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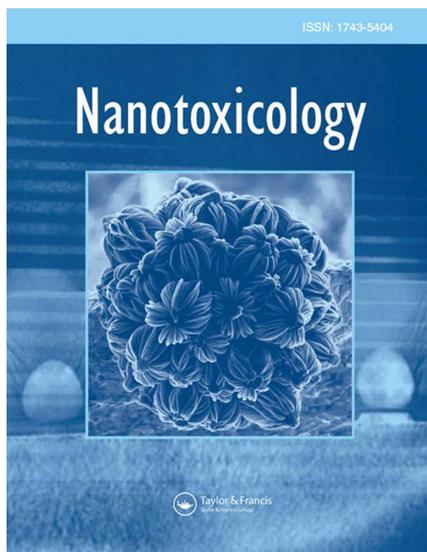
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## Integrated assessment of ceria nanoparticle impacts on the freshwater bivalve *Dreissena polymorpha*

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### Abstract

Exposures in realistic environmental conditions are essential to properly assess the effects of emerging pollutants on ecosystems. While ceria nanoparticles ( $n\text{CeO}_2$ ) production and use are expanding quickly, ecotoxicity studies remain very scarce. In this work, we set up experimental systems reproducing a simplified ecosystem to assess the effects of a chronic exposure to citrate-coated  $n\text{CeO}_2$  (ci- $\text{CeO}_2$ ) and bare  $n\text{CeO}_2$  (ba- $\text{CeO}_2$ ) on the freshwater mussel *Dreissena polymorpha* using an integrated multibiomarker approach. The fate of nanoparticles was tightly monitored to properly characterize the exposure. Organisms were exposed for three weeks and sampled weekly for biomarker analysis. Mussel filter-feeding activity resulted in significant removal of  $n\text{CeO}_2$  from the water column. At the same time, bioaccumulation was low, reaching its maximum in the first week. Mussels bioaccumulated ci- $\text{CeO}_2$  three times more than ba- $\text{CeO}_2$ , probably due to coating-related differences in their behavior in the water column and in organisms. Meanwhile, biomarker results were integrated and synthesized using linear discriminant analysis, highlighting that pi-glutathione-S-transferase (piGST) mRNA, catalase (CAT) activity, and lysosomal system were the most impacted of the seven biomarkers singled out by the discriminant analysis. These biomarker responses indicated that mussels exposed to both forms of  $n\text{CeO}_2$  were stressed and differentiate from the controls. Moreover, they responded differently to ba- $\text{CeO}_2$  and ci- $\text{CeO}_2$  exposure. However, biomarkers used in the experimental conditions of the present study did not indicate severe  $n\text{CeO}_2$  toxicity on mussels, as cellular damage biomarkers and mussel filtering activity were left unimpaired. However, further studies are needed to investigate if the slight perturbations observed could lead to populational impacts in the long-term.

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# **Integrated assessment of ceria nanoparticle impacts on the freshwater bivalve *Dreissena polymorpha***

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## Abstract

Exposures in realistic environmental conditions are essential to properly assess the effects of emerging pollutants on ecosystems. While ceria nanoparticles ( $\text{nCeO}_2$ ) production and use are expanding quickly, ecotoxicity studies remain very scarce. In this work, we set up experimental systems reproducing a simplified ecosystem to assess the effects of a chronic exposure to citrate-coated  $\text{nCeO}_2$  (ci- $\text{CeO}_2$ ) and bare  $\text{nCeO}_2$  (ba- $\text{CeO}_2$ ) on the freshwater mussel *Dreissena polymorpha* using an integrated multibiomarker approach. The fate of nanoparticles was tightly monitored to properly characterize the exposure. Organisms were exposed for three weeks and sampled weekly for biomarker analysis. Mussel filter-feeding activity resulted in significant removal of  $\text{nCeO}_2$  from the water column. At the same time, bioaccumulation was low, reaching its maximum in the first week. Mussels bioaccumulated ci- $\text{CeO}_2$  three times more than ba- $\text{CeO}_2$ , probably due to coating-related differences in their behavior in the water column and in organisms. Meanwhile, biomarker results were integrated and synthesized using linear discriminant analysis, highlighting that pi-glutathione-S-transferase (piGST) mRNA, catalase (CAT) activity, and lysosomal system were the most impacted of the seven biomarkers singled out by the discriminant analysis. These biomarker responses indicated that mussels exposed to both forms of  $\text{nCeO}_2$  were stressed and differentiate from the controls. Moreover, they responded differently to ba- $\text{CeO}_2$  and ci- $\text{CeO}_2$  exposure. However, biomarkers used in the experimental conditions of the present study did not indicate severe  $\text{nCeO}_2$  toxicity on mussels, as cellular damage biomarkers and mussel filtering activity were left unimpaired. However, further studies are needed to investigate if the slight perturbations observed could lead to populational impacts in the long-term.

## 1. Introduction

Production and use of nanomaterials have been expanding quickly in the recent decades, raising concern about their potential impacts on the environment (Moore, 2006). Due to their use as catalysts in fuel additives or as UV absorber in paints (Auffan *et al.*, 2014a), CeO<sub>2</sub> nanoparticle (nCeO<sub>2</sub>) production has risen sharply, potentially increasing environmental exposure (Sun *et al.*, 2014). This led to the recent calculation of predicted environmental concentrations as high as 1 µg/L in surface waters (O'Brien and Cummins, 2011). To date, studies on nCeO<sub>2</sub> ecotoxicity have focused on acute toxicity assessment in standardized conditions, generally reporting low toxicity in bacteria, algae, invertebrates and fishes (Collin *et al.*, 2014a). However, those standardized studies failed to incorporate environmental parameters likely to impact exposure and effects of nCeO<sub>2</sub> in the environment (Auffan *et al.*, 2014b).

Recent studies have stressed the importance of conducting investigations under more environmentally relevant conditions, i.e. working in complex media, at realistic concentrations, in long-term exposure, to correctly assess the potential impacts of nanoparticles on organisms (Bour *et al.*, 2015). It has been shown that interactions between nCeO<sub>2</sub> and natural inorganic or organic colloids could modify their chemical and colloidal stability, therefore modulating their bioavailability and toxicity (Conway *et al.*, 2014; Van Hoecke *et al.*, 2011). Consequently, there is a need to better characterize the fate of nanoparticles in the exposure media and to relate that to their bioaccumulation, biotransformation and subsequent biological effects. To meet these goals, the French ANR MESONET program was launched with the aim of setting up adaptable freshwater mesocosms (Auffan *et al.*, 2014b) in three French laboratories to study the fate and effects of manufactured nanoparticles in simplified ecosystems, using species belonging to different

functional groups in each laboratory allowing relevant inter-species comparisons of nanoparticle effects.

In the present study, biological effects were assessed using a multibiomarker approach, including responses related to various organism functions, and ranging from the molecular to the individual scale, allowing a better understanding of stressor mechanisms of action (Garaud et al., 2015). Such multibiomarker studies on the effects of nanoparticles on aquatic organisms are scarce, probably due to the difficulty to synthesize and analyze properly the complex data set (Guerlet *et al.* 2010). Linear discriminant analysis could simplify multibiomarker data analysis by integrating them into a model allowing (i) the discrimination of experimental groups as a function of the global biological response pattern and (ii) the isolation of a minimal biomarker battery allowing group discrimination.

