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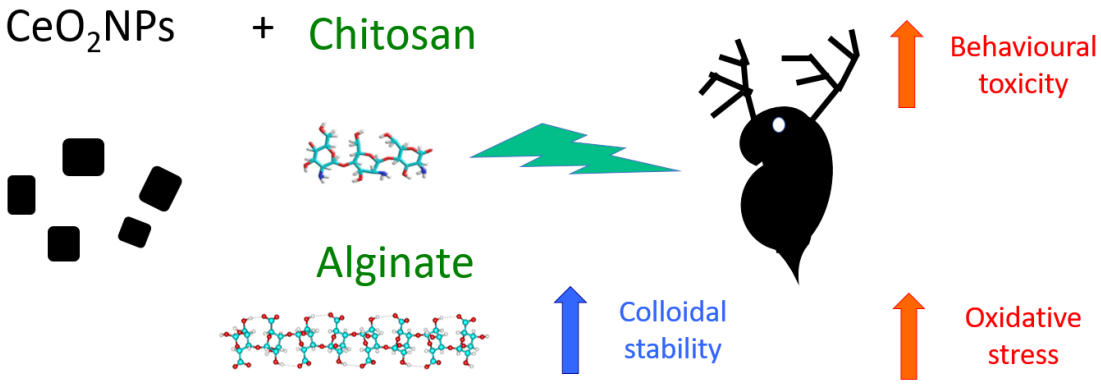

Natural molecule coatings modify the fate of Cerium dioxide nanoparticles in water and their ecotoxicity to *Daphnia magna*

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Abstract

The ongoing development of nanotechnology has raised concerns regarding the potential risk of nanoparticles (NPs) to the environment, particularly aquatic ecosystems. A relevant aspect that drives NP toxicity is represented by the abiotic and biotic processes occurring in natural matrices that modify NP properties, ultimately affecting their interactions with biological targets. Therefore, the objective of this study was to perform an ecotoxicological evaluation of CeO_2NPs with different surface modifications representative of NP bio-interactions with molecules naturally occurring in the water environment, to identify the role of biomolecule coatings on nanoceria toxicity to aquatic organisms. *Ad hoc* synthesis of CeO_2NPs with different coating agents, such as Alginate and Chitosan, was performed. The ecotoxicity of the coated CeO_2NPs was assessed on the marine bacteria *Aliivibrio fischeri*, through the Microtox® assay, and with the freshwater crustacean *Daphnia magna*. Daphnids at the age of 8 days were exposed for 48 h, and several toxicity endpoints were evaluated, from the molecular level to the entire organism. Specifically, we applied a suite of biomarkers of oxidative stress and neurotoxicity and assessed the effects on behaviour through the evaluation of swimming performance. The different coatings affected the hydrodynamic behaviour and colloidal stability of the CeO_2NPs in exposure media. In tap water, NPs coated with Chitosan derivative were more stable, while the coating with Alginate enhanced the aggregation and sedimentation rate. The coatings also significantly influenced the toxic effects of CeO_2NPs. Specifically, in *D. magna* the CeO_2NPs coated with Alginate triggered oxidative stress, while behavioural assays showed that CeO_2NPs coated with Chitosan induced hyperactivity. Our findings emphasize the role of environmental modification in determining the NP effects on aquatic organisms.

Keywords: Ceria nanoparticles; *Daphnia magna*; Microtox; functionalization; environmental modifications

Capsule: Interactions with environmental molecules influence the fate and ecotoxicity of NPs

1. Introduction

The ongoing use of products containing nanomaterials (NMs) results in a relevant voluntary or involuntary release of these contaminants into the environment with adverse consequences for natural ecosystems (Klaine et al., 2008). In fact, the unique features that render NMs advantageous for technological applications, such as antimicrobial activity, electronic, catalytic and reactivity properties, can be deleterious in an environmental context (Klaine et al., 2012). The aquatic ecosystem, in particular, is highly susceptible to such contamination, and it is the sink of the NMs leached from soil and discharged in wastewater (Selk et al., 2016). An extremely important feature of NMs lies in the fact that they do not follow elemental physicochemical features of other classes of human-made pollutants, thus creating new challenges in ecotoxicology and risk assessment (Gottshalk et al., 2013). A relevant aspect that drives NM toxicity is represented by the complex dynamics of abiotic and biotic processing occurring in natural matrices (Zhang et al., 2018). In fact, upon being released in the environment, NMs undergo several transformations, such as aggregation, dissolution, and redox reactions, which alter their fate, transport and potential toxicity (Amde *et al*., 2017).

Several studies have shown that the interaction with natural organic matter (NOM) modifies the surface properties and behaviour of NPs in water media (Quik et al., 2012; Sani-Kast et al., 2017 and citation therein). Nevertheless, either stabilizing or destabilizing effects have been reported depending on NOM composition, the type of NP, and the different water media where the interactions are tested. In addition, the ecotoxicological outcomes of interactions with natural molecules remain largely unknown.

The surface modifications, in particular, represent a key driver for NM cellular uptake and toxicity. Indeed, in aquatic media and biological fluids, NMs tend to bind biomolecules from the surroundings, implying the modification of their properties and eventually affecting their interactions with biological targets (Canesi and Corsi 2016; Surette and Nason, 2018). While this concept is widely considered in toxicological/pharmacological studies, it is hardly addressed in nano-ecotoxicological research. To overcome such limitations, proper nano-ecotoxicological studies should address the chemical, biological and ecological transformations of NMs and their final impact on biological targets (Zhang et al., 2018).

Among NMs, cerium oxide nanoparticles (CeO_2NPs) are extensively employed as excellent catalysts in diesel fuel oil production, in energy conversion and storage, polishing powders and in biomedicine as antioxidant agents and as UV absorbents (Sun et al., 2012). The use of CeO_2NPs is foreseen to further increase in the upcoming with a forecasted estimated upper limit of production volumes at 10,000 t/a in 2050 (Giese et al., 2018). The consequent release of CeO_2NPs in 2050 is estimated to be up to 300 t/a in the ecosphere (waters, soil, air) and up to 4000 t/a in the technosphere (i.e., landfills, waste treatment) (Giese et al., 2018).

The predicted environmental concentration of CeO_2NPs in surface waters is in the ng L^{-1} range (Gottshalk et al., 2015), but a release of up to μg L^{-1} in hotspots, such as wastewater treatment plants, could occur (Keller 2014).

The toxicity of CeO_2NPs has been documented among several taxa (Collin et al., 2014). The effects are described at different biological scales, such as oxidative stress (Koehlé-Divo et al., 2018; Garaud et al., 2015; Zhang et al., 2011; Rodea Palomares et al., 2012), immunomodulation (Ciacci et al., 2012 ; Auguste et al., 2019 ; Falugi et al., 2012), altered swimming performance (Artells et al., 2013 ; Garaud et al., 2015), impaired growth and development (Van Hoecke et al., 2009 ; Manier et al., 2013 ; Conway et al., 2014) and lethality (Van Hoecke et al., 2009; Bour et al., 2015). Nonetheless, the cellular mechanisms underlying the toxicity of CeO_2NPs are far from understood. Most of this evidence arose from ecotoxicity tests carried out on pristine NPs. Nevertheless, several studies emphasized that environmental modifications might affect the physicochemical features of CeO_2NPs (Quik et al., 2010; Auffan et al., 2014; Tella et al., 2014), which in turn influence the bioavailability and toxic outcomes for aquatic organisms (Garaud et al., 2016).

