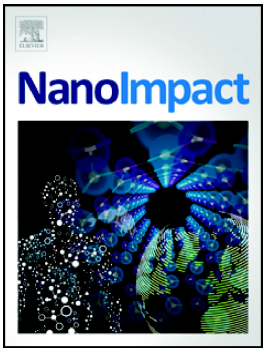

Coating with polysaccharides influences the surface charge of cerium oxide nanoparticles and their effects to Mytilus galloprovincialis

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Coating with polysaccharides influences the surface charge of cerium oxide nanoparticles and their effects to *Mytilus galloprovincialis*

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ABSTRACT

This study focused on the effects of surface coating, acquired through the interaction with natural biomolecules, on the behavior and ecotoxicity of nanoparticles (NPs). To this aim, the effects of Cerium Oxide Nanoparticles (CeO_2NPs) naked and coated with chitosan and alginate on the marine mussel *Mytilus galloprovincialis* were compared. Mussels were exposed for 7 days to 100 µg L^{-1} of CeO_2NPs and for 28 days to 1 µg L^{-1} of CeO_2NPs. In both experiments CeO_2NPs were used naked and coated with the two polysaccharides. The lowest tested concentration allowed to understand the environmental relevance of this biological process. A set of biomarkers related to oxidative stress/damage and energy metabolism was applied to assess the ecotoxicity of CeO_2NPs. The aggregation and stability in water of CeO_2NPs were measured through dynamic light scattering analysis and the levels of Ce in the exposure media and in mussels soft tissues were determined by inductively coupled plasma-mass spectrometry. Results showed a different hydrodynamic behavior and stability of CeO_2NPs in saltwater related to the different coatings. Despite this, no differences in the bioaccumulation of CeO_2NPs were observed among the experimental groups. Different coatings affected also CeO_2NPs toxicological outcomes in both 7- and 28-days exposures. Coating with chitosan enhanced antioxidant enzyme activities while coating with alginate triggered oxidative damage. Although the oxidant pathways did not differ that much among the exposures, biomarkers of energetic supplies suggested a different strategy of defense in response to CeO_2NP exposure at a lower concentration and for a longer period of time. The obtained results are in line with findings of a previous study on freshwater mussels, suggesting that the coating with biomolecules, which impart negative charge to the NPs, might enhance their biological effects. This study highlighted that interactions of NPs with natural biomolecules largely present in the aquatic environment could affect NP toxicity altering interaction towards organisms.

Keywords: Cerium oxide nanoparticles; eco-corona; marine mussels; chitosan; alginate; energy metabolism; oxidative stress

1. INTRODUCTION

The large development in the use of nanoparticles (NPs) in a vast range of applications resulted in their release in environmental matrices, rising concern about the possible impacts on human and environmental health (Wiesner et al., 2006; Wang et al., 2016). The marine environment, in particular, is the final sink of NPs from direct application and land-based sources (Selck et al., 2016). The intrinsic proprieties of NPs (which depend on the chemical composition, size, shape, surface charge among others) drive their behaviour, fate and impacts on biota (Christian et al., 2008; Klaine et al., 2009; Selck et al., 2016; Lead et al., 2018). These parameters are largely affected by abiotic and biotic transformations occurring once NPs are released into the environment (Keller et al., 2010; Lowry et al., 2012; Zhang et al., 2018; Markiewicz et al., 2018). In particular, the adsorption of biomolecules from the surrounding environments is one of the transformations that play a key role in NP fate, implying a significant modification of their properties and affecting their interactions with biological targets (Lowry et al., 2012; Canesi et al., 2017; Corsi et al., 2020; Della Torre et al., 2021). When NPs are released in water, biological macromolecules such as humic substances, proteins, exudates and polysaccharide acid compounds, could cover NP surface creating a corona of biological macromolecules called eco-corona (Canesi and Corsi, 2016; Canesi et al., 2017). This eco-corona could alter NP aggregation state and distribution in the water column (Huber and Stoll, 2018) having an impact on the toxicity towards marine organisms (Wang et al., 2016).

Although it is very important to predict how the eco-corona can influence the fate and toxicity of NPs, there are still many knowledge gaps on how different NPs released into the environment can interact with the huge diversity of biological macromolecules present in different compartments, and on the consequent effects on NP behaviour, fate and biological impacts (Canesi and Corsi, 2016; Ren et al., 2016; Baalousha, 2017). Previous studies focused on this issue showed conflicting results as the effects are dependent on the type of NPs and its mechanism of toxicity, the specific natural organic matter (NOM) composition, the different water media where the interactions are tested, and the biological model used (Xu et al., 2020). Indeed, some studies reported a decrease in adverse effects of different kinds of NPs such as AgNPs, CuONPs, ZnONPs and TiO_2NPs, due to adsorption of NOM (Wang et al., 2016; Noventa et al., 2018; Yu et al., 2018). However, enhanced toxicity has also been reported (Dasari and Hwang, 2010; Wang et al., 2011b; Yang et al., 2013a). Among NPs, CeO_2NPs are used in a wide variety of industrial and

consumer products and biomedical applications (De Marchi et al., 2019). The production of CeO_2NPs is forecasted increase in the next decades with a consequent release in the natural compartments predicted to reach up to 300 tons per years in 2050 (Giese et al., 2018), rising concern on the adverse impacts that these NPs can pose to wildlife, given also their high biological reactivity. CeO_2NPs have been included as one of the 13 priority listed representative manufactured nanomaterials by the Organization for Economic Co-operation and Development (OECD) Working Party on Manufactured Nanomaterials (WPMN) established a to assess the human health and environmental safety implications of manufactured nanomaterials (ENV/JM/MONO(2008)13/REV).

Several studies highlighted that surface coating with organic molecules could enhance CeO_2NP stability in the water column reducing bioavailability and hence decreasing the toxicity to organisms (Van Hoecke et al., 2011; Collin et al., 2014). On the contrary, other studies conducted by Garaud and co-authors (2016) investigated how coating could enhanced the uptake and bioaccumulation of CeO_2NPs by filter feeders (Garaud et al., 2016; Briffa et al., 2018). Our recent study showed that CeO_2NPs coated with chitosan and alginate could alter the interactions of these NPs with the freshwater mussel *Dreissena polymorpha*. In detail, even if the coatings did not affect the bioaccumulation of NPs in mussels, alginate-coated CeO_2NPs resulted more effective than Naked and chitosan-coated CeO_2NPs, targeting key metabolic pathways (Della Torre et al., 2021).