In the light of the shortcomings on current nano-ecotoxicity research, the aims of this study were to assess nCeO<sub>2</sub> effects on the freshwater mussel *Dreissena polymorpha* by (i) working in complex experimental systems at low concentrations to enhance environmental relevance, (ii) linking the fate of nCeO<sub>2</sub> to their bioaccumulation, biotransformation and biological effects, and (iii) using a wide multibiomarker battery integrated using linear discriminant analysis to obtain an overview of nCeO<sub>2</sub> impacts under more environmentally realistic conditions. We hypothesized that linear discriminant analysis could reveal global biological response to nCeO<sub>2</sub> and isolate the main physiological functions impacted. We investigated during four weeks the fate and effects of citrate-coated (ci-CeO<sub>2</sub>) and bare (ba-CeO<sub>2</sub>) nCeO<sub>2</sub> in freshwater experimental systems containing bacteria, algae, and the mussel *Dreissena polymorpha*, which as a filter-feeding bivalve could be a primary target for nanoparticle accumulation and toxicity (Canesi et al., 2012). The multibiomarker approach used to assess nCeO<sub>2</sub> effects on mussels measured responses at different levels of the biological organization and covered the main physiological functions (figure 1), and the results were

integrated using a linear discriminant analysis. The stability of nCeO<sub>2</sub> was followed in the water column, and the organism exposure was characterized measuring bioaccumulation and biotransformation of nCeO<sub>2</sub> in mussel tissues.

## **2. Materials and methods**

### ***2.1 Test material***

nCeO<sub>2</sub> suspensions were supplied and characterized by the CEREGE (UMR CNRS 7600 Aix en Provence, France). Detailed characterization is available in Tella et al. (2014). Briefly, ba-CeO<sub>2</sub>, commercially available, and ci-CeO<sub>2</sub>, purchased from Byk as a  $2.2 \times 10^5$  mg Ce/L stock suspension in  $5.1 \pm 0.3 \times 10^3$  mg/L of citrate, are both crystallites of cerianite (3–4 nm TEM diameter) with an average hydrodynamic diameter centered on 8 nm in stock suspensions. In milli-Q water; the point of zero charge (PZC) of ba-CeO<sub>2</sub> was measured between 7 and 7.5, while PZC could not be measured for ci-CeO<sub>2</sub> as it exhibits a negative zeta potential from pH 3 to 10 ( $\approx$ -20 mV between pH 7 and 8; (Auffan et al., 2014a)).

### ***2.2. Collection and acclimatization of mussels***

*D. polymorpha* (2-2.5 cm length) were hand-collected (Vadonville, Meuse, France), transferred to the laboratory, cleaned up and placed on ceramic tiles (10x15 cm) allowing byssus attachment and acclimatized during 10 days by increasing gradually the spring water (Volvic<sup>®</sup>) percentage to 100% and the temperature to  $17 \pm 2^\circ\text{C}$ .

### ***2.3. Micro-organism inoculum preparation and analyses***

The bacterial *inoculum*, sampled from the freshwater-aquarium filters of the Museum-Aquarium of Nancy (France), was prepared and characterized by pyrosequencing as described in the Supplementary Material (SM). The algal *inoculum* was composed of

*Chlorella vulgaris*, *Raphidocelis (Pseudokirchneriella) subcapitata*, and *Scenedesmus capricornus*, which were cultured independently and added to the experimental systems to reach an initial concentration of  $1 \times 10^5$  cells/mL ( $3.3 \times 10^4$  cells/mL per species). Microorganisms were used as food sources for mussels. Water was sampled weekly for algal and bacterial biomass determination (Figure 2, details in SM).

#### **2.4. Experimental design**

The experimental design is summarized in Figure 2. The experiment was conducted in nine aquariums (75x20x60cm) containing 7 kg synthetic OECD sediments (89% sand - 10% kaolin - 1%  $\text{CaCO}_3$ ) and filled with 56L of Volvic® (composition in SM) supplemented with 70 mg/L  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  (final conductivity = 350  $\mu\text{S}/\text{cm}^2$ ). Details on experimental systems are given in Auffan *et al.* (2014b). Temperature was kept at  $17.5 \pm 0.2^\circ\text{C}$ , 18:6 photoperiod was applied and water was continuously circulated by pumps (70 L/h flow rate). Temperature,  $\text{O}_2$ , conductivity and pH were continuously recorded at few centimeters from below the surface (Kit Ponsel Odéon open X with PHEHT, C4E and ODOT probes) and were not influenced by  $\text{nCeO}_2$  (Table S3).

Aquatic systems were run in triplicate for each experimental condition (control, ba- $\text{CeO}_2$  and ci- $\text{CeO}_2$ ). Organisms were introduced sequentially and  $\text{nCeO}_2$  was added regularly in 12 injections (84  $\mu\text{g}/\text{L}$  per injection) to reach a final nominal concentration of 1 mg/L. Water and organisms were sampled for  $\text{nCeO}_2$  content, microorganism counting and biomarker analysis as indicated in Figure 2.

#### **2.5. Water and biota Ce concentrations**

Total water [Ce] (in  $\mu\text{g}/\text{L}$ ) was measured weekly by ICP-MS (PerkinElmer Elan DRc) from samples taken before  $\text{nCeO}_2$  injection (Figure 2) and microwave mineralized (40 bars and

220°C max; Anton Paar Multiwave PRO) with HNO<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> mixture (1.75/0.75 mL + 3 mL of sample). Bioaccumulation was measured in the lyophilized digestive glands of three mussels per aquarium following the same analytical procedure. [Ce] were expressed in µg Ce/g dry weight. Ce mass balance was defined as % of Ce measured in a given compartment as a function of the total mass of Ce introduced. Based on the assumption that the % of Ce in the water column at day 0 would be similar at days 7, 14 and 21 without the mussel filtering activity, which is a rather conservative hypothesis given the fact that as Ce concentrations were measured three days after injections during which mussels could be exposed to and filtrate higher nCeO<sub>2</sub> concentrations, we calculated the removal % of Ce from water column due to mussel filtration activity as the difference between expected and real % of Ce in the water column.

## **2.6. Tissue nCeO<sub>2</sub> speciation**

At the end of the exposure, one pool of three digestive glands per exposure condition was freeze-dried. Samples were ground, pressed in thin pellets and Ce L3 (5.723 keV) X-ray Absorption Near Edge Structure (XANES) measurements were performed on the FAME beamline at the ESRF (Grenoble, France) as described in Tella *et al.* (2014).

## **2.7. Biomarker measurements – Detailed protocols in SM**

Gill mRNA expressions of metallothionein (RNA MT), pi-glutathione-S-transferase (RNA piGST) and selenium-dependent glutathione peroxidase (RNA SeGPx) were measured by RT-qPCR (Pain-Devin *et al.*, 2014). Multi-xenobiotic resistance efflux activity (MXR) was assessed in gill tissues (Kurelec *et al.*, 2000).

Hemocyte viability, phagocytosis, ROS production, lysosomal system size and CSP-3 induction were measured by flow-cytometry (FACScalibur™, BD Biosciences) using protocols adapted from Minguez *et al.* (2012).

Prior to biomarker analysis on the automated chemistry analyzer Konelab 20 Xti (Thermo Scientific), digestive glands were treated as described in Sroda and Cossu-Leguille (2011). Antioxidant and antitoxic defenses, i.e. total antioxidant capacity (TAC; Erel (2004)), total glutathione peroxidase (GPx), glutathione-S-transferase (GST) and the acid phosphatase activities (ACP) were measured using colorimetric methods adapted and developed on the Konelab. Catalase activity (CAT) was measured spectrophotometrically (Beer and Sizer, 1952). Lipid hydroperoxide concentration ([LOOH]) was measured following the automated method developed by Arab and Steghens (2004) and Caspase-3 activity (CSP-3) activity was assessed following manufacturer instructions (Euromedex). Protein, triglyceride, cholesterol concentrations ([prot], [trigly.] and [chol.]) and Lactate Dehydrogenase (LDH) activity were measured using Thermo-Scientific Konelab ready-to-use reagents. Electron Transport System (ETS) mitochondrial activity was measured following the method from Coen and Janssen (1997). Finally, mussel filtration rate was measured according to Palais (2011).

