂ NPs and Hg²⁺ individually and in combination. Significant differences ($p \leq 0.05$) among exposure conditions were represented with different lowercase letters for groups exposed at 17°C and upper case letters for groups exposed at 22°C. In addition, an asterisk represents significant differences between groups exposed to different temperatures (17°C and 22°C).

Fig. 2. A: Catalase (CAT) activity; B: Glutathione peroxidase (GPx) activity; C: Glutathione reductase (GR) activity; D Glutathione S-transferases (GSTs) activity (mean + standard deviation) (n=9), in *M. galloprovincialis* exposed to CTL, as well as CeO₂ NPs and Hg²⁺ individually and in combination. Significant differences ($p \leq 0.05$) among exposure conditions were represented with different lowercase letters for groups exposed at 17°C and upper case letters for groups exposed at 22°C. In addition, an asterisk represents significant differences between groups exposed to different temperatures (17°C and 22°C).

Fig. 3. A: Lipid peroxidation (LPO) level; B: Protein carbonylation (PC) level (mean + standard deviation) (n=9), in *M. galloprovincialis* exposed to CTL, as well as CeO₂ NPs and Hg²⁺ individually and in combination. Significant differences ($p \leq 0.05$) among exposure conditions were represented with different lowercase letters for groups exposed at 17°C and upper case letters for groups exposed at 22°C. In addition, an asterisk represents significant differences between groups exposed to different temperatures (17°C and 22°C).

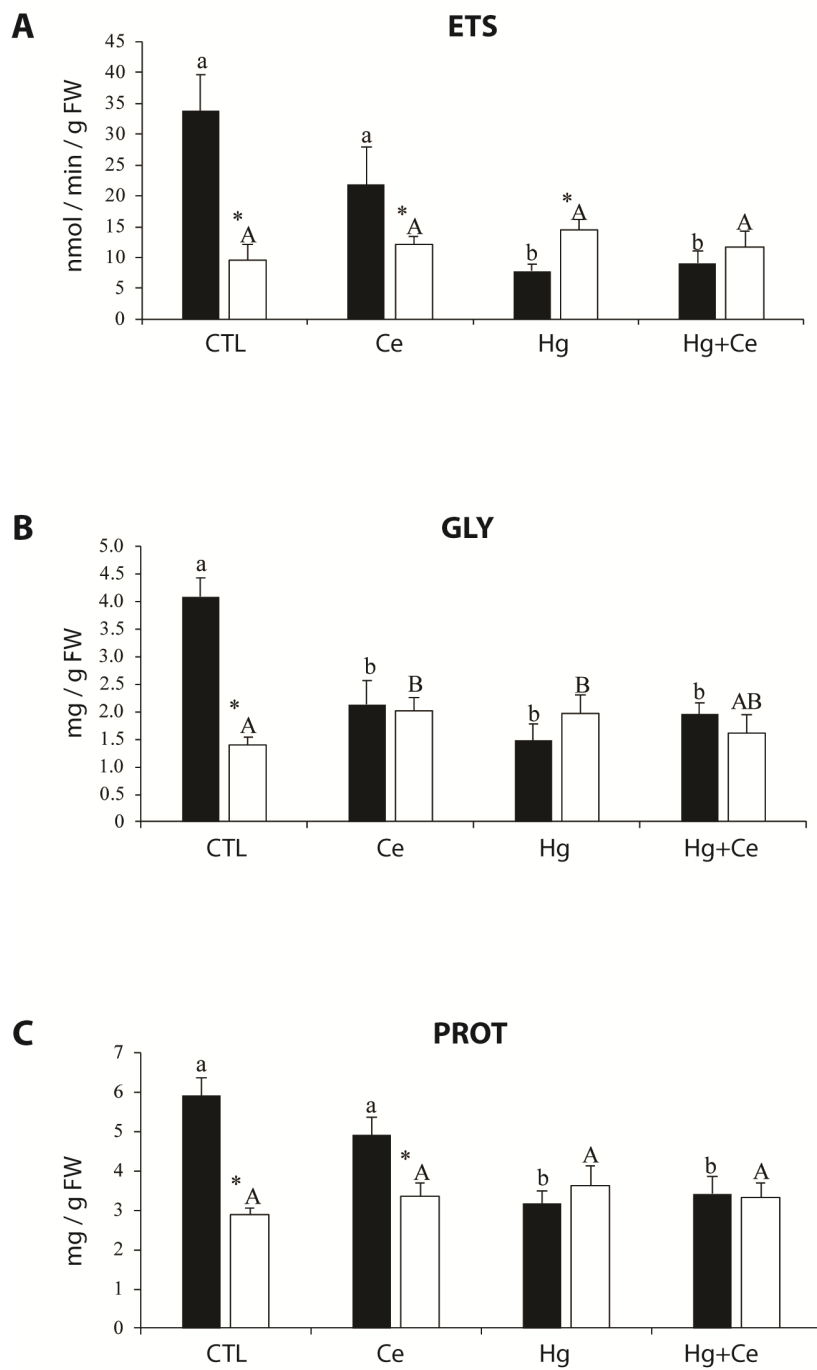
Fig. 4: Glutathione (GSH) content (mean + standard deviation) (n=9), in *M. galloprovincialis* exposed to CTL, as well as CeO₂ NPs and Hg²⁺ individually and in combination. Significant differences ($p \leq 0.05$) among exposure conditions were represented with different lowercase letters for groups exposed at 17°C and upper case letters for groups exposed at 22°C. In addition, an asterisk represents significant differences between groups exposed to different temperatures (17°C and 22°C).