While most of the studies carried out so far provided information about the role of interplay between NPs and biomolecules towards freshwater organisms, there is a lack of data about the environmental implications in the marine environment. In the saltwater matrix, further environmental parameters (i.e. pH and ionic strength) could influence the interactions of NPs with organisms. For instance, Manier and co-authors (2011) comparing different media for ecotoxicity tests (ISO, moderately hard water and OECD 201) showed that in media with higher ionic strength the increase in the suspension could screen the particle surface charges reducing the electrostatic repulsion and enhancing the agglomeration process.

In this view, the present study was carried out with the objective to evaluate the biological effects of CeO_2NPs coated with alginate (Ce@Alginate) and chitosan (Ce@Chitosan) using the marine bivalve *Mytilus galloprovincialis*. This species is largely used as sentinel organism in pollutant bio-concentration/bioaccumulation and toxic-kinetic studies, as well as in the monitoring of anthropogenic

pollution trends in coastal waters (Farrington et al., 2016). Moreover, *Mytilus* represents a suitable model for testing the toxicity of NPs in marine invertebrates (Canesi et al., 2012).

Alginate and chitosan were chosen as coating agents representative of biomolecules abundant in water systems. Alginate is a polysaccharide highly abundant in the extracellular polymeric substances produced by biofilm (Ostermeyer et al., 2013) and is structural element of cell walls of brown algae (Phaeophyceae). Chitosan is a chitin derivative produced from crustacean shells, which is the second most abundant natural polysaccharide on earth (Komi and Hamblin 2016). The two polysaccharides are characterized by repetition units containing functional groups (COOH and NH_2, respectively) that can be easily protonated or deprotonated as a function of pH. Thus, at the typical pH values of the marine environment chitosan results mainly protonated in its amine sites, hence resulting positively charged. On the contrary, alginate is negatively charged since its carboxylic groups are deprotonated to carboxylate groups. By employing these two polymers for covering the Naked Ceria NPs, we intended to change the overall charge present at the NP surface level.

The aggregation and stability in ocean water of CeO_2NPs (CeO_2NPs naked (Ce Naked), coated with alginate (Ce@Alginate) and coated with chitosan (Ce@Chitosan)) were characterized to understand how different coatings could affect NP aggregation state. The levels of Ce in exposure media and mussels soft tissues were determined, to evaluate the bioavailability and bioaccumulation of the different CeO_2NPs. A set of biomarkers related to oxidative stress, oxidative damage, redox balance and energetic metabolism was applied, to investigate the potential adverse effects of the three types of CeO_2NPs. We investigated biomarkers related to oxidative stress, since NPs including CeO_2NPs are known to increase ROS generation. This mechanism of toxicity is well described in several biological models including bivalves (Canesi and Corsi 2016 and citations therein). The energetic metabolism was also investigated as the modulation of metabolic activities will influence the expenditure of energy reserves that are linked to the health status of individuals.

2. MATERIALS AND METHODS

2.1 *Nanoparticles synthesis and characterization*

CeO_2NPs were synthesized and characterized by modifying a literature procedure (Plakhova et al., 2016), as described by Villa et al. (2020). This manuscript provides also information on the shape and dimensions of the NPs, assessed through observation at Transmission Electron Microscopy. The mean size of Naked CeO_2NPs and Ce@Chitosan was ~5 nm, while Ce@Alginate had larger size of ~50 nm. The hydrodynamic size and ζ-potential of NPs in ocean water were characterized through dynamic light scattering, using a Zetasizer Nano ZS instrument (Malvern) equipped with a 633 nm wavelength solid-state He-Ne laser. The scattered light was gathered at an angle of 173 °, setting the operating temperature at 25 °C. ζ-potential is the overall charge developed at the interface between the NP surface and the liquid medium in which it is suspended, and it is measured in millivolt. Prevailing a positive or negative charge at the NP surface, this will influence the content of the counterions in the nearby region. The ζ -potential is hence a function of the NP surface charge that can be experimentally measured by a particular electrophoretic measurement that employs the scattering of the light to detect the rate and the direction of the movement of the NP under the applied electric field effect. The ζ-potential is measured to characterize the surface composition. The NP samples were suspended in ocean water (the same used in the *in vivo* experiments) at a concentration ranging between 0.1 and 1 mg mL^{-1} and carrying out the analyses by the Malvern disposable "Folded capillary" cells, which enable the measurement of the ζ-potential also for those samples characterized by a very high ionic strength, by using the so-called "diffusion barrier technique". This last one consists of the introduction of a small plug of sample at the bottom of the folded capillary cell, which contains the same buffer used for the preparation of the sample (in our case ocean water). In this way the sample is isolated from the electrodes, protecting the sample itself and the electrodes (Corbett and Jack, 2011). The analyses were mediated over at least three repeated measurements.

2.2 *Mytilus galloprovincialis experimental setup*

About 200 individuals of *M. galloprovincialis* were collected at Ria de Aveiro lagoon (Portugal). After the acclimation period (15 days), organisms were exposed to CeO_2NPs naked (Ce Naked) or coated with alginate (Ce@Alginate) and chitosan (Ce@Chitosan) as well as used as control (CTRL). Two different

experiments were performed. In the first experiment, mussels were exposed to 100 µg L^{-1} of CeO_2NPs naked and coated with the two polysaccharides for 7 days. In the second experiment, 1 µg L^{-1} of CeO_2NPs naked and coated with alginate and chitosan were administered and the experiment lasted 28 days. The rationale beyond the choice of the two treatments is that the first concentration (100 µg L^{-1}) and the duration of exposure was comparable to a previous treatment carried out using the freshwater bivalve *Dreissena polymorpha* (Della Torre et al., 2021). In the second experiment, the treatment was protracted for a longer exposure period, with a concentration closer to the environmental one. Indeed, even if the latter concentration is above the predicted environmental concentration of CeO_2NPs in marine environments, that is estimated to be at 0.03-2 pg L^{-1} in seawater and 0.04-2 µg kg^{-1} in marine sediments (Giese et al., 2018), a concentration up to 1 µg L^{-1} was reported as a worst-case scenario such as direct discharge of wastewater treatment plant (Keller and Lazareva 2013) and represents the upper limit of natural aquatic mean exposure concentration modelled by O'Brien and Cummins (2011).