## **2.8. Statistical analysis**

Statistical analyses were conducted using R (R Core Team, 2014). Homoscedasticity and normality were verified respectively by Levene and Shapiro-Wilk tests, then two-ways ANOVA were performed to evaluate combined effects of nCeO<sub>2</sub> exposure and exposure duration with a threshold of  $p \leq 0.05$  considered as significant. Post hoc Tukey HSD tests were done to verify differences between pairs of values. Non-parametric data were analyzed using Kruskal-Wallis test followed by post-hoc tests.

## **2.9. Discriminant analysis**

In order to (i) define whether the exposure conditions led to different patterns of biological responses and (ii) select a minimal battery of biomarkers that allow identification of the exposure condition and effects, we performed a linear discriminant analysis (functions `lda` and `discDA` of the `MASS` and `DiscrMiner` packages in R). A preliminary analysis of variance partitioning (functions `varpart` and `rda` of the `vegan` library) revealed that the time factor did not contribute significantly in explaining the variance (explained variance of 1.5%,  $p=0.32$ ). The time factor was thus neglected in our discriminant analysis.

To simplify effect assessment, we had to look for the smallest battery while preserving its discriminant ability. To achieve this, we dropped one explanatory variable at each step based on its individual significance in the discriminant analysis. We controlled at each step the overall signification of the analysis and the classification error rate among our dataset and stopped when the classification errors started to increase. At this step, we controlled our model for (i) multicollinearity, (ii) multivariate normality and (iii) variance-covariance homogeneity.

### 3. Results

#### 3.1. [Ce] in water column

The concentrations of Ce in the water column were below limit of detection in all of the experimental systems before the first nCeO<sub>2</sub> injection (day -7) and in the control tanks during the whole exposure period. Just before mussel introduction (day 0), 47±6 % (ba-CeO<sub>2</sub>; 96±12 µg/L) and 92±5 % (ci-CeO<sub>2</sub>; 187±11 µg/L) of total introduced Ce remained in the water column (Figures 3 and 4A). [Ce] dropped significantly after mussel insertion : from day 7 to day 21, [Ce] averaged 39% (ba-CeO<sub>2</sub>; 37±4 µg/L) and 69% (ci-CeO<sub>2</sub>; 128±9 µg/L) of initial [Ce], even though the total amount of Ce introduced was gradually increased by nCeO<sub>2</sub> injections. At the end of the experiment Ce left in the water column represented only 4±1% (ba-CeO<sub>2</sub>) and 14±3% (ci-CeO<sub>2</sub>) of the total Ce introduced (Figure 3). Water [Ce] was 1.9-fold and 3.2-fold higher in aquariums contaminated with ci-CeO<sub>2</sub> at days 0 and 7 respectively (Figure 4A).

Estimation of Ce removal by mussel filtration showed that after 7, 14 and 21 days of exposure respectively, at least 79±13%, 88±12% and 91±14% of the suspended ba-CeO<sub>2</sub>, and 64±15%, 76±17% and 84±13% of the suspended ci-CeO<sub>2</sub> could have been removed due to mussel filtration activity.

#### 3.2. Ce bioaccumulation and speciation in mussel tissues

Results showed a significant coating-dependent Ce bioaccumulation in digestive glands following nCeO<sub>2</sub>-exposure (Figure 4B and Table 1), with significantly higher accumulation for ci-CeO<sub>2</sub> (2.7-folds). After 21 days of exposure, 0.3% (ba-CeO<sub>2</sub>; 10.2±1.3 µg/gdw) and 0.8% (ci-CeO<sub>2</sub>; 29.1±10.3 µg/gdw) of the total Ce introduced were found in digestive glands (Figure 3). ba-CeCe bioaccumulation was unaffected by exposure duration.

After 21 days of exposure, almost total reduction of the  $\text{Ce}^{\text{IV}}$  originally constituting  $\text{nCeO}_2$  into  $\text{Ce}^{\text{III}}$  was reported in the digestive gland (Figure 5). Percentages of  $\text{Ce}^{\text{III}}$  reached  $82 \pm 8$  % (ba- $\text{CeO}_2$ ; R factor = 0.0029) and  $78 \pm 8$  % (ci- $\text{CeO}_2$ ; R factor = 0.0029).

### 3.3. Biomarker responses

Only significantly impacted biomarkers are described in this section (see detailed values in Tables S5 to S7).

Significant interactive effects of  $\text{nCeO}_2$  exposure and exposure duration were observed on SeGPx, piGST and MT mRNA expressions and immuno-efficiency (Table 1). Compared to control, SeGPx mRNA was transiently decreased (-75% at day 7, Table S5) in mussel exposed to ba- $\text{CeO}_2$  ( $p=0.01$ ). A 90% reduction of piGST mRNA expression was observed in mussels 14 and 21 days-exposed to ci- $\text{CeO}_2$  ( $p<0.001$ ), reaching levels significantly lower than in ba- $\text{CeO}_2$ -exposed mussels ( $p=0.035$  and  $0.002$ ), which exhibited a 75% decrease at day 14 ( $p=0.09$ ). MT mRNA expression was transiently induced by ba- $\text{CeO}_2$  (14-folds,  $p<0.001$ ) and ci- $\text{CeO}_2$  (3.4-folds,  $p<0.001$ ) at day 7, and ba- $\text{CeO}_2$  induced MT mRNA 4-folds more than ci- $\text{CeO}_2$  ( $p<0.001$ ). Higher immuno-efficiency was reported in ba- $\text{CeO}_2$ -exposed mussels between days 7 and 21 (+55%,  $p=0.021$ , Table S6).

Healthy cells % was impacted by exposure duration (Table 1), with an increase at day 21 compared to day 14 ( $p=0.003$ ). Increase in hemocyte lysosomal system size was reported for ci-Ceci- $\text{CeO}_2$ -exposed mussels compared to ba- $\text{CeO}_2$ -exposed (+18%,  $p=0.035$ ) and control (+16%,  $p=0.052$ ) mussels.

CAT activity was strongly affected by  $\text{nCeO}_2$  exposure and exposure duration (Table 1). Lower CAT activities in ci-Ceci- $\text{CeO}_2$ -exposed mussels compared to control ( $p=0.04$ ) and ba- $\text{CeO}_2$ -exposed mussels ( $p=0.004$ , Table S6) were measured. All conditions combined, CAT activities decrease at day 21 compared to days 7 ( $p=0.006$ ) and 14 ( $p=0.034$ ). Exposure

duration also impacted TAC (decrease at day 14 compared to day 7,  $p=0.026$ ), GST activity (reduction between days 14 and 21,  $p=0.014$ ) and [LOOH] (reduction between days 7 and 14;  $p=0.04$ ).

### **3.4. Discriminant analysis**

We first get a model with no classification errors with only 9 biomarkers among the 24 initially considered. However, multicollinearity was observed, leading to drop two additional variables (MT mRNA and ACP). The final classification error rate was 3.7% (i.e., a classification error among 27 individuals). The final set of variables achieved multivariate normality (all  $p$ -values above 0.05, E-statistic test (Székely and Rizzo, 2005)). The Box M test revealed slight differences of variance-covariance between groups ( $p=0.008$ ), but that did not induce major bias in the LDA, considering the method robustness.

