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Vanessa Koehle-Divo, Carole Cossu-Leguille, Sandrine Pain-Devin, Cécile Simonin, Carole Bertrand, et al.. Genotoxicity and physiological effects of CeO₂ NPs on a freshwater bivalve (*Corbicula fluminea*). *Aquatic Toxicology*, 2018, 198, pp.141-148. 10.1016/j.aquatox.2018.02.020 . hal-01726355

HAL Id: hal-01726355

<https://hal.univ-lorraine.fr/hal-01726355>

Submitted on 8 Mar 2018

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Accepted Manuscript

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PII: S0166-445X(18)30189-9
DOI: <https://doi.org/10.1016/j.aquatox.2018.02.020>
Reference: AQTOX 4868

To appear in: *Aquatic Toxicology*

Received date: 2-10-2017
Revised date: 20-2-2018
Accepted date: 26-2-2018

Please cite this article as: Koehle-divo, Vanessa, Cossu-Leguille, Carole, Pain-Devin, Sandrine, Simonin, Cécile, Bertrand, Carole, Sohm, Bénédicte, Mouneyrac, Catherine, Devin, Simon, Giamberini, Laure, Genotoxicity and physiological effects of CeO₂ NPs on a freshwater bivalve (*Corbicula fluminea*). *Aquatic Toxicology* <https://doi.org/10.1016/j.aquatox.2018.02.020>

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Genotoxicity and physiological effects of CeO₂NPs on a freshwater bivalve (*Corbicula fluminea*)

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Highlights

- Potential impacts of CeO₂ NPs on widespread freshwater bivalve *Corbicula fluminea* were studied
- Realistic exposure concentrations (10 & 100 µg/L) during 6 days was carried out
- Genotoxic, biochemical and physiological effects were assessed at short term
- Induction of caspase pathway and DNA damages appeared significant for exposed organisms

Abstract

The rapid development of nanotechnology and the increased use of nanomaterials in products used in everyday life have raised the question of the potential release of nanoparticles into the aquatic environment. Their fate and effects in natural ecosystems are not currently well understood but harmful effects of nanoparticles have been demonstrated at low concentrations on some freshwater and marine species. Cerium dioxide nanoparticles (CeO₂NPs) are produced in large quantities and used in products in many different fields, such as automotives or optics. Because of their widespread use in daily products, CeO₂ NPs are included in the OECD priority

list of manufactured nanomaterials for human and environmental assessment. Indeed some studies have been conducted to assay various enzymatic biomarkers, which showed the CeO₂ NPs potential to modify anti-oxidative defenses and cellular membrane stability. Nevertheless, only a few studies were performed on their genotoxic potential. The aim of this work was to evaluate the genotoxic and physiological effects of CeO₂ NPs on a widespread freshwater bivalve *Corbicula fluminea* by using comet assay and a multi-enzymatic biomarker approach. Exposure to two CeO₂ NP concentrations during a short term experiment (6 days) was set up. The first one (10 µg/L) was chosen in order to work with low but measurable concentrations whereas the second one was ten times higher (100 µg CeO₂ NPs/L). DNA damage was significantly more pronounced compared with control for both concentrations tested as early as two days of exposure and seemed to increase with time. Some enzymatic biomarkers of anti-oxidative defenses (total antioxidant capacity, catalase activity), anti-toxic mechanisms (glutathione-S-transferase activity, caspase-3 activity) or metabolism (lactate dehydrogenase activity) tended to increase after 6 days of exposure but only the induction of caspase pathway and DNA damages appeared significant for exposed organisms. In this study, time and concentration effects of CeO₂ NPs were highlighted by coupling genotoxic and cellular biomarker assessments.

Keywords: cerium dioxide nanoparticles (CeO₂ NPs); biomarker; comet assay; genotoxic; apoptosis; *Corbicula fluminea*.

1. Introduction

Production and use of engineered nanomaterials (ENMs) have been significantly growing for several decades because of their unique and hopeful properties that allow their use in very diverse areas such as industry, energy, agriculture, medicine and the environment. Nanoparticles (NPs) are part of ENMs and their behavior depends on properties related to the NPs themselves such as size, surface properties, synthesis method and initial concentration.

Nevertheless external environment has a key role on their behavior and can highly modify it by a single parameter change such as pH, temperature, turbidity, ionic strength or presence of other components like natural organic matter (NOM) (Van Koetsem *et al.*, 2015; Rocha *et al.*, 2015; Peralta-Videa *et al.*, 2011; Wiesner *et al.*, 2009). Because of multiple properties of NPs, their synthesis and use in the last two decades has led to an increasing diversity of existing ENMs included in daily life products. The environment and human beings are nowadays exposed to unknown amount of various ENMs for which effects cannot be predicted by classical ecotoxicological tests performed on corresponding macroscopic size material. Furthermore, no analytical method is currently available to quantify ENM concentrations in the natural environment due to the complexity of such matrices. Most information about environmental concentration is then estimated by modeling (Gottschalk *et al.*, 2015). Thereby, fate, transformations and effects of nanomaterials into environment are poorly known and more studies are essential to assess their environmental repercussion.