In the experiment of 7 days the water medium was changed daily, and the exposure conditions were completely re-established (concentration of NPs, salinity, temperature). With the aim to maintain constant the conditions of the exposure medium, in the experiment of 28 days, the water medium was changed weekly with the reestablishment of all conditions (concentration of NPs, salinity, temperature). In both experiments, 24 organisms were distributed for each treatment: 3 replicates per treatment, with 8 organisms per replicate. Each aquarium (4 L) was filled with artificial seawater (addition of artificial sea salt - Tropic Marin® Sea Salt - to deionized water). Individuals were reared with oxygenators at 19.0 ± 1 °C and salinity 30 (resembling the sampling area), under a 12h/12 h light/dark photoperiod and they were fed three times a week with Algamac protein plus (1500,000 cells/animals/day). At the end of the exposure period, organisms (N=5 per aquarium) were frozen with liquid nitrogen and stored at -80 °C; the remaining organisms (N=3 per aquarium) were pooled and stored at -20 °C for the measurement of Ce concentration.

Furthermore, 1 h after spiking with CeO_2NPs, an aliquot of 15 mL of water was sampled from both the control and the exposure tanks and stored at -20°C to quantify the Ce concentrations.

2.3 *Cerium analyses in exposure water and mussel soft tissue*

The soft tissue of *M. galloprovincialis* individuals from the control and exposure tanks was lyophilized and then solubilized by acid digestion by the following method: 3 mL HNO_3 and 0.5 mL H_2O_2 (ultrapure reagents) were added to about 300 mg of pools consisting of 9 individuals per each treatment. Solubilization was carried out in Teflon bombs in a Milestone Ethos 900 microwave lab station.

The Ce concentrations in the exposure water and mussels' soft tissues from each treatment were determined by inductively coupled plasma-mass spectrometry (ICP-MS) using the Perkin Elmer NexION 350 spectrometer. The standard reference materials SRM 3110 (Cerium Standard Solution) and SRM 2977 (Mussel Tissue) of National Institute of Standards and Technology (USA) were analyzed to check the accuracy of Ce determinations in exposure water and mussel soft tissue, respectively. Recoveries were in the range 95.6-98.2% for SRM 3110 and 94.6–98.9% for SRM 2977. The Ce concentrations in exposure water and mussel soft tissue were expressed as $\mu g\ L^{-1}$ and $\mu g\ g^{-1}$ dry weight (d.w.), respectively.

2.4 Biomarker analyses

The individually whole body of frozen organisms (3 for each aquarium) was pulverized with liquid nitrogen and divided into 0.5 g fresh weight (FW) aliquots and used for biochemical analyses: the activity of the electron transport system (ETS); protein (PROT) and glycogen (GLY) contents; the activity of the enzymes superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), glutathione S-transferases (GSTs); levels of lipid peroxidation (LPO) and protein carbonylation (PC); reduced glutathione content (GSH). Homogenates for biomarkers were obtained following the methods described by Coppola et al. (2018); the extraction for each biomarker was performed with specific buffers. The potassium phosphate buffer extraction (50 mM potassium dihydrogen phosphate; 50 mM dipotassium phosphate; 1 mM ethylenediamine tetra-acetic acid disodium salt dihydrate (EDTA); 1% (v/v) Triton X-100; 1 mM dithiothreitol (DTT); pH 7.0) was used for SOD, CAT, GSTs, GPx, GR, CP, PROT and GLY assays. For GSH assay samples were extracted using 0.6% (w/v) sulfosalicylic acid in potassium phosphate buffer as described in Coppola et al. (2017). LPO assay supernatants were extracted in 20% (v/v) trichloroacetic acid (TCA) (1:2, w/v). Tissues were lysated using TissueLyser II set at frequency 20 $1s^{-1}$, during 1.30 min. and then centrifuged at 10,000 x g at 4 °C, during 20 min. For the quantification of metabolic capacity (assessed by the ETS activity), supernatants were extracted in homogenizing buffer containing 0.1 M Tris-HCl pH 8.5

with 15% (w/v) PVP, 153 µM magnesium sulphate ($MgSO_4$) and 0.2% (v/v) Triton X-100, in a 1:2 proportion. Tissues were lysates using TissueLyser II set at frequency 20 $1s^{-1}$, during 1.30 min. and then centrifuged at 3.000 x g at 4 °C, for 20 min.

All supernatants were then reserved and stored at -80 °C to determine metabolic capacity and energy reserves (ETS, PROT, GLY), activity of antioxidant and biotransformation enzymes (SOD, CAT, GPx, GSTs and GR), oxidative damage (LPO and CP) and oxidative status (GSH), as described in details in the supporting information.

2.5 Statistical analyses

All biochemical analysis and quantification data were analysed 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 ($p < 0.05$) were considered as significantly different and are reported in Table SI.1. Different letters represent significant differences among tested conditions (uppercase letter for the 7-days experiment; lowercase letter for the 28-days experiment).

3. RESULTS

3.1 CeO_2NP characterization

CeO_2NP suspensions were analyzed in saltwater by means of dynamic light scattering (DLS) and ζ-potential, which are the election techniques to characterize dynamic suspended nanoparticles, provided that the morphology has been already analyzed by electronic microscopy. While DLS provides information about the mean hydrodynamic diameter of the suspended material, the surface charge is obtained by means of the measurement of the ζ-potential. In general, a high absolute value of ζ-potential means high stability of the suspended nanoparticles, nevertheless macromolecular stabilizing agents, as the one employed in this study, can impart a steric stabilization due to the hindrance at the surface of the particles, hampering the NPs to approach on to each other, collapse and precipitate. The three synthesized CeO_2NPs (Ce Naked, Ce@Alginate and Ce@Chitosan) showed some differences in terms of aggregation state in saltwater. The