The discriminant power of our analysis was assessed basing on the Wilk's Lambda (0.063,  $p$ -value  $<0.001$ ). It evidenced that the deployed biomarker battery was able to describe the effect of exposure to nCeO<sub>2</sub>, and to depict the two nCeO<sub>2</sub> types using two discriminant functions (DF) DF1 and DF2, with respective discriminant powers of 0.75 and 0.25. We managed to significantly reduce the biomarker dataset from 24 to 7, which were sufficient to depict nCeO<sub>2</sub> exposure.

The reduced data set of seven biomarkers is shown in Table 2. To consider the relative importance of the different variables, we used the standardized coefficients and associated  $p$ -values. The three most discriminating biomarkers ( $p < 0.05$ ) on the two axes were the piGST mRNA expression, the catalase activity and the hemocyte lysosomal system size, which were also among the biomarkers the most impacted individually. The four other biomarkers were less significant but nevertheless critical to avoid classification errors.

When plotting the 27 replicates (9 per experimental condition with the exposure duration factor neglected), the three experimental conditions were clearly separated from each other (Figure 6). ci-CeO<sub>2</sub> and control only differ according to the first DF, while ba-CeO<sub>2</sub> differs from the two other groups on the two axes. Therefore, the reduced set of seven biomarkers was sufficient to discriminate the three experimental conditions.

## 4. Discussion

### 4.1. [Ce] in the water column

ba-CeO<sub>2</sub> and ci-CeO<sub>2</sub> presented distinct behaviors in the water column. At day 0, ci-CeO<sub>2</sub> displayed high stability, with more than 90% of the introduced ci-CeO<sub>2</sub> still in suspension compared to 50% for ba-CeO<sub>2</sub>. Tella *et al.* (2014) also showed that ci-CeO<sub>2</sub> was relatively stable in the short term in freshwater mesocosms but aggregated after few days due to coating degradation. Enhanced ci-CeO<sub>2</sub> stability could result from their citrate coating which endows them with negative zeta potential at pH of the experiment (Auffan *et al.*, 2014a). Therefore, homo and hetero-aggregation processes, with negatively charged clay particles or microorganisms, could be hindered by electrostatic repulsions, reducing ci-CeO<sub>2</sub> aggregation and sedimentation (Tella *et al.*, 2014). On the contrary, ba-CeO<sub>2</sub> were uncharged at the pH of the experiment, and therefore aggregated faster (Tella *et al.*, 2014). ba-CeO<sub>2</sub> could also interact strongly with suspended matter (Conway *et al.*, 2014) and the larger hetero-aggregates produced could sediment faster.

As filter feeding organisms, mussels could greatly impact nanoparticle concentrations in water column (Conway *et al.*, 2014; Montes *et al.*, 2012). In fact, based on the review of zebra mussel filtration rate made by Elliott *et al.* (2008), a median value of ~83 mL/mussel/h was calculated, meaning that 120 L/day and 60 L/day (compared to a total volume of water of 56L in aquariums) were filtered by mussels in our experimental systems at the beginning and

at the end of the experiment respectively. During this filtration process, mussels could trap nanoparticles, ultimately resulting in their immobilization in the sediment as *pseudofaeces* or *faeces*, which could explain why after mussel introduction at day 7, only 10% (ba-CeO<sub>2</sub>) and 33% (ci-CeO<sub>2</sub>) of the introduced Ce were still in suspension compared to 47% and 92% at day 0. Estimation of nCeO<sub>2</sub> removal due to mussel filtering activity showed that more than 70% of the Ce was removed from the water column, similar to other studies (Conway *et al.*, 2014; Montes *et al.*, 2012). Slightly higher removal of ba-CeO<sub>2</sub> could arise from its behavior in water (see below). In fact, while zebra mussel can filter out sub-micron particles (down to 0.4 μm) from water, particle retention efficiency decrease with size, with a maximum efficiency between 5 and 35 μm (Sprung and Rose, 1988). Consequently, ba-CeO<sub>2</sub> homo-aggregated or hetero-aggregated with algae and suspended matters could have been more efficiently trapped by mussel gills (Baker *et al.* 2014; Ward and Kach, 2009).

As a result of nCeO<sub>2</sub> immobilization in *faeces* and *pseudofaeces*, natural aggregation and sedimentation processes, the percentage of Ce still suspended in water or accumulated by mussels at the end of the experiment was low for both nanoparticles. Previous mesocosm studies confirmed that sediments would be the primary sink of nCeO<sub>2</sub> (Auffan *et al.*, 2014b; Tella *et al.*, 2014; Zhang *et al.*, 2012a) and while it was not measured in our experiment, the sediment probably made up most of the balance of Ce indicated as unknown in Figure 3.

#### **4.2. Ce bioaccumulation**

Mussels accumulated both types of nCeO<sub>2</sub> in their digestive gland, the preferential site of nanoparticle accumulation in filter-feeding organisms (Baker *et al.* 2014; Browne *et al.*, 2008; Canesi *et al.*, 2010).. Ce bioaccumulation presented two distinctive features. Firstly, it seemed that nCeO<sub>2</sub> surface properties influenced bioaccumulation, ci-CeO<sub>2</sub> accumulating three times more than ba-CeO<sub>2</sub>. Differences in ba-CeO<sub>2</sub> and ci-CeO<sub>2</sub> surface properties could

modulate bioaccumulation at several levels. As evidenced in the previous paragraph, ci-CeO<sub>2</sub> could be more stable in the water column, making it more bioavailable to mussels than ba-CeO<sub>2</sub>. Once filtrated and trapped by the gills, nCeO<sub>2</sub> could undergo chemical sorting (Baker *et al.*, 1998), and the organic citrate-coating of ci-CeO<sub>2</sub> could be recognized as a potential food source and promote its ingestion compared to ba-CeO<sub>2</sub>. Furthermore, according to Ward and Shumway (2004), suspended sediments with organic coating had longer gut retention time than uncoated ones in *M. edulis*. Retention time could also be increased for smaller particles (100 nm) compared to larger (10 µm) ones (Ward and Kach, 2009). Such an increase in ci-CeO<sub>2</sub> retention time could explain part of the observed higher bioaccumulation, and could facilitate cellular internalization. Once inside digestive glands, nCeO<sub>2</sub> coating and surface charge could modulate their internalization inside cells (Patil *et al.*, 2007). *In vitro* data comparing uptake of negatively charged and of neutral nanoparticles are contradictory, some works showing increased uptake of negative nanoparticles (Patil *et al.*, 2007), which could support the observed higher ci-CeO<sub>2</sub> bioaccumulation, but other works show the opposite trend (Asati *et al.*, 2010).

The second distinctive feature of nCeO<sub>2</sub> bioaccumulation is that for both nCeO<sub>2</sub> types, internal [Ce] remained stable between day 7 and 21, even though total [nCeO<sub>2</sub>] in experimental systems increased by repeated injections. A similar pattern was observed for bivalves exposed to nCeO<sub>2</sub> or to citrate-Au (Conway *et al.*, 2014; García-Negrete *et al.*, 2013). nCeO<sub>2</sub> hetero-aggregation with algae and suspended matters (Tella *et al.*, 2014) could have increased nCeO<sub>2</sub> retention during the first week of exposure, when algae and suspended matter contents were higher (Ward and Kach, 2009). For example, Conway *et al.* (2014) reported a transient increase in nCeO<sub>2</sub> bioaccumulation in the presence of algae in five days-exposed mussels. Authors also reported concentration-dependent increases in mussel clearance rate and *pseudofaeces* production, probably as a response to low quality food,

therefore limiting nCeO<sub>2</sub> ingestion and bioaccumulation. These findings could explain the low recovery (<1%) of total introduced Ce in digestive gland at the end of exposure, coherent with other works (Conway *et al.*, 2014; Montes *et al.*, 2012).