In particular, the surface interactions of CeO_2NPs with organic and inorganic molecules can enhance stability in water and modify their biological consequences to organisms. For instance, poly(acrylic acid)-stabilized CeO_2NPs remained more dispersed in water and generated higher toxicity to the freshwater algae *Pseudokirchneriella subcapitata* than non-stabilized CeO_2NPs (Booth et al., 2015). Citrate-coated CeO_2NPs were more stable in the water column than bare CeO_2NPs in a simulated pond ecosystem (Tella et al., 2015). A further investigation on the bivalve mussel *Dreissena polymorpha* showed that this coating enhanced the accumulation of CeO_2NPs (Garaud et al., 2016). In addition, the citrate-coated CeO_2NPs significantly reduced the expression of the pi-glutathione-S-transferase gene and the activity of the catalase (CAT) enzyme and increased the lysosomal system.

In this context, the objective of this study was to perform an ecotoxicological evaluation of CeO_2NPs with different surface modifications that represent NP bio-interactions with molecules naturally occurring in the water environment to identify the influence of biomolecule coatings on the nanoceria fate and toxicity to aquatic organisms.

To this end, CeO_2NPs were *ad hoc* synthesized with two different coating agents such as Alginate and Chitosan. Alginate and Chitosan were selected as representative biomolecules present in natural aquatic systems. Alginate is a natural polysaccharide that represents up to 30% of natural organic matter (NOM) in lake water (Buffle et al., 1998) and is a model of extracellular polymeric substances produced by biofilms (Ostermeyer et al., 2013). Chitosan is a biopolymer, a chitin derivative that is produced from crustacean shells, which is the second most abundant natural polysaccharide on earth (Komi-Hamblin 2016). Alginate is a negatively charged polymeric species in a wide range of pH due to carboxylate functionalities present in each repeating unit. In contrast, Chitosan is a polymer characterized by amine groups that are mainly protonated until pH 7-8. The ζ-potential curves for the two natural polymers (Figure S1, together with the chemical structures of the repeating units) show the different overall charge of these two natural macromolecules.

The impacts of bare and coated CeO_2NPs were assessed in the marine bacteria *Aliivibrio fischeri* and in the freshwater crustacean *Daphnia magna*. Different toxicity endpoints were evaluated, such as the inhibition of luminescence in *A. fischeri* and the imbalance of antioxidant mechanism, inhibition of acetylcholinesterase

activity and swimming performance in *D. magna*. Such metrics would allow linking the effects observed at the molecular-cellular level to toxic outcomes at the individual level.

2 Methods

2.1 Nanoparticle synthesis

The CeO_2NPs (Naked Ceria) were prepared by modifying a literature procedure (Plakhova et al., 2016) as described in detail in the supporting materials.

The preparation of ceria-chitosan NPs (Ce@Chitosan) and Ce@Alginate nanocomposites was carried out starting from freshly prepared naked CeO_2NPs as fully described in the supporting materials.

The three suspensions were stored at 4 °C. The final concentrations of ceria NPs (9.3 mg mL^{-1}), Ce@Chitosan NPs (13.0 mg mL^{-1}) and Ce@Alginate NPs (10.1 mg mL^{-1}) were determined on the basis of thermogravimetric analysis (TGA) carried out with a lyophilized sample of a known suspension volume.

2.2 Nanoparticle characterization

The morphology of the CeO_2NPs was observed with Zeiss LEO 912ab Energy Filtering TEM operating at 100 kV using a CCD-BM/1 K system. TEM samples were prepared by depositing a drop of diluted aqueous ceria suspensions on a carbon-coated copper grid (CF300-Cu), allowing the contact for 15 min and then carefully removing the drop from the surface grid, and allowing it to dry at room temperature overnight.

DLS and ζ-potential measurements were carried out on 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 and at the natural pH of S. Benedetto tap water, typically dissolving samples at a concentration of 1 mg mL^{-1} or less in dependence on the conductivity of the solution and/or the scattering power. The size and charge analyses were averaged from at least three repeated measurements.

The stability over time of the three colloidal suspensions was carried out by acquiring UV−vis absorption spectra on an Agilent model 8543 spectrophotometer at room temperature and using standard quartz cells with a 1.0 cm path length.

Thermogravimetric analysis (TGA) was carried out using a Mettler-Toledo thermogravimetric balance (TGA/DSC 2 Star® System), analysing ~ 10-15 mg of lyophilized samples operating in air and spanning in the temperature range of 50–800 °C with a heating rate of 5 °C min^{-1}.

Infrared spectra were acquired for lyophilized samples on a PerkinElmer Frontier spectrometer equipped with an ATR accessory with a diamond/ZnSe crystal.

2.3 Microtox bioassay

The acute toxicity for the bioluminescent bacterium *Aliivibrio fischeri* was measured using the Microtox bioassay in accordance with the test conditions and operating protocol of the Microtox® system operating manual, the Acute Toxicity Basic Test procedures (Azur Environmental 1998). The freeze-dried bacteria and the reconstituent solution were purchased from Ecotox LDS (Milan, Italy). Luminescence inhibition was

measured using a Microtox model 500 analyser (Ecotox) in acute mode. Bacteria were exposed for 15 min at 15 °C. Toxicity tests were carried out on a control and nine serial dilutions of the three CeO_2NP suspensions (0.0039-1 mg L^{-1}) in a 2% NaCl solution buffered at pH 7. Each test was performed three times in duplicate.

2.4 Daphnia magna culture and exposure

Adult *D. magna* individuals came from a single clone obtained from the Istituto Superiore di Sanità (Roma, Italy). They were reared as described in detail in the supplementary materials. Eight-day-old individuals were exposed under the same rearing conditions to 10 µg L^{-1} and 100 µg L^{-1} of each type of CeO_2NPs for 48 h in 50 mL glass beakers under semi-static conditions, with renewed media after 24 h. The selected concentrations did not induce acute toxicity for *D. magna* (Collin et al., 2014), but were able to modulate sub-lethal toxicity endpoints in another crustacean species, the amphipod *Gammarus roeseli* (Garaud et al., 2015). Individuals were not fed during the experiments. Five experimental replicates of 25 individuals each were performed for each experimental condition. A control beaker containing only culture water was included in all replicates. At the end of the 48-h exposure, individuals were frozen in liquid nitrogen and stored at -80 °C prior to biomarker analyses.

2.4 Biomarker analysis

Pooled individuals from each replicate were homogenized in a 100 mM potassium phosphate buffer (added with KCl 100 mM, EDTA 1 mM, dithiothreitol 1 mM and 1:100 v/v protease inhibitors, pH 7.4). The homogenates were centrifuged at 15,000 x g for 15 min at 4 °C, then the supernatant was collected and processed for enzyme activity measurements through spectrophotometric methods as described in (Parolini et al., 2018) and reported in the supplementary materials using a 6715 UV/Vis spectrophotometer (Jenway, UK). All the enzymatic activities were measure in triplicate per pool.

2.5 Behavioural assay

The behavioural tests were performed on 24 daphnids for each treatment, as fully described in supplementary materials. Three behavioural endpoints distance moved, average speed and activity time, were monitored according to Villa et al. (2018).

2.6 Statistical analysis

Biomarker data were compared through one-way analysis of variance (ANOVA) after checking for normality and homoscedasticity, taking $p<0.05$ as a significance cut-off. The LSD *post hoc* test was applied to evaluate significant differences between exposure groups. To evaluate whether the different coatings lead to distinct effects on the antioxidant response pathway, all biomarkers of oxidative stress were analysed through discriminant function analysis (DFA). The analyses were performed using the STATISTICA 7.0 software package. Statistical analysis of behavioural results was performed using GraphPad Prism 6 software (version 6.01). The one-way analysis of variance (ANOVA) was applied (Dunnett's multiple comparisons

test; 95% confidence interval). Alternatively, a non-parametric test (Dunn's multiple comparisons test; 95% confidence interval) was performed when the data did not follow a normal distribution.