colloidal stability of Ce@Alginate was greatly affected by the salinity of the environment and most of the NPs copiously precipitated. As to the fraction remaining in suspension, DLS measurements showed a population with a very large hydrodynamic diameter (3105 ± 460 nm) (Table 1). The hydrodynamic diameter of Ce Naked was close to micron size (1760 ± 270 nm) while in case of Ce@Chitosan two much smaller populations were detected (220 ± 40 and 880 ± 190 nm) (Table.1; Fig. SI.1) whose size distribution by numbers clearly stated that the most numerous population was the smaller one (Fig. SI.1). As to ζ-potential, the very strong ionic strength of the ocean water medium forced us to use the "diffusion barrier technique" to collect reliable measurements of the surface charge (see experimental part). The values of the ζ-potential for the three nanoparticles are reported in Table 1, showing that whilst Ce@Alginate NPs were a negatively charged, the ζ-potential for Ce@Chitosan was neutral in ocean water. Nevertheless, these last ones showed to be the most stable, indicating that Chitosan is able to highly stabilize also by steric hindrance the cerium oxide NPs. On the contrary, the Naked CeO_2NPs in ocean water were positively charged, possibly for the selective interaction of the naked surface with some ions forming a non null surface charge.

3.2 Cerium in exposure water and mussel soft tissue

A fast sedimentation occurred for all three administered CeO_2NPs. This behavior was expected, due to the strong tendency of aggregation of CeO_2NPs once released in water media (Singh et al., 2014). The concentration of Ce in the water spiked with 100 μg L^{-1} in the 7-days experiment was lower in the Ce Naked group (13.51 ± 0.40 μg L^{-1}) and higher in the Ce@Alginate one (51.28 ± 0.48 μg L^{-1}; Table.2). In the 28-days experiment, regardless of the tested condition, the medium showed very low Ce concentrations (<0.1 μg L^{-1}) due to the low concentration of CeO_2NPs used for this experiment (1 μg L^{-1}).

In the 7-days experiment, the Ce levels in mussels' soft tissues were rather similar in all exposure groups and higher than the control group (CTRL; Table 2). At the end of the 28-days experiment, due to the low concentration of CeO_2NPs in the water column, the Ce levels in mussels of all exposure groups were lower (from 0.21 ± 0.03 to 0.26 ± 0.03 μg g^{-1} d.w.) than in the 7-days experiment, and similar to the control group (0.19 ± 0.03 μg g^{-1} d.w.; Table 2).

3.3 Effects of CeO_2NPs in Mytilus galloprovincialis

3.3.1 Experiment 1 (7-days)

Concerning the metabolic capacity and energetic reserves, ETS values were significantly lower in mussels exposed to Ce@Chitosan and Ce@Alginate in comparison with the control group (CTRL). A significant difference was observed between mussels exposed to Ce@Alginate in comparison with the Ce Naked group (Fig. 1A; Table SI.1). Regarding GLY content, no significant differences between CTRL and the three NPs treated groups have been observed, yet the GLY content of mussels exposed to Ce@Chitosan was significantly lower than Ce@Alginate group (Fig. 1B; Table SI.1). Mussels exposed to the three types of CeO_2NPs showed significantly lower protein content than CTRL group. Moreover, Ce@Chitosan showed significantly lower PROT content in comparison to Ce Naked group (Fig. 1C; Table SI.1).

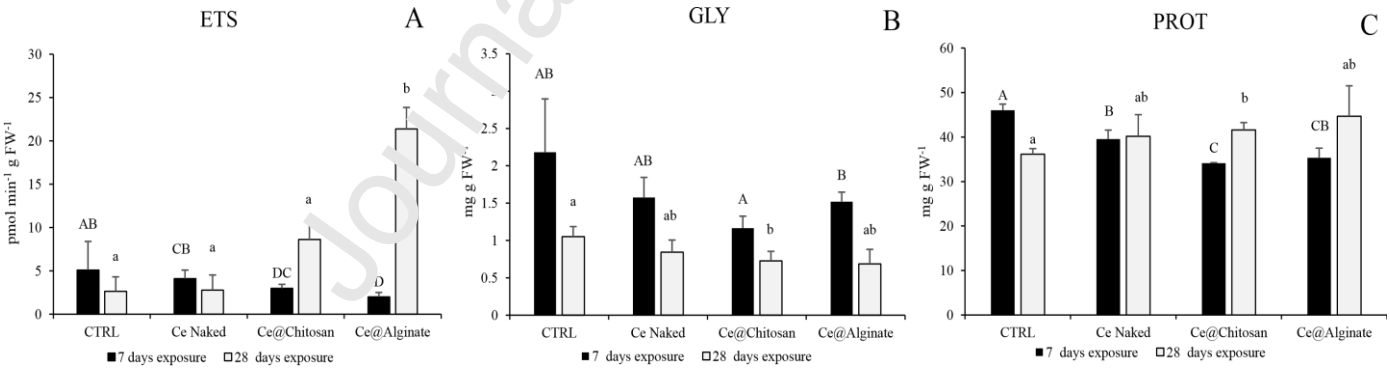
Concerning enzymes involved in the antioxidant response, a general increase of SOD activity was observed in mussels exposed to Ce Naked and Ce@Chitosan compared to CTRL group. The SOD activity was significantly higher in mussels exposed Ce Naked and to Ce@Chitosan compared to CTRL, and the activity in the Ce@Chitosan group was significantly higher than in the Ce@Alginate one (Fig. 2A; Table SI.1). The CAT activity was significantly reduced in mussels exposed to all CeO_2NPs compared to CTRL group; the inhibition was more pronounced upon exposure to Ce@Alginate, which resulted significantly lower than Ce@Chitosan group (Fig. 2B; Table SI.1). The GPx activity was significantly higher in mussels exposed to Ce Naked compared to CTRL and Ce@Alginate groups (Fig. 2C; Table SI.1), while the GR activity was significantly enhanced in mussels exposed to Ce@Chitosan and Ce@Alginate compared to CTRL group. Furthermore, the Ce@Chitosan group showed significantly higher activity compared to the Ce Naked group (Fig. 2D; Table SI.1). The activity of GSTs was significantly higher in mussels exposed to both coated NPs compared to CTRL and Ce Naked groups (Fig. 2E; Table SI.1), with a higher activity detected in individuals exposed to Ce@Alginate in comparison with Ce@Chitosan group.