Finally, even in the case of nCeO<sub>2</sub> internalization, bivalves are able to regulate internal concentrations of some metals (Marigómez *et al.*, 2002; Viarengo and Nott, 1993) and the strong increase in MT mRNA levels after one week suggests that some regulation mechanisms were activated and could have also contributed to the stabilization of internal [Ce].

#### **4.3. Internal nCeO<sub>2</sub> speciation**

We observed an almost complete reduction of the original Ce<sup>IV</sup> into Ce<sup>III</sup>, which was also observed in the freshwater snail *P. corneus* (Tella *et al.*, 2014) and in the nematode *C. elegans* (Collin *et al.* 2014b). In both experiments, no reduction occurred in the water column, meaning that Ce reduction occurred after particle trapping by the mussels, in the lumen and/or in contact with the cell lining the gut, the stomach and the digestive tubules, which are loaded with free amino acids, proteins and digestive enzymes (Zhong and Wang, 2006). The cystein and thiol groups contained in the digestive fluid are known to be strong metal complexation agents (Zhong and Wang, 2006). However, Collin *et al.* (2014b) and Liu *et al.* (2013) showed that no reduction occurred when nCeO<sub>2</sub> was incubated at neutral pH with a wide range of biological components, suggesting that other mechanisms and molecules played a role in Ce<sup>IV</sup> reduction. To unveil reduction mechanisms, the effects of mussel digestive enzyme mixtures at relevant pH should be investigated thoroughly.

As hypothesized by Zhang *et al.* (2012b), the other possible mechanism for Ce<sup>III</sup> presence in digestive glands could be the dissolution of nCeO<sub>2</sub> following contact with biological molecules. Solubility of nCeO<sub>2</sub> in environmentally relevant aqueous media has been shown

to be negligible (Collin et al., 2014a), , while in the range of pH observed in zebra mussel stomach (6.6-8.8 (Morton, 1969)), Ce predominant form is supposed to be solid  $Ce^{IV}O_2$ , or solid  $Ce^{III}(OH)_3$  in the case of highly reducing conditions (Tamilmani *et al.*, 2003). Furthermore,  $nCeO_2$  have been retrieved in *faeces* following experiments on marine mussels, even after transit in digestive apparatus, supporting the hypothesis that no dissolution occurred (Montes *et al.*, 2012). Formation of  $Ce^{III}$  oxides, but also possibly as  $CePO_4$ , a stable Ce form observed in phosphate-rich medium (Singh et al., 2011; Zhang et al., 2012b) as is the intracellular environment, could then arise from surface reduction of  $nCeO_2$ . Alternatively,  $nCeO_2$  internalized in lysosomes could experiment dissolution due to acidic conditions (Cornelis et al., 2011) and the soluble  $Ce^{III}$  could then reprecipitate into oxides or phosphates or be trapped in metal-rich insoluble granules (Marigómez et al., 2002; Viarengo and Nott, 1993).

#### **4.4. Biological effects of $nCeO_2$**

We recently stressed the benefit of using integrated biomarker responses to study  $nCeO_2$  effects and to identify biomarkers of interest (Garaud et al., 2015). In the present work, a very synthetic overview of the effects of  $nCeO_2$  on mussel biology was provided using discriminant analysis and we were able i) to clearly differentiate control groups from  $nCeO_2$ -exposed groups, ii) to show differential responses depending on  $nCeO_2$  type and iii) to identify a reduced set of biomarkers sufficient to discriminate groups and their respective significance. However, due to a reduced dataset, this model has no predictive value, and should only be considered for its ability to synthetize and describe links between nanoparticle exposition and biomarker responses. Of the seven biomarkers identified, three were of particular significance ( $p < 0.05$ ; piGST mRNA levels, catalase activity, and hemocyte lysosomal system size) confirming our previous results (Garaud et al., 2015).

Of these most discriminant biomarkers, the gill piGST mRNA expression was the most impacted biomarker and decreased following nCeO<sub>2</sub> exposure, while greater impact was reported for ci-CeO<sub>2</sub>. GST are involved in endogenous molecule and xenobiotic detoxication, and piGST, the principal cytosolic GST, could deactivate lipid hydroperoxides and their by-products, preventing lipoperoxidation, and directly detoxify ROS through their cysteine groups (Doyen *et al.*, 2008). GST gene transcription can be induced directly by several xenobiotics or indirectly following oxidative stress (Blanchette *et al.* 2007). Park *et al.* (2008) observed GST mRNA up-regulation following *in vitro* nCeO<sub>2</sub> exposure (40 mg/L, human bronchial cells) associated with increased ROS production, GSH decrease and cytotoxicity. Conversely, in an nCeO<sub>2</sub> *in vitro* exposure on rat neuronal cells, Ciofani *et al.* (2014) reported the absence of effects on piGST mRNA expression, but highlighted the down-regulation of several genes related to ROS presence and inflammatory processes, such as GPx genes expression, also transitory decreased at day 7 during our experiment. Down-regulation of piGST and SeGPx genes could result from a trapping of endogenous ROS by nCeO<sub>2</sub>, whose antioxidant properties have been observed in many studies (Das *et al.*, 2007; Garaud *et al.*, 2015). That could be linked to the observed reduction of Ce<sup>IV</sup> into Ce<sup>III</sup>, which implied redox reactions impacting redox balance of the cell.

The second most discriminative biomarker was the catalase activity, which was reduced throughout the exposure for ci-CeO<sub>2</sub>, correlating with the stable internal [nCeO<sub>2</sub>]. As an important player in the cellular antioxidant defenses, catalase is classically induced following pollutant exposure, while inhibition is generally observed in cases of severe toxicity (Osman *et al.* 2007). However, no impacts on lipoperoxidation, caspase-3 induction or energy reserve depletion were observed in ci-CeO<sub>2</sub>-exposed mussels, refuting the existence of severe ci-CeO<sub>2</sub> toxicity. A similar decrease in catalase activity, associated with reduced cellular damages, was observed in Garaud *et al.* (2015) and could arise from nCeO<sub>2</sub> ROS scavenging

through catalase mimetic activity (Das *et al.*, 2007), or gene down-regulation resulting from decreased intracellular H<sub>2</sub>O<sub>2</sub> levels (Ciofani *et al.*, 2014). The observed reduction of Ce<sup>IV</sup> to Ce<sup>III</sup> could also explain the observed decrease in catalase activity, as Ce<sup>III</sup> phosphate has been shown to exert catalase mimetic activity (Singh *et al.*, 2011).

Finally, the third most discriminative biomarker was the lysosomal system in hemocytes, more developed following ci-CeO<sub>2</sub> exposure. In invertebrates, hemocytes are circulating cells implied in important physiological processes, namely xenobiotic sequestration and detoxication, immunity, intracellular digestion of nutrients and transport, and endogenous and exogenous waste disposal (Giamberini and Pihan, 1997). Various nanoparticles were internalized by mussel hemocytes following short-term *in vitro* and *in vivo* exposures (Ciacci *et al.*, 2012; Couleau *et al.*, 2012). Direct nCeO<sub>2</sub> uptake could occur in the haemolymph, in which nanoparticles were shown to penetrate quickly in marine mussel (Browne *et al.*, 2008), or in the digestive tubule lumen, in which hemocytes can penetrate to absorb nutrients before re-infiltrating in the tissues to distribute them into the organism (Cheng, 1981). Alternatively, a classic detoxication mechanism is the disintegration of mussel digestive cells to eliminate the indigestible material phagocyted, the distal parts of the cell forming spherules eliminated through the *faeces*, and the basal parts forming circulating cells to transport wastes to excretory organs (Morton, 1969; Viarengo and Nott, 1993). Both direct uptake and digestive cell fragmentation could explain the presence of nCeO<sub>2</sub> inside hemocytes. However, lysosomal system enlargement is only observed in hemocytes of ci-CeO<sub>2</sub>-exposed mussels, which could arise from a weaker ba-CeO<sub>2</sub> bioaccumulation. This could also support the hypothesis that negatively charged nanoparticles accumulate preferentially inside lysosomes (Asati *et al.*, 2010; García-Negrete *et al.*, 2013; Singh *et al.*, 2010). Despite potential exposure of hemocytes to nCeO<sub>2</sub>, no adverse effects on the other hemocyte parameters were observed, whereas several nanoecotoxicological studies using

other metallic nanoparticles showed impacts on phagocytosis, lysosomal membrane stability, and ROS production following *in vitro* and *in vivo* exposures of hemocytes at high concentrations (Canesi *et al.*, 2010; Ciacci *et al.*, 2012; Couleau *et al.*, 2012).