3 Results and Discussion

3.1 Nanoparticle characterization

The full characterization of NPs highlighted that the coating with natural polymers altered the stability and hydrodynamic behaviour of the NPs in water, with Ceria@Alginate being more prone to sedimentation compared to the other two NPs.

Synthesized Naked Ceria NPs were homogeneous in size with a mean diameter centered at ~5 nm (TEM micrograph, Fig. 1a). From these NPs, the Chitosan and Alginate nanocomposites have been obtained (see the Experimental Part for details). The three synthesized nanoceria showed some differences in size, shape or aggregation state (Fig. 1). TEM images of Naked Ceria showed some aggregates, but most of the freshly prepared NPs appeared well spread on the sample holder. In contrast, Ceria@Chitosan NPs appeared in the TEM micrographs as irregular NP clusters composed of NPs identical in size with the naked NPs, and the Chitosan shell was not visible. The Ceria@Alginate was composed of particles with rounded shapes and larger sizes (~50 nm). In this nanocomposite, we can observe the Alginate shell surrounding the NPs in the TEM images (Fig. S2 of the Supporting Information), which explains the observed differences by TEM both in shape and size.

To confirm the formation of the coating and quantify it, we carried out thermogravimetric analysis (TGA). While in the TG profile of Naked Ceria, a continuous mass loss until 800 °C is present, for both the Chitosan and Alginate derivatives, there is a defined weight loss step (from 150 to 400 °C), stating the presence of the polymer together with Ceria NPs. The starting weight loss is due to the loss of hydration water (~2% and ~9% for Chitosan and Alginate, respectively). Then, while in the Ceria@Chitosan curve the decomposition of the polymer accounts for only ~9% of weight loss (T onset = 230 °C), for Ceria@Alginate, the percentage of polymer reached ~49% (T onset = 210 °C), in line with what was observed by TEM, which showed that the Alginate amount surrounding the Ceria NPs, compared to that of Chitosan, was much higher (Fig. S3).

Another indicator of the presence of polymers on Ceria NPs was observed in FTIR-ATR spectroscopy. The spectra are reported in Figure S4 (panel (a) Chitosan; panel (b) Alginate), in which a comparison between Naked Ceria NPs, the polymers and the relative Ceria derivatives are reported. The attribution of the bands relative to the various species is reported in Table S1. From this comparison, it can be seen that in the blue traces, the signals of Chitosan (Fig. S4a) and Alginate (Fig. S4a) are evident (vertical grey dashed lines) together with the intense and partially visible band of the Ceria lattice (750-400 cm^{-1}).

The three samples suspended in milliQ water appeared different to the naked eye. While Ceria@Chitosan NPs were well suspended, both the Naked Ceria and Ceria@Alginate NPs showed a certain amount of precipitate settling down over time, with the Ceria@Alginate the sample having the highest visible sediment formation. Fist, we tentatively tried to characterize the three samples by DLS in milliQ water at a

concentration as low as 0.1 mg mL^{-1}, but the insufficiency of the results, due to a very low scattering power, prompted us to increase the concentration to 1 mg mL^{-1}. At these concentration levels, the analyses were robust and reproducible but far from the concentrations used for the assessment of the effects on bacteria as well as on animal models (vide infra). Nevertheless, DLS is a useful tool to compare the colloidal behaviour of the different nano-derivatives and to collect information on their actual state, provided that the suspension is stable enough.

The DLS results (Table 1) in milliQ water showed that Ceria@Chitosan NPs present only one peak, which was centered at ~200 nm, while in the case of the Naked Ceria two populations were detected (at ~150 and ~400 nm), indicating that incipient aggregation began to occur. The instability over time of some suspensions produced DLS outputs that were not always representative of the whole sample since even during the short time of the measurements, dynamic aggregation events occurred, as in the case of the Alginate derivative, which showed the most aggregated situation. This last sample started to flocculate in a few minutes, and one of the two peaks detected by DLS was centered at much higher sizes (~790 nm). This behaviour can be ascribed to the ability of this polymer species to crosslink several NPs to each other. Hence, it is clear that the hydrodynamic diameter is representative of just the fraction of material remaining in suspension.

Through measurements of the ζ-potential, the surface charge was also investigated. As expected, the different coatings heavily modified the surface charge, from the negative value -28 mV for Ceria@Alginate to positive values for Naked Ceria (+36.7 mV) and Ceria@Chitosan (+42.8 mV).

In tap water, all the CeO_2NPs exhibited increased aggregation and colloidal instability (see sizes in Table 1, entries 5-7). In general, the increase of the ionic strength affects the diffused layer at the NP surface by reducing it, thus lowering the ζ-potential values and consequently enhancing aggregation. Moreover, in the case of Alginate, Ca^{2+} ions can heavily affect the aggregation crosslink of different chains since carboxylate groups effectively bind this cation. In this medium, the hydrodynamic diameter of Naked Ceria resulted close to micron size, Ceria@Chitosan formed aggregates of ~506 nm, while Ceria@Alginate copiously precipitated, leaving in suspension few smaller aggregates as 98% of the population showed a hydrodynamic diameter of ~210nm. In addition, NPs showed a consistent ζ-potential change in tap water (whose natural pH is 7.9). Naked Ceria acquired a negative surface charge (-12 mV), the Ceria@Chitosan ζ-potential was approximately zero (-2.7 mV), while Ceria@Alginate still showed a negative surface charge (-17.0 mV). All of these values were within the range of +20/-20 mV and very near zero, which explains the deterioration of the colloidal stability. A much greater instability was finally observed in NaCl water media, where larger hydrodynamic diameters were observed for all NPs, again affecting the Alginate derivative more than the Chitosan derivative, with the following descending order: Ceria@Alginate > Naked Ceria > Ceria@Chitosan. The Naked Ceria and Ceria@Chitosan acquired a positive surface charge, while the Ceria@Alginate ζ-potential was still negative (-18.2 mV).

We also evaluated the sedimentation of the NPs by absorption UV-vis spectroscopy, following the decrease over time of the absorption band at ~ 300 nm in the ceria matrix. Cue to the high sensitivity of this

spectroscopic technique, these measurements were carried out at a concentration as low as 0.01 mg mL^{-1} to still detect the adsorption band of ceria while implementing similar concentration conditions used in the biological tests. In tap water and at this low concentration, the Ceria@Chitosan derivative was more stable and decreased slowly and almost linearly, and a similar profile was also observed also for the Naked Ceria (Fig. 2). In contrast, the Ceria@Alginate NPs were prone to aggregate and flocculate much faster, and in 50 min their concentration was halved (Figure 2).

The results showed that in tap water, the coating with Chitosan seemed to increase the dispersion and stability of CeO_2NPs, while the coating with Alginate enhanced sedimentation, leaving smaller aggregates in the water column with respect to those of the other NPs.

A different trend was observed in saltwater, where the very high ionic strength provokes substantial aggregation of all the NPs, in agreement with previous observations (Keller et al., 2010; Quik et al., 2014). The most evident and fast aggregation was observed, once more, for Alginate derivative (hydrodynamic diameter of ~1860 nm, surface charge of -18 mV), followed by the Naked NPs (~1590 nm, ~ zero charge) and the Chitosan derivative (~780 nm, +20 mV) which, once more, resulted in the most stability among the three. Overall, the evidence underlined that the interaction with biomolecules influenced the fate of CeO_2NPs, and this could reflect the NPs' bioavailability to aquatic organisms.