Concerning oxidative damage, the LPO levels showed no significant differences among all treatments (Fig. 3A; Table SI.1), while PC content increased in mussels exposed to Ce@Alginate compared to CTRL and Ce@Chitosan groups (Fig. 3B; Table SI.1). As for the oxidative status, the GSH content was significantly lower in Ce@Chitosan group in comparison to CTRL (Fig. 3C; Table SI.1).

3.3.2 Experiment 2 (28-days experiment)

Concerning the metabolic capacity an increase of ETS activity was observed in mussels exposed to Ce@Chitosan and Ce@Alginate. The latter group showed significantly higher levels in comparison to all the other treatments (Fig. 1A; Table SI.1). A significant decrease in GLY content was observed in mussels exposed to Ce@Chitosan compared to CTRL group (Fig. 1B; Table SI.1). The PROT content was higher in contaminated mussels, with significant differences between individuals exposed to Ce@Chitosan and CTRL treatment (Fig. 1C; Table SI.1).

Concerning the oxidative stress enzymes, mussels exposed to Ce@Chitosan showed a significantly higher SOD activity compared to CTRL and Ce@Alginate groups (Fig. 2A; Table SI.1) while the CAT activity was similar among all treatments (Fig. 2B; Table SI.1). The GPx activity of mussels exposed to Ce@Chitosan was significantly induced in comparison to all other treatments. The Ce Naked group also showed significantly higher activity than CTRL (Fig. 2C; Table SI.1). A general decrease of GR activity was detected in mussels exposed to all CeO_2NPs compared to CTRL group, with a significant difference only between individuals exposed to Ce Naked and CTRL treatment (Fig. 2D; Table SI.1). The activity of GSTs increased in Ce@Chitosan and Ce@Alginate groups, with significant differences between Ce@Alginate and



all other exposure conditions (Fig. 2E; Table SI.1).

Concerning oxidative damage, a significant increase of LPO was observed in mussels exposed to Ce@Alginate in comparison to all other treatments (Fig. 3A; Table SI.1) while no significant differences were observed in PC content upon all the treatments (Fig. 3B; Table SI.1). Regarding to GSH content, no significant differences were observed among all treatments (Fig. 3C; Table SI.1).

Fig. 1. Metabolic capacity and energy-related biomarkers. Electron transport system (ETS) activity (A), Glycogen (GLY) content (B) and Protein (PROT) content (mean ± standard deviation) (N = 9) in *M. galloprovincialis* exposed to the three CeO_2NPs in the 7 days exposure and in the 28 days exposure. Different letters represent significant differences among tested conditions (uppercase letter for the 7-days exposure; lowercase letter for the 28-days exposure).

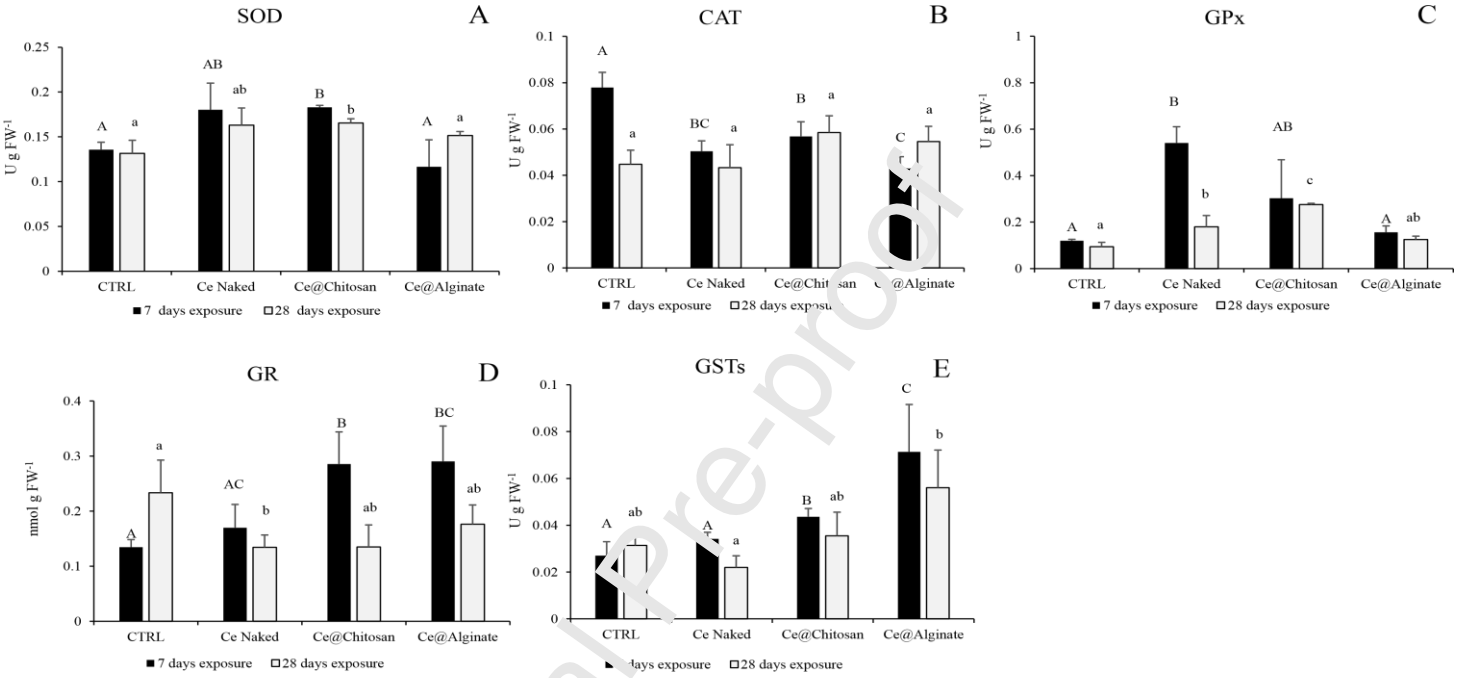


Fig. 2. Oxidative stress and detoxification enzymes. Activity of Superoxide dismutase (SOD) (A), Catalase (CAT) (B), Glutathione peroxidase (GPx) (C), Glutathione reductase (GR) (D), Glutathione S-transferases (GSTs) (E) (mean ± standard deviation) (N = 9), in *M. galloprovincialis* exposed to the three CeO_2NPs in the 7 days exposure and in the 28 days exposure. Different letters represent significant differences among tested conditions (uppercase letter for the 7-days exposure; lowercase letter for the 28-days exposure).

