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Alginate coating modifies the biological effects of Cerium oxide nanoparticles to the freshwater bivalve *Dreissena polymorpha*

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Abstract

The adsorption of biomacromolecules is a fundamental process that can alter the behaviour and adverse effects of nanoparticles (NPs) in natural systems. While the interaction of NPs with natural molecules present in the environment has been described, their biological impacts are largely unknown. Therefore, this study aims to provide a first evidence of the influence of biomolecules sorption on the toxicity of cerium oxide nanoparticles (CeO_2NPs) towards the freshwater bivalve *Dreissena polymorpha*. To this aim, we compared naked CeO_2NPs and coated with alginate and chitosan, two polysaccharides abundant in aquatic environments. Mussels were exposed to the three CeO_2NPs (naked, chitosan- and alginate-coated) up to 14 days at 100 μg L^{-1}, which is a concentration higher than the environmental one predicted for this type of NP. A suite of biomarkers related to oxidative stress and energy metabolism was applied, and metabolomics was also carried out to identify metabolic pathways potentially targeted by CeO_2NPs. Results showed that the coating with chitosan reduced NP aggregation and increased the stability in water. Nonetheless, the Ce accumulation in mussels was similar in all treatments. As for biological effects, all three types of CeO_2NPs

reduced significantly the level of reactive oxygen species and the activity of superoxide dismutase, glutathione peroxidase and glutathione-S-transferase. The effect was more pronounced in individuals exposed to CeO_2NPs coated with alginate, which also significantly induced the activity of the electron transport system. Metabolomics analysis of amino acid metabolism showed modulation only in mussels treated with CeO_2NPs coated with alginate. In this group, 25 metabolites belonging to nucleotides, lipids/sterols and organic osmolytes were also modulated, suggesting that the nanoparticles affect energetic metabolism and osmoregulation of mussels. This study highlights the key role of the interaction between nanoparticles and natural molecules as a driver of nanoparticle ecotoxicity.

Keywords: Cerium oxide nanoparticles; eco-corona; oxidative stress; metabolomics; osmoregulation

1. Introduction

Nanoparticles (NPs), particles falling in a range of dimension from 1 to 100 nm, are nowadays quickly developing as materials applied in manufacturing and manipulation of several products, with a broad range of possible applications (Piccinno et al., 2012; Vance et al., 2015). This production and large-scale use of NPs inevitably lead to their release into the environment, reaching different types of ecosystem compartments (water, sediments and biota; Selck et al., 2016). This has raised concern about the possible consequence of NPs on wildlife and environmental health, particularly for aquatic ecosystems, being the final sink for NPs release.

The behaviour and fate of NPs in aquatic systems can be subjected to several physical, chemical and biological modifications that could affect their uptake and potential toxicity for organisms (Zhang et al., 2018; Spurgeon et al., 2020). Among these transformations, the adsorption of biomolecules from the surroundings represents a phenomenon which should be deeply addressed. When NPs come in contact with natural media, their surface is covered by a corona of biological macromolecules, called eco-corona (Canesi and Corsi, 2016). In water, one of the main biological components is the natural organic matter (NOM). The NOM is constituted by humic substances, proteins, exudates and polysaccharide acid compounds (Sharma et al., 2015). These organic substances can alter NP aggregation state in the water column, and the consequent

impacts on biological targets (Huber and Stoll, 2018). For instance, some studies have shown a reduced aggregation of NPs in freshwater due to the presence of NOM which increases the particle ζ-potential, thus increasing electrostatic repulsion (Baker et al., 2014; Lawrence et al., 2016). Although it is very important to understand how the eco-corona can influence the ecotoxicity of NPs, nowadays studies concerning this topic are very limited and either alleviation or enhancement of NP toxicity have been reported (Canesi and Corsi, 2016; Ren et al., 2016; Wang et al., 2016; Baalousha et al., 2017; Yu et al., 2018). This depends on several factors such as the NP-specific toxicity mechanism, the NOM composition, the water chemistry and the biological model used (Lundqvist et al., 2008; Qiu et al., 2010; Hajipour et al., 2014; Mahmoudi et al., 2013; Pozzi et al., 2015; Tenzer et al., 2013).

Cerium oxide nanoparticles (CeO_2NPs) are one of the most promising NPs used in several applications such as paint coating, polishing powder, catalysts, luminescent materials, coatings, glass polishers, oxygen gas sensors, fuel cells, fuel additives and pharmacology (Celardo et al., 2011). In CeO_2NPs both Ce^{3+} and Ce^{4+} oxidative states coexist on the surface and the presence of redox reaction between Ce^{3+}/Ce^{4+} generates oxygen vacancies which confer catalytic and electrical properties and biological reactivity (Sun et al., 2012). The actual levels of CeO_2NPs in the natural ecosystem are still unknown, but several models attempted to establish a predicted environmental concentration in natural matrices. The predicted environmental concentration of CeO_2NPs in freshwaters is in the ng L^{-1} range (Giese et al., 2018) and it is expected to rise to 0.02–300 ng L^{-1} in the next decades, mostly due to their large use in the diesel fuels (Johnson and Park, 2012; Sun et al., 2014). A calculation of predicted environmental concentrations (PECs) as high as 1 µg L^{-1} in surface waters has been suggested in some worst-case scenarios, such as effluents of wastewater treatment plant (O'Brien and Cummins, 2010; 2011).

Ecotoxicological studies have described the adverse effects of CeO_2NPs in several aquatic species at different levels of the food-web (Zhang et al., 2011; Rodea-Palomares et al., 2011; Fairbairn et al., 2011; Collin et al., 2014; Bour et al., 2015; Callaghan et al., 2017). CeO_2NPs have been reported to trigger oxidative imbalance (Rodea Palomares et al., 2012; Garaud et al., 2015; Koehlé-Divo et al., 2018), immunomodulation (Sendra et al., 2018; Auguste et al., 2019), alteration of feeding and swimming capacity (Artells et al., 2013; Garaud et al., 2015), impairment of growth and development, and death (Van Hoecke et

al., 2009, 2011; Manier et al., 2013; Conway et al., 2014). Nonetheless, uncertainties remain concerning the molecular mechanisms underlying these effects.

While the interaction of CeO_2NPs with macromolecules such as alginate present in natural compartments has been described (Oriekhova et al., 2017: 2018), the influence on biological impacts of these NPs is largely unknown, except for a study carried out on the algae *P. subcapitata*, which showed that NOM enhanced the stability of CeO_2NPs in suspension and decreased their toxicity (Van Hoecke et al., 2011).