3.2 Effects on A. fischeri

No significant effect was observed on luminescence activity measured on the gram-negative bacterium *A. fischeri* through the Microtox® bioassay, as the percentage of inhibition was always maintained below 20% in bacteria exposed to all CeO_2NPs (Fig. S5). The toxicity of nano ceria towards bacteria has been postulated and could occur through direct contact with the cellular membrane without cellular internalization. A cytotoxic effect has been observed in the gram negative bacterium *Escherichia coli* exposed to positively charged CeO_2NPs with LC_{50} at 5 mg L^{-1} (Thill et al., 2006). Exposure to CeO_2NPs of different sizes triggered inhibition of luminescence in the cyanobacterium *Anabaena CPB4337* with EC_{50} ranging from 38 to 70 mg L^{-1} after 1 h of exposure, but no evidence of NP uptake was observed (Rodea-Palomares et al., 2012). The toxicity of the CeO_2NPs stabilized with hexamethylenetetramine was previously assessed through Microtox® bioassay, showing IC_{50} at 21.76 mg mL^{-1}, a concentration well above the highest used in this study (Garcia et al., 2011). The absence of toxic effects observed in our study could therefore be related to the lower concentrations of CeO_2NPs tested in the short exposure time, as the assay conditions are probably not enough to determine any significant interactions of the NPs with the cell membrane and the induction of signalling cascades that determine the emergence of toxic outcomes.

3.3 Effects on D. magna

The effects of the three NPs, assessed at different biological scales on *D. magna*, highlighted that the modification of the physicochemical features of CeO_2NPs due to coatings with biomolecules reflects different toxicological outcomes for *D. magna*.

The three CeO_2NPs at both tested concentrations did not induce significant acute toxic effects as immobilization/mortality was below 10% in all replicates. To assess whether the different coatings may interfere with cellular pathways, the activity of enzymes involved in the antioxidant response machinery was investigated. The exposure to the three CeO_2NPs did not modify superoxide dismutase (SOD) activity (Fig. 3). The CAT activity was significantly reduced in individuals exposed to Ceria@Chitosan at concentrations of 100 μg L^{-1} with respect to that of controls and of groups exposed to Naked Ceria and Ceria@Alginate (Fig. 3). A significant induction of glutathione-S-transferase (GST) activity was observed in daphnids exposed to Ceria@Alginate at concentrations of 100 μg L^{-1} compared to that of all the other exposure conditions. A similar profile was also observed for the reactive oxygen species (ROS) content, which was significantly higher only in the group exposed to Ceria@Alginate at concentrations 100 μg L^{-1} compared to that of controls (Fig. 3). Discriminant function analysis further pointed out that the Ceria@Alginate and Ceria@Chitosan at concentrations of 100 μg L^{-1} were distinguished from the other treatments (Fig. 3). Wilk's Lambda value of 0.125 ($p<0.0001$) confirmed the significant power of the analysis. The first and second axes explained 78% and 18% of the total variance, respectively. The most discriminating biomarkers on the first axis were GST and CAT, and CAT and ROS on the second axis, which also resulted in significant modulation of individual biomarkers.

The CAT enzyme plays a key role in mitigating the toxicity of hydrogen peroxide (H_2O_2) and its inhibition could occur as toxic effects of pollutants related to ROS overproduction. Therefore, the decrease of CAT activity observed in daphnids exposed to Ceria@Chitosan at 100 μg L^{-1} without a concomitant ROS increase suggests ROS scavenging behaviour for these NPs. In line with our evidence, exposure of the freshwater bivalve *Dreissena polymorpha* to bare and citrate-coated CeO_2NPs induced downregulation of the *gpx* and *gst-pi* genes and decreased CAT activity, thus suggesting an antioxidant protective behaviour for this species (Garaud et al., 2016). In contrast, the increase in ROS levels paralleled the induced GST activity upon exposure to Ceria@Alginate at 100 μg L^{-1}, suggesting that this coating triggers an oxidative stress condition in daphnids. Similar to our results, an increase in ROS levels and GST activity has been observed in the marine bivalve *Mytilus galloprovincialis* exposed *in vivo* (Auguste et al., 2019).

Therefore, our results highlight that the molecules adsorbed on the NP surface might significantly modify the oxidative properties of CeO_2NPs, shifting from ROS scavenging activity to the induction of oxidative stress as a function of different surface coatings. Indeed, the CeO_2NPs have either prooxidant or antioxidant properties. Several studies have shown that CeO_2NPs can act as ROS scavengers, protecting cells from oxidative damage and mimicking the activity of the CAT and SOD enzymes (Korsvik et al., 2007; Ciofani et al., 2014). In contrast, other studies showed the ability of CeO_2NPs to trigger an imbalance of the oxidative status in different biological models (Yokel et al., 2014). CeO_2NPs are insoluble and highly stable upon environmental conditions (Xia et al., 2008; Briffa et al., 2018). Nevertheless, in internal tissue and body fluids, a significant release of Ce^{3+} ions might occur. The dissolved Ce^{3+} ions could have a relevant role in generating oxidative stress, as suggested in previous study (Pulido-Reyes et al., 2015, Sendra et al., 2017). A change in the extent of Ce^{3+} release from the NPs might therefore explain the opposite effect of

Ceria@Alginate and Ceria@Chitosan NPs on the oxidative system. Further investigations are warranted to identify the properties/characteristics responsible for such an effect. A similar behaviour has been also described in a study that compared the toxicity of CeO_2NPs with different surface charges on three algal species (Sendra et al., 2017). Authors observed both protective effects against ROS and toxicity related to the NP surface charge and species-specific susceptibility. The different interactions of the three CeO_2NPs with the ROS homeostasis pathway could impact the health status of the organism. In fact, oxidative imbalance can generate oxidative damage to cellular macromolecules, resulting in alteration of their structure and functionality and disruption of cellular activity, eventually incurring organ damage.

To understand whether these effects have implications for higher hierarchical levels, we assessed an endpoint of neurotoxicity as acetylcholinesterase inhibition and behavioural parameters. The neurotoxic potential of NPs has been postulated as one of the most worrisome side effects of NPs. Neurotoxicity mechanisms suggested thus far involve mostly the generation of oxidative stress, but mechanisms underpinning behavioural effects are far to be understood (Win-Shwe and Fujimaki, 2011; Feng et al., 2015). To understand the potential mechanism underlying the observed alteration of swimming performance we assessed acetylcholinesterase activity. This endpoint, in fact, is considered a potential bridge to link the sub-cellular effects of pollutants with symphtoms of toxicity at the individual level, such as the impairment of behavioural performances (Beauvais et al., 2000; Amiard Triquet et al., 2009; Khalil et al., 2017; Parolini et al., 2018). Some studies have suggested that AChE could be a target of the toxic action of NPs. Metal- and carbon-based NPs have been shown to adsorb/inhibit AChE activity *in vitro* (Wang et al., 2009). The inhibition of AChE activity has also been observed in zebrafish erythrocytes after short-term and prolonged exposure *in vivo* to AgNPs in the mg L^{-1} range (Katuli et al., 2014). The inhibition of cholinesterase activities has been measured in coelomocytes of the sea urchin *Paracentrotus lividus* exposed to metal NPs including CeO_2NPs (Falugi et al., 2012). In this study, the acetylcholinesterase activity in *D. magna* was not affected upon all exposure conditions (Fig. 4). This result suggests that the alteration of antioxidative stress enzymes observed did not affect the cholinergic system.

Concerning the swimming performance, our results showed a different profile upon exposure to the three CeO_2NPs at the behavioural level. Naked Ceria and Ceria@Alginate did not affect swimming performance in daphnids. In contrast, CeO_2NPs coated with Chitosan exhibited the ability to trigger behavioural effects in daphnids. The Ceria@Chitosan NPs at both concentrations induced hyperactive behaviour by increasingthe average speed and acceleration, without increasing the distance moved (Fig. 4).