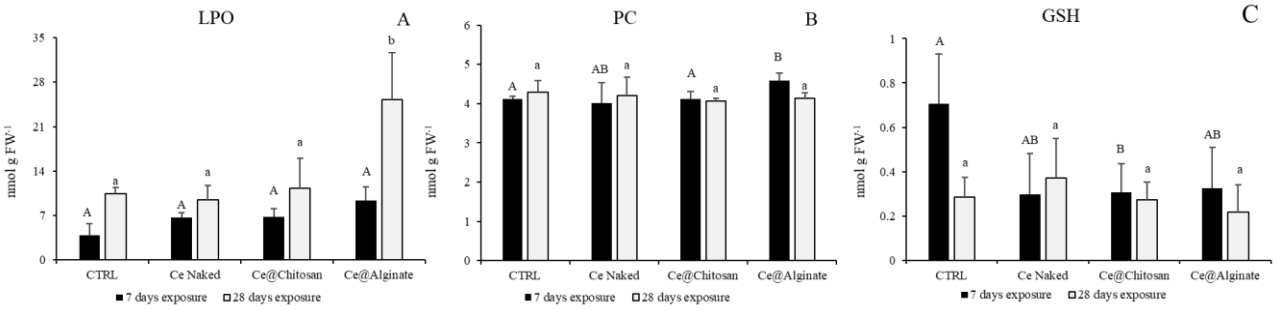


Fig. 3. Oxidative damage and Oxidative status. Lipid peroxidation (LPO) level (A); Protein carbonylation (PC) level (B) and Glutathione (GSH) content (C) (mean ± standard deviation) (N = 9), in *M. galloprovincialis* exposed to the three CeO_2NPs in the 7 days exposure and in the 28 days exposure. Different letters represent significant differences among tested conditions (uppercase letter for the 7-days exposure; lowercase letter for the 28-days exposure).

4. DISCUSSION

4.1 Influence of surface coating on CeO_2NPs behavior in ocean water and accumulation in mussels

Results obtained through DLS analysis showed that different surface coatings could significantly affect the aggregation and stability of CeO_2NPs in saltwater. In detail, Ce@Chitosan seems to be the most stable, while Ce Naked and Ce@Alginate were more prone to aggregation, and hence, they showed fast sedimentation. This could be due to the fact that the polymers present a different surface charge to CeO_2NPs, leading to an alteration of the electrostatic repulsion forces between NPs (Villa et al., 2020). Alginate seems more effective than chitosan in shielding the surface charge of NPs, making the electrostatic repulsion lower and hence inducing NP aggregation and sedimentation. Furthermore, the dispersion of CeO_2NPs in saltwater increased the state of aggregation and colloidal instability in comparison with freshwater. Similarly, in previous studies it has been observed how the saltwater could affect the diffuse layer at the NP surface by making it thinner, thus lowering the ζ-potential at the NP surface and enhancing aggregation (Keller et al., 2010; Manier et al., 2011; Quik et al., 2014). Indeed, in this study, a higher aggregation has been observed in both naked and coated NPs, in comparison with previous analyses performed on the same type of NPs through DLS analysis in different freshwater types (Table SI.2). The comparison of values of the ζ-potential for the three NPs with the values collected for the very same NPs in different media, indicates that whilst Ce@Alginate NPs showed a constant slightly negative value in all the media, Ce@Chitosan passed from highly positive in MilliQ water to neutral in tap water/millQ water 1:1 as well as in seawater. This is explainable by taking into account that Chitosan can interact with negatively charged species through the amine groups that, in these conditions, are positively charged. On the contrary, the Naked CeO_2NPs in seawater became slightly positively charged while in the other two media, which contain a much less content

of ionic species, they basically remained unchanged and with a very small negative charge. This can be due to a preferential interaction of positive ions whose tenor in the seawater is much higher than in tap water. The quantification of Ce in exposure water used for the 7-days experiments suggested a fast and heavy sedimentation of the three CeO_2NP in the exposure tanks regardless the constant stirring.

Even though the three CeO_2NPs have different hydrodynamic behavior, such changes are not significant enough to cause any difference in bioavailability and the Ce accumulation in *M. galloprovincialis* resulted low (7-days experiment) or negligible (28-days experiment) regardless the NP tested. Since the measurement of Ce accumulation was made at the end of the exposure in both experiments, it is likely that the NPs retained in the gut have been excreted before the measurements. Moreover, a study by Montes and co-authors (2012) showed that a large portion of CeO_2NPs filtered by *M. galloprovincialis* was rejected in pseudofeces. This could explain the negligible Ce accumulation, given the low level of NPs administered in the second experiment. But this does not mean that the NPs have exerted a biological effect. Indeed, a very low bioaccumulation of CeO_2NPs has been reported also in other experiments carried out on bivalves (1-2% of the administered NPs), despite impacts on several biological functions have been observed (Garaud et al., 2015; Koehlè-Divo et al., 2019). Therefore, it is likely that the effects observed upon exposure to the three CeO_2NPs could be determined by the interactions of the NPs with biological targets rather than by their environmental fate, as already hypothesized for freshwater mussels in our previous study, where the coating with alginate made the CeO_2NPs more reactive compared to Naked and chitosan-coated NPs inducing mussel energetic metabolism, and affecting osmoregulation (Della Torre et al., 2021).