In this view, the objective of this study was to perform an ecotoxicological evaluation of CeO_2NPs coated with the two polysaccharides, alginate and chitosan, using the freshwater bivalve zebra mussel as animal model (*Dreissena polymorpha)*. The two polymers were selected as representative of biomolecules occurring in aquatic systems. The CeO_2NPs tested in this study have been already used in our previous research aimed to investigate if the coating with natural polysaccharides could affect the behaviour in water towards the freshwater crustacean *Daphnia magna* (Villa et al., 2020) and their toxicity. This first study showed that the coating with alginate and chitosan affected significantly the aggregation state and stability of the CeO_2NPs in water. On the other hand, it was not possible to assess the NP uptake and accumulation in organisms. Moreover, Ce@Chitosan induced hyperactivity in daphnids, while biomarkers related to oxidative stress showed controversial results. Starting from this first evidence, this work has been carried out to test the hypothesis that the different coating could affect also the uptake/accumulation of nanoceria by aquatic organisms, and to deepen our understanding on the potential mechanisms underlying the ecotoxicological effects induced by the different coatings. To this aim, we used as biological model *D. polymorpha*. Indeed, due to their ecophysiological features, bivalves represent an excellent model to investigate the accumulation and impacts of NPs (Canesi et al., 2012).

The stability and aggregation state of bare and coated CeO_2NPs in the water column has been evaluated. To assess NP bioavailability, Ce bioaccumulation in mussels was measured. Moreover, a biomarker suite related to oxidative stress and energy reserves has been applied, to investigate the toxicity mechanisms induced by naked and coated CeO_2NPs. Finally, metabolomics analysis has been carried out to provide a broad picture of the metabolic condition of the organisms and to identify the cellular pathways potentially affected by CeO_2NPs.

2. Materials and methods

2.1 Chemicals and reagents

All standards, solvents and reagents used were of highest purity (LC–MS grade where available). The reagents used for biomarkers were purchased by Sigma Aldrich (St. Louis, MO, USA). Solvents and reagents used for metabolomics and Ce analysis were purchased by Carlo Erba (Milano, Italy). Amino acid standards were purchased by Sigma Aldrich and Cambridge Isotope Laboratories (Andover, Inc. MA USA) (see SM).

2.2 Nanoparticles synthesis and characterization

CeO_2NPs, naked and coated with chitosan (Ce@Chitosan) and alginate (Ce@Alginate), were synthesized as described in detail by Villa and co-authors (2020). The thorough characterization of the three NPs is also reported in the paper. The hydrodynamic size and ζ-potential of NPs in exposure water 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 and equipped with the device for the ζ-potential measurement. Disposable cuvettes were used for DLS measurements and the universal deep-cell kit for the ζ-potential measurements. The size and charge analyses were averaged from at least three repeated measurements.

2.3 Dreissena polymorpha exposure

About 1200 individuals of *D. polymorpha* were collected in Lake Maggiore by SCUBA diving. They were maintained for acclimation in aquaria filled with tap water and distilled water (1:1, total volume 12 L) for 1 week at 20 ± 1 °C, with a natural photoperiod and an oxygen saturation >90%. After the acclimation, 75 mussels of similar size were placed per tank (4 L) to perform the exposure. Organisms were exposed to four different conditions: control group and mussels exposed to three different types of CeO_2NPs (naked Ceria, Ce@Alginate and Ce@Chitosan) at concentrations of 100 µg L^{-1}. The concentration was selected based on a previous study, which showed a significant accumulation of Ce in *D. polymorpha* and the occurrence of biological effects at this concentration after 4 days (Garaud et al., 2015). Three tanks were used for each

experimental condition. Exposure was carried out in semi-static conditions with a daily renewal of water and spiking with CeO_2NPs suspensions.

Organisms were maintained with oxygenators at 20 ± 1 °C under a 12 h/12 h light/dark photoperiod and they were fed daily with a suspension of *Spirulina spp* 1 h before the water renewal. Besides oxygenators, to potentiate the maintenance of NPs in the water column a constant magnetic stirring was placed at the tank bottom. Mussels were collected after 7 days and at the end of the exposure period, and soft tissues of 3 mussels from each tank were pooled (3 pools *per* treatment), frozen in liquid nitrogen and stored at -80 °C, before biomarker analysis. Other three mussels were collected and pooled for metabolomics analysis only after 14 days of exposure. At the end of the experiment, a pool of about 30 individuals was also collected from each tank and stored at -20 °C for Ce analysis in soft tissues. Furthermore, 1 h after the contamination, an aliquot of 15 mL of water was sampled from the control and the exposure tanks, then stored at -20 °C, to quantify the Ce concentrations.

2.4 Quantification of Ce in exposure waters and soft tissues of Dreissena polymorpha

The soft tissues of *D. polymorpha* were solubilized by acid digestion by adding 3 mL HNO_3 and 0.5 mL H_2O_2 (ultrapure reagents) to 125 to 250 mg of pools consisting of about 30 individuals from the control and exposure tanks. Solubilization was carried out in Teflon bombs in a Milestone Ethos 900 microwave lab station. The solutions resulting from acid digestion were filtered and ultra-pure water was added to a final volume of 50 mL. The Ce concentrations in *D. polymorpha* soft tissue and waters in each experimental condition were determined by inductively coupled plasma-mass spectrometry (ICP-MS) using a Perkin Elmer NexION 350 spectrometer. The analytical accuracy was checked through the analysis of Ce concentration in the following–standard reference materials of National Institute of Standards and Technology (USA): SRM 3110 (Cerium Standard Solution) and SRM 2977 (Mussel Tissue) for the analysis of waters and freshwater bivalve, respectively. The Ce concentrations measured in these standard reference materials were in good agreement with the certified values with recoveries of 99.2-102.5% for SRM 3110 and 93.5-96.8% for SRM 2977. The analytical precision was evaluated using the percentage relative standard deviation (% RSD) of five replicate analyses of each water sample and pool of *D. polymorpha*. The values of

RSD were in the range 0.3-6.7% and 1.4-3.9% for Ce analysis in water samples and soft tissue of *D. polymorpha* individuals, respectively.

2.5 Biomarker analysis

The biomarker analyses were carried out on pooled soft tissue following the procedures described in Magni et al. (2020). The following biomarkers suite was measured for each experimental condition: electron transport system activity (ETS), reactive oxygen species content (ROS), superoxide dismutase activity (SOD), catalase activity (CAT), glutathione peroxidase activity (GPx), glutathione S-transferases activity (GSTs). Each method is described in detail in the Supplementary Materials.