The four other biomarkers of the reduced data set (i.e. LDH, ETS and GPx activities, and cholesterol content) were less significant but nonetheless essential to obtain a correct classification. Taken individually, their trends were not clear, but the discriminant analysis suggests that they could be impacted by nCeO<sub>2</sub> exposure. For example, ETS activity helped discriminate mussels exposed to ba-CeO<sub>2</sub> on the DF2 axis (decreasing trend), suggesting an impact on mussel metabolic activity. Cholesterol content and LDH activity also played a role in the discriminant analysis, supporting the hypothesis of an impact on metabolism. Similarly, GPx activity, linked to nonspecific antioxidant defense systems, could be impacted by nCeO<sub>2</sub> exposure.

MT mRNA was not maintained in the discriminant analysis due to collinearity problems, but considered individually, this variable exhibited significant variations and is of high biological significance when studying the effects of metallic contamination on organisms. MT mRNA levels were strongly induced at day 7 for both nanoparticle types, then dropped back to basal values. This feature could be linked with the levelling off of the internal [Ce] indicating that some regulation mechanisms were activated and equilibrated. MT gene transcription responds to a wide spectrum of stresses, permitting heavy metal chelation and reducing their cytotoxicity (Viarengo and Nott, 1993). ba-CeO<sub>2</sub> induced MTs four-times more than ci-CeO<sub>2</sub>, although ci-CeO<sub>2</sub> was three times more accumulated by mussels. Two hypotheses could explain this paradox. Firstly, the citrate coating of ci-CeO<sub>2</sub> could hide the metallic core and therefore reduced MT binding to CeO<sub>2</sub> and subsequent mRNA induction. Secondly, MTs are mostly localized in the cytosol and nucleus (Cherian, 1994), and neutral nCeO<sub>2</sub> could be mostly localized in the cytosol while negatively charged nanoparticles

accumulated in lysosomes (Asati et al., 2010; Singh et al., 2010), then ba-CeO<sub>2</sub> would be more likely to bind to and induce MT.

As a whole, although the discriminant analysis showed a clear separation between control and exposed mussels, we were unable to evidence severe toxicity of nCeO<sub>2</sub> since the number of impacted biomarkers and the variations observed were quite low. Furthermore, on the impacted biomarkers, MT mRNA levels and hemocyte lysosomal system are considered as exposure biomarkers, but provide no clues on the toxicity resulting from this exposure. In the same way, the results on the other two most impacted biomarkers, piGST mRNA levels and catalase activity, and on the four less significant biomarkers of the reduced data set, are insufficient to conclude on hypothetical toxic impacts of nCeO<sub>2</sub> on mussel biology. No biomarkers of toxicity (lipoperoxidation, caspase-3) were activated by nCeO<sub>2</sub> exposure, nor was the functional filtering activity of mussels affected, even after a three week exposure. It suggests that, in our experiment, nCeO<sub>2</sub> did not exert severe deleterious effects on mussels.

## 5. Conclusions

This work aimed at assessing the fate and effects of ba-CeO<sub>2</sub> and ci-CeO<sub>2</sub> nanoparticles on *D. polymorpha*, in simplified ecosystems and at low concentrations, using an integrated multibiomarker approach. ba-CeO<sub>2</sub> and ci-CeO<sub>2</sub> presented distinct behavior in the water column, the citrate-coating enhancing ci-CeO<sub>2</sub> colloidal stability. This led to different bioaccumulation patterns, mussels accumulating three times more ci-CeO<sub>2</sub> than baCeO<sub>2</sub>. However, the same bioreduction of Ce<sup>IV</sup> into Ce<sup>III</sup> was observed inside mussel digestive glands, but mechanisms pertaining to this biotransformation remain to be uncovered. An integrated discriminant analysis on the measured biomarkers was performed to assess the effects of nCeO<sub>2</sub> bioaccumulation and biotransformation on mussel biology. This powerful tool allowed the separation of exposed and control mussels, and also showed that mussels responded differently to ba-CeO<sub>2</sub> and ci-CeO<sub>2</sub> exposure. The discriminant analysis also allowed us to single out a set of seven biomarkers sufficient to discriminate experimental groups. We showed that two of the three most significant biomarkers, lysosomal system size and catalase activity, were related to those isolated as the most responsive in a previous short-term exposure to nCeO<sub>2</sub> (Garaud et al., 2015), confirming the usefulness of these biomarkers in nCeO<sub>2</sub> ecotoxicity assessment. MT mRNA induction and lysosomal enlargement were more indicative of the exposure and stress responses by the organisms, while the lack of impact on cellular damages, energetic reserves and filtration activity suggested the absence of serious adverse effects of nCeO<sub>2</sub> following more environmentally realistic exposure. However, further studies are needed to investigate if the slight perturbations observed could lead to populational impacts in the long-term.