4.2 Influence of surface coating on CeO_2NPs toxicity to mussels

The significant decrease of the ETS activity observed in organisms exposed to Ce@Alginate and Ce@Chitosan in the first experiment of 7 days at 100 μg L^{-1} suggests that CeO_2NPs could affect the metabolic capacity of mussels as already hypothesized by Garaud et al. (2016). Indeed, the ETS activity provides information about the energy demand that could be also required to increment cellular defense in response to contaminants (Della Torre et al., 2021). This result is further supported by the observed decrease of protein content in all mussels exposed to CeO_2NPs, probably due to either high detoxification investment

or lower investment in the production of proteins (Morosetti et al., 2020; Pytharopoulou et al., 2008; Kalpaxis et al., 2004). Nevertheless, the GLY content was maintained at levels similar to controls. This could be due to the need of maintaining the GLY storage as energetic fuel for other physiological investments as for instance reproduction (Timmins et al., 2014) or the fact that reducing their metabolism mussels do not need to use this energy reserve. Concerning oxidative stress, in the first experiment both Naked and coated CeO_2NPs seemed to trigger oxidative stress as already observed in other aquatic species (Xia et al., 2013). Anyhow, the mechanism of oxidative stress in the treatments resulted fairly different depending on the different coatings. Regarding the Naked CeO_2NPs, the induction of GPx activity is probably due to an excess of H_2O_2 content generated by the NPs (Correia et al., 2020). This excess could also be responsible for the inhibition of CAT activity (Regoli et al., 2011). Similarly, a significant decrease of the CAT activity was observed also in mussels exposed to Ce@Chitosan with a significant increase of the SOD and GSTs activity. In this group, the decrease of GSH content in comparison to the control group could be due to its consumption as a substrate of the GSTs reaction (Regoli and Giuliani, 2014). The GSH consumption by GSTs might also lead to the significant rise of the GR activity that is used to maintain the balance between GSH and oxidized glutathione (GSSG) (Trevisan et al., 2014; Rao et al. 2006). The antioxidant response of mussels exposed to Ce@Alginate is more similar to mussels exposed to Ce@Chitosan rather than to Ce Naked. Moreover, Ce@Alginate resulted as the only CeO_2NPs generating significant oxidative damage as PC, suggesting that these NPs are the most effective, in line with a previous study carried out on freshwater mussels (Della Torre et al., 2021). Despite the high concentration tested, the oxidative damage in Ce@Alginate was not so relevant suggesting that the induction of antioxidant enzymes as GR and GSTs were able to prevent the occurrence of lipid peroxidation. Besides, the high detoxification capacity especially in Ce@Chitosan NPs could have prevented damages while the GPx activities may explain the absence of damages in Naked NPs. However, the general lowering GSH content in all contaminated mussels suggests that a loss of redox balance occurred.

In 28-days experiment the concentration of CeO_2NPs was set to 1 $\mu g\ L^{-1}$ that represents the predicted environmental concentration (PEC) for the worst case scenario (Keller and Lazareva 2013) and the upper limit of natural aquatic mean exposure concentration likelihoods modelled by O'Brien and Cummins (2011). The results obtained by this second experiment are in line with those of the 7-days experiment with the two

coated NPs that triggered major oxidative stress in comparison to Naked NPs, being Ce@Alginate the only CeO_2NP type able to induce oxidative damage. Nevertheless, data related to the metabolic activity showed a different pattern. Indeed, a general trend of increased ETS activity was observed as well as the protein content, while the GLY content decreased. These findings suggest a different defense strategy in response to NP exposure at a lower concentration and for a longer period of time. Indeed, in this case, it seems that mussels activated their metabolism (identified by increased ETS activity) using the GLY content as energy storage, and induced the synthesis of proteins required to increment cellular defense mechanisms in response to contaminants. Concerning the antioxidant system, it is possible to observe also at 1 µg L^{-1} the pro-oxidant effects of CeO_2NPs, with specific effects observed depending on the different coatings. For instance, in mussels exposed to Ce Naked the increase of the GPx activity accompanied by a significant reduction of the GR activity suggested a variation of oxidative status in the maintenance of GSH/GSSG balance (Regoli and Giuliani, 2014). In mussels exposed to Ce@Chitosan, the activity of SOD and GPx was induced significantly revealing that also at this concentration the antioxidant defense system was enhanced probably due to an excess of ROS content generated by CeO_2NPs. Finally, mussels exposed to Ce@Alginate showed an increase in the GSTs activity and a significant generation of LPO, suggesting that, in this case, the antioxidant system was not able to counteract oxidative stress thus confirming the major effects of these NPs (Villa et al., 2020). Higher LPO levels in Ce@Alginate group might be associated with higher ETS activity, as mitochondrial respiration is responsible for the generation of ROS (Freitas et al., 2020). The absence of modulation of GSH further revealed that regardless the Ce NPs redox balance was maintained.

Overall, the results from the two exposure experiments carried out on *M. galloprovincialis* showed the ability of CeO_2NPs to trigger an imbalance of the oxidative status and alter mussel metabolic capacity. This finding is in line with results from previous studies testing the effects of CeO_2NPs on Mediterranean mussels. Specifically, Auguste and co-authors (2019) showed an increase in ROS levels and the GSTs activity in *M. galloprovincialis* exposed *in vivo* to CeO_2NPs. Park and co-authors (2008) also observed an up-regulation of the GSTs mRNA following exposure of CeO_2NPs, which increased when the GSH/GSSG ratio decreased. Similarly, genotoxic effects induced by 100 µg L^{-1} and 10 µg L^{-1} CeO_2NPs has been detected in *Corbicula fluminea* (Koehle-Divo et al., 2018) while Ciacci and co-authors (2012) showed that CeO_2NPs induced ROS in *M. galloprovincialis* and had inflammatory and/or immunosuppressive effects at a concentration higher

than the one used in the present study (10 μg mL^{-1}). In detail, the results here presented underline that in the 28-days experiment the CeO_2NPs act more evidently on different biochemical targets depending on the coating in comparison to the 7-days experiment, in which the oxidant pathways were more similar upon all the three NPs. Besides, the oxidant pathways did not differ much among the two experiments, with the Ce@Alginate that seem to be the most effective in both experiments to affect energy metabolism and oxidative status. These findings suggest that the best way to analyze the effect of CeO_2NPs on mussels should be through long time exposure and at low concentrations, which represents the best approach to predict the risk of environmental pollutants for wildlife.