Cerium dioxide nanoparticles (CeO_2 NPs) are among the most produced nanoparticles in the world, at the rate of 10,000 tons/year (Keller *et al.*, 2013). They are used for commercial applications in several fields such as electronic and optic, painting or catalysis. These uses lead inevitably to both direct and indirect release of nanoparticles in the environment and ultimately in the aquatic compartment (Weinberg *et al.*, 2011), which may lead to hazards to humans and the environment. That's the reason why cerium dioxide nanoparticles are among the NPs selected for priority testing by the Organization for Economic Co-operation and Development (OECD). Cerium (Ce) is one of the most abundant rare earth element and European median stream water concentration is about 55 ng/L (www.gsf.fi/publ/foregsatlas/). CeO_2 NP concentrations in natural waters are nevertheless only estimated by modeling studies because of the difficulty of assessing measurements in this complex media. Few studies were conducted in order to model the potential environmental concentration of CeO_2 NPs in water but they

indicate values in the range of ng/L- μ g/L (Keller *et al.*, 2014; O'Brien *et al.*, 2011; Gottschalk *et al.*, 2015). Even if current aquatic concentrations are estimated to be very low, present or future effects should not be neglected considering their increasing incorporation in industrial and commercial products but also their emerging applications in biomedical fields.

In most studies, assessment of NM toxicity was conducted at environmentally unrealistic concentrations of exposure and with standard ecotoxicological tests that are not always representative of the environmental matrix considered. Furthermore, several studies showed that standardized tests are not fully adapted to NMs exposures because of multiple interactions with assay components or detection systems (Azqueta *et al.*, 2015). Aquatic compartment is frequently characterized as the ultimate sink of contamination, however little data is available about the effects of CeO₂ NPs on non-standardized organisms, like widespread filter-feeder bivalves, that are considered as relevant test organisms for nanoparticle toxicity (Conway *et al.*, 2014; Baun *et al.*, 2006; Canesi *et al.*, 2012). Toxicity tests using bivalves in order to assess ENM toxicity were mainly conducted with marine species, therefore data for freshwater species is scarce (Rocha *et al.*, 2015). Contradicting results have been obtained for CeO₂ NPs that may lead either to damaging, scavenging or no apparent effects (Lee *et al.*, 2009; Bour *et al.*, 2015; Garaud *et al.*, 2015; Golbamaki *et al.*, 2015). ROS overproduction is one of the most discussed toxic effect of NPs. This overproduction can lead to the activation of anti-oxidative enzymes, like SOD, CAT and GPx, or genotoxic effects. Assessment of such enzymes, antitoxic enzymes and fitness biomarkers is useful to understand NP effects and side effects at biochemical level (Vale *et al.*, 2016). Moreover, genotoxicity assessment of NPs is recommended by OECD for regulatory purposes asking more data for risk assessment (Nanogenotox, 2013; ANSES, 2014). The concern about potential genotoxicity is reinforced by studies revealing genotoxic effects of CeO₂ NPs in human cell lines (Auffan *et al.*, 2009; Benameur *et al.*, 2015). This also raises the question about genotoxicity of NPs in aquatic

ecosystems, for which, as usual, data are lacking (Lee *et al.*, 2009). In bivalves, studies of DNA damages induced by chemicals are frequently performed by the alkaline comet assay, using hemocytes or gill cells (Rocha *et al.*, 2015). It permits the measurement of DNA strand breaks in individual eukaryotic cells and different injuries on DNA depending on the pH used during lysis and electrophoresis (Azqueta *et al.*, 2015; Jha, 2008; Lee *et al.*, 2003; Golbamaki *et al.*, 2015). However, since the digestive gland is the main organ for ENM accumulation in bivalve mollusks (Hull *et al.*, 2011; Rocha *et al.*, 2015), DNA damage assessment in this organ is particularly relevant. Integrative vision of NP effects coupling different scales of organization should be a good way to understand their potential toxicity. Thus, monitoring both genotoxicity and other cellular responses to stress should allow a broad and complementary view of NP effects.

The aim of this work was to study the potential impacts of CeO₂ NPs on non-target aquatic organisms like bivalves, known as efficient bioaccumulators of contaminants. Therefore, we selected a widespread freshwater sediment-dwelling bivalve *Corbicula fluminea* to achieve this goal. In order to perform a more realistic exposure while staying in a quantifiable range of concentrations, concentrations representative of 10 to 100 times (10 & 100 µg/L) the maximal Predicted Environmental Concentration (PEC) values were employed (O'Brien *et al.*, 2011). A short-term exposure was carried out for 6 days and genotoxic effects were followed at 2 and 6 days and coupled with a multi-biomarker approach at biochemical and physiological scales. The alkaline comet assay was performed to detect DNA damage, and a battery of biomarkers involved in response to oxidative stress, detoxification, cellular damages and energy metabolism was used to determine biochemical and physiological effects. A Partial Least Square Discriminant Analysis (PLS-DA) was employed to elicit an integrative vision of responses of control and exposed organisms according to NPs concentration and exposure time.

2. Materials and methods:

2.1. Chemicals

Cerium dioxide uncoated nanomaterials (CeO_2 ; NM-212) of 20 to 25 nm in diameter were provided as powder from the Joint Research Center (JRC) Institute (Ref: CeO₂-JRCNM02102a003903). Observations made using Dynamic Light Scattering were in accordance to those reported by the JRC. They were monodispersed in ultra-pure water and had a mean size of 225.4 ± 92.47 nm. A stock suspension of 1 g/L was prepared in deionized water in accordance with the guideline described by the JRC Science and Policy Report (Singh *et al.*, 2014). An intermediate solution of 100 mg/L was prepared in deionized water from the stock suspension and used to inoculate media of bivalve exposure (artificial water) in order to have 0, 10 and 100 $\mu\text{g CeO}_2\text{NPs/L}$. Nanoparticle solutions were sonicated in ice for 20 secs at 90% amplitude before use.