2.6 Statistical Analysis

Biomarker data were compared through one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison tests; 95% confidence interval, after checking for normality and homoscedasticity and using $p \leq 0.05$ as the significance cut-off. Before analysis, the outlier values were determined through a calculator performing Grubbs' test, also called the ESD method (extreme studentized deviate). To evaluate if the different coating leads to a distinct effect on the antioxidant response pathway, all biomarkers were analysed through the discriminant function analysis (DFA). The analyses were carried out using the STATISTICA 7.0 software package.

2.7 Metabolomics analysis

2.7.1 Amino Acid analysis

Amino acids were extracted from 10 mg of lyophilized zebra mussel pooled tissues with 1 mL methanol/acetonitrile mixture (1:1, v:v). Samples were vortexed for 30 seconds, centrifuged at 14000 g for 10 min at 4 °C and the organic phase was evaporated under a stream of nitrogen. The residues were dissolved in 100 µL methanol and 10 µL used for amino acid derivation.

Amino acid derivation was achieved with AccQ•Tag reagents (Waters Corporation, Milford, MA, USA) according to the manufacturer's protocol. Briefly, 10 µL of either a standard amino acid mix solution or a biological extract was mixed with 65 µL of AccQ•Tag Ultra borate buffer, 5 µL of "Metabolomics Amino

Acid Mix Standard" from Cambridge Isotope Laboratories (Andover, Inc. MA USA), and 20 µL of AccQ•Tag reagent was added. The reaction was allowed to proceed for 10 min at 55 °C.

Samples have been analysed at Unitech OMICs (University of Milano, Italy) using ExionLC™ AD system connected to TripleTOF™ 6600 System equipped with Turbo V™ Ion Source with ESI Probe (SCIEX, MA, USA).

Samples were separated on CORTECS UPLC T3 column 1.6 µm, 2.1 x 150 mm, (Waters, Franklin, MA, USA). The temperature was set at 40 °C. The analytes were eluted using as mobile phase A, water with 0.1% formic acid and, as mobile phase B, acetonitrile with 0.1% formic acid. The flow was 500 µL min^{-1} and the elution gradient was set as below: 0–1 min (1% B), 1–2 min (1–13%B), 2–8 min (15% B), 8–9.50 min (15–20% B), 9.50–10 min (20-95% B), 10–11 min (95% B), 11–11.01 min (95-1% B) and 11.01-15 min (1%B). Two microliters of solution were injected in the LC-MS/MS. All samples were analysed in triplicate.

MS spectra were collected, in positive polarity, in Full-mass Scan from 75 to 600 Da (250 ms accumulation time) and in IDA® mode (Information Dependent Acquisition) from 50 to 500 Da (100 ms accumulation time, top 10 spectra per cycle 1.3 s). Nitrogen was used as a nebulizing gas (GS1, 55 psi), turbo spray gas (GS2, 65 psi), and curtain gas (CUR, 35 psi). Spray Voltage was fixed at 4.5 Kv, declustering potential (DP) was 50 eV, the collision energy was 30 eV with a collision energy spread (CES) of 10 eV and source temperature was 350°C.

Data were acquired using Analyst® TF version 1.7.1 and processed using MultiQuant™ Software 3.0.2 (SCIEX, MA, USA) with MQ4 integration algorithm. The linearity was tested by verifying the coefficient of determination (R^2) of analytes. Compounds were quantitated using the internal standard method and calibration curves were plotted using a weighted regression ($1/x$) only for Histidine, Alanine, Proline, Lysine, Leucine, Cystine. For all compounds was determinate a $R^2 > 0.994$ (see table S1). The exact mass of each AA is reported in table S3.

All samples were analysed in triplicate and quantified. For every sample Mean, Standard Deviation (SD) and coefficient of variation (CV%) were measured. The values of concentration obtained were normalized per mg of sample weighted.

Heatmap representation of normalized amino acid levels was obtained by the heatmap.2 function from the ggplot R package (Gregory et al., 2020). Clustering of amino acid level profiles and experimental conditions

was carried out using the hierarchical clustering algorithm, as implemented in the R cluster package (Maechler et al., 2019), with median linkage and using Euclidean distances.

2.7.2 Untargeted metabolomics

A further untargeted analysis was carried out to compare control and Ce@Alginate group, as only this exposure condition induced a significant difference in amino acid content. Two microliters of each sample, extracted as described before, were injected in LC-MS/MS. Samples were separated on ACQUITY UPLC® BEH C18 - 2.1 x 50 mm, 1.7µm (Waters®). The temperature was set at 40 °C. The analytes were eluted with the following gradient: from 95% buffer A (0.1% formic acid in water) to 95% buffer B (0.1% formic acid in acetonitrile) in 9 min. Constant flow rate: 400 µL min^{-1}. Total run: 15 min. MS spectra were collected, in positive polarity, in Full-mass Scan from 50 to 1300 Da (100 ms accumulation time) and in IDA® mode (Information Dependent Acquisition) from 50 to 1300 Da (40 ms accumulation time, top 16 spectra per cycle 0.75 s). Nitrogen was used as a nebulizing gas (GS1, 55 psi), turbo spray gas (GS2, 65 psi), and curtain gas (CUR, 35 psi). Spray Voltage was fixed at 5.5 Kv, declustering potential (DP) was 50 eV, the collision energy was 35 eV with a collision energy spread (CES) of 15 eV and source temperature was 350°C. The minimum absolute height required was set at 100 counts.

Raw data files (.wiff) were converted in .abf format and processed using the MS-DIAL software ver. 3.98, setting the Database MSMS-Public-Pos-VS12 composed by a total of 29.269 records (spectra MS/MS). Data elaboration was initially carried out applying the LOWESS normalization, which supports the LOWESS algorithm. The identifications (ID) were obtained based on the value m/z (parent ion) achieved and determined in high resolution. As a further filter, we considered only ID that showed a CV% < 30%. For each identified ID we measured: mean, SD and CV%. To these ID the function "TESTT" was applied and results were further filtered for values TESTT ≤ 0.05. In identified ID, we selected those with fragmentation spectrum MS/MS matched.

3. RESULTS

3.1 NP characterization

The three synthesized nano-ceria showed clear differences in terms of aggregation state. In the exposure water, naked CeO_2NPs showed one population centred at 1385 ± 170 nm, while for Ce@Chitosan NPs the population observed was at 270 ± 150 nm. The Ce@Alginate NPs tended to aggregate, with a mean hydrodynamic diameter centred at 1124 ± 110 nm, and to precipitate much faster than the other coated NPs (Table S2). The different coatings also modified the surface charge, shifting from positive for Ce@Chitosan (+6.2 ± 3.9 mV) to a negative value for the other two NPs (-10.6 ± 1.6 for naked CeO_2, -19.0 ± 2.4 mV for Ce@Alginate).