Some studies have reported the ability of NPs to alter swimming performance in *Daphnia* and both hyperactivity and hypoactivity have been documented. For instance, a decrease in swimming velocity has been observed in *D. pulex* and *D. similis* exposed to CeO_2NPs in the mg L^{-1} range (Artells et al., 2013). The effect was species-specific and was ascribed to the strong NP adsorption/accumulation in the cuticle, which impairs animal locomotion. Similarly, a decreased swimming velocity has been observed in *D. magna* exposed to multi-walled carbon nanotubes and graphene (Stanley et al., 2016; Cano et al., 2017). A reduction of swimming activity has been reported in *D. magna* exposed to C_{60} but not to C_{60} functionalized with (1,2-

methanofullerene C_{60})-61-carboxylic acid and this difference might be attributable to the different size, which was smaller in the fC_{60} (Brausch et al., 2011). In contrast, exposure to C_{60} and functionalized C_{60} ($C_{60}HxC_{70}Hx$) increased hopping frequency and appendage movement (Lovern et al., 2007).

Our results suggest that the different particle sizes measured in water could be a driving factor for the distinct behavioural effects. Indeed, particles with dimensions of 500 nm are preferentially taken up by daphnids (Gophen and Geller, 1984), and this size corresponds to the hydrodynamic range measured for Ceria@Chitosan NPs. Therefore, the observed effect could be related to the fact that daphnids mistake these NPs as food sources and therefore increase their movement to obtain more particles. A behavioural response related to feeding has already been suggested by Noss and coauthors (2013), who observed that exposure to $nTiO_2$ triggered swarming behaviour. Another mechanism responsible for altered swimming performance could be an escape reaction against NPs, which drives daphnids to avoid the toxicant by swimming faster. Further studies performed with longer exposure times, would allow us to clarify whether other mechanisms related to neurochemical alterations induced by NPs could be involved in the observed behavioural effect.

Overall, the tracking of swimming performance proved to be a suitable and sensitive ecotoxicity tool that could be used to assess the sub-lethal toxicity of NMs. The alteration of swimming performance is indicative of neurophysiological events that could have implications at the population level. In fact, swimming behaviour is a crucial element of the prey/predator relationship and for the capture of food (Uttieri et al., 2014).

Since an increase of movement of the zooplankton could increase the risk of predation, making the prey more visible for the predator (Zaret, 1980), and given the key role of *D. magna* in the aquatic food web, the observed modification of swimming behaviour might reflect ecological consequences at the population level.

4 Conclusions and implications for environmental risk assessment

This study aimed to provide insight into our current understanding of the exposure, hazard, and risk of NPs in the aquatic environment, particularly for CeO_2NPs.

Our results showed that interactions of CeO_2NPs with biomolecules (Alginate and Chitosan), largely present in the aquatic environment, are able to influence the processes of aggregation/agglomeration, sedimentation and dissolution of the different forms of CeO_2NPs. These interactions introduce further bias and uncertainties in the predictive capabilities of exposure models, as, at the time being, the entity of such interactions are highly unpredictable.

The potential formation of CeO_2@Chitosan and CeO_2@Alginate could provide new "ecotoxicological properties" to nano Cerium oxide. However, in our study, at the tested concentrations no acute effects (neither photoinhibition in *A. fischeri* nor mortality in *D. magna*) were observed on the tested organisms. These results are in line with many published data, which reported acute effects in different species at concentrations much higher than those utilized in our study, which approach predicted environmental levels. Thus using the traditional risk ratio approach (exposure/toxicity ratio), CeO_2NPs should not be a concern for aquatic species. However, we demonstrated that the coating with biomolecules conferred new biological

407 reactivity to the NPs which targeted the antioxidant stress system and swimming activity in *D. magna*,
408 indicating potential sub-lethal toxicity of these compounds, which could hamper the fitness of the exposed
409 populations.
410
411 **References**
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Figure captions

590 **Figure 1**. TEM micrographs of (a) Naked Ceria NPs; (b) Ceria@Alginate NPs; (c) Ceria@Chitosan NPs.

591 **Figure 2.** Sedimentation test, followed by UV-vis spectroscopy, of the three Ceria NP suspensions in tap
592 water, monitored by the absorbance peak variation of the Ceria core (λ_{max} = 300-305 nm) over time.

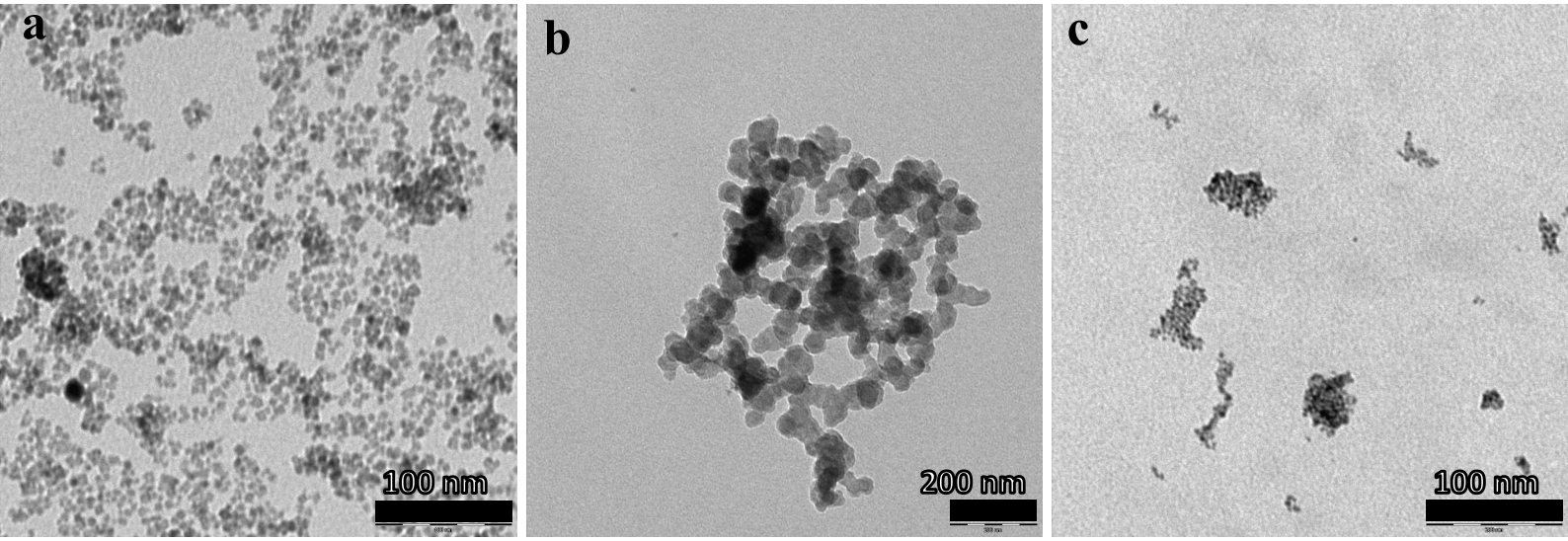
593 **Figure 3.** Activity of SOD, CAT, GST and amount of ROS measured in *D. magna* exposed to the three
594 CeO_2NPs at 10 and 100 µg L^{-1} for 48 h. Data are expressed as Mean ± SD (N = 5). Different letters indicate
595 significantly different values $p<0.05$. Plot of DFA performed on oxidative stress biomarkers.