Artificial seawater was made using Tropic Marine® sea salt (Tropicarium Buchshlag Dreieich Germany) to allow reproduction of medium for all experiments (Mouneyrac *et al.*, 2002). All other chemicals were purchased from Sigma Aldrich (Chemical Co, St Louis, MO) and Thermo-Scientific (Waltham, Massachusetts).

2.2. Collection of clams and acclimation to the laboratory

Freshwater clams *Corbicula fluminea* (2 cm length) were hand-collected (Moselle, Metz, France) in May 2015, transported to the laboratory and acclimated in a temperate room progressively for 10 days by gradually increasing the artificial water percentage from 0 to 100%. Artificial water was made using commercial salt (TropicMarin®) and adjusted to 2.9 ± 0.06 PSU. Forty-three clams were placed in 5 L of artificial water with bubbler and glass beads. Temperature was 17.5 ± 0.4 °C with a 16:8 (day:night) photoperiod for the entire acclimation period.

2.3. Exposure design

The experiment was conducted in artificial water for 6 days. Temperature was kept at 15.4 ± 1.3 °C. A 16:8 (day:night) photoperiod was applied. No renewal of water was carried out and no oxygenation was ensured during the experiment in order to avoid interference with nanoparticles. Temperature, conductivity, O₂ and pH were measured a few centimeters under the surface at each sampling date with ODEON® probes (Kit Ponsel Odéon open X with PHEHT, C4E and ODOT probes). Fourteen organisms were exposed per treatment. Water was sampled for [Ce] whereas organisms were sampled for [Ce], genotoxic and physiological responses analysis after 0, 2 and 6 days of exposure.

2.4. Water and digestive gland Ce concentrations

Total water [Ce] (in µg/L) was measured by ICP-MS Agilent 7700 (Micropolluants Technologies SA, Saint-Julien-lès-Metz, France) after 0, 2 and 6 days of exposure. Bioaccumulation of Ce was measured in the digestive gland (DG) of three clams per treatment after a 24 hour depuration time in clean artificial water. Tissues were dry weighed. Then 1 mL HNO₃ 32.5% was added to dry tissues and left in the oven during 72 hour at 65°C. Finally 4 mL MilliQ water was added to each sample and [Ce] was measured by ICP-MS. [Ce]_{dg} was expressed in µg Ce/g dry weight (µg/g dw).

2.5. Digestive gland dissection and homogenate preparation

After removal of the shell, the digestive gland (DG) of 14 clams per treatment were excised, frozen in liquid nitrogen and stored at -80°C until analysis. Whole gland of 6 clams per treatment were used for comet assay while 3 glands were used for Ce bioaccumulation measurements (see 2.4.). The 5 remaining digestive glands were then treated as described in Sroda and Cossu-Leguille (2011) for cellular biomarker measurement. Digestive gland were defrosted, weighed and crushed at 4°C in a 50 mM phosphate buffer (pH 7.6) supplemented with 1 mM phenylmethylsulfonyl fluoride (PMSF) and 1 mM L-serine borate mixture as

protease inhibitor at a 8/1 volume/weight ratio. The mixture was then centrifuged for 5 min at 4°C at 250xg and each supernatant was divided in two parts: one was used for the assays of total protein, LOOH content, ACP and ETS activity, while the other part was centrifuged 1,000xg for 20 min at 4 °C. The supernatant was collected and a second 50 min centrifugation was performed at 20,000xg at 4°C. The final supernatant, corresponding to the cytosolic fraction, was used for enzyme activity.

2.6. Biomarker measurements

2.6.1 Biochemical and physiological biomarker assessment

Biomarker analysis was performed on the automated spectrophotometer analyser (Konelab 20 XTi, Thermo Scientific) using protocols described in Garaud *et al.* (2016) and adapted for *C. fluminea* by Bertrand *et al.* (2016). Total protein content ([prot]), lipid hydroperoxide concentration ([LOOH]), acid phosphatase activities (ACP) and mitochondrial electron transport system (ETS) activity were measured using the whole homogenate, whereas total protein content, total antioxidant capacity (TAC), total glutathione peroxidase (GPx), glutathione-S-transferase (GST) and lactate dehydrogenase (LDH) activity, and caspase-3 activity (Casp-3) were measured in cytosolic fractions. Catalase activity (CAT) was measured spectrophotometrically according to Garaud *et al.* (2015) and Bertrand *et al.* (2016).

2.6.2 Comet assay – DNA damage assessment

The comet assay was performed under alkaline conditions adapted from Singh *et al.* (1988) for *C. fluminea* DG cells. Cellular suspension from DG was obtained by mixing small pieces of DG with 1.5 mL 0.01 M phosphate-buffered saline solution (PBS) under agitation for 5 min at room temperature and centrifuged for 1 min at 23xg. The resulting supernatant was centrifuged for 7 min at 50xg; the resulting supernatant was centrifuged for 7 min at 100xg. The resulting pellet was then suspended with 200 µL 0.01 M PBS. Viability of digestive gland cells was

microscopically checked using trypan blue (0.5%: W/V) dye exclusion test for each treatments before conducting the comet assay. According to Tice *et al.* (2000), the test can be performed when cell viability is equal or more than 80% as in all our experiments.