3.2 Cerium in exposure waters and soft tissues of Dreissena polymorpha

The concentration of Ce measured in exposure waters collected 1 h after NPs addition was lower than the nominal one, suggesting that all three CeO_2NPs are prone to fast sedimentation. This result indicates that the actual CeO_2NPs bioavailable for mussel during the exposure are lower than the one expected based on the nominal concentration. Nevertheless, a different stability as a function of different coatings could be observed. A higher concentration of Ce was measured in water collected from Ce@Chitosan tanks (7.16 ± 0.44 µg L^{-1}), while similar Ce levels were determined in waters from the Ce Naked (4.91 ± 0.33 µg L^{-1}) and Ce@Alginate (4.85 ± 0.05 µg L^{-1}) tanks (Tab. 1).

Upon all exposure conditions, Ce concentration in *D. polymorpha* soft tissues was comparable and higher than in individuals of the control group ($p = 0.002$) (Tab. 1). This finding indicates that Ce accumulation was similar in freshwater mussels exposed to the three types of CeO_2NPs.

3.3 Effects of CeO_2NPs in Dreissena polymorpha

3.3.1 Biomarkers

The ETS values were significantly higher in mussels exposed to Ce@Alginate in comparison with the control group ($p = 0.022$) and the group exposed to Ce@Chitosan ($p = 0.049$) in mussels collected after 7 days of exposure. Any significant modulation of ETS was observed in mussels collected at the end of the exposure (14 days) upon all exposure conditions (Fig. 1A).

The ROS content was lower in the group exposed to all three NPs compared to the control group in mussels collected at 7 days, albeit differences were not significant. The effect became more evident in mussels collected at the end of the exposure and a significant decrease of ROS content was observed compared to the control group in mussels exposed to both naked CeO_2 (p = 0.004), Ce@Chitosan (p = 0.008) and Ce@Alginate (p = 0.002) NPs (Fig. 1B).

A general decrease of SOD activity was observed in mussels collected after 14 days of exposure, in groups exposed to all CeO_2NPs compared with controls, which resulted significantly lower only in mussels treated with naked CeO_2 NPs (p = 0.021) (Fig. 1C).

The profile of CAT activity resembled the SOD activity, but any significant difference was observed upon all exposure conditions (Fig. 1D).

Concerning GPx, mussels collected after 7 days showed lower activities in CeO_2NP treated groups compared to the controls. These differences were statistically significant in organisms exposed to Ce@Chitosan (p = 0.021) and Ce@Alginate (p = 0.022). Mussels collected at 14 days showed significantly lower GPx activities in organisms exposed to Ce@Alginate NPs compared to the control group (p = 0.031) (Fig. 1E).

As for the GSTs, organisms collected at 7 days of exposure showed lower activities in mussels exposed to CeO_2NPs compared to the controls with significant differences observed only in mussels exposed to Ce@Aginate NPs (p = 0.049). A similar trend was observed in mussels collected at 14 days, albeit not significant (Fig. 1F).

The integration of all the biomarkers by DFA showed that in mussel collected after 7 days the Ce@Alginate distinguished from the other treatments (Fig. 2). The Wilk's Lambda value (0.005 p = 0.025) confirmed the significant power of the analysis. The first axis explained 58% of the total variance, where GPx and SOD were the most discriminating biomarkers. The second axis explained 31% of the total variance with GST and ROS being the most discriminating biomarkers. This difference was not evident in mussel collected at the end of the exposure, as the Wilk's Lambda was not significant (0.070 p = 0.089).

3.3.2 Effects on the metabolome

The analysis of amino acid metabolism highlighted a clear separation in the pattern of amino acid modulation in mussel exposed to CeO_2NPs compared to controls. Naked CeO_2 and Ce@Chitosan clustered together, while Ce@Alginate showed a standalone profile (Fig. 3). The heatmap showed also that, in the latter group, the amino acids Ser, Gly, Thr, Pro, Tyr, Met, Val, Ile, Leu, Phe and Trp clustered together and their concentration increased compared to controls, and Ser, Gly and Pro content was significantly higher in the Ce@Alginate group compared to controls. Based on this result we carried out untargeted metabolomics analysis comparing only the group Ce@Alginate with control.

The untargeted analysis was carried out using MS-DIAL software ver. 3.52 as described in the 2.7.2 section and, based on the m/z values (parent ion), 6893 hits were obtained in high resolution analysis. The statistical analysis showed a significant modification in the level of 25 ID known and 1382 ID Unidentified metabolites, which are shown in table 2 and table S3 and table S4, respectively. Concerning the known metabolites, several amino acids (Pro, Ile, His, Phe, Tyr and Trp), nucleotides (namely adenosine, inosine, guanosine, adenosine 3'-monophosphate, uracil, hypoxanthine and guanine), lipids/sterols (nicotinic acid, lysophosphatidylcholine 18:2 and 18:1 and cholesterol) and organic osmolytes (choline, betaine, carnitine, O-Acetyl-L-carnitine and propionyl-carnitine) were significantly modulated by the exposure to Ce@Alginate.

4. DISCUSSION

Results obtained through DLS analysis pointed out that the different coatings significantly affected the CeO_2NPs hydrodynamic behaviour and their colloidal stability. The two polysaccharides impart a different surface charge to the nanoceria, which could reflect in a different ecotoxicity, as observed in previous studies (Sendra et al., 2018; Collin et al., 2013). The measured ζ-potentials indicated also that all three NPs are poorly stable. These measurements were in line with the quantification of Ce in exposure tanks since low Ce concentrations were measured in water column of each experimental condition. This finding is indicative of a relevant sedimentation, regardless of the stirring and oxygenation, which should have avoided NP settling at the bottom of the exposure tanks. Anyhow the CeO_2NPs coated with chitosan were the most stable in the

exposure tanks in line with DLS analysis. Following the results obtained from these studies, alginate and chitosan should affect their bioavailability for mussels, changing the aggregation state and stability of NPs. Nevertheless, all NPs were bioaccumulated by *D. polymorpha* in the same quantity and to an extent comparable to what has been measured in the same species by Garaud et al., (2015). Therefore, this could mean that the different toxicity 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.