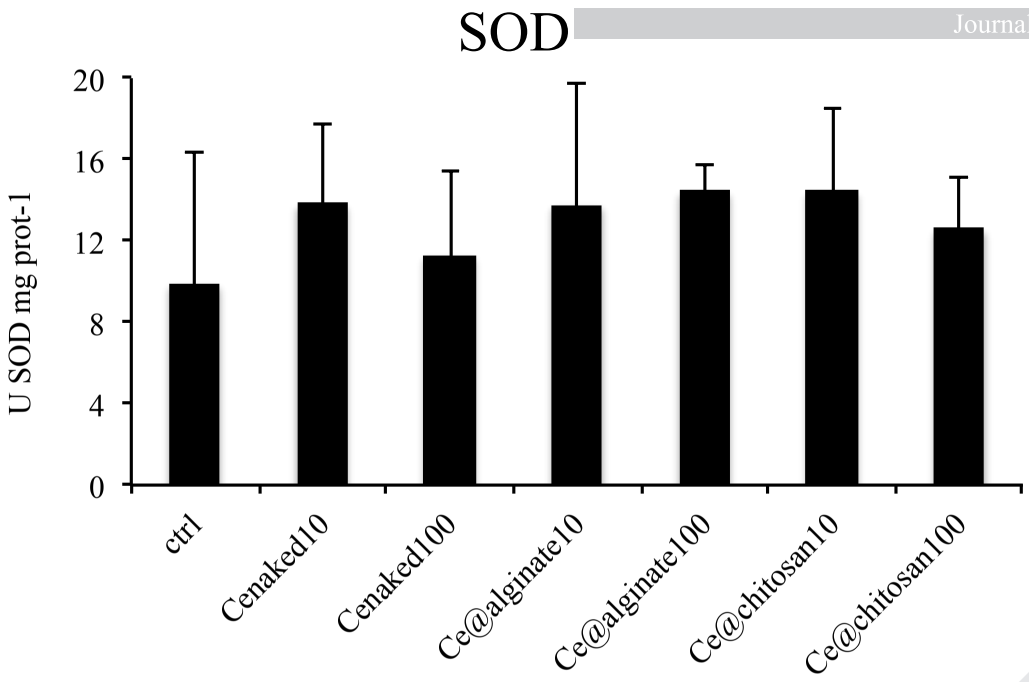
596 **Figure 4.** Swimming activity, swimming velocity and total distance and Achetylcholinesterase activity
597 measured in *D. magna* exposed to the three CeO_2NPs at 10 and 100 µg L^{-1} for 48 h. Data are expressed as
598 Mean ± SD (N = 5). Different letters indicate significantly different values $p<0.05$.

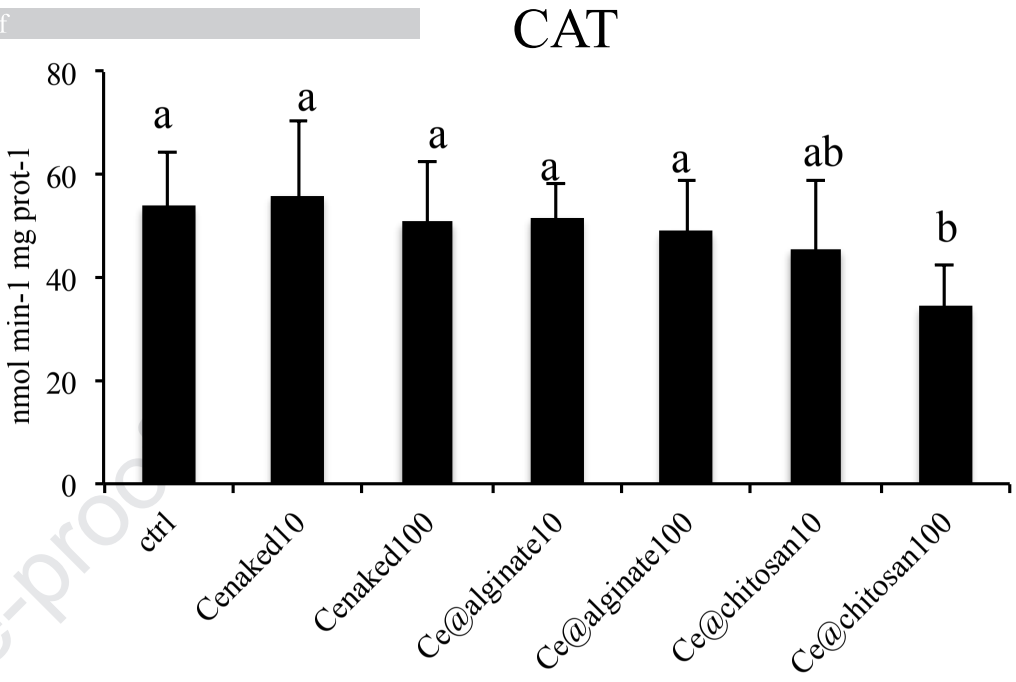
599

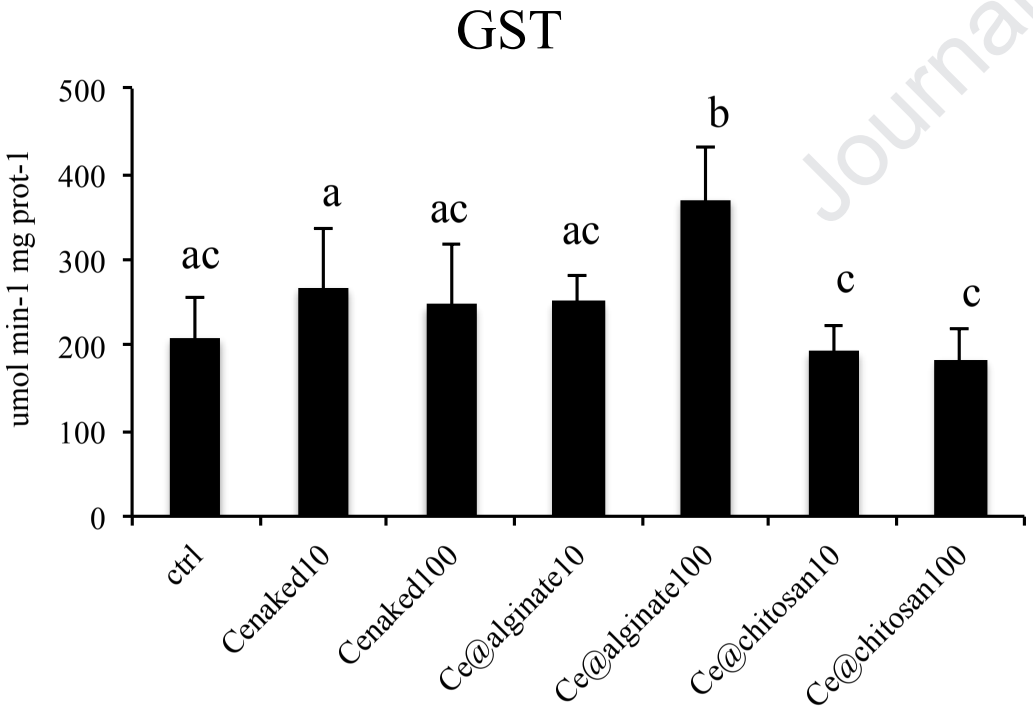
Table 1. Hydrodynamic diameters (nm) and z-potential values of the three CeO_2NPs in milliQ water, tap water and NaCl water.

