1 ***Corbicula fluminea* gene expression modulated by CeO₂**

2 **nanomaterials and salinity**

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Abstract

Cerium dioxide nanomaterials (CeO₂ NM) are used in different fields and incorporated in daily products. Several studies highlighted their effects on organism physiology, although molecular studies remain scarce. NM behavior is strongly dependent on the environment but few data are available using complex exposure media, raising the question of its environmental impacts. The aim of the present work was to assess the toxic potential of three CeO₂ NM in *Corbicula fluminea* at molecular level by RT-qPCR under a more realistic scenario of exposure, in a multistress context at two different salinities (1.5 and 15 psu). *C. fluminea* was exposed for 28 days to pulses of the three selected NM (reference, manufactured and aged manufactured). In bivalves, the gills and digestive gland are two key organs used for ecotoxicological studies. The expression change of 12 genes was measured in control organisms after 28 days in both organs, allowing us to clearly separate the responses for both organs and salinities. As gills come in contact with the environment first, we monitored gene expression at intermediate time points (7, 14 and 21 days) for this organ in order to highlight clams responses to NM and salinity. Two genes (Se-GPx, MnSOD) had a salinity dependent level of expression. HSP70, Se-GPx and Trxr mRNAs presented significant changes in their expressions in presence of NM. This study was completed by using an integrated statistical approach. The exposed organisms were differed more from control at field salinity than those exposed to hyper-saline conditions. At 15 psu, salinity pressure seems to cause the first molecular impact. At 1.5 psu, gene expression patterns allowed the effect of each NM to separate clearly. These results confirmed the usefulness of gene expression studies. Moreover, we highlighted the necessity to assess environmental toxicity of the different form of manufactured NM.

Keywords: *Corbicula fluminea*, gene expression, CeO₂ pristine and aged nanomaterials, mesocosm, salinity

1. Introduction

Engineered nanomaterial (NM) production and use are still growing despite the lack of robust information regarding their environmental fate and effects. Their increasing use necessarily leads to an elevation of their concentration in the environment. Because of their specific physicochemical properties and associated reactivity, their presence in environmental matrices might conduct to interaction with the biota and harmful effects can be expected (Park et al., 2007). Hence, some widely used NMs were listed in OECD priority lists of manufactured nanomaterials for safety evaluation (OECD, 2010). Cerium dioxide nanoparticles are part of the priority lists. As cerium dioxide nanoparticles are mostly found in polishing agents and in fuel additives, their use results in immediate environmental release (Wang & Nowack, 2018a). Their environmental predicted concentrations (PEC) are 2 ng/L in European surface waters and 46 µg/kg in sediments while their predictive no effect concentration (PNEC) in freshwaters was estimated to be 2.62 µg/L. According to the estimated release, no risk is predicted until 2020. However, their production continues to increase substantially (Wang & Nowack, 2018a, 2018b). Therefore, their putative interaction with the biota is of major concern and their effects in relevant biological matrices has to be assessed.

When assessing fate and effects of NM, one major concern lies in the difficulty of understanding and anticipating their behavior in complex matrices. In laboratory experimental studies, NM behavior may be affected by the physico-chemical characteristics of the NM itself (size, coating...), of the surrounding media, by the exposure duration or by the presence and the activity of test organisms themselves, making comparisons between studies particularly difficult. In the case of CeO₂ NMs, the Joint Research Center (JRC) proposed a well-characterized representative cerium dioxide nanomaterial (NM-212) allowing comparison between results from different laboratories and projects (Singh et al., 2014). Providing such a reference material is a useful step for NM characterization and risk assessment, but this

comparative approach is not sufficient. Alteration occurring after NM incorporation in the product and after daily-life use may change their properties and then their toxic potential. It was thus demonstrated that CeO₂ NMs from a fuel additive (Envirox™) were subjected to some modifications after combustion, mimicking changes occurring after consumer use. These modifications affected NM properties such as size, coating, morphology and the presence of surfactant that could considerably change their physicochemical properties (Auffan et al., 2017; Dale et al., 2017). Therefore, alteration and aging have to be investigated to better understand NM potential effects on environment (Auffan et al., 2017).

Aquatic ecosystems are the ultimate sink of a wide range of contaminants. Intentional or incidental release of NMs may enhance their introduction in freshwaters that flow to the ocean with approximately 40 000 km³/year, resulting in NM transportation to marine waters. The modification of salinity between freshwaters and marine systems may influence NM physico-chemistry and needs to be taken into account for a full environmental evaluation (Bertrand et al., 2016). The high ionic strength in salt waters will encourage CeO₂ NMs sedimentation that will be quickly removed from the water column (Keller et al., 2010). Indeed, NM fate and behavior in the field diverge considerably depending on medium complexity but most laboratory studies so far have mostly used simplified and standardized exposure media. Experiments assessing low levels of NM effects using exposure conditions closer to the environmental ones (e.g. water, sediment, food and bacteria) are currently scarce in spite of their relevance (Bottero et al., 2015).