Slides were prepared in duplicate for each sample. Microscope slides were pre-coated with a first layer composed by 90 μL Normal Melting Point Agarose (NMPA, 0.8 % diluted in 0.01 M PBS) and were allowed to dry for 7 days. Then, 80 μL of a mixture of cell suspension and 1% Low-Melting Point Agarose (LMPA) (v/v ratio) were transferred onto slides to compose the second layer. A third layer composed of 75 μL 0.5% LMPA was formed. The slide was finally given 10 min at 4°C for polymerization. The cells were then lysed for 1 hour at 4°C in lysing solution (2.5 M NaCl, 100 mM Na₂EDTA, 100 mM Tris pH 10, 1% N-laurylsarcosinate, 1% Triton X-100, 10% DMSO). Then, slides were placed in an electrophoresis unit for 20 min in the electrophoretic solution (300 mM NaOH, 10 mM Na₂EDTA, pH > 13) in order to induce DNA denaturation. Electrophoresis was thereafter performed for 20 min at 300 mA and 20 V.cm⁻¹ in the same electrophoretic solution. Afterwards, slides were removed from the electrophoresis bath and kept to dry 10 min before being submerged twice for 10 min in a porcelain tank containing neutralization solution (0.4 M Tris-HCl, pH 7.5) followed by a 5 min submersion in 95% ethanol porcelain tank and then let to dry 10 mins. Slides were stained with 40 μL of 30 $\mu\text{g}\cdot\text{mL}^{-1}$ ethidium bromide before analysis and then observed using an epifluorescence microscope (BX60; Olympus) linked to an image analysis system (Comet Assay IV Image analysis system, PI, UK). Cells were blindly analyzed at the central part of each slide. A hundred cells per slide were scored out of a total of 200 cells per clams. The percentage of DNA in comet tail was chosen to express DNA damage. Arbitrary classes of DNA damage were made to present the distribution of cells as a function of the tail DNA percentage: 0-20 %, 20-40 %, 40-60 %, 60-80 %, 80-100 %. The tail length was selected to complete the integrative model using all biochemical and physiological biomarkers with

genotoxicity data. The tail length was also measured on organisms directly after sampling in the river.

2.7. Statistical analysis

Data were collected and treated using R (R core team, 2014). Homoscedasticity and normality were checked for each test by using Levene and Shapiro-Wilk tests respectively. When the conditions were confirmed, a one-way ANOVA was performed in order to compare time effect on non-exposed organisms and two-way ANOVA was performed to compare experimental groups 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. Nonparametric data were analyzed using Kruskal-Wallis test.

2.8. PLS-DA

A Partial Least Square Discriminant Analysis (PLS-DA) was performed in order to obtain a global description of the relationships among the whole battery of biomarkers, comet tail length and the different treatments over time. A representation of exposure conditions was made on a factorial plane while only variables with $VIP > 0.8$ (Variables of Importance in the Projection) were plotted on a correlation circle in order to focus on important biomarkers. Finally, Hotelling T^2 tests with a Benjamini and Hochberg risk correction (Benjamini and Hochberg, 1995) were performed between exposure conditions to assess the effect of the two studied factors.

3. Results

3.1 Ce concentration in the water column

Due to the low dissolution of CeO_2 NPs (Singh *et al.*, 2014), the majority of measured Ce should come from NPs and so, total [Ce] were measured. [Ce] in the control sample was under the quantification limit ($0.5 \mu\text{g/L}$) at each date. At the beginning of the exposure, $0.5 \pm 0.4 \mu\text{g/L}$

and $7.2 \pm 6.7 \mu\text{g/L}$ were retrieved in the water column for $10 \mu\text{g CeO}_2\text{NPs/L}$ and $100 \mu\text{g CeO}_2\text{NPs/L}$ respectively (Figure 1). After 2 and 6 days of exposure, the nominal concentration for the group $10 \mu\text{g CeO}_2\text{NPs /L}$ was under the quantification limit. Ce concentration decreased in the $100 \mu\text{g CeO}_2\text{NPs /L}$ media between the beginning and 2 days of exposure and reached $2.7 \pm 1.3 \mu\text{g/L}$. All treatments remained relatively stable between 2 and 6 days for all exposure concentrations.

Other parameters such as temperature, pH, salinity or redox potential (Table S1) were very similar among all treatments suggesting that the only difference between exposures was CeO_2 NPs injection.

3.2 Ce bioaccumulation in digestive gland

At the beginning of the experiment, control mussels had mean Ce concentrations of $0.21 \pm 0.16 \mu\text{g/g dw}$ (Figure 2). After 2 days of exposure, clams reached mean values of 0.14 ± 0.07 , 0.2 ± 0.08 and $0.46 \pm 0.46 \mu\text{g Ce/g dw}$ for 0, 10 and $100 \mu\text{g CeO}_2\text{/L}$ exposure respectively. After 6 days of exposure, means internal concentrations were 0.19 ± 0.10 , 0.16 ± 0.02 and $0.23 \pm 0.08 \mu\text{g Ce/g dw}$ for 0, 10 and $100 \mu\text{g CeO}_2\text{/L}$ exposure respectively. Results showed no significant differences in Ce bioaccumulation under CeO_2 NP exposure (Kruskal-Wallis test, $p = 0.5388$).

3.3 Comet assay (Determination of DNA strand breaks)

Significant differences in the percentage of DNA in comet tails (Figure 3) and in tail length (Figure 4) were observed after 2 and 6 days of exposure in exposed groups compared to control ones. Figure 3 shows that control organisms presented a similar profile throughout the time with $96 \pm 1 \%$ of cells displaying 0 to 40 % DNA in tail and $4 \pm 1 \%$ presented 40 to 60%. Organisms exposed to $10 \mu\text{g CeO}_2\text{NPs /L}$ presented 75% of cells containing 0 to 40% tail DNA and 25% with more than 40 % tail DNA at day two. At day six, the proportion of cells containing more than 40% tail DNA increased whereas those containing 0 to 20% tail DNA disappeared.