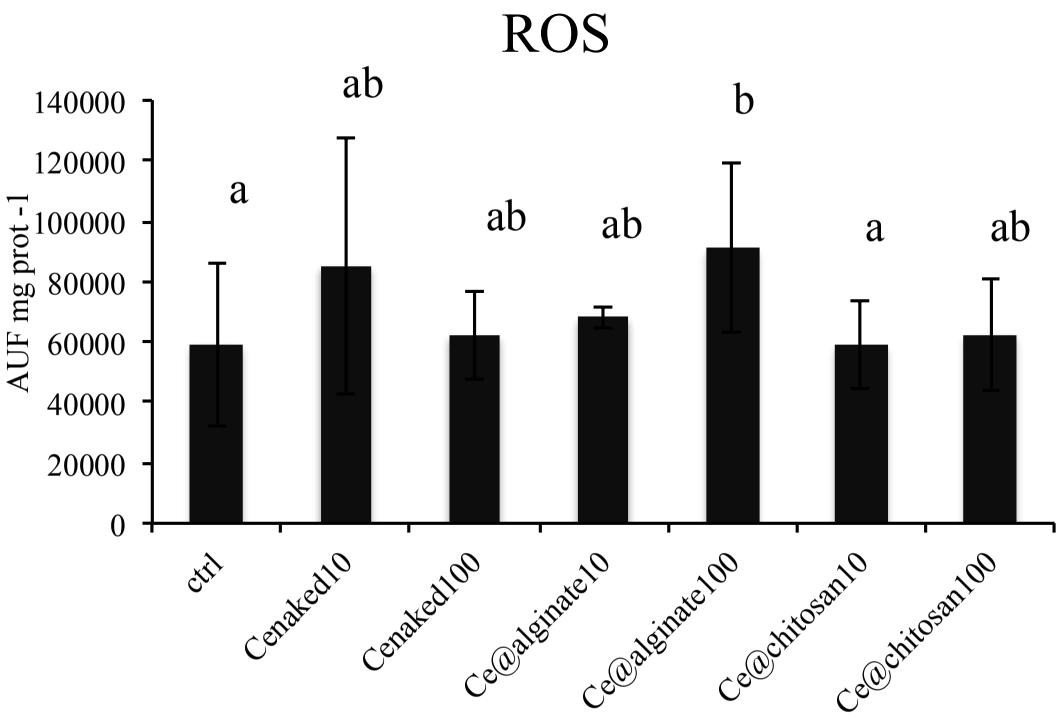
	Sample	Dynamic light scattering			ζ potential (mV)
		d_h (nm, intensities)	Vol %	Numb.%	
milliQ water	**Naked Ceria (1 mg/mL)**	146 ± 15	78.3	100	+36.7 ± 2.3
		400 ± 75	21.7	0	
	Ceria@Chitosan (1 mg/mL)	209 ± 46	100	100	+42.8 ± 3.2
	Ceria@Alginate (1 mg/mL)	786 ± 115	79	2	-28 ± 2.2
		144 ± 14	21	98	
Tap water	**Naked Ceria (1 mg/mL)**	973 ± 115	100	100	-12 ±2.8
	Ceria@Chitosan (1 mg/mL)	506 ± 20	100	100	-2.7 ± 2.4
	Ceria@Alginate (1 mg/mL)	210 ± 25	66	98	-17.0 ± 2.0
		790 ± 150	33	2	
NaCl water	**Naked Ceria (0.1 mg/mL)**	1590 ± 110	100	100	+ 2.8
	Ceria@Chitosan (1 mg/mL)	780 ± 80	100	100	+ 20.2
	Ceria@Alginate (0.05 mg/mL)	1857 ± 200	100	100	-18.2