In order to evaluate the potential cellular stresses, analysis of gene expression at mRNA level is an appropriate tool (Pina et al., 2007, Calzolari et al., 2007) because it enables to detect early warning signals of chemical impact (Bigot et al., 2011; Chen et al., 2015). Indeed, first responses of organisms exposed to contamination take place at the cellular level by the change of molecular components. Then, cascade events lead to impact at the level of tissues, organisms

and even at the level of population and community on a longer timescale. Relatively few studies were performed using molecular tools in aquatic ecotoxicology in the case of NM effect assessment and were mainly done on fish, crustacean and algae species. To date, only about twenty studies have monitored their effects on gene expressions in bivalves, which represents key species for the monitoring of aquatic pollution by NMs (Rocha et al., 2015). Some published works reported strong impacts in a variety of pathways as those related to metal exposure (metallothioneins (MT)), to antioxidant and antitoxic defenses (MnSOD, CAT, Se-GPx, GSTpi, HSP70, HSP90, CYP450), to excretion processes (MRP, PGP), to ionic exchange (Na/K ATPase), to immune system (lysozymes), to cellular damages (p53, TNF- α , Ras) and to DNA repair mechanisms (rad51, Gadd45) using a variety of metallic NMs such as TiO₂ (D'Agata et al., 2014; Canesi et al., 2014; Barmo et al., 2013, Banni et al., 2016; Balbi et al., 2014; Guan et al., 2018, Shi et al., 2017), CuO (Ruiz et al., 2015; Châtel et al., 2018; Volland et al., 2018), ZnO (Li et al., 2018), Au (Volland et al., 2015; Renault et al., 2008) and Ag NMs (Ringwood et al., 2010). The only study assessing CeO₂ NMs effects in a bivalve at molecular scale monitored 3 genes expressions (Se-GPx, GSTpi and MT) which allowed to differentiate the NM according to their coating and were related to their antioxidant properties (Garaud et al., 2016). The screening of a battery of genes implicated in numerous different pathways will thus provide useful information about the mechanism of action of NMs.

Despite the observed antioxidant properties of CeO₂ NMs (Chen et al., 2006; Das et al., 2007; Niu et al., 2007; Garaud et al., 2015), several studies highlighted harmful effects on cells of non bivalves eukaryotic organisms. These effects are mainly oxidative stress (Park et al., 2008; Eom & Choi, 2009; Cheng et al., 2013; Lin et al., 2006; Zhang et al., 2011), inflammation (Morimoto et al., 2015), autophagy and apoptosis (Hussain & Garantziotis, 2013).

According to these previous results, our study focused on genes involved in several pathways that could be affected like (i) antioxidant and detoxification mechanisms (SOD, CAT, GPx or

thioredoxin reductase (Trxr), MT, GST, PGP, MRP), (ii) general stress response (HSP70) or even (iii) autophagy (GABARAP). The monitoring of gene expression following NM exposure and salinity changes was done in a freshwater bivalve, *Corbicula fluminea*. *C. fluminea* is an endobenthic filter-feeder organism living at the water-sediment interface and supporting large salinity variations. Two salinities were employed representative from continent (1.5 psu) to brackish water (15 psu) in order to get insight on NM effects at salinities occurring from rivers to sea. In order to get closer to environmental conditions and to mimic interactions occurring in nature, we chose to expose the bivalves in tanks fills with water, sediment and algae as a food source. The present work focused on two organs: the gills and the digestive gland. Gills constitute the first line in contact with environment because of their anatomic position. They ensure multiple exchanges and have to maintain cellular homeostasis (Cerqueira & Fernandes, 2002). The digestive gland is the main organ for biotransformation and detoxification of contaminants.

Thereby, the aim of the study was to evaluate the molecular impacts of NM under a complex exposure scenario on *C. fluminea* exposed to (i) different forms of NM encountered under their life cycle, (ii) different salinities and (iii) the combination of both. Gene expressions linked to antioxidant, antitoxic, autophagy and general stress responses were analyzed after 28 days in gills and digestive gland. As gills are in the first place during exposure, we additionally monitored gene expression at intermediate time points (7, 14 and 21 days) in this organ. The data were analyzed by using both individual and multivariate analyses.

2. Materials and methods

2.1.Sampling and acclimation

Individuals of *C. fluminea* of 15-20 mm length were hand-collected in September 2014 in Argancy in the Moselle River (49° 19'48'58''W, 6 19'81.11 N, France). The collected clams were transported to the laboratory in the sediment and water from the collection site. The physico-chemical parameters of the collection site were pH: 7.3, temperature: 13.9°C, salinity: 1.2 psu. The organisms were then quickly placed into aerated artificial water (Tropic Marine®) at 1.5 psu at 15°C for 3 days. Then, a progressive acclimation process was performed as described in Bertrand *et al.* (2017). Briefly, half of the bivalves were progressively acclimated in a 3-day step by step process to increasing salinity at 5, 10 and 15 psu (9 days). The other half was kept at 1.5 psu water for the same duration. Organisms (n=96) were then placed in each experimental unit for 3 days according to their acclimation salinity, in order to stabilize in the mesocosm unit (photoperiod: 16:8, temperature: 19.3°C, oxygen saturation: 8.14 mg O₂/L, pH: 8.1, redox potential: 232.9 mV).

2.2.Tested nanomaterials

Envirox™ product was purchased from Energenics Europe Ltd. Envirox™ is a fuel additive that enhances combustion efficiency and reduces emission of soot in diesel engines during catalytic combustion (Park *et al.*, 2007). CeO₂ is incorporated in this product at the concentration of 26.7 ± 0.8 g.L⁻¹. Two different states of Envirox™ were prepared and characterized by the CEREGE (UMR CNRS 7600 Aix en Provence, France). Envirox™ suspension was centrifuged for one hour at 20°C at 396 750 x g. After being lyophilized, the pellet was divided into two parts. One was directly used to prepare the Envirox™ exposure media. The other part was combusted in an oven for 20 minutes at 850°C to simulate the car engine combustion and by this way, mimic the aging of Envirox™. The combusted pellet was used to prepare the Combusted Envirox™ exposure media. The characterization of both Envirox™ states was performed by Auffan et al.

(2017). Briefly, Envirox™ particles were monodispersed and spherical with a mean size measured by Transmission Electron Microscopy (TEM) of 7.6 ± 1.2 nm while Combusted Envirox™ particles were polyhedral with a mean size of 19.0 ± 10.4 nm. These sizes were in accordance with those obtained by X-Ray Diffraction (XRD). The crystalline nature observed by XRD was identical between both states and corresponded to cerianite. There was no detectable Ce(III) atoms at both Envirox™ states using X-Ray Adsorption Near Edge Structure (XANES) spectra. The organic matrix surrounding the CeO₂ particles of Envirox™ was characterized by Attenuated Total Reflection-Fourier Transform Infrared (ATR-FTIR) spectroscopy and a complete loss of the organic matrix surrounding the CeO₂ particles was shown after the 850 °C Combustion (Combusted Envirox™). The Dynamic Light Scattering (DLS) technique indicated that the Envirox™ NMs were monodispersed with an average hydrodynamic diameter at 20 nm after dilution in heptane (200 mg/L). The Combusted Envirox™ showed 2 populations of size of 90 and 530 nm (milli-Q water, pH 5.9). In different water salinities made using the TropicMarine™ sea salts, Auffan et al. (2017) showed that the zeta potential was salinity dependent ranging from -25 mV at 0.1 g/L (pH 6.5) to -5 mV at 30 g/L (pH 8.3). No data are available on their hydrodynamic diameters due to the colloidal instability of the aggregates (Auffan et al., 2017). The dissolution was low (< 1.5 %, 48h, with and without light) at each salinities tested (0.1, 1, 10, 30 g/L) for both Envirox™ states and was slightly less in the combusted form (Combusted Envirox™) than in the uncombusted one (Envirox™). The dissolution was also decreased at higher salinity (10 and 30 g/L) compared to lower ones (0.1 and 1 g/L) (Auffan et al., 2017).

NM-212 Representative Manufactured Nanomaterials (Ref: CeO₂-JRCNM02102a/JRCNM02102a004014) were provided by the Joint Research Centre (JRC). This NM was well characterized by Singh et al. (2014). Briefly, NM-212 were polyhedral with irregular morphology and had a non-homogenous size distribution ranging from below 10 nm to well in

excess of 100 nm (TEM). The crystallite sizes assessed by XRD were in the range of 33.3 / 49 nm. The X-Ray Photoelectron Spectrometers (XPS) results showed that the Ce^{4+} was the predominant form (Singh et al., 2014). Measurements by Dynamic Light Scattering (DLS) indicate that they were monodispersed in ultra-pure water and had a mean size of 225.4 ± 92.5 nm. NM-212 powder was used to prepare 15 mL of stock suspension at 1g/L every week during the whole exposure duration. An ultrasonication (90% amplitude in ice tank, 20 seconds) was done on the stock suspension before each introduction in mesocosm as described in Singh *et al.* (2014) guidelines.

2.3. Mesocosm setup

After the progressive acclimation, clams were placed in eight mesocosms (750 x 200 x 600 mm) filled with 14 kg of synthetic sediment (90 % sand, 9 % kaolin, 1 % $CaCO_3$) and 45 liters of artificial water (ultra-pure water and Tropic MarinTM sea salts (Tropicarium Buchshlag Dreieich, Germany)) made at each tested salinity (1.5 and 15 psu). In order to assess the salinity effect, four mesocosms per salinity were used, 1.5 psu corresponded to the field salinity and 15 psu to the hyper-saline condition. Clams were exposed to three treatments: NM-212, EnviroxTM and Combusted EnviroxTM for each tested salinity. Eleven injections of 4.1 mg CeO_2 NM for each treatment were made into the mesocosm water column (every 3 days) in order to reach a final concentration of 1 mg/L after 28 days of exposure. One uncontaminated mesocosm per salinity (Controls) was set up. The organisms were fed during the whole experiment with 1×10^5 cells *Chlorella sp*/mL. Physico-chemical parameters in the water column (temperature, salinity, pH, redox potential and dissolved oxygen) were continuously monitored during the whole duration of exposure.

2.4. RT-qPCR

Digestive gland and gills were used at 28 day for gene expression assessment in the different tissues and a time scale analysis of gene response was also conducted for gills at 7, 14 and 21 days.

2.4.1. Preparation of RNA extract

Both organs were dissected, snapped in frozen liquid nitrogen and stored at -80°C until subsequent analysis. Total RNA was extracted from tissues using an RNeasy kit (Qiagen) according to the manufacturer protocol. After extraction, contaminated DNA was digested with DNase I (Sigma Aldrich) and total RNA was purified with RNeasy kit. RNA purity and quantity were assessed by optical density measurements (OD 260 nm and OD ratio 260/280 and 260/230) and RNA integrity was checked using Bioanalyser 2100 (Agilent, CA, USA).

2.4.2. RT-qPCR analysis

RT-qPCR was performed on 13 genes. The primer sequences are listed in Supporting Information Table S1. Primers were designed using Primer3Plus. The cDNA was synthesized in a final volume of 20 µl by using 900 ng of RNA, 2.5 µM of random hexamer primers, and SuperScript® III reverse transcriptase according to the manufacturer's instructions (Invitrogen). The qPCR reaction was conducted with 45 ng of cDNA, water (no template control) or total RNA (45 ng/reaction; no RT control) as a template, from 200 to 300 nM of primers (see table S1) and Fast SYBR® green master mix (Applied Biosystem®, CA, USA) in a final volume of 20 µL. The cycling conditions were 20 s at 95°C, followed by 40 cycles of 3 s at 95°C and 30 s at 60°C. Specificity of amplifications was checked using a melting curve generated at the end of the reaction and the size of the amplicons was verified on agarose gel. All PCR amplifications were performed on each biological replicate using the StepOnePlus RT-PCR system (Applied Biosystems®). Gene expression levels were analyzed using the relative

quantification method ($\Delta\Delta C_t$) (Livak *et al.*, 2001) with the ribosomal protein S3 gene as a reference for normalization. Condition control 1.5 psu was chosen as reference condition for the gills' relative gene expression presentation. The final treated/control ratio and pooled standard deviation were calculated based on the mean ΔC_t of biological replicates. Due to qPCR plate constraint, the four times of exposure were treated separately.

2.5. Cerium quantification in bivalves

The digestive glands of organisms (n=6 per treatments) were dissected and placed in oven (45°C) during 48 h. After dry weight determination, digestive glands were added with 1 mL of HNO₃ (32.5 %) and placed at 65°C during 48 h to release total Ce contained in the tissues. Quantifications of Ce into homogenate were performed by Micropolluants Technologie S.A. (Metz, France) using ICP-MS (Agilent 7700, Agilent Technologies France). Results were expressed in ng Ce/ g dry weight.

2.6. Statistical analysis

Statistical analyses have been conducted using R (R Core Team, 2014). For the individual gene expression data, homoscedasticity and normality have been checked by Levene and Shapiro tests respectively. As the conditions were respected, a two-way anova test was conducted to assess treatment and salinity effects. We only considered variations between groups that presented a fold change (FC) between groups compared to the control 1.5 psu $\pm$ 1.5 (FC_{log2} $\pm$ 0.58). Tukey HSD post-hoc analysis was performed when significant differences were depicted with the anova.

The multivariate structure of gene expression dataset was required to apply a specific set of statistical tools. First, a manova - multivariate ANOVA was set up to assess if significant differences accorded to the tested factors could be depicted. These results help us to design each Partial Least Square Discriminant Analysis (PLS-DA), that highlight genes explaining those

differences. Only a variable (*i.e.* gene) with a VIP (Variable Importance in Projection) score above 0.8 was considered. Finally, Hotelling T² tests were performed on PLS-DA results to explore more thoroughly differences between exposure conditions.

3. Results

3.1 Comparative gene expression between gills and digestive gland

After 28 days, the set of monitored genes allowed us to clearly differentiate digestive gland and gill gene expressions in control organisms (Fig. 1A). The manova analysis pointed out the significant impact of both salinity ($F=16.6_{2,14}$ df, $p < 0.001$) and organ ($F=102_{2,14}$ df, $p < 0.001$) but no significance for their interaction ($F=0.8_{2,14}$ df, $p= 0.48$). These results are confirmed by Hotelling tests (Fig. 1B). Digestive glands presented more mRNA for MT, CAT, Se-GPx and to a lesser extent GST-Pi whereas gills had more mRNA for MRP1, PGP and HSP70. MnSOD transcript appeared more pronounced in both organs in the organisms exposed to the highest salinity (15 psu) compared to the field salinity (1.5 psu).

Manova analysis made on the entire gene expression data from non-exposed and exposed organisms after 28 days (Fig. 2) indicated significant differences among organs ($F=542_{2,68}$ df, $p < 0.001$) and contaminations ($F=2_{6,138}$ df, $p=0.037$) while their interaction was not significant ($F=1.6_{6,138}$ df, $p=0.16$). Pairwise comparisons demonstrated a clear organ effect, but no contamination effect (Table S2). NM responses seemed nonetheless to be more pronounced in gills with both EnviroxTM that tend to respond differently from NM-212 and control, while in the digestive gland, no tendency was observable.

3.2 Temporal monitoring in gills

3.2.1 Individual gene expression

Four genes (HSP70, MnSOD, Se-GPx and Trxr) presented significant changes during the time course of the experiment (Fig.3). However, no clear patterns were evidenced considering neither salinity nor exposure to NMs.

For HSP70, mRNA levels in Combusted Envirox™ were significantly different from control, NM-212 or both, at both salinities and at all time points except at day 28 at 15 psu. Even if it is not systematic, we observed that both Envirox™ products induced similar response, often different from the response in the two other conditions. Actually, Envirox™ products tended to lead to decreased mRNA levels, except at day 14. With respect to salinity, we did not observe any difference except at day 21, when every exposure condition led to decreased mRNA levels at 15 psu.

For MnSOD, we did not record any change in mRNA levels in organisms exposed to the NM conditions at each salinity. However, the highest salinity (15 psu) induced an increase of mRNA levels in each exposure condition at 7 and 14 days and in Envirox™ at 28 days while a decrease is observed in combusted Envirox™ at day 21.

For Se-GPx, mRNA levels significantly decreased (at least nine-fold) in NM-212, Envirox™ or both in most time points at 1.5 psu. At 15 psu, the only difference was found at 21 days for the combusted Envirox™. When comparing results obtained between both salinities, we observed, at 15 psu, a systematic decrease in the control relative to the control at 1.5 psu and a decrease in combusted Envirox™ at day 14 and day 28, relative to the same condition at 1.5 psu. To summarize, the overall response pattern to both NM conditions and salinity was a decrease, except in combusted Envirox™ where the decrease did not occur at day 7, 14 and 28 at 1.5 psu and at day 21 at 15 psu.

For Trxr, mRNA levels did not change at day 7, 21 and 28 (except a significant difference between NM-212 and combusted Envirox™ at 15 psu). However, at day 14 for both salinities, both Envirox™ products led to a significant increase of mRNA levels when compared to control. For 15 psu, NM-212 also led to an increase when compared to the control. When comparing results obtained between both salinities, we did not observe any change except at day 7 for Envirox™ at 15 psu, where an increased level was recorded relative to the same condition at 1.5 psu.

The other genes presented fewer or no expression changes during the exposure (Table S3 & S4). Briefly, PGP, Beta-actin and CAT mRNA levels did not change during the exposure neither between contaminations nor between salinities. The other genes (CuZnSOD, MT, GST-Pi, GABARAP and MRP1) presented some changes on just a few dates.

3.2.2 PLS-DA analysis

Manova analyses have been conducted at each sampling time and showed some significant effects according to the salinity ($F=40_{2,32}$ df, $p < 0.001$ for 7 days; $F= 89_{2,38}$ df, $p < 0.001$ for 14 days; $F=12.5_{2,39}$ df, $p < 0.001$ for day 21; $F=53.5_{2,31}$ df, $p < 0.001$ for day 28), to contamination ($F=2.46_{6,66}$ df, $p= 0.037$ for 7 days; $F= 7.86_{7,78}$ df, $p<0.001$ for day 14; $F= 1.96_{8,80}$ df, $p= 0.096$ for day 21; $F=3.66_{6,64}$ df, $p= 0.004$ for 28 days) and to their interaction ($F=3.2_{6,66}$ df, $p= 0.007$ for 7 days; $F=1.3_{6,78}$ df, $p= 0.28$ for day 14; $F=5.7_{6,80}$ df, $p < 0.001$ for day 21; $F= 2.9_{6,64}$ df, $p=0.015$ for day 28). In order to focus on the effects of NM, we decided to analyze the results obtained for each salinity separately.

PLS-DA analyses were summarized in Fig. 4 and Fig. 5 for 1.5 and 15 psu respectively and pairwise comparison results were shown in Table S5. The PLS-DA approach indicated that NM exposures induced higher modifications of gene expression profile at field salinity (1.5 psu) than in hyper saline condition (15 psu), whatever the sampling date.

320 For day 7 at 1.5 psu, all conditions were different from each other except control and NM-212
321 ($p= 0.11$). The horizontal axis clearly separated Control and NM-212 groups from both
322 Envirox™ products, with both Envirox™ products presenting less CAT, HSP70, GST-Pi, Beta-
323 actin and GABARAP mRNA. The vertical axis separated both Envirox™ exposures, with PGP
324 and MRP1 being higher in Combusted Envirox™ while the opposite was observed for MnSOD
325 mRNA.

326 After day 14 at 1.5 psu, all exposures appeared different from each other. The horizontal axis
327 separated both Envirox™ from Control and NM-212 treatments with more MnSOD, MT,
328 HSP70, GST-Pi and Trxr expressed in both Envirox™. The vertical axis separated Control from
329 NM-212 due to Se-GPx mRNA content.

330 After 21 days at 1.5 psu, all groups were different from the others except Envirox™ and NM-
331 212. The horizontal axis pointed to differences between NM treatments with NM-212 and
332 Envirox™ having less MnSOD, CuZnSOD, CAT, Beta-actin and HSP70 than the combusted
333 form. The other axis separated Control exposure from the others with control presenting more
334 MRP1, GST-Pi, Se-GPx but less MT mRNA than NM exposed organisms.

335 After 28 days at 1.5 psu, Envirox™ and NM-212 were different from control mainly due to Se-
336 GPx expression that was higher in Control. The only other difference appeared between NM-
337 212 treatment and Combusted Envirox™ with NM-212 presenting more MRP1, GABARAP,
338 HSP70, MnSOD, Trxr while less Se-GPx mRNA. The vertical axis separated both NM-212 and
339 Envirox™ from control with less Se-GPx mRNA measured in these treatments.

340 Regarding the overall pattern at 1.5 psu, two gene expressions (MnSOD, HSP70) were
341 systematically discriminant for the PLS-DA models at all exposure times. All NM conditions
342 appeared different from control condition during all exposure time except for NM-212 at 7 days
343 and Combusted Envirox™ at 28 days.

344 For 7 days at 15 psu, no significant difference was observed except for Envirox™ and control.
345 The horizontal axis separated control from Envirox™ ($p < 0.05$) and Combusted Envirox™
346 (NS, $p = 0.06$) with control presenting less Se-GPx mRNA but more HSP70 and GABARAP
347 mRNA. Gene expression of Trxr, PGP and MRP1 also allowed a separation between control
348 and Envirox™.

349 After 14 days at 15 psu, Envirox™ appeared significantly different from control while the only
350 other difference pointed out was between NM-212 and Combusted Envirox™. Envirox™
351 presented more HSP70, Trxr, GST-Pi, CuZnSOD and MnSOD mRNA than Control. The
352 vertical axis underlined some differences between NM-212 and Combusted Envirox™ with this
353 last group presenting more PGP, MRP1 and Se-GPx mRNA.

354 After day 21 at 15 psu, the only group significantly different from the others was Combusted
355 Envirox™. The horizontal axis allowed a clear separation between Combusted Envirox™ and
356 the other groups, Combusted Envirox™ presenting less HSP70, GABARAP, MnSOD and
357 CuZnSOD mRNA but more MRP1 mRNA. The vertical axis participated to this separation only
358 between Combusted Envirox™ and Control/Envirox™, by modulation of GST-Pi and Se-GPx
359 mRNA content.

360 After 28 days at 15 psu, the model that was mainly explained by HSP70, CuZnSOD,
361 GABARAP, PGP, MRP1 and Beta-actin gene expressions, pointed out no clear pattern.

362 Regarding the overall pattern at 15 psu, one gene (HSP70) appeared systematically important
363 for the PLS-DA models in all sampling dates. Differences from control were observed from 7
364 to 14 days for Envirox™, from 14 to 21 days for Combusted Envirox™ while never for NM-
365 212.

3.3. Bioaccumulation of Ce into digestive gland of organisms

After 7, 14, 21 and 28 d of exposure, total Ce concentrations in the digestive gland of bivalves exposed in the different mesocosms are illustrated in Figure 6.

At 1.5 psu (Figure 5-A), significant greater Ce concentrations were measured into the organisms exposed to Envirox™ and NM-212 compared to Control and Combusted Envirox™ only after 28 days. At 15 psu (Figure 5-B), only organisms exposed to NM-212 exhibited significant higher accumulation of Ce compared to Control after 7, 14 and 28 days of exposure.

Comparing both tested salinities, digestive Ce accumulation was greater in the organisms exposed to Combusted Envirox™ at 14 days and to Envirox™ at 21 and 28 days, under field salinity (1.5 psu) than under hyper-saline condition (15 psu).

4. Discussion

The aim of the present work was to evaluate the potential toxicity of CeO₂ NMs in *C. fluminea* exposed to different salinities (1.5 & 15 psu) by monitoring their impact at molecular scale. In order to increase the environmental realism, a complex exposure (indoor mesocosm) was designed with pulse dosing by the addition of standardized (NM-212) or commercially available NM (Envirox™) undergoing or not an aging process in order to take into consideration different forms of NM during their life cycle.

4.1. Comparative gene expression between gill and digestive gland

Our results showed a strong difference of gene expression between gills and the digestive gland in the freshwater Asiatic clam. Several studies have documented differential expression of genes or differential levels of enzymatic activities between both organs in other freshwater and marine bivalve species (Doyen *et al.*, 2006, 2008; Bigot *et al.*, 2009, Cossu *et al.*, 1997; Metian *et al.*, 2008; Guo *et al.*, 2017), relative to their physiological functions and their implication in stress response. In our study, gills presented enhanced mechanisms of direct action against xenobiotics through excretion pathways (MRP1, PGP) or by the role of chaperone protein (HSP70). As gills are in direct contact with surrounding media, they are vulnerable to particles and overexpress these kinds of early defenses. The digestive gland expressed higher content of MT, CAT, Se-GPx and GST-Pi mRNAs that are involved in detoxification mechanisms through complexation or interactions with xenobiotics (*eg.* metals, drugs) or oxidative components. As they are able to accumulate various contaminants including NMs (Canesi *et al.*, 2012; Canesi & Corsi, 2016), they are the main organ for detoxification and biotransformation, explaining the expression of such mechanisms of stress response. The strong difference of expression between both organs remained after the application of NM contamination during 28 days. In our study, we selected gills in order to monitor the temporal variations of gene expression throughout the exposure duration. As gills are in direct contact with surrounding media, they

may react first to environmental modifications. In the bivalve *Dreissena polymorpha*, Navarro *et al.* (2011) reported that the expression of genes involved in metal stress response was affected in gills earlier than in digestive gland.

4.2. Effect of salinity and NM in gills throughout the exposure duration

Salinity appeared to be the main variable explaining differences in gene expression in gills. MnSOD mRNA level was increased at high salinity and could be correlated to ROS generation (An & Choi, 2010). The generation of H₂O₂ by MnSOD should have required detoxification mechanisms such as Se-GPx or CAT. However, Se-GPx mRNA decreased in hyper-saline condition while CAT mRNA level did not change. As MnSOD represents a small proportion of total SOD inside cells, its increasing expression may not be sufficient to require more CAT or Se-GPx, even though it indicates some mitochondrial perturbations.

Once the salinity effect has been set aside, we could reveal the effect of CeO₂ NMs that differed according to the type of NM. When *C. fluminea* were exposed to NMs at field salinity (1.5 psu; i.e no salt stress), overall gene expression in gills was affected by NM-212 from 14 days until the end, and at each time point by both Envirox™ products (except in Combusted Envirox™ at 28 days). Even if it was not systematic, the three NMs gave different patterns of response. The main trend in all NM conditions was a decrease of the expression for most of the studied genes. Exposure to NM-212 and Envirox™ induced the decrease of Se-GPx mRNA compared to the control at field salinity. Ciofani *et al.* (2013) also showed a down-regulation of genes coding for some members of the GPx family, but also a down-regulation of other genes involved in cellular protection. They suggested that CeO₂ NM presented a potential role of ROS scavenger. Garaud *et al.*, (2015; 2018) also supported this possibility in *Dreissena polymorpha* since CeO₂ NM were already reported as having antioxidant properties such as SOD, CAT, peroxidase and oxidase capacities (Singh, 2016). On the contrary, CeO₂ NMs were also reported to damage DNA molecules (Koehl-Divo *et al.*, 2018; Auffan *et al.*, 2009) but sequences of genes involved

in DNA damage response pathway are not available so far in *C. fluminea* and therefore their mRNA levels have not been evaluated here. However, this decrease of Se-GPx mRNA during the entire time of the experiment rather supports the antioxidant properties of CeO₂ NMs. For the combusted form, only weak impact on Se-GPx gene expression has been noted, while a decrease of HSP70 mRNA content in most sampling dates was monitored. The inhibition of HSP70 synthesis was already observed after metal exposure and was correlated to inhibition of stress protein synthesis (Boutet *et al.*, 2003; Luo *et al.*, 2014). HSP70 down-regulation was also shown after Ag NPs exposure and could be related to the induction of a caspase-independent apoptosis (Gomes *et al.*, 2013). The differences observed between both Envirox™ products showed that transformations occurring during NM life cycle had significant impacts on *C. fluminea* gene expressions. The 850°C combustion led to modifications on NM physico-chemical properties (surrounding matrix, size and form) that can considerably change their bioavailability and their potential effects on living organisms (Auffan *et al.*, 2017). The measurements performed in the digestive gland of the exposed organisms suggest that the 850°C combustion decreased the bioavailability of the contaminant since it was the only one that has not been bioaccumulated in this organ. The absence of bioaccumulation in the digestive gland does not preclude a contact of the contaminant with the gills, that may explain the observed effects. NM physico-chemical properties and biological effects monitored are difficult to link accurately in this study.

When *C. fluminea* were exposed to NMs at high salinity (15 psu; salt stress), we showed an overall absence of response compared to the control. Salinity may have strongly influenced and changed the behavior of NMs. In our study, it seems that the increase in salinity led to a removal of biological response, with gene expressions close to the control. NM interactions with high concentrations of salt (Cl⁻ ions; Devoille *et al.*, 2018) may enhance their aggregation and sedimentation (Baker *et al.*, 2014). The rapid sedimentation of NMs in our experimental unit

451 may have reduced their filtration by *C. fluminea*. This may explain why gills seem not to react
452 in the present context of contamination: salinity may have limited NM bioaccessibility and
453 bioavailability. Another explanation is that salinity may have stressed the bivalves as shown in
454 Bertrand *et al.* (2017). The isolation of organisms from their surrounding environment was
455 demonstrated as a response against salinity stress (Kholodkevich *et al.*, 2009). Indeed, some
456 bivalves are able to escape from stressful conditions by closing their shell valves (Ferreira-
457 Rodriguez & Pardo, 2016; Tran *et al.*, 2003). Isolation of organisms might cause a drastic
458 reduction of general metabolism (Carregosa *et al.*, 2014) and might affect gene expression level
459 by decreasing it due to its high energetic cost (Phillip *et al.*, 2012). In the same way, in hyper-
460 saline conditions, osmoregulation processes may have been more intensively required and the
461 regulation of metabolic trade-offs may have led to the result that gene expression was disturbed
462 and some pathways were depressed or shut down. This may explain why the usual defense
463 mechanisms were not responding at high salinity in comparison to what we observed at field
464 salinity.

Conclusion

This study has demonstrated significant impacts of CeO₂ NMs on *C. fluminea* gene expressions that were salinity dependent. The set of monitored genes was sufficient to clearly differentiate responses to NM contamination at field salinity. However, it did not reveal NM effects at hyper-saline exposure (15 psu). This could be due to the fate and behavior of both NM or/and organism physiology. Since they should respond in priority to osmotic stress in order to conserve cellular functions, an adequate reaction against contamination should not have been implemented.

While few molecular studies have been performed using NMs (Wiesner & Bottero, 2017), classical molecular observations of NMs effects were linked to the over-expression of oxidative stress related genes (Klaper *et al.*, 2014). As CeO₂ NMs were reported to have both pro- and anti-oxidant roles, the decrease of gene expressions observed in our study could be linked both to harmful and beneficial effects. Nevertheless, the significant modifications of expression profiles by NMs remain worrisome especially since the organisms were exposed in complex media using a final concentration below the no observed effect concentrations (NOECs) values of CeO₂ NMs determined in aquatic invertebrates (Hoecke *et al.*, 2009). In addition, our results often showed significant differences between NM-212 and Envirox™ conditions, with more pronounced effects in the last one originating from a commercial form. This finding reinforces the need to focus on NMs as a part of daily-life products. In the same way, some significant differences were shown between pristine and aged Envirox™ products, highlighting the need to consider the entire life cycle of NMs. While previous nanotoxicology studies have allowed the understanding of many essential features of nanomaterials, there is always a great lack of information regarding their environmental fate and impact on organisms.

The integration of “omic” tools in aquatic ecotoxicology have been done primarily with species having their full genome sequenced, while as yet only a few bivalves have been sequenced despite their ecological relevance. Nonetheless, such an approach provides a valuable

assessment of the molecular responses of organisms to emergent contaminant such as NMs (Châtel *et al.*, 2018; Wiesner & Bottero, 2017). In this study, we provided fundamental knowledge required to understand molecular mechanisms by comparing two key organs (gills and the digestive gland) before assessing NM toxicity that might be useful to aid comprehension in AOP approaches. Furthermore, the level of mRNAs was actually impacted by NMs in each condition highlighted the usefulness of this approach in such complex exposure design. The integrative vision of effects also showed the importance of weak expression modifications to reveal differences between NMs. By this study, we have contributed to deepen this knowledge, demonstrating the necessity to perform exposures closer to environmental scenarios (*i.e.* media complexity, low and cumulative NM concentrations).

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Conflict of Interest

The authors declare that they have no conflict of interest.

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