The 100 $\mu\text{g CeO}_2\text{NPs/L}$ treatment led to an absence of cells containing 0 to 20 % DNA in tail as early as two days of exposure with the apparition of cells containing more than 80% tail DNA.

Figure 4 shows that the tail length measured in control organisms remained stable all along the exposure and was about $3.5 \pm 1 \mu\text{m}$, close to the value measured in organisms just after sampling ($3.1 \pm 0.7 \mu\text{m}$). In exposed organisms, the mean tail length was about $6.5 \pm 3.5 \mu\text{m}$ and $9.01 \pm 4.5 \mu\text{m}$ after 2 days of exposure and about $8.9 \pm 4.2 \mu\text{m}$ and $11.2 \pm 5.2 \mu\text{m}$ after six days of exposure to 10 $\mu\text{g CeO}_2\text{ NPs /L}$ and 100 $\mu\text{g CeO}_2\text{ NPs /L}$, respectively.

3.4 Biochemical and physiological biomarker responses

Only one biomarker (Casp-3) of the whole battery (Table S2) showed significant changes in exposed organisms compared to controls. Significant effects of time and CeO_2 NP exposure were observed in Casp-3 activity (Figure 5). No significant difference of Casp-3 activity was observed during the whole experiment in control organisms or during the first two days for all exposures. However, Casp-3 activity was significantly increased at day 6 in the highest CeO_2 NP exposure concentration compared to the control group.

3.5 Probabilistic Linear Discriminant analysis (PLS-DA)

A PLS-DA (Figure 6) was performed in order to discriminate each exposure group according to time and treatment. The most important parameters were comet tail length and Casp-3, ACP, GST, LDH, TAC and CAT activities. ACP, GST, TAC, CAT and LDH levels (Table S2) decreased over exposure time in control organisms. The most important variable that allowed the discrimination of exposure conditions was tail length that increased with concentration and time. A concentration effect was linked to the tail length changes, whereas time effect was mainly explained by Casp-3 activity induction in exposed organisms. Groups exposed to both tested concentrations also appeared different between 2 and 6 days of exposure. Hotelling T^2

test (Table 1) highlighted significant differences between exposed and control groups at day 2 and day 6. Significant differences were also observed as a function of time and concentration for exposed organisms whereas no difference seen in control ones over the exposure period.

4. Discussion

This study was conducted in order to evaluate the potential genotoxic and physiological effects of low CeO₂ NP exposure concentrations in the freshwater bivalve *C. fluminea*. First we focused on the fate of CeO₂ NPs in the exposure medium. A strong decrease of total [Ce] in the water column was observed just after introduction and represents approximatively a 90% loss for both concentrations. The JRC report (Singh *et al.*, 2014) reported that cerium dioxide nanoparticles used in our study showed high agglomeration/aggregation when dispersed in deionized water (DI water). Furthermore, these CeO₂ NPs dispersed in ecotoxicological test media such as fish and daphnia test media and seawater showed larger agglomerates than DI water, with the largest found in seawater (Singh *et al.*, 2014). Aggregation could have occurred in our more complex experimental system because of the presence of other components (anions, cations, nutrients, trace elements) that could interact with NPs. In addition to these observations, bare CeO₂ NPs were found to homoaggregate in a few minutes in Volvic water (Tella *et al.*, 2014). That could potentially explain the strong loss of cerium concentration just after inoculation.

NP bioavailability is supposed to be very low given their small size, even for aggregates up to 0.5 µm. However, the major route of internal exposure in suspension-feeding bivalves is capture and ingestion that could be enhanced by aggregation (Ward *et al.*, 2009). Clams and mussels are known to filter large volumes of water with high efficiency, even when NP contamination occurs. Based on a post-feeding clearance experiment which followed the decrease of phytoplankton concentration, Conway *et al.* (2014) calculated that up to 99.9% of CeO₂ could be filtered from the water column over 2 days by *M. galloprovincialis*. Accordingly, Montes *et*

al. (2012) showed that almost all of the Ce mass was removed from the water column by this mussel after 24 hours of exposure and was mainly transferred in pseudo-feces.

Although bivalves are able to strongly bioaccumulate a large range of contaminants, no significant bioaccumulation of Ce was depicted in this study and only a tendency could be observed in bivalves exposed to the highest concentration. As CeO₂ NP dissolution was reported to be low (Singh *et al.*, 2014) the major part of measured Ce should come from NPs. The combination of both NP properties (as discussed above: homo-aggregation, surface reactivity...) and filtration by bivalves may favor sedimentation, explaining the low recorded bioaccumulation. Finally, it seems that CeO₂ NPs were weakly bioaccumulated in aquatic organisms (Zhang *et al.*, 2012; Tella *et al.*, 2014) and some studies indicated that CeO₂ NPs were not internalized into organisms even if they were found in the digestive gland of aquatic organisms (Artells *et al.*, 2013; Auffan *et al.*, 2013; Manier *et al.*, 2011; Van Hoecke *et al.*, 2009). In accordance with these observations, Montes *et al.* (2012) showed a significant bioaccumulation only at the highest concentration of CeO₂ NPs in marine bivalve *Mytilus galloprovincialis* exposed over 4 days to high concentrations (1.0, 2.5, 5.0 and 10.0 mg/L), a hundred times higher than our tested concentrations. Their mass balance indicated that Ce accumulation in mussel tissues was nevertheless very low (1-3%) whereas nearly all the injected CeO₂ NPs were found in pseudo-feces. In our exposure media, Ce concentrations decreased between 0 and 2 days and stayed relatively stable until the end of the experiment. Considering that initial exposure concentrations measured in our media were very low compared to most studies, the absence of significant bioaccumulation was not surprising.