The results obtained further suggest that the highest effectiveness of Ce@Alginate with respect to the other CeO_2NPs depends on bio-interactions towards mussels rather than by their behavior in saltwater. This hypothesis is supported by our previous work in which it seems that Ce@Alginate might compromise the integrity and functionality of cell membrane affecting lysosome permeability to ions K^+/H^+ and thus generating an osmotic imbalance (Della Torre et al., 2021). Moreover, Della Torre and co-authors (2021) showed that the alginate and chitosan coatings were able to impart a different surface charge to CeO_2NPs, one of the several factors/properties that could influence the interaction, disposition of NPs in the cell and the subsequent toxicity to organisms. In fact, the surface charge is a key property of NPs by which depends on their colloidal stability, dispersion and transport in environmental and biological systems (Collin et al., 2014). Furthermore, the surface charge could define the physical interaction between NPs and cellular membranes (El Badawy et al., 2011). In general, positively charged NPs are taken up more easily by cells and generate higher toxicity (Frohlich, 2012), but some studies showed stronger impacts for negatively charged NPs. For instance, negatively charged CeO_2NPs were internalized in lysosomes more than positive and neutral NPs and resulted more toxic (Asati et al., 2010). Similarly, a previous *in vitro* study on *M. galloprovincialis* (Sendra et al., 2018), showed how the negative-charged CeO_2NPs caused more deleterious effects in respect to neutral charged NPs, underlining how the surface charge is one of the main properties that could influence the toxic outcomes of NPs. In other type of NPs, such as polystyrene NPs, the surface functionalization with COOH (negative charge) increased the toxicity to Caco-2 cells with respect to amine functionalized (positive charge) NPs (Thubagere and Reinhard, 2010). Another feature that could affect the toxicity of CeO_2NPs is the Ce^{3+}: Ce^{4+} ratio, which could lead to the formation of ROS, specifically H_2O_2

(Celardo et al., 2011), interfering with nutrient transport function of the membrane (Zeyons et al., 2009) or leading to membrane disruption (Rogers et al., 2010; Palomares et al., 2011). Therefore, more investigation should be carried out in future studies focusing on the influence of the coating on the Ce^{3+}: Ce^{4+} ratio and how this could be reflected in the interactions between NPs and organisms.

5. CONCLUSIONS

Our study highlighted that the fate of CeO_2NPs in saltwater is highly dependent on the interactions with biomolecules, affecting the processes of aggregation/agglomeration and sedimentation. While this interaction could impact planktonic or pelagic organisms, it did not seem to affect the bioavailability of NPs and their uptake by a benthic filter feeder as the mussel species *Mytilus galloprovincialis*. The coating with alginate, which imparted a negative surface charge, made the CeO_2NPs more reactive compared to NPs Naked and coated with chitosan also in the long-term experiment at close-environmental concentration. Our results do not allow to establish the specific mechanism of action of polysaccharide-coated CeO_2NPs, therefore more investigation are warranted focusing on the potential factor, such as surface charge, that drives the toxicity of NP to organisms. The present findings confirm the need to investigate how the ecotoxicity of NPs could be influenced by eco-corona to properly predict their impacts on the aquatic ecosystems.

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Table 1. Hydrodynamic diameters (nm) and ζ-potential values of the three CeO_2NPs in artificial seawater.

	Conditions	Dynamic light scattering			ζ potential (mV)
		d_h nm	Vol %	Numb %	
Artificial seawater	Ce Naked (0.1 mg mL^{-1})	1760 ± 270	100	100	+23.8 ± 4.8
	Ce@Chitosan (0.1 mg mL^{-1})	220 ± 40 880 ± 190	100	100	0.0 ± 4.2
	Ce@Alginate (0.1 mg mL^{-1})	3105 ± 460	100	100	-16.1 ± 3.5

Table 2. Cerium concentrations in exposure water and *Mytilus galloprovincialis* soft tissue from each treatment (mean ± standard deviation).

Exposure conditions		Ce exposure water ($\mu g\ L^{-1}$)	Ce mussel soft tissue ($\mu g\ g^{-1}$ d.w.)
7d	CTRL	1.01 ± 0.005	0.32 ± 0.154
	Ce Naked	13.51 ± 0.400	5.58 ± 4.251
	Ce@Chitosan	23.73 ± 0.497	3.41 ± 0.557
	Ce@Alginate	51.28 ± 0.479	3.30 ± 2.501
28d	CTRL	0.29 ± 0.036	0.19 ± 0.034
	Ce Naked	0.07 ± 0.007	0.25 ± 0.113
	Ce@Chitosan	0.03 ± 0.006	0.26 ± 0.029
	Ce@Alginate	0.01 ± 0.002	0.21 ± 0.030

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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.

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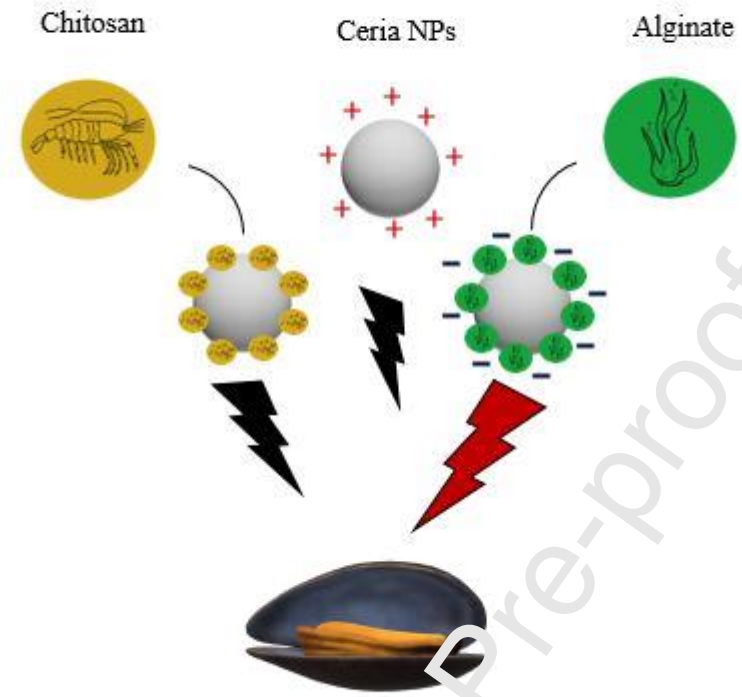
Graphical Abstract



Highlights

- Coating with Alginate and Chitosan modifies surface charge of CeO_2NPs.
- Different hydrodynamic behaviour and stability of CeO_2NPs with different coatings.
- No difference observed in CeO_2NP uptake by *M. galloprovincialis*
- CeO_2NPs coated with Alginate resulted more toxic to *M. galloprovincialis*
- The long-term exposure to CeO_2NPs coated with Alginate induced lipidic peroxidation.