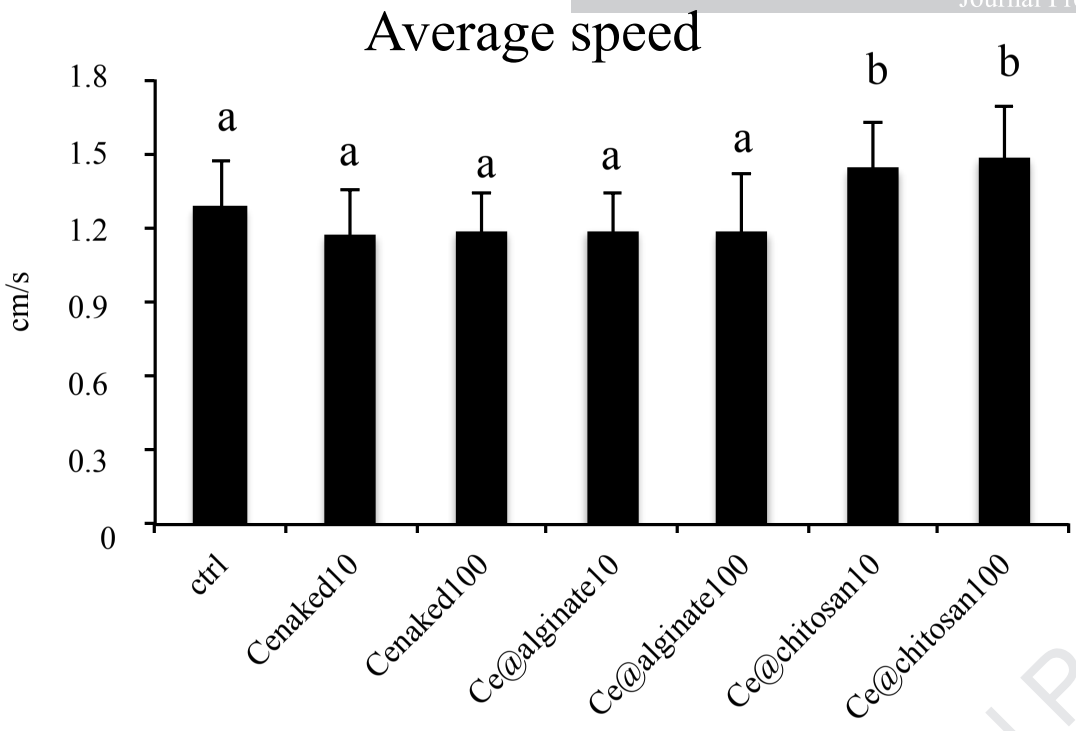


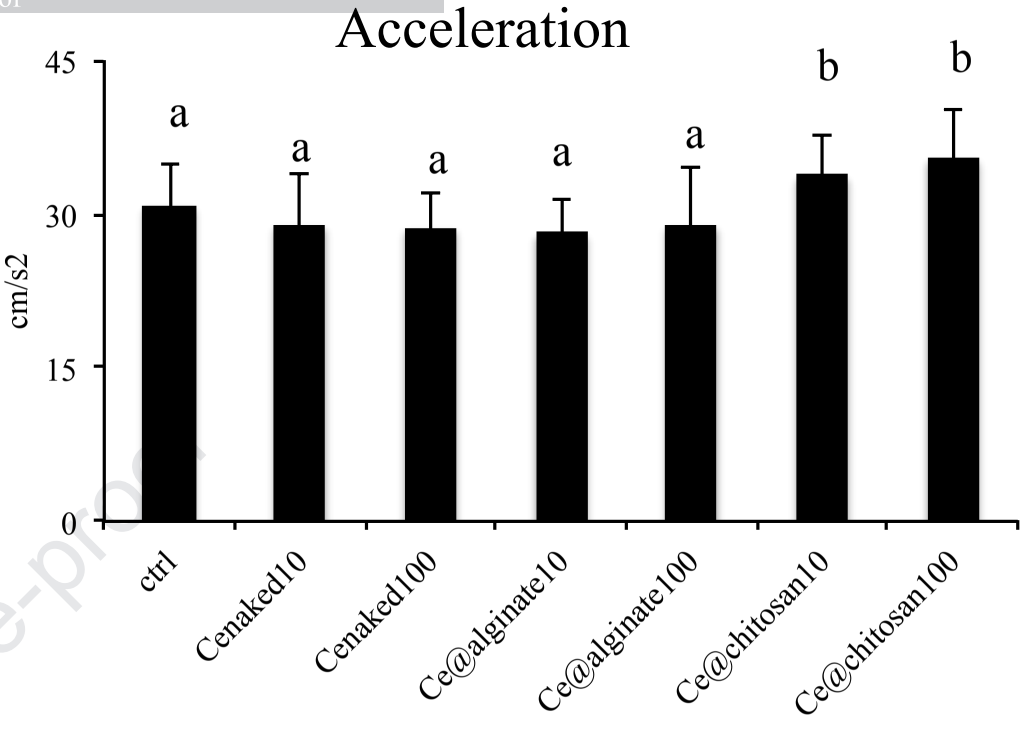


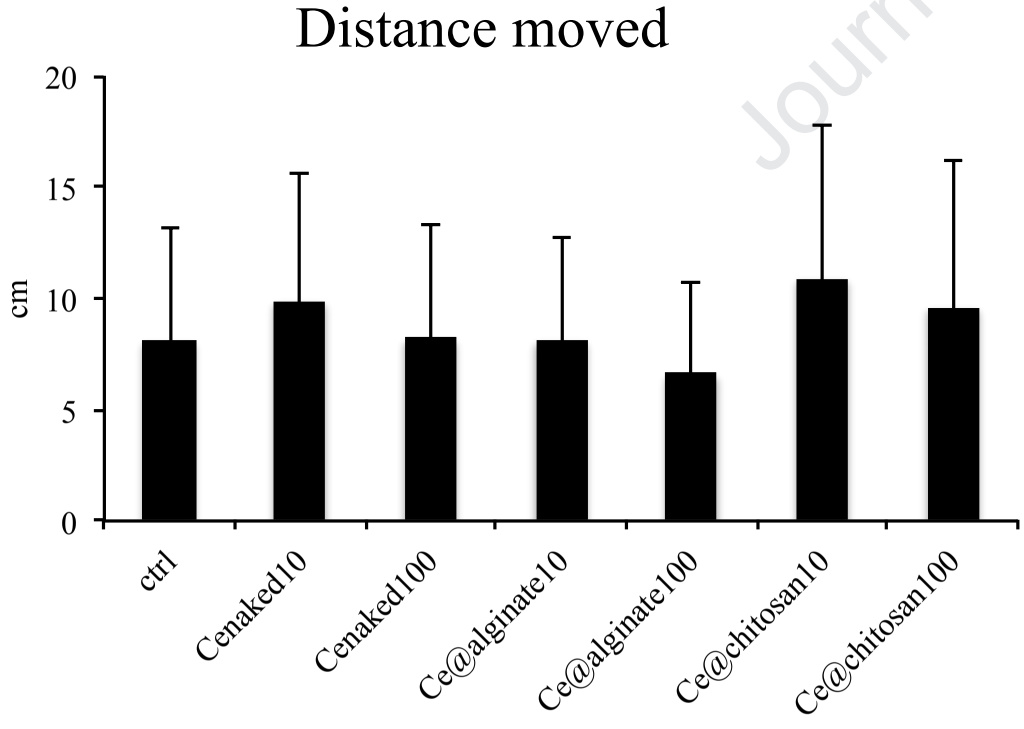


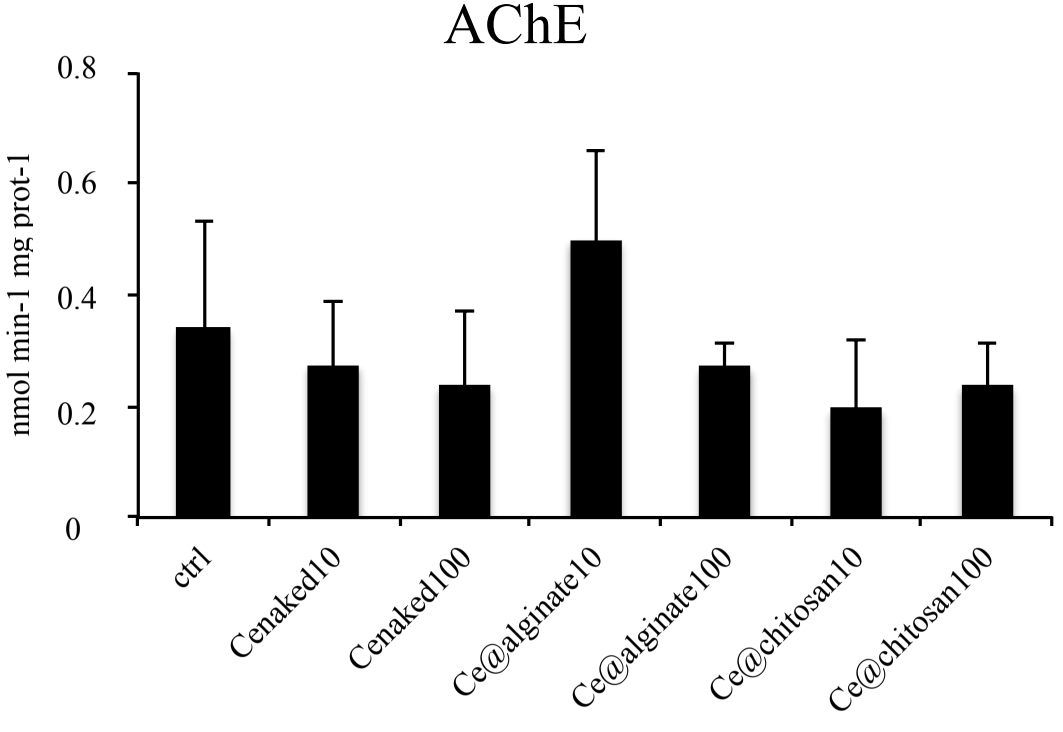


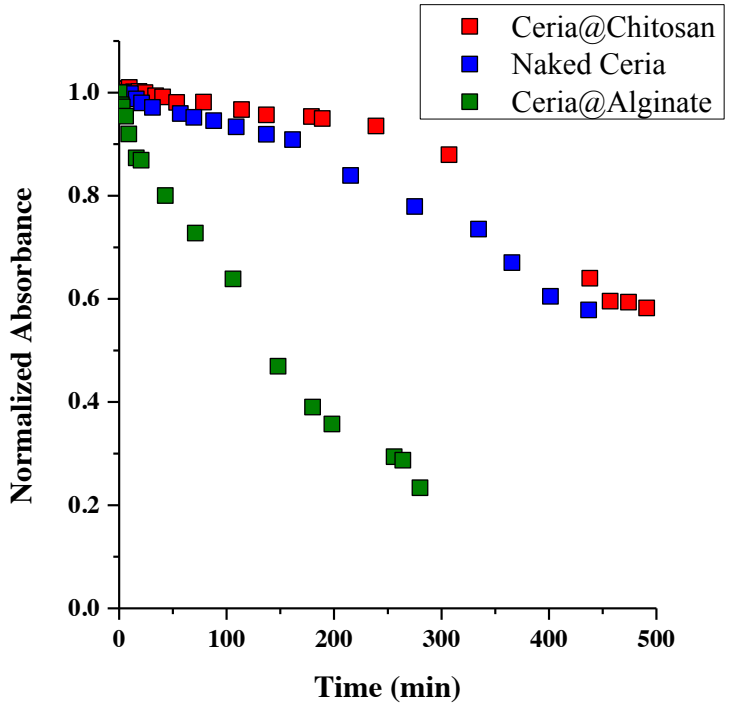












Highlights

CeO_2NPs were coated with Alginate and Chitosan and their ecotoxicity was assessed

The coating with natural molecules modified the stability and hydrodynamic behaviour of CeO_2NPs

Natural coating conferred new ecotoxicological properties towards *Daphnia magna*

The interaction between NPs and natural molecules is a driver of NPs' ecotoxicity

Conflict of interest

Authors declare that there are no conflict of interest