In order to study the potential CeO₂ NP effects on *C. fluminea*, a two dimensional approach coupling genotoxic and physiological measurements was performed. Genotoxic effects were assessed by Single Cell Gel Electrophoresis assay (SCGE), also known as Comet assay, frequently used to measure and evaluate DNA damages owing to its sensibility, rapidity and

relative simplicity. In this study, Comet assay was performed at pH > 13 in order to allow the detection of single and double-strand breaks but also alkali-labile sites. It has been demonstrated that a large tail length can be associated with both a high and a weak intensity of tail. For this reason, both the tail length and the intensity of the tail were used here as Lee *et al.*, 2003 proposed. Cells are constantly exposed to DNA damage caused by various sources and have developed a molecular and cellular set of defense systems to handle the damage. However, exposure to external or environmental sources such as UV light, ionizing radiations or genotoxic agents can amplify damages. Some of them could be repaired. Nevertheless some are irreparable and affected cells can trigger cell death by activation of apoptosis in order to eliminate potentially damaged cells (Matt *et al.*, 2016). Our results indicated that DNA damage increased with time and concentration of exposure in both exposed groups but stayed constant in control organisms. CeO₂ NPs induced significant DNA degradation in other organisms like *Daphnia magna* and *Chironomus riparius*, which was associated with other higher biological level perturbations (24h, 1mg/L) (Lee *et al.*, 2009). Extensive DNA damage can also induce apoptosis that is considered an irreversible process, in contrast to most of the lesions revealed by the classical comet assay that could be potentially repairable (Lee *et al.*, 2003). Casp-3 is a common enzyme involved in two apoptotic pathways and can enable us to follow activation of apoptosis (Romero *et al.*, 2015). Apoptosis may be activated by direct interactions of NPs with DNA or DNA-repair or intracellular enzymes but also by indirect ways, like induction of Reactive Oxygen Species (ROS) production. They are highly reactive products derived from the reduction of oxygen involved in the respiration process. In the present study we showed a tendency of Casp-3 to be differentially expressed at day 2, but a significant induction of Casp-3 activity at day 6 indicated a probable cell death induction *via* apoptosis. Genotoxic effects leading to Casp-3 response were already shown (Chen *et al.*, 2000). Apoptosis induction after CeO₂ NP exposure was also reported (Park *et al.*, 2008) and acts as a protective mechanism

when extensive damages occur (Singh *et al.*, 2008). Biological mechanisms inducing NPs genotoxicity are less clear. NPs are reported to have the ability to damage the genetic material by direct physical interactions as well as indirectly by the ROS generation (Li *et al.*, 2013; Peng *et al.*, 2017). In our study, the presence of NPs and ROS within tissues, even in low proportion, may be partly responsible for this genotoxicity leading to apoptosis. However, knowledge regarding the genotoxicity mechanisms of NPs is still in progress and complementary information could arise from future studies at molecular level.

Defense and damage biomarkers taken individually have not yet shown any significant induction of antioxidant process but only a slight tendency to activation after 6 days of exposure. Nevertheless, the integrated vision obtained by a PLS-DA showed a role of oxidative and antitoxic mechanisms as CAT, TAC, ACP and GST activities, as well as LDH, Casp-3 activities and tail length in the discrimination of groups by time and concentration of exposure. This approach allowed the discrimination of exposed organisms from non-exposed ones as a function of time and even as a function of low tested concentrations, highlighting the sensitivity and the usefulness of this approach. The combination of our observations indicated that exposed organisms are not responding in the same way even if concentrations of CeO₂ NPs in the exposure medium are low.

In this study we used OECD recommended CeO₂ NPs and also a recommended genotoxicity test for risk assessment (comet assay) because of the lack of such data in ecotoxicology. Exposure to concentrations close to the PEC values has activated genotoxicity and cellular stress and can be mostly explained by induction of DNA damages involving enzymatic machinery and cell apoptosis that can have further impacts on the global health of the organism. These observations indicate a possible risk for Asiatic clams and underlined the need of further studies in nano-geno-ecotoxicology.

5. Conclusion

Genotoxic effects in *C. fluminea* exposed to low CeO₂ NP concentrations were underlined as earlier as two days of exposure and has increased with time, inducing apoptosis at the highest tested concentration (100 µg/L) after six days of exposure. DNA damage could induce further injuries in organisms and finally lead to death. However, defense mechanisms involved were anti-oxidant and anti-toxic enzymes but also a non-reversible process inducing programmed cell death, called apoptosis. Our results indicate a potential risk to *C. fluminea* exposed to concentrations of CeO₂ NPs close to estimated aquatic PEC values. However, the complexity of the environmental matrix may greatly modify bioavailability of NPs and interactions between NPs and other components. Further studies should be carried out with more realistic exposures such as chronic assays with more complex exposure media, in order to clarify what process occurs at the molecular scale and what is the long-term impact of cerium dioxide nanoparticles on living organisms.

Acknowledgements

Financial supports were provided by the French National Agency (ANR-3-CESA-0014/NANOSALT project) for running costs, CPER Lorraine-ZAM (Contrat Projet Etat Région Lorraine, Zone Atelier Moselle). This work is a contribution to the Labex Ressources 21 (ANR-10-LABX-21-01, Strategic metal resources of the 21st century). The authors gratefully acknowledge CNRS for funding the iCEINT International Consortium for the Environmental Implications of NanoTechnology. KOEHLE-DIVO Vanessa received financial support for salary from French Research ministry. Sharon Kruger is gratefully acknowledged for her English corrections.