Fig. 5: Acetylcholinesterase (AChE) activity (mean + standard deviation) (n=9), in *M. galloprovincialis* exposed to CTL, as well as CeO₂ NPs and Hg²⁺ individually and in combination. Significant differences ($p \leq 0.05$) among exposure conditions were represented with different lowercase letters for groups exposed at 17°C and upper case letters for groups exposed at 22°C. In addition, an asterisk represents significant differences between groups exposed to different temperatures (17°C and 22°C).

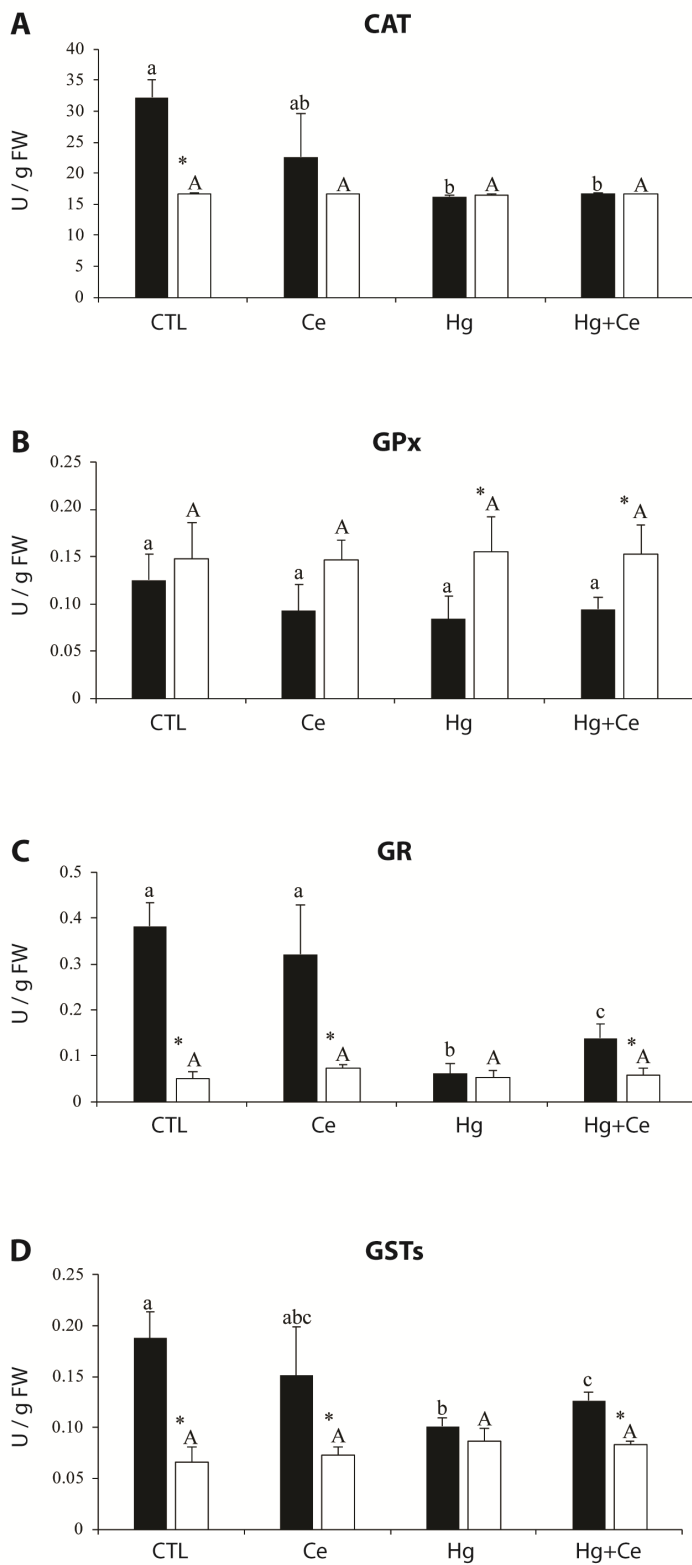
Table 1. Hg^{2+} and CeO_2 NPs concentrations ($\mu\text{g g}^{-1}$) in *M. galloprovincialis* exposed to CTL, Hg and CeO_2 NPs individually and in combination (mean \pm standard deviation). Different letter means statistically difference $p < 0.05$.

| Condition | Hg | | Ce | |
|--------------------|-------------------|-------------------|-----------------|-----------------|
| | 17 °C | 22 °C | 17 °C | 22 °C |
| CTL | 0.15 ± 0.01^a | 0.13 ± 0.01^a | 0.28 ± 0.09 | 0.32 ± 0.12 |
| CeO_2 NPs | - | - | 0.43 ± 0.12 | 0.24 ± 0.03 |
| Hg | 4.93 ± 1.11^b | 4.44 ± 0.95^b | - | - |
| Hg+Ce NPs | 4.98 ± 1.39^b | 7.66 ± 1.37^b | 0.30 ± 0.18 | 0.34 ± 0.15 |