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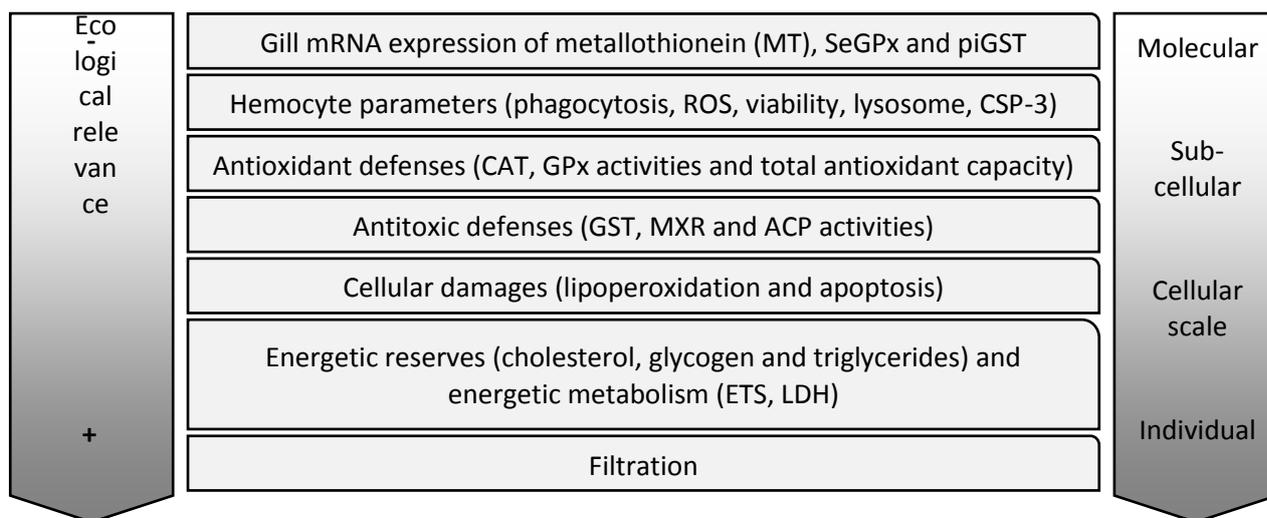
Fact or	exposure duration (df=2)		nCeO <sub>2</sub> exposure (df=2)			interaction (df=4)		f		
	F	Pr(F)	F	Pr(F)	F	Pr(F)				
<b>Ce bioacc.</b>	0.03	0.9661								
<b>RNA MT</b>	162.80	0.0000	***	173.23	0.0000	***	93.62	0.0000	***	18
<b>RNA piGST</b>	7.45	0.0044	**	53.79	0.0000	***	4.48	0.0109	*	18
<b>RNA SeGPx</b>	15.56	0.0001	***	1.10	0.3535		7.27	0.0011	**	18
<b>Cv</b>	7.41	0.0045	**	0.18	0.8339		1.94	0.1478		18
<b>Ce</b>	3.00	0.0749		0.57	0.5781		1.50	0.2453		18
<b>Cm</b>	1.93	0.1745		1.01	0.3827		0.54	0.7095		18
<b>ROS</b>	0.82	0.4562		0.74	0.4930		0.96	0.4515		18
<b>Lyso</b>	0.07	0.9350		3.73	0.0442	*	0.52	0.7225		18
<b>phag. 1b</b>	0.53	0.4801		0.62	0.5538		0.15	0.8602		12
<b>phag. 3b</b>	8.94	0.0113	*	2.16	0.1585		3.93	0.0486	*	12
<b>H. CSP-3</b>	Kruskal-Wallis chi-squared = 16.7407, df = 8, p-value = 0.0329*									
<b>GPx</b>	1.80	0.1936		1.86	0.1836		0.87	0.4996		18
<b>CAT</b>	7.00	0.0056	**	7.34	0.0047	**	1.12	0.3784		18
<b>TAC</b>	4.42	0.0274	*	0.10	0.9027		2.79	0.0577		18
<b>GST</b>	5.10	0.0176	*	0.42	0.6645		0.97	0.4500		18
<b>ACP</b>	1.71	0.2089		1.07	0.3634		1.01	0.4271		18
<b>[LOOH]</b>	3.93	0.0384	*	0.96	0.4021		1.41	0.2715		18
<b>CSP-3</b>	0.59	0.5639		0.72	0.4996		0.57	0.6850		18
<b>[trigly.]</b>	3.38	0.0568		0.35	0.7115		0.48	0.7519		18
<b>[chol.]</b>	0.03	0.9750		1.36	0.2809		0.43	0.7828		18
<b>[prot.]</b>	1.29	0.2986		0.04	0.9646		0.52	0.7213		18
<b>ETS</b>	2.89	0.0818		1.89	0.1796		0.25	0.9081		18
<b>LDH</b>	1.26	0.3085		2.68	0.0956		0.68	0.6133		18
<b>MXR</b>	0.20	0.8222		0.45	0.6432		0.92	0.4760		18

**Table 1 : Results of the statistical analysis (2 ways-ANOVA or Kruskal Wallis when non-parametric data) to assess respective effects of nCeO<sub>2</sub> exposure, exposure duration and their interaction on Ce internal concentration and measured biomarkers after 7, 14 and 21 days of exposure to ba-CeO<sub>2</sub> and ci-CeO<sub>2</sub> - \* indicates significant effect of the studied factor (\*p<0.05; \*\*p<0.01; \*\*\*p<0.001)**

Biomarker	Raw coefficients		Standardized coefficients		F	p value
	DF1	DF2	DF1	DF2		
RNA piGST	0.027	0.029	1.224	1.299	27.49	<0.001
CAT	0.040	-0.036	0.948	-0.837	4.82	0.017
Lyso	-0.057	0.040	-0.901	0.627	4.42	0.023
LDH	0.009	0.010	0.213	0.235	2.77	0.083
ETS	-0.053	0.098	-0.506	0.931	1.84	0.181
GPx	0.992	2.677	0.110	0.296	1.54	0.190
[chol.]	-0.097	0.052	-0.443	0.236	1.78	0.234

**Table 2 : Results of the discriminant analysis – final set of biomarkers and their raw coefficients on discriminant functions 1 and 2 (DF1 and DF2), useful for predicting the group for a new replicate, their standardized coefficients, useful to hierarchize relative influence of the variables, and their significance.**

JUST ACCEPTED



**Figure 1**

JUST ACCEPTED

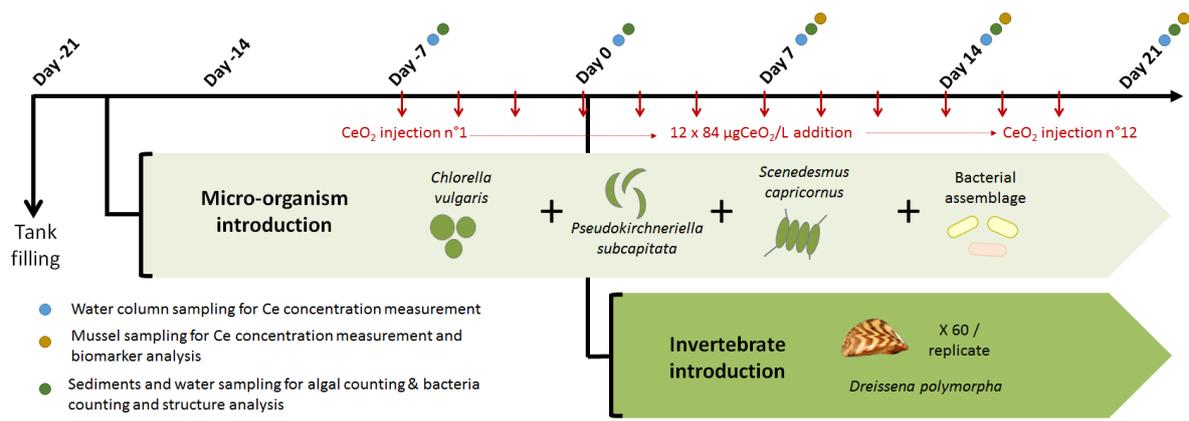


Figure 2

JUST ACCEPTED

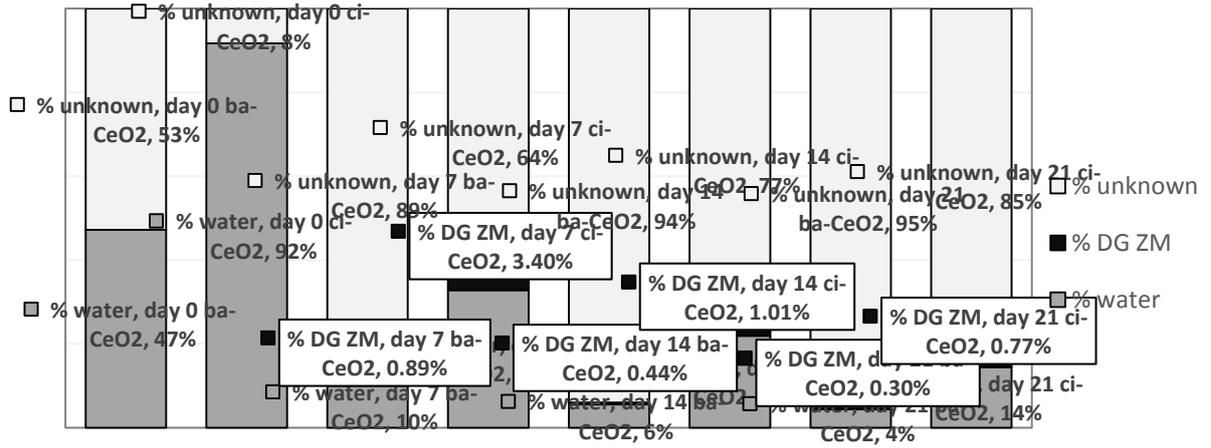


Figure 3

JUST ACCEPTED

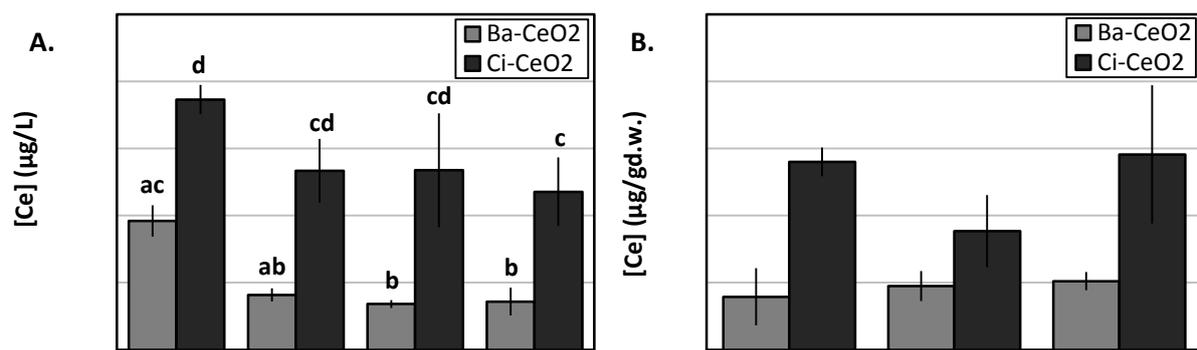


Figure 4

JUST ACCEPTED

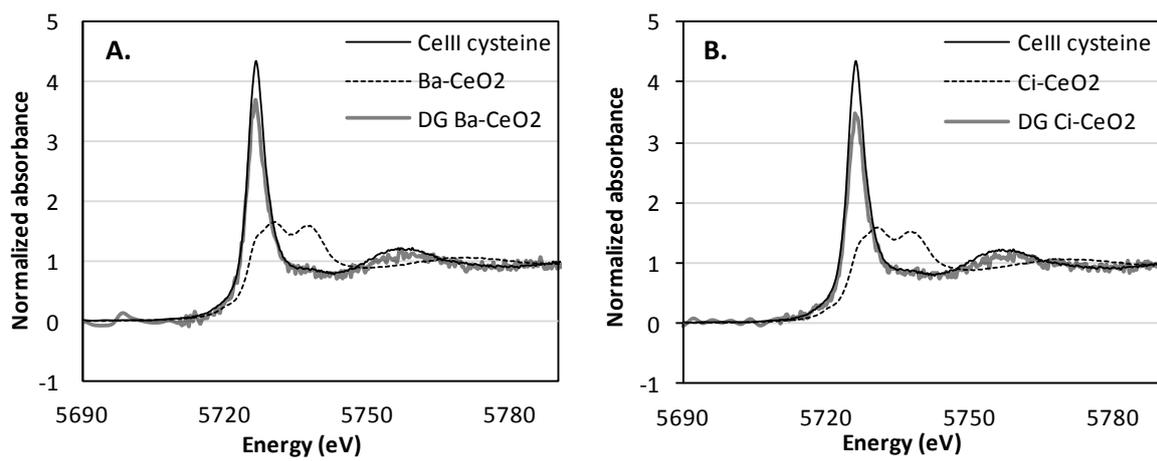


Figure 5

JUST ACCEPTED

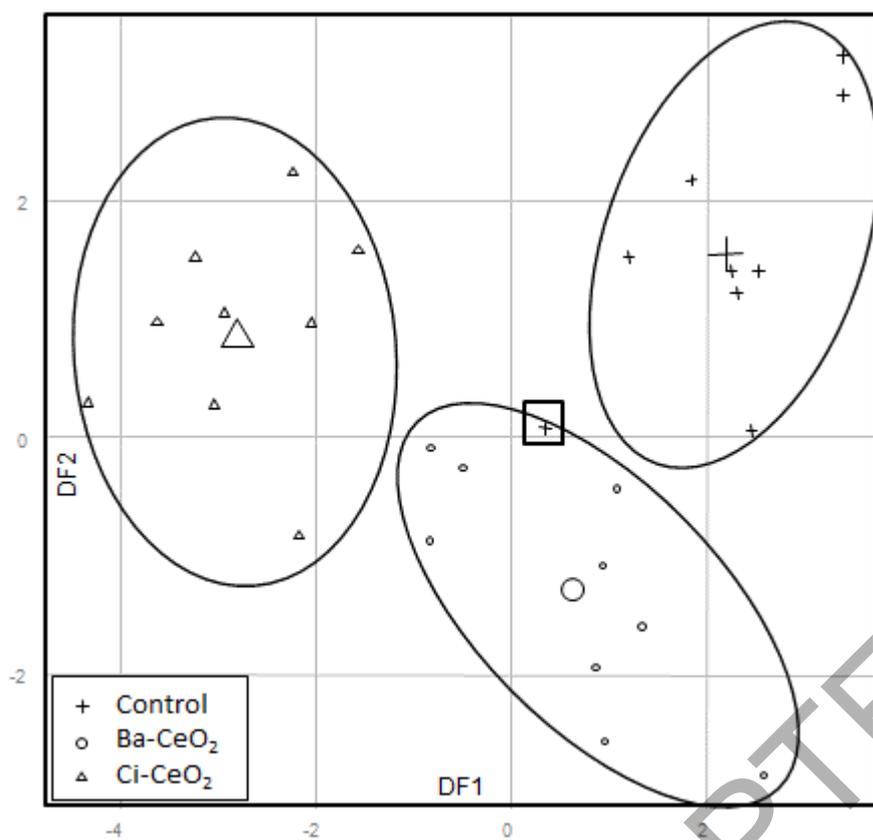


Figure 6

## Figure captions

Figure 1: Multibiomarker approach used and physiological functions involved, ecological relevance (left arrow) and level on the biological scale (right arrow).

Figure 2 : Schematic representation of the experimental design.

Figure 3 : Mass balance of Ce in experimental systems at days 0, 7, 14 and 21 (d0, d7, d14 and d21) expressed as % of the total mass of introduced Ce retrieved in water column and mussel digestive glands (% of Ce in mussel DG are indicated in the with boxes)

Figure 4: Measured [Ce] in experimental systems water column at day 0 (just before mussel introduction), and after 7, 14 and 21 days of contamination (A) and total Ce bioaccumulation in mussel digestive glands after 7, 14 and 21 days of exposure to ba-CeO<sub>2</sub> and ci-CeO<sub>2</sub> (B) – data are represented as mean  $\pm$  SD (n=3) – groups that do not share a common letter are statistically different (p<0.05)

Figure 5 : Ce L3-egde XANES in mussel digestive glands (DG) after 7, 14 and 21 days of exposure to ba-CeO<sub>2</sub> (A) and ci-CeO<sub>2</sub> (B) - spectra of the original nCeO<sub>2</sub> and of Ce<sup>III</sup> cysteine standard are represented on each graph.

Figure 6 : Discriminant analysis plot of the 27 replicates (9 per exposure condition) against the two discriminant functions DF1 and DF2 – larger symbols represent group barycenters. The individual in a square corresponds to the unique classification error (a control individual classified in the Ba-CeO<sub>2</sub> group).