To assess the biological effects of the different nanoceria, the ETS activity was investigated as a marker of metabolic capability in mussels, including *D. polymorpha* (Fanslow et al., 2001). In bivalves, the increase of ETS has been often correlated to the high energy demand required to boost cellular defence in response to chemical contaminants (Gagnè et al., 2006; 2007) and also carbon-based NPs (De Marchi et al., 2017). The higher ETS was observed only in the Ce@Alginate group after 7 days of exposure. This suggests that NPs coated with this polysaccharide trigger a cellular response, being more reactive compared to the other two NPs. However, this response was transient as the ETS returned to control levels in mussels collected at 14 days of exposure. This is in line with results of Garaud et al., (2016), who did not observe any significant modulation of ETS in *D. polymorpha* after 3 weeks of exposure to bare and citrate-coated CeO_2 NPs (ci-CeO_2NPs) in a small freshwater mesocosm system, even if authors underlined that the ETS could be targeted by CeO_2NPs.

Concerning the antioxidant response, our results suggested that the three nanoceria behaves as ROS scavengers. Several studies showed that CeO_2NPs have antioxidant properties, protecting cells from oxidative damages, mimicking the activity of the CAT and SOD (Das et al., 2007; Korsvik et al., 2007; Pirmohamed et al., 2010). The decrease of ROS levels upon exposure to nanoceria reflected also in a depletion of CAT and GPx activities, suggesting that the CeO_2NPs might behave as scavenger towards H_2O_2, which is the substrate of both enzymes. The lack of activation of CAT and GPx could be also linked to the fact that the inhibition of SOD, due to the mimic action of NPs, does not allow the production of the specific substrate (H_2O_2) for the two enzymes. Indeed, the activity of SOD was lower in all NP exposed groups compared to controls at both times of exposure. Our results are in line with data reported by Gauraud and co-authors (2015) in which the exposure to CeO_2NPs led to a decrease in the CAT activity and lipoperoxidation

in *D. polymorpha* digestive glands. Again, a significant decrease of CAT and GST activities have been observed in the zebra mussel upon exposure to bare and ci-CeO_2NPs by Garaud et al., (2016).

A further confirmation of the antioxidant properties of CeO_2NPs derives from the fact that antioxidant biomarkers were lowered also in the Ce@Alginate group, although in this group the ETS levels were higher than in the controls. Since the ETS activity is one of the main generators of cellular ROS (Liu et al., 2002), we would have expected an increase of oxidative stress in this group, which, on the contrary, showed the highest level of inhibition.

Though the observed effects suggest a protective behaviour for the organisms by CeO_2NPs, an imbalance in ROS levels could be detrimental in the long term. Since ROS are essential signalling molecules (Finkel and Holbrook, 2000), their reduction below physiological levels might generate cytostatic effects and could impair some physiological functions of mussels (Mittler, 2017).

Concerning results from metabolomics analysis, the first screening focused on amino acid metabolism, highlighted clearly that only Ce@Alginate induced a modulation in the levels of free amino acids (FAA). In bivalves, FAA are involved in several pathways contributing to osmotic regulation, energetic metabolism and immune response (Hosoi et al., 2008; Cappello et al., 2018). The alteration of FAA level was observed in bivalves upon exposure to different classes of environmental pollutants both under laboratory and field conditions (Wu and Wang, 2010; Kwon et al., 2012; Leonard et al., 2014; Watanabe et al., 2015; Cappello et al., 2017; Serra Compte et al., 2019; Campillo et al., 2019), suggesting that these metabolites could represent early signals of metabolic imbalance due to the toxic actions of contaminants. The only study available on NPs reported an increase of several FAA, as Gly, Tyr, Pro, Arg, Trp, Leu and Ile in *M. galloprovincialis* exposed to low doses of fullerene NPs (Sanchis et al., 2018). In line with what has been reported for other contaminants, our results suggest that Ce@Alginate can affect relevant physiological functions of bivalves.

Therefore, the untargeted metabolomics analysis was carried out to investigate further the metabolic effects of Ce@Alginate. The high number of unknown metabolites modified in the Ce@Alginate group with respect to the controls confirmed that these NPs can induce metabolic imbalance in the organisms. Concerning the known metabolites, results highlighted the alteration in the level of some organic osmolytes that, together with the increase of FAA, suggest that Ce@Alginate could target the mussel osmotic regulation. The ability of some metal-based NPs to affect the iono-osmoregulatory system has been already documented in fish

(Shaw et al., 2012; Shultz et al., 2012) and invertebrate species. For instance, Gürkan (2019) showed that CuO NPs, α-Al_2O_3 NPs and α-Fe_2O_3 NPs inhibited Na /K -ATPase activity and modulated the level of ions in the hemolymph of the crab *Carcinus aestuarii*. Similarly, Völker et al., (2014) observed disruption of Na/K -ATPase activity by AgNPs in the freshwater clam (*Sphaerium corenum*). Concerning CeO_2NPs the only evidence of this effect derives from the study by Garaud et al., (2015) who reported a decrease in the Na^+ content in the hemolymph of zebra mussels, confirming our hypothesis.

The exposure to Ce@Alginate modulated also the level of several metabolites involved in energetic metabolism. Carnitine is the carrier of fatty acids inside the mitochondria for ß-oxidation (Connor and Gracey, 2011). The level of free carnitine is increased through the conversion of Propionylcarnitine, catalyzed by the carnitine acetyltransferase (Vanella et al., 2000). Therefore, the increase of both metabolites suggested an induction of ß-oxidation to sustain the cellular energetic demand in response to Ce@Alginate treatment. The result is consistent with the increase of ETS activity observed in this group. On the other hand, the decrease of O-Acetyl-L-carnitine might be related to a depletion of Acetyl-CoA, which is consumed by the citric acid cycle (TCA). This hypothesis is supported by the increase of FAA such as Pro, Ile, Phe and Tyr, which are synthesized starting from intermediates produced during the glycolysis and the TCA, suggesting that both metabolic pathways are also induced in mussels treated with Ce@Alginate. A further metabolite with an energetic role is the Nicotinic Acid (NA), a precursor of NAD^+ and $NADP^+$ (Liu et al., 2015), whose increase indicates again the activation of energetic metabolism upon exposure to Ce@Alginate.

The alteration of cholesterol levels and the lysophosphatidylcholine (LPC 18:1 and LPC 18:2) suggested that Ce@Alginate might modify the composition of cell membranes, with consequent disruption of their integrity and functionality, a toxic effect already described for other contaminants (i.e. metals by Fokina et al., 2013). In particular, the LPCs are a key component of the lysosomal membrane and therefore their reduction, observed in the Ce@Alginate treated organisms, might affect lysosome permeability to ions K^+/H^+ (Hu et al., 2007), generating an osmotic imbalance in accordance to what has been hypothesized previously.

Another pathway affected by Ce@Alginate was the purine/pyrimidine metabolism. In particular, Ce@Alginate lowered the level of adenosine and adenosine 3'-monophosphate (AMP), while the levels of inosine, guanine, guanosine, hypoxanthine and uracil were increased. An imbalance of nucleotide

metabolism has been reported in zebra mussels collected from contaminated harbour site, albeit the trend is opposite to our results, as levels of adenosine and AMP increased compared to individuals from an unpolluted site (Watanabe et al., 2015). Similarly, the level of adenosine increased in *M. galloprovincialis* exposed to wastewater treatment plant effluent extract, while guanine and uracil were depleted and inosine was unaffected (Dumas et al., 2020). Also, a significant decrease of uracil level was observed in *M. galloprovincialis* subjected to petrochemical contamination (Cappello et al., 2017). This discrepancy might be due to a peculiar interaction of Ce@Alginate with the nucleotide metabolism, differently from the other organic pollutants.

Overall, the results of this study showed that the coating with alginate increased the biological effects of CeO_2NPs. Though it is not possible to establish the factor/property responsible of this outcome, we hypothesize that the difference observed among the three CeO_2NPs can be ascribed to the different surface charge, which does not influence the accumulation by mussels but might affect the NP disposition in the cell and the consequent toxicity. For instance, it was demonstrated that the negatively charged CeO_2NPs are internalized into lysosomes more than neutral and positively charged NPs, becoming more toxic (Asati et al., 2010). Results of untargeted metabolomics seem to support this hypothesis and to suggest that the coating with alginate imparts a surface reactivity which enhances the NP accumulation into lysosomes and to induce osmotic stress through the imbalance of the lysosomal system, with consequent deregulation of other physiological functions as energy and nucleotide metabolism. A further *in vitro* study developed to assess this specific mechanism would verify this assumption.

5. CONCLUSIONS

Taken together, our results demonstrated that macromolecules occurring in the water media might interact with NPs providing new ecotoxicological properties. In particular, the coating with alginate makes the CeO_2NPs more reactive compared to bare and chitosan-coated NPs. Untargeted metabolomics showed that Ce@Alginate NPs induce energetic metabolism, and might affect the osmoregulation of the mussels. These mechanisms deserve more investigation in future studies focusing on the toxic potential of metal-based NPs. Our study confirms the need to deeply investigate the influence of the eco-corona on the fate and ecotoxicity of NPs to provide a more realistic assessment of their impacts on the aquatic ecosystems.

Figure captions

Fig. 1 Electron transport system (ETS) activity (A); ROS content (B); Superoxide dismutase (SOD) activity (C); Catalase (CAT) activity (D); Glutathione peroxidase (GPx) activity (E) and Glutathione S-transferases (GST) activity (mean ± standard deviation) in zebra mussel exposed to the three CeO_2NPs for 7 (7 d) and 14 days (14 d). Different letters mean significant differences ($p \leq 0.05$) among exposure conditions obtained through Anova followed by Dunnett's post-hoc test (N = 3).

Fig. 2 Plot of Discriminant Function Analysis carried out on biomarkers measured in zebra mussel exposed to the three CeO_2NPs for 7 and 14 days.

Fig. 3 Heatmap of amino acid. Normalized amino-acid levels, as obtained from MS analysis are represented using an orange to a blue colour gradient, as shown in the colour key legend. Amino acids are reported on the columns, experimental conditions on the rows. MS read-outs of individual measurement are indicated in the corresponding cells. The row dendrogram is used to group experimental conditions with similar patterns of amino acid levels. The column dendrogram delineates amino acids that show a similar variation across experimental conditions. Amino acids abbreviations: Arginine (Arg), Lysine (Lys), Histidine (His), Glutamine (Gln), Asparagine (Asn), Alanine (Aln), Cystine (Cistin), Cysteine (Cys), Aspartic Acid (Asp), Glutamic Acid (Glu), Threonine (Thr), Methionine (Met), Tryptophan (Trp), Proline (Pro), Glycine (Gly) Serine (Ser), Phenylalanine (Phe), Isoleucine (Ile), Leucine (Leu), Tyrosine (Tyr), Valine (Val).

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author contribution

Camilla Della Torre: Conceptualization; Funding acquisition; Supervision; Writing. **Daniela Maggioni**: Resouces; Methodology; Writing - review & editing. **Lara Nigro**: Investigation; Writing - original draft. **Hady Hamza**: Investigation. **Giuseppe Protano**: Resources, Investigation, Writing - review & editing. **Stefano Magni**: Investigation. **Manuela Fontana**: Investigation; Formal analysis. **Nicoletta Riccardi**: Resouces. **Matteo Chiara**: Formal analysis. **Fiorenza Farè**: Methodology; Writing - review & editing. **Donatella Caruso**: Resources; Methodology; Writing - review & editing. **Andrea Binelli**: Funding acquisition; Writing - review & editing.

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:

Table 1. Ce level measured in exposure waters and zebra mussel soft tissue after14 days of exposure to CeO_2 NPs ± standard deviation. Different letters mean significant differences ($p \leq 0.05$) among exposure conditions obtained through Anova followed by Dunnett's post-hoc test (N = 3).

Exposure group	Ce water (µg L^{-1})	Ce soft tissue (µg g^{-1})
CTRL	< lod	0.38 ± 0.17^{a}
Naked Ce	4.91 ± 0.33	74.18 ± 12.13^{b}
Ce@Chitosan	7.16 ± 0.44	85.71 ± 25.93^{b}
Ce@Alginate	4.85 ± 0.05	89.73 ± 24.25^{b}

Table 2: Changes in the abundance of metabolites in zebra mussel exposed to Ce@Alginate for 14 days with respect to controls

Metabolite	m/z	Adduct type	Formula	Folds variation	P-value
Amino acids					
Proline	116.0706	$[M+H]^+$	C5H9NO2	↑ 2.16	0.00000008
Isoleucine	132.1020	$[M+H]^+$	C6H13NO2	↑ 1.66	2.0908E-07
Histidine	156.0759	$[M+H]^+$	C6H9N3O2	↑ 1.81	2.8284E-07
Phenylalanine	166.0862	$[M+H]^+$	C9H11NO2	↑ 1.94	5.9523E-07
Tyrosine	18	$[M+H]^+$	C9H11NO	↑	3.8983E

	2.0803		3	2.18	-05
Tryptophan	205.0971	$[M+H]^+$	C11H12N2O2	↑ 2.30	5.3478E-05
Nucleosides					
Adenosine	268.1038	$[M+H]^+$	C10H13N5O4	↓ 8.00	2.2396E-08
Inosine	269.0885	$[M+H]^+$	C10H12N4O5	↑ 1.71	0.00116192
Guanosine	284.0971	$[M+H]^+$	C10H13N5O5	↑ 1.60	0.00866846
Adenosine 3'-monophosphate	348.0677	$[M+H]^+$	C10H14N5O7P	↓ 1.18	0.0019769
Uracil	113.0345	$[M+H]^+$	C4H4N2O2	↑ 1.14	0.02297335
Hypoxanthine	137.0457	$[M+H]^+$	C5H4N4O	↑ 1.66	6.1692E-05
Guanine	152.0567	$[M+H]^+$	C5H5N5O	↑ 1.53	0.0033041
Osmolytes					
Choline	104.1075	$[M]^+$	C5H14NO	↓ 1.77	0.00000008
Betaine	118.0862	$[M+H]^+$	C5H11NO2	↑ 1.24	1.4198E-07
Carnitine	162.1121	$[M+H]^+$	C7H15NO3	↑ 1.17	8.1415E-06
O-Acetyl-L-carnitine	204.1217	$[M]^+$	C9H18NO4	↓ 1.61	5.536E-10
Propionylcarnitine	218.1395	$[M+H]^+$	C10H19NO4	↑ 1.47	6.9305E-05
Lipids/sterols					

Nicotinic acid	124.0394	$[M+H]^+$	C6H5NO2	↑ 1.63	1.8296E-09
lysophosphatidylcholine 18:2	520.3401	$[M+H]^+$	C26H50NO7P	↓ 2.10	7.4993E-07
lysophosphatidylcholine 18:1	522.3554	$[M+H]^+$	C26H52NO7P	↓ 1.93	6.6E-05
Cholesterol	369.3503	$[M-H2O+H]^+$	C27H46O	↑ 1.17	0.00303638
Miscellaneous					
NCGC00347704-02	415.2105	$[M-H2O+H]^+$	C24H32O7	↑ 2.63	0.00044548
3-Formylindole	146.0599	$[M+H]^+$	C9H7NO	↑ 3.89	0.00111679
Erucamide	338.3382	$[M+H]^+$	C22H43NO	↓ 2.34	0.02110945

Highlights

Coatings with alginate or chitosan modified hydrodynamic behavior of CeO2NPs

The different coatings did not affect Ce accumulation in zebra mussels

Ce@Alginate NPs increased the level of several free amino acid in zebra mussels

Ce@Alginate NPs seem to affect mussel energetic metabolism and osmoregulation

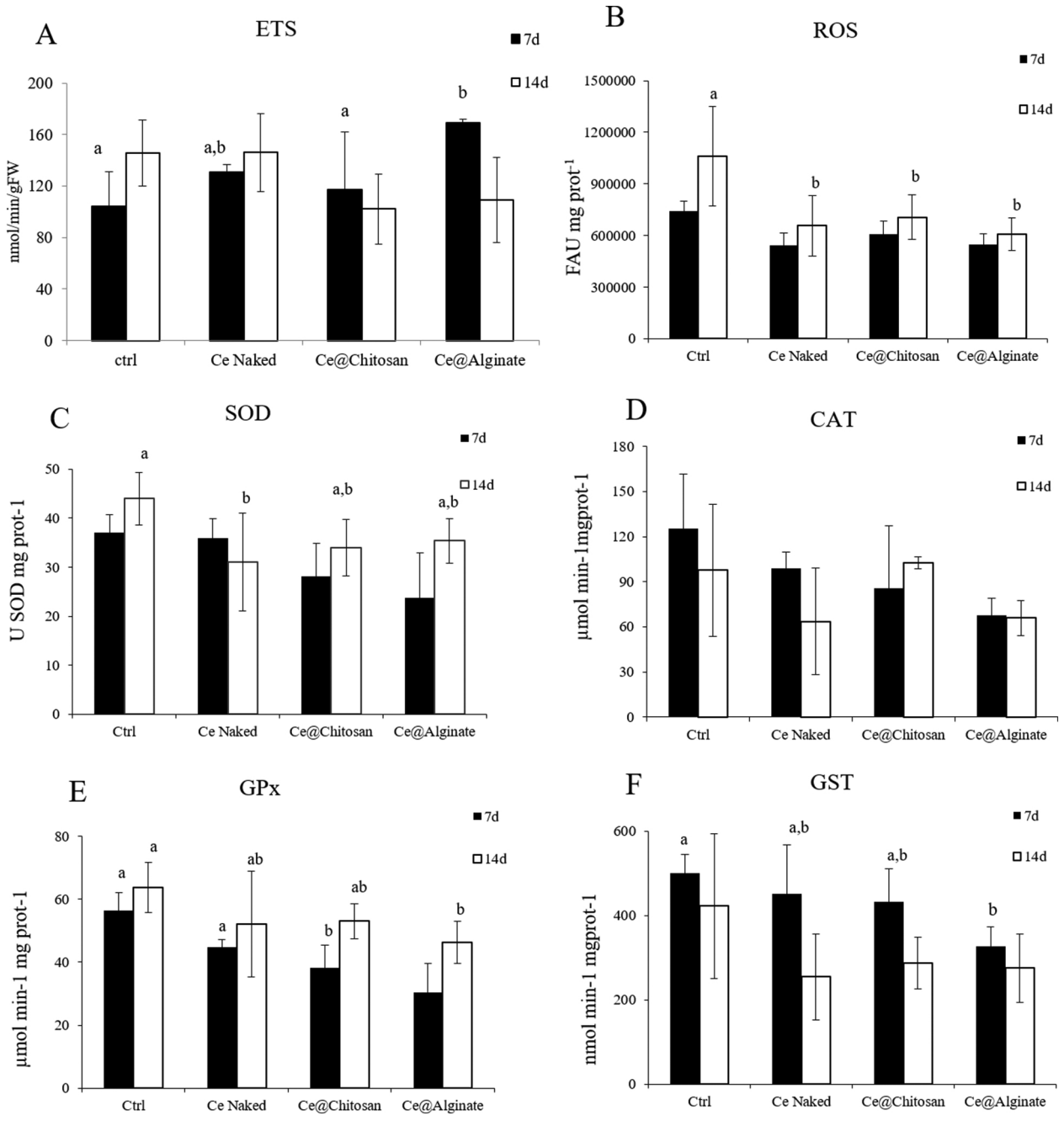

A
ETS
7d
14d
nmol/min/gFW
200
160
120
80
40
0
a
a,b
a
b
ctrl
Ce Naked
Ce@Chitosan
Ce@Alginate
B
ROS
7d
14d
FAU mg prot-1
1500000
1200000
900000
600000
300000
0
a
b
b
b
Ctrl
Ce Naked
Ce@Chitosan
Ce@Alginate
C
SOD
7d
14d
U SOD mg prot-1
50
40
30
20
10
0
a
b
a,b
a,b
Ctrl
Ce Naked
Ce@Chitosan
Ce@Alginate
D
CAT
7d
14d
µmol min-1mgprot-1
180
150
120
90
60
30
0
Ctrl
Ce Naked
Ce@Chitosan
Ce@Alginate
E
GPx
7d
14d
µmol min-1 mg prot-1
80
60
40
20
0
a
a
a
ab
b
ab
b
Ctrl
Ce Naked
Ce@Chitosan
Ce@Alginate
F
GST
7d
14d
nmol min-1 mgprot-1
600
400
200
0
a
a,b
a,b
b
Ctrl
Ce Naked
Ce@Chitosan
Ce@Alginate

Figure 1

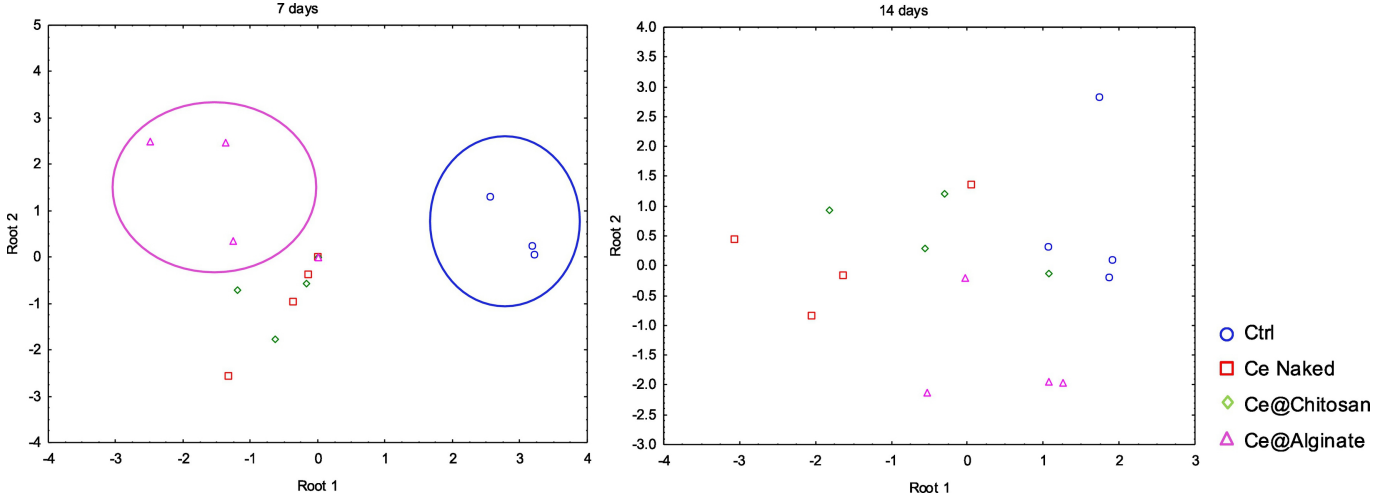
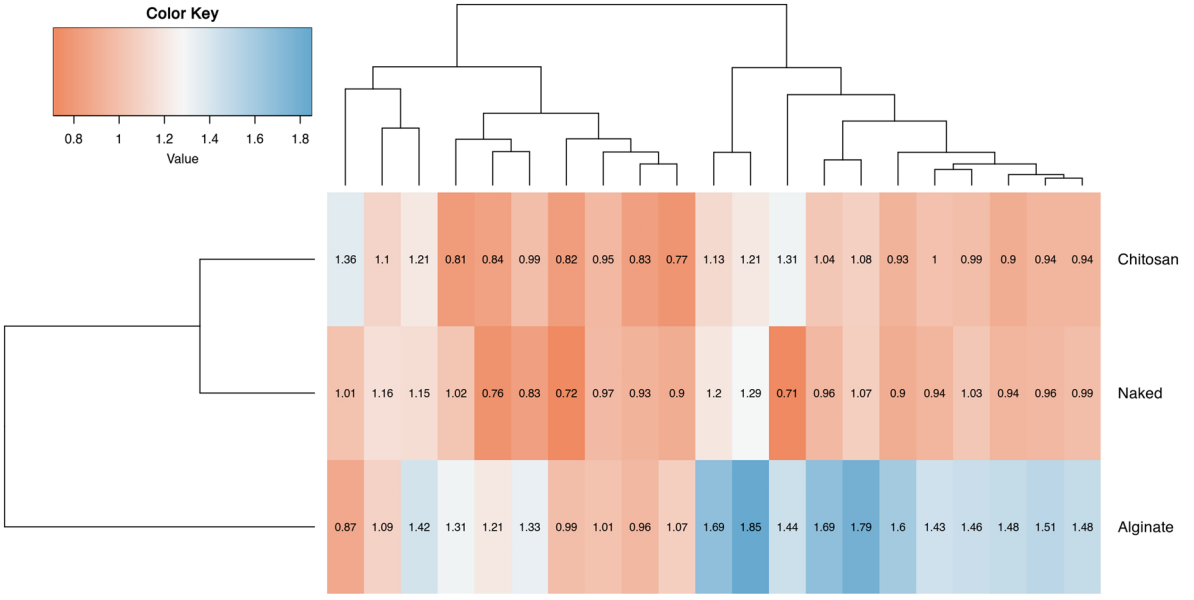

7 days
Root 2
Root 1
14 days
Root 2
Root 1
Ctrl
Ce Naked
Ce@Chitosan
Ce@Alginate

Figure 2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1
1.2
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Value
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Chitosan
1.01 1.16 1.15 1.02 0.76 0.83 0.72 0.97 0.93 0.9 1.2 1.29 0.71 0.96 1.07 0.9 0.94 1.03 0.94 0.96 0.99
Naked
0.87 1.09 1.42 1.31 1.21 1.33 0.99 1.01 0.96 1.07 1.69 1.85 1.44 1.69 1.79 1.6 1.43 1.46 1.48 1.51 1.48
Alginate
Arg
Lys
His
Gln
Asn
Ala
Cistin
Cys
Asp
Glu
Thr
Met
Trp
Pro
Gly
Ser
Phe
Ile
Leu
Tyr
Val

Figure 3