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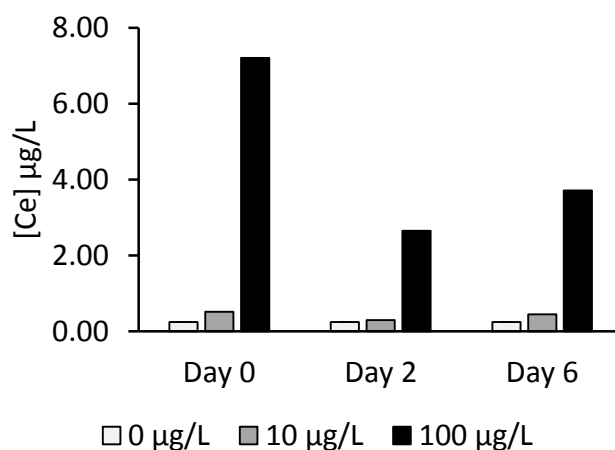


Figure 1: Total [Ce] in the water column after introduction (Day 0) and after 2 and 6 days of exposure to nCeO₂ NPs at 0, 10 and 100 µg/L.

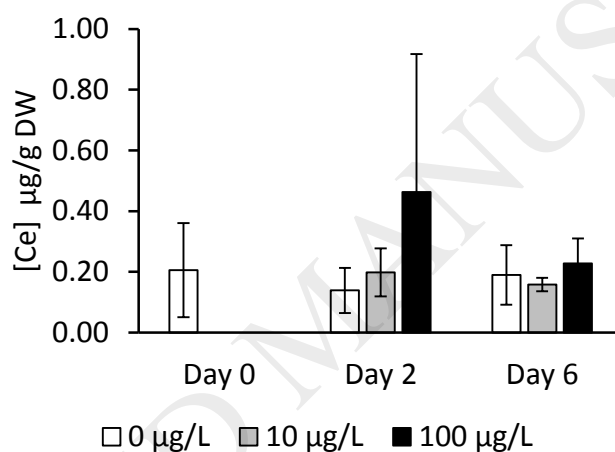


Figure 2: Mean (\pm SD, n=3) total [Ce] concentrations in the digestive gland of *C. fluminea* after 0, 2 and 6 days of exposure to nCeO₂ NPs at 0, 10 and 100 µg/L. No significant differences between groups were pointed out by statistical tests.

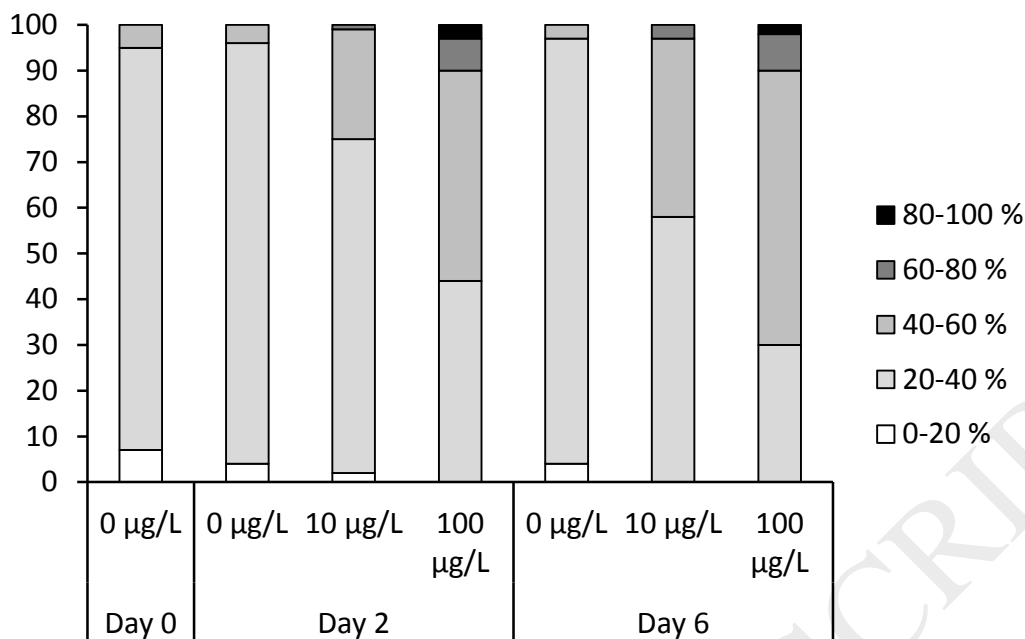


Figure 3: Proportion of cells included in 5 arbitrary classes based on DNA in comet tail (in percentage) measured in digestive gland cells of *C. fluminea* after 0, 2 and 6 days of exposure to nCeO₂ NPs at 0, 10 and 100 µg/L.

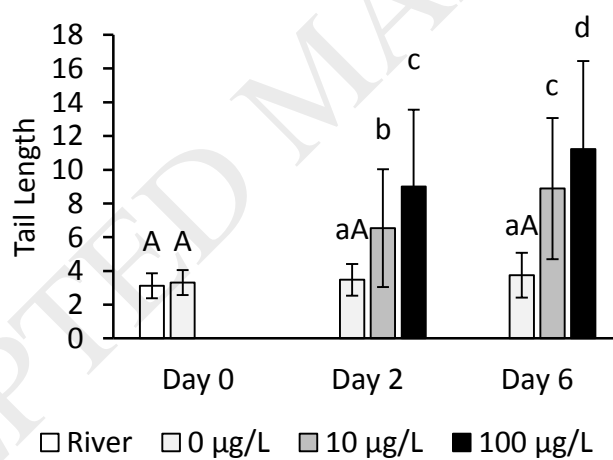


Figure 4: Mean (\pm SD, n=6) Tail length measured in digestive gland cells of *C. fluminea* after 0, 2 and 6 days of exposure to nCeO₂ NPs at 0, 10 and 100 µg/L. Results obtained for the one-way and the two-way ANOVA analysis are shown in capital and lower case letters, respectively.

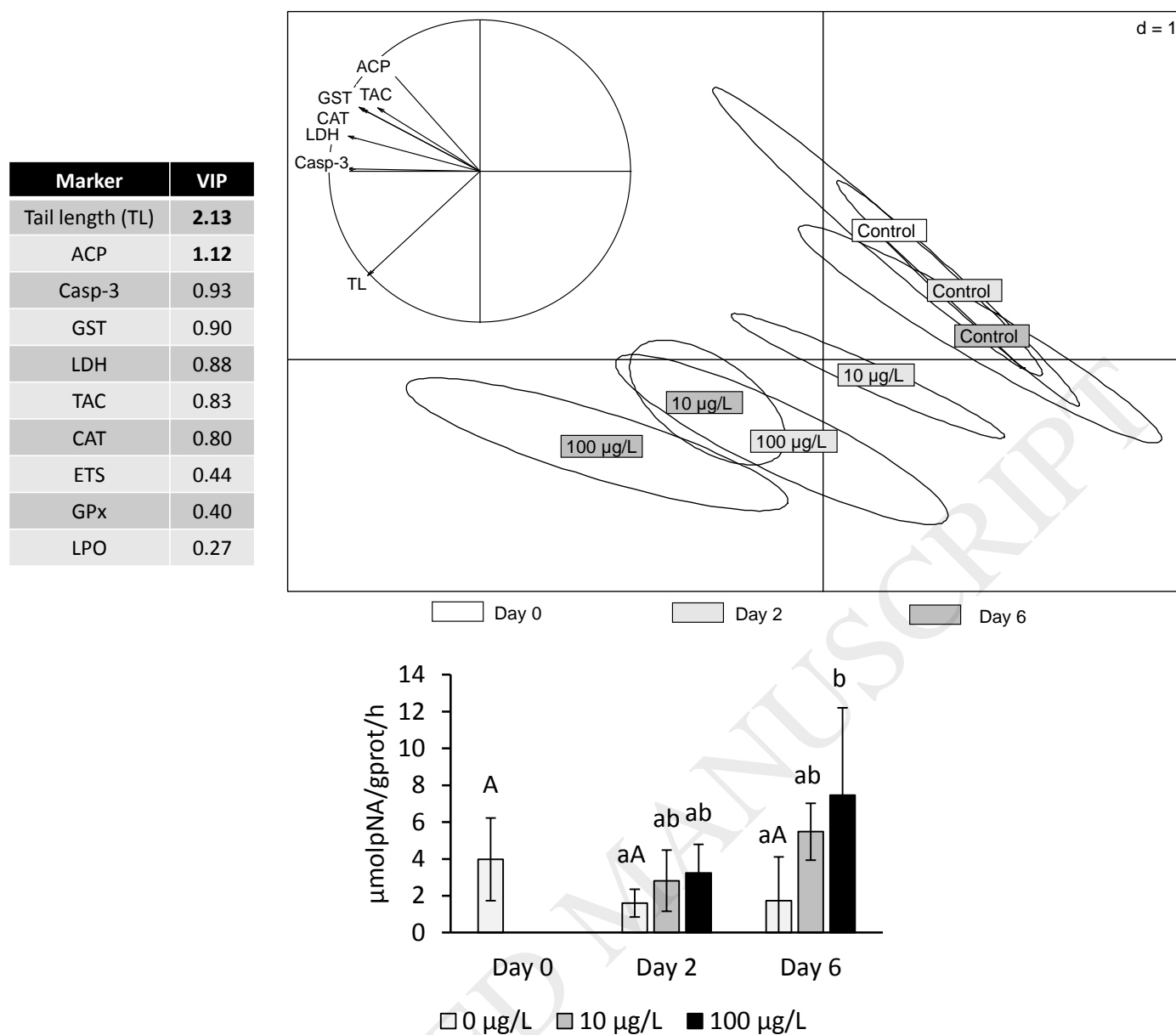


Figure 5: Mean (\pm SD, $n=5$) Casp-3 activity in the digestive gland of *C. fluminea* after 0, 2 and 6 days of exposure to nCeO₂ NPs at 0, 10 and 100 µg/L. Results obtained for the one-way and the two-way ANOVA analysis are shown in capital and lower case letters, respectively.

Figure 6: PLS-DA performed with the whole set of physiological biomarker data and comet tail length values. Only VIP > 0.8 (Variable Importance in the Projection) are shown on the correlation circle.

Table 1: Hotelling T² test performed on PLSDA data set in order to assess effects of time and exposure concentrations on the biomarker battery. Using a threshold of $p \leq 0.05$, significant values were bolded.

Hotelling T ² test		Day 0	Day 2			Day 6	
		Control	Control	10 µg/L	100 µg/L	Control	10 µg/L
Day 2	Control	0.38					
	10 µg/L		<0.001				
	100 µg/L		<0.01	<0.01			
Day 6	Control	0.18	0.3				
	10 µg/L			<0.01		<0.001	
	100 µg/L				<0.01	<0.001	<0.01