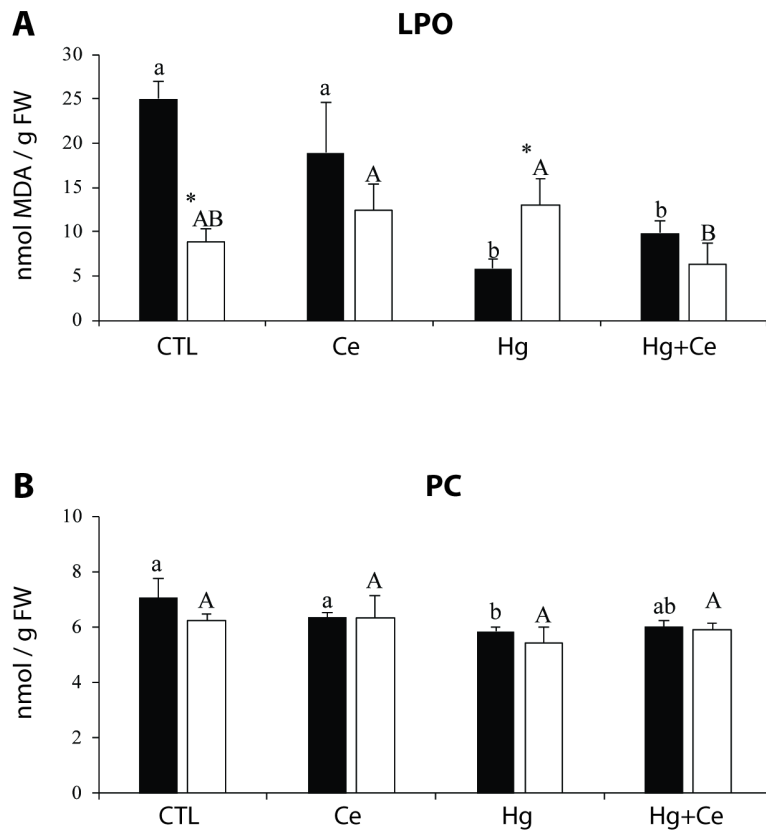
■ 17 °C □ 22 °C



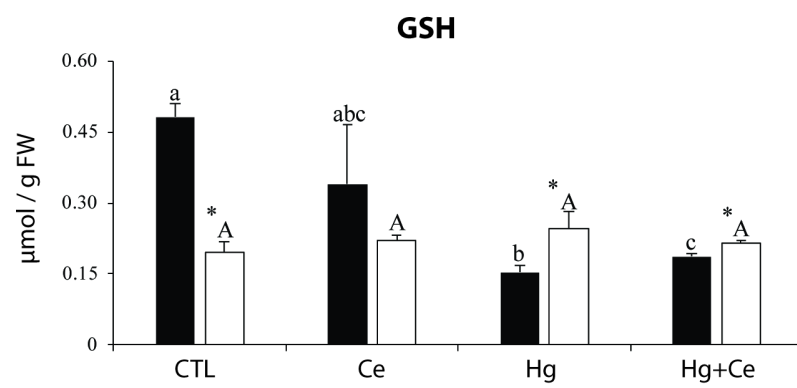
■ 17 °C □ 22 °C



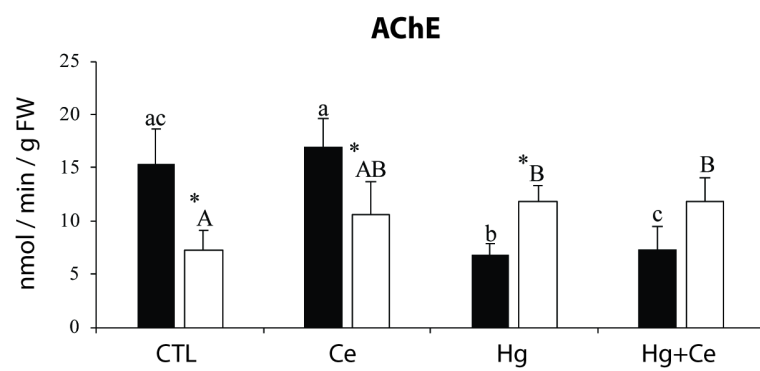
■ 17 °C □ 22 °C



■ 17 °C □ 22 °C



■ 17 °C □ 22 °C



Highlights

Hg determined reduction of energetic metabolism and antioxidant activities in mussels

CeO₂ NPs did not affect Hg bioavailability and accumulation in mussels

Co-exposure with CeO₂ NPs partially recovered the Hg inhibition of GR, GSTs and AChE

At 22 °C metabolic functions and biochemical activities were lowered

The adverse outcomes induced by temperature overcome the effects of pollutants

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Daniela Maggioni: methodology, writing/ review and editing.

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Declaration of interests

☒ 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.

☐ The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: