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RESEARCH REPORT

Combined effects of surface waters and CeO nanoparticle in zebra mussels

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Abstract

Cerium oxide nanoparticles (nCeO) are currently used in many sectors of our economy, for instance as fuel additives and in ceramics for catalytic converters. As a result, there are concerns about their release and resulting toxicity in the aquatic environment. The purpose of this study was to examine the bioavailability and toxicity of nCeO and Ce(IV) in zebra mussels (*Dreissena polymorpha)* in various types of surface water differing in organic matter, conductivity and pH. Mussels were exposed to 100 µg/L Ce as either nCeO or Ce(IV) for 96 h in 4 types of water: 1) green water (high conductivity and low total organic carbon), 2) brown water (low conductivity and high natural total organic matter), 3) 10% municipal effluent (high conductivity and high anthropogenic organic matter) and 4) controls, which consisted of dechlorinated tap water. After the exposure period, the mussels were analyzed for morphological changes, resistance to survive in air, triglycerides (fat reserves), oxidative stress (arachidonate cyclooxygenase and lipid peroxidation) and DNA damage. Evidence of aggregation was observed with nCeO in most types of water, with the exception of the diluted municipal effluent. The data revealed that some of the effects of nCeO were influenced by surface water properties. The mussels were more sensitive to air emersion when exposed to nCeO in green water but not in the other water types and Ce(IV) to all types of water, although a marginal decrease was observed in mussels co-exposed to the diluted municipal effluent. A general decrease in oxidative stress and lipid levels was observed with both forms of Ce and all water types. Ce(IV) in brown water did not reduce the levels of DNA strand breaks compared with the controls. In conclusion, the sublethal toxicity of nCeO could be modulated by the surface water from which the nanoparticle is suspended.

Key Words: cerium; genotoxicity; lipids; nanoparticles; oxidative stress; toxicity

Introduction

Inorganic nanoparticles have attracted commercial attention owing to their special properties. For example, cerium oxide nanoparticles (nCeO) are widely used in the biomedical area as antioxidants in biological systems (Brunner *et al*., 2006), in the textiles and color industry (Dawson, 2008) and as an additive in diesel fuel and in catalytic converters (Trovarelli *et al*., 1997; Jemec *et al*., 2012). Ce is the most abundant element in the rare earth family and is increasingly used by our economy owing to the high redox properties of Ce(III)/Ce(IV). The many industrial applications of this element have given rise to some concerns about its release into the environment and the possible impacts on aquatic life. Indeed, Ce pollution originates from solid waste (electronic device disposal) leachates, industrial (ceramic industry) waste, municipal sludge and wastewaters (Limbach *et al*., 2008; Keller and Lazareva, 2014). The persistence of nCeO and other nanoparticles in the aquatic environment, bioavailability and toxicity are an important part of the risk assessment of nanomaterials (Gagné *et al*., 2007). Ce nanoparticles have a positive charge at the surface with a zeta potential of 30 mV, making this particle susceptible to aggregation from surface charge neutralization in a low-salt environment usually found in freshwater. However, the occurrence of natural organic matter was shown to limit aggregation by capping the nanoparticle (Grillo *et al*., 2015). Humic acid, a major constituent of organic matter, was used to stabilize engineered nanoparticles from aggregation. According to a recent survey, 6% of the Ce was released in municipal plant effluent and in the receiving waters (Limbach *et al*., 2008), perhaps through stabilization

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by the dissolved organic matter. The issue of the interaction of not only the organic matter content but other properties such as conductivity, pH and suspended matter could complicate the behavior of the nanoparticle and resulting toxicity in commonly found types of surface water.

The aquatic toxicity of Ce and nCeO revealed that exposure to Ce leads to detection in fish liver and that it is not very toxic given the antioxidant properties (Van Hoecke *et al*., 2009; Gonzalez *et al*., 2014). The antioxidant properties arise from the Ce(III) which could form at the surface of nCeO. Ingestion of nCeO could compromise the digestive system in bivalves, which readily feed on suspended matter, including nanoparticles (Gagné *et al*., 2007; Canesi *et al*., 2012). Indeed, bivalves are considered species at risk of nanoparticle contamination because they are filter feeders, sessile and live for a relatively long time. There is some evidence that Ce could influence DNA integrity depending on the redox properties of nCeO. On the one hand, Ce(IV) could lead to DNA scission in the presence of sodium triphosphate, which forms the basis of a DNA assay (Wu *et al*., 2005). On the other hand, the presence of Ce(III) could protect DNA against oxidative stress as observed in Caco-2 cells *in vitro,* which lead to a decreased amount of oxidized nucleotides (Vila *et al*., 2018). Given that the organic matter or other ligands could interact at the surface of nCeO, the equilibrium between Ce(III)/Ce(IV) at the surface of the nanoparticle could be changed and modulate its toxicity in freshwater mussels or other organisms. Hence, the toxic outcome of nCeO in representative surface waters with differing chemistries, and the nature of organic matter are difficult to predict at this time.

The purpose of this study was therefore to examine influence of 4 types of surface waters on the behavior of dissolved Ce and toxicity of nCeO in the freshwater mussel *Dreissena polymorpha*. The surface water types were green (high conductivity, high pH and low in total organic carbon), brown (low conductivity, low pH and high natural organic matter), a 10% dilution of municipal effluent in green water (high conductivity, low pH and high “urban” organic matter) and the laboratory control water. Health effects were determined by monitoring mortality during exposure to nCeO and Ce(IV) because nCeO mainly consists of Ce(IV) from CeO_2. Resistance to air emersion and weight loss were also evaluated to determine the mussels’ resilience against an additional stress. Sublethal effects comprise triglyceride levels (energy reserves), oxidative stress and DNA damage. An attempt was made to compare the effects of nCeO and Ce(IV) in the 4 types of surface water on the freshwater mussels.

Materials and Methods

Cerium oxide nanoparticle

A stock solution of Cerium oxide nanoparticle (CeO_2) from Sigma Chemical Company (Ontario, Canada) was used in this study. According to the manufacturer’s specifications, NP CeO_2 suspension has a size ≤ 25 nm and forms a homogeneous suspension at a concentration of 10% wt in water. For the exposure regime, zebra mussels (*Dreissena polymorpha*) were exposed to a nominal concentration of 100 µg/L total Ce as either nCeO or Ce(IV) in dechlorinated tap water (controls), and three other types of surface water as described below. The Ce concentration was well below the reported solubility for $Ce(SO_4)_2$ at 9.84 g/L (20 ^oC). The hydrodynamic diameter and zeta potential of nCeO nanoparticles were measured at least three times using a dynamic light scattering instrument (Mobius Instrument, Wyatt Technologies, Santa Barbara, CA, USA) operating with a laser at a wavelength of 532 nm. The instrument was previously calibrated with standard suspensions of silver nanoparticles (calibrators for electron microscope) from TedPella (USA).

Three types of water were sampled in the St. Lawrence River: green, brown and a 10% dilution of physico-chemical–treated municipal effluent to simulate urban pollution. Controls consisted of UV and charcoal-treated dechlorinated tap water. In each of the surface waters, total organic carbon (TOC), pH and conductivity were determined according to standard methods (APHA, 2010). Green water is characterized by a relatively high conductivity (200-290 µS/cm), low TOC (< 4 mg/L) and a slightly alkaline pH (pH > 7.2) (Table 1).

Table 1 Surface water basic properties

Water Type	Conductivity (µS/cm)	Total Suspended Matter (> 0.7 µm; mg/L)	pH	Total Organic Carbon (mg/L)	% Measured Ce (µg/L)[1]
Aquarium	310 ± 10	<1	7.97 ± 0.3	2.1 ± 0.1	75%
Brown	83 ± 5	5 ± 1	8.12 ± 0.4	6.5 ± 0.4	100%
Green	276 ± 25	13 ± 1	8.12 ± 0.3	2.9 ± 0.2	82%
Effluent 10%	375 ± 20	3 ± 0.5	8.07 ± 0.5	3.5 ± 0.3	78%

Measured concentration in the aquarium after 1 h equilibrium (aeration, 15 ^oC). The data are expressed as the percentage of added total Ce as nCeO

Brown water is characterized by low conductivity (100–160 µS/cm), higher TOC levels (> 5 mg/L) and

a slightly acidic pH (pH < 7). The surface waters (20 L) were collected 2–3 weeks before the onset of exposure; they were stored in the dark at 4 °C until the exposure experiments at 15 °C. The total levels of Ce in the 4 surface waters were determined after 1 h of dissolution of nCeO using ICP-MS spectrometry after acidification with 1% v/v HNO_3 seastar grade BC. Zebra mussels (n = 30) were exposed to each type of water with and without 100 µg/L total Ce as either nCeO or $Ce(SO_4)_2$ (CeIV) for 96 h at 15 °C under constant aeration. The control mussels were exposed to each type of surface water only. The exposure experiment was repeated 3 times. The surface waters were not renewed and the mussels were not fed during the exposure period. A subgroup of 10 mussels was kept aside for air-stress survival while the remaining 20 mussels were processed as follows. For tissue Ce assessments, a subgroup of 10 mussels were placed in clean aquarium water overnight as a depuration step and the soft tissues were removed for Ce determination using ICP-mass spectrometry as described above. The tissues were acid-digested in 10% HNO3 at 70-80 °C for 12 h and diluted to 1% with MilliQ water. For the biomarkers, the remaining group of 10 mussels were processed on ice for the shell length, total weight and the soft tissues weight were determined. The soft tissues were then stored at -85 °C with the homogenization buffer (at 20% weight/volume). The homogenization buffer consisted of 100 mM NaCl, 25 mM Hepes-NaOH, pH 7.4, 1 µg/mL apoprotin and 1 mM dithiothreitol.

Air survival test

After the exposure period, 10 mussels were kept aside to determine the air-time survival as previously described (Gagné *et al.*, 2015). In brief, the mussels were weighed and the shell length determined, and they were placed individually in open plastic containers at 80% humidity at 20 °C. They were maintained as such and weighed each day until evidence of mussel mortality (opened shells, no decrease in mussel weight). The time of death in days was recorded and the data were expressed as the mean day of mortality for each individual over the 12 treatment groups: 4 types of surface water alone, 4 types of surface water with Ce(IV) and 4 types of surface water with nCeO.

Biomarker analyses

The soft tissues were allowed to thaw on ice for 30 min and homogenized still in melting ice using a Teflon pestle tissue grinder at 4 °C. A portion of the homogenate was set aside for lipid peroxidation (LPO), total triglycerides (TG) and total proteins. The remainder of the homogenate was centrifuged at 15000 x *g* for 20 min at 4 °C and the supernatant (S15) was removed for arachidonate cyclooxygenase (COX) evaluation. Total proteins were determined in the homogenate and S15 fraction using the protein-dye binding principle using standard solutions of serum bovine albumin for calibration (Bradford, 1976).

Lipid peroxidation (LPO) was determined in soft tissue homogenates using the thiobarbituric acid method (Wills, 1987). A volume of 25 µL of the homogenate was mixed with 175 µL of 10% trichloroacetic acid containing 1 mM $FeSO_4$ and 50 µL of 0.7% thiobarbituric acid. The mixture was heated at 75 °C for 10 min. The mixture was cooled to room temperature and centrifuged at 10000 x g for 5 min to remove any precipitates. A 200 µL volume was transferred to a 96-well dark microplate, and fluorescence readings were taken at 540 nm excitation and 600 nm emission. Standard solutions of malonaldehyde (tetramethoxypropane, Sigma Chemical Company, ON, Canada) were made for calibration in the homogenization buffer. Results were expressed as µg thiobarbituric acid reactants (TBARS)/mg total proteins in the homogenate. The activity of arachidonate-dependent cyclooxygenase activity was determined in the S15 fraction of soft tissues using a fluorescence microplate approach (Gagné, 2014). Briefly, 25 µL of the S15 fraction was added to 175 µL of the assay mixture composed of 50 µM arachidonate, 2 µM of dichlrofluorescein and 0.1 µg/mL of horseradish peroxidase in 50 mM Tris-HCl, pH 8.0 and 0.05% Tween-20. The reaction was allowed to proceed for 20 min at 20 °C and fluorescence readings were made at each 5-min interval at 485 nm excitation and 528 nm emission in dark microplates (Synergy 4, Biotek microplate reader, USA). The data were expressed as the increase in fluorescence/min/mg proteins in the S15 fraction. Finally, total triglycerides in soft tissue homogenates were determined using an AdipoRed fluorescent reagent (Lonza; Walkersville, MD, USA). A volume of 5 µL of AdipoRed reagent was added to 100 µL of homogenate in a black 96-well microplate. After 10 min incubation time, fluorescence was measured at 485 nm excitation and 535 nm emission (Synergy 4, Biotek microplate reader, USA). Data were expressed as relative fluorescence units (RFU)/mg proteins in the homogenate.

Data analysis

The study design examines the influence of surface waters on two chemical forms of Ce (nCeO and Ce(IV)) in terms of bioavailability and toxicity in zebra mussels. In this study, there were 12 treatments: mussels exposed to each type of water: aquarium, brown, green, effluent 10% alone (treatments 1-4); mussels exposed to 100 µg/L Ce as nCeO in each type of water (treatments 5-8); and mussels exposed to 100 µg/L of Ce as $Ce(SO_4)_2$ in each type of water (treatments 9-12). A bioavailability factor (L/kg) was calculated as follows: Ce in tissues (ug/Kg) / Ce in water (ug/L). Data normality and homogeneity of variance were verified using the Shapiro-Wilk and Bartlett tests respectively. The influence of surface water types (aquarium, green, brown and 10% effluent) and Ce forms (control, Ce(IV) and nCeO) were examined using 2-way factorial analysis of variance. Critical differences between treatments were determined using the Least Square Difference (LSD) test. The trends between the data were also analyzed using the Pearson moment correlation test. The biomarker data were also analyzed by principal component analysis to determine which effects explained most of the observed variance. Significance was set at $p < 0.05$. All statistical analyses were performed with the SysStat software package (version 13.2, USA).

Results

The surface water characteristics were determined (Table 1). The aquarium water consisted of green water for drinking water from the city of Montréal treated with charcoal filtration and UV radiation to remove chlorinated products. The diluted municipal effluent was prepared with aquarium water. The green water in the St. Lawrence River originated from the Great Lakes (Canada) and is characterized by low total organic carbon (< 4 mg/L) and high conductivity (> 200 µS/cm). The brown water originated from the forest mountains of the Laurentian shield and is characterized by high organic carbon content (> 4 mg/L), low conductivity (< 200 µS/cm) and a mildly acidic pH (6.8-7). However, the green water contained relatively high amounts of total suspended matter compared with the brown water and the diluted municipal effluent. Although the total organic carbon contents of the green water and the diluted municipal effluent were statistically similar (t test, $p > 0.05$), they differed in their nature: where the organic matter of the green water consisted mainly of humic and fulvic acids, the diluted municipal effluent was composed of proteinaceous matter. The addition of nCeO to these waters revealed that the nanoparticles remained in suspension at > 75% after 1 hr exposure time. This proportion increased to 100% in the organic-rich brown water, as expected. The properties of nCeO in the various types of water were examined by dynamic light scattering analysis (Table 2). Pre-dilution of nCeO suspension to 1000 µg/L in MilliQ water produced no significant changes in the mean diameter distribution and zeta potential of nCeO. Dilution at this concentration produced no changes when the green and brown waters and diluted municipal effluent were used. In the aquarium water, the size distribution was somewhat increased compared with the other types of surface water. Based on these data, we selected MilliQ water to pre-dilute the nCeO suspension before the final concentration of 100 µg/L in each of the 4 types of water. Diluting the nCeO suspension at the exposure concentration for mussels in surface waters revealed no important changes in size distribution and zeta potential in the presence of aquarium brown and green waters after 1 h. The mean size increased from 50 to 81 nm in the presence of diluted municipal effluent (10%). After staying for 48 h in the water samples, the mean size of the nanoparticles increased to 81 nm (brown water), 108 nm (green water), 103 nm (aquarium water) and 322 nm (10% municipal effluent). This suggests that nCeO tends to form aggregates in time, whereas natural organic matter tends to favor small aggregates of nCeO (81-108 nm) compared with urban municipal effluents (322 nm).

The bioavailability of Ce was assessed in mussels exposed to Ce(IV) and nCeO in the various water types (Table 3). In mussels placed only in the various water samples, the levels of Ce ranged between 0.4 to 4.5 ng/g and were somewhat higher in brown waters (3.5 ± 0.7 ng/g) and diluted municipal effluents (2.3 ± 0.8 ng/g) compared to aquarium water (0.8 ± 0.3 ng/g). In mussels exposed to CeIV, total levels of Ce in tissues significantly increased from the corresponding water controls. Mussels exposed to CeIV in brown waters and diluted municipal effluent accumulated more Ce compared to mussels exposed to CeIV in aquarium and green waters reaching 155 ± 35 ng Ce/g soft tissues. The bioavailability factor was 0.4, 0.36, 1.4 and 1.6 kg/L in aquarium, green, the diluted municipal effluent and brown waters respectively. In mussels exposed to nCeO, total Ce levels were modestly increased for all water types compared to water controls reaching 22 ± 3 ng Cd/g in mussels placed in brown water and the diluted

Table 2 nCeO characterization in surface waters

Water sample	nCeO	Diameter (nm) 1 h	Zeta potential (mvolt) 1 h	Diameter (nm) 48 h	Zeta potential (mvolt) 48 h
Brown water	1000 µg/L	51 ± 3	-15.2 ± 3.1	50.0 ± 1.5	-16.1 ± 3.8
Green water	1000 µg/L	52 ± 1	-19.1 ± 1.8	72.1 ± 4.5*	-19.1 ± 1.5
10% municipal effluent	1000 µg/L	54 ± 2	-13.7 ± 3.0	235 ± 111*	-12.5 ± 2.4
Aquarium	1000 µg/L	88 ± 7	-14.0 ± 1	258 ± 22*	-14.5 ± 1.0
Brown water	100 µg/L	50 ± 3	-13.6 ± 3.8	81 ± 10*	-20.0 ± 2.9
Green water	100 µg/L	53 ± 6	-17.1 ± 3.8	108 ± 31*	-19.2 ± 1.8
10% municipal effluent	100 µg/L	81 ± 24	-14.0 ± 1.7	322 ± 200	-14.5 ± 2.1
Aquarium	100 µg/L	60 ± 15	-10.2 ± 2.5	103 ± 6*	-14.7 ± 4.4

*Indicates significant difference between 1 and 48 h.

Table 3 Tissue Ce loadings in mussels

Waters/Treatment	Control (ug/g)	CeIV (ng/g)	Ce(IV) bioavailabilty[3]	nCeO (ng/g)	nCeO bioavailabilty
Aquarium	0.8 ± 0.3	40 ± 7[1]	0.4	13 ± 3[1]	0.13
Green waters	1.7 ± 0.5	36 ± 6[1]	0.36	9 ± 4[1]	0.09
Brown waters	2.3 ± 0.8	155 ± 35[1,2]	1.55	22 ± 6[1,2]	0.22
10 % Municipal effluent	3.5 ± 0.7	138 ± 30[1,2]	1.38	22 ± 3[1,2]	0.22

[1]Significantly different from water control
[2]Significantly different from aquarium water within CeIV or nCeO treatments
[3]Bioavailability in Kg/L: concentration in tissues/concentration in water.

municipal effluent. Mussels exposed to nCeO in brown water and diluted municipal effluent contained more Ce in tissues compared to mussels exposed to nCeO in aquarium and green waters. The bioavailability factor for the nanoparticulate form was low with factors of 0.1, 0.09, 0.22 and 0.22 kg/L in mussels exposed to the nanoparticle in aquarium, green, brown waters and diluted municipal effluent respectively.

The resistance to air emersion in post-exposed mussels was examined as a means of assessing the general health status of the mussels exposed to nCeO and Ce(IV) in the 4 types of surface water (Figure 1A-C). Factorial 2-way ANOVA revealed a significant ($p < 0.05$) interaction between surface water types and cerium form [*i.e.*, nCeO and Ce(IV)]. In the mussels exposed to surface waters only, there was a significant decrease between time of death in brown water (11 days) and green water (13.2 days). In the mussels exposed to Ce(IV), no changes were observed in the time of death in the mussels placed in aquarium water compared with the aquarium water alone. A decrease in time of death was observed for the mussels exposed to Ce(IV) and the municipal effluent (10 days) compared with the mussels exposed to Ce(IV) in the controls (13.2 days, $p < 0.01$) and in the mussels exposed to aquarium water only (13 days, $p < 0.05$). In the mussels exposed to nCeO in aquarium water, no significant change in the time of death was observed compared with aquarium water. There was a significant change in time of death in the mussels exposed to nCeO in aquarium water (13.2 days) compared with those exposed to nCeO in green water (9 days, $p < 0.05$) and nCeO in brown water (12 days, $p = 0.05$). Of the mussels exposed to the municipal effluent, the mussels exposed to Ce(IV) survived marginally less to air stress (9 days) compared with the mussels exposed to nCeO and the municipal effluent (12 days; $0.1 < p < 0.05$). Weight loss after 4 days in air was also determined in the mussels exposed to the Ce forms and different surface waters (Figure 1D-F). Two-way factorial ANOVA revealed that only surface water types produced a significant effect in mussels ($p < 0.05$). With respect to the mussels treated with aquarium water, the weight loss was higher in the mussels in brown water ($p < 0.05$) and diluted municipal effluent ($p < 0.05$). The mussels in brown water displayed more weight loss than the mussels exposed to green water ($p < 0.05$). Correlation analysis revealed that air-time survival was significantly correlated with initial weight loss ($r = -0.32$; $p < 0.01$) *i.e.*, the mussels that lost more weight initially (after 4 days) survived less time in the air.

The condition factor (mussel weight g/shell length in cm) was also evaluated (Figure 2). Two-way factorial ANOVA revealed a significant interaction between surface waters and Ce form ($p < 0.001$). With respect to aquarium water only, the other types of water (*i.e.*, municipal effluent, brown and green waters) did not significantly influence the condition factor. The condition factor of the mussels placed in the diluted municipal effluent was higher compared with the brown water ($p < 0.01$) and the controls ($p = 0.05$). In the mussels exposed to Ce(IV) in green water, the condition factor was marginally lower relative to the mussels placed in aquarium water only ($0.1 < p < 0.05$). In the mussels exposed to nCeO in aquarium water, the condition factor was marginally higher compared with that of the mussels in aquarium water only ($0.1 < p < 0.05$). The condition factor was significantly higher in mussels exposed to nCeO in green water ($p < 0.05$) and lower in the mussels in the diluted municipal effluent and nCeO ($p < 0.05$) compared with the mussels in aquarium water alone. The mussels exposed to Ce(IV) in aquarium water had a significantly lower condition factor than the mussels exposed to nCeO in aquarium water ($p = 0.05$). The condition factor was significantly higher in the mussels exposed to nCeO in green water compared with those exposed to nCeO in the diluted municipal effluent ($p < 0.001$), but lower when compared with the mussels exposed to Ce(IV) in

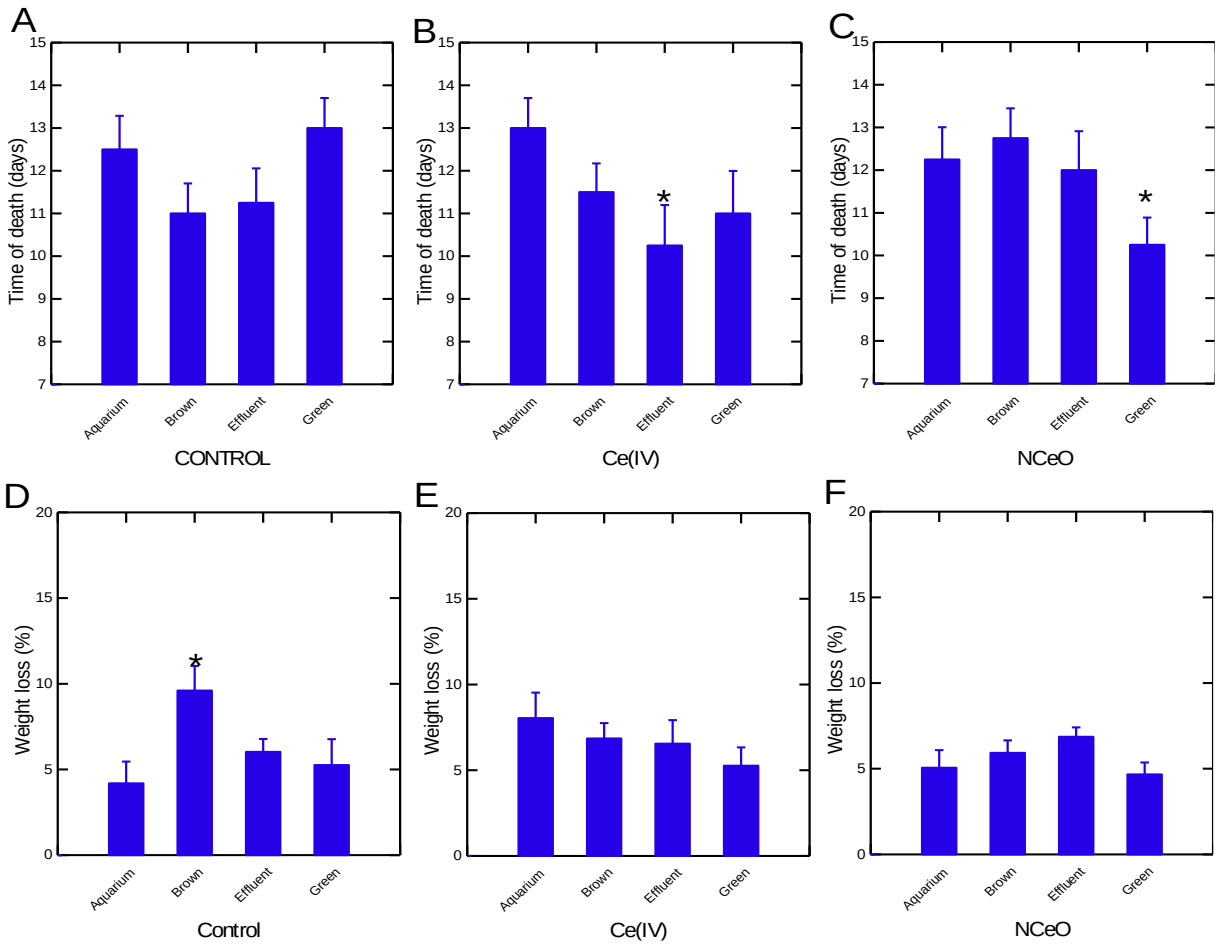

Fig. 1 Influence of Cerium form and surface waters on air-time survival. The mussels were exposed to Ce(IV) and nCeO in the 4 types of water for 96 h at 15 ^{o}C. After the exposure period, a subgroup of mussels was processed for assessment of air-time survival (A, B, C) and initial weight loss after 4 days (D, E, F), as described in Materials and Methods. The star * indicates significance from aquarium water

green water ($p < 0.001$) and those exposed to green water only ($p < 0.001$). Correlation analysis revealed that the condition factor was marginaly correlated with Ce tissue concentration (r=-0.21, $0.1 < p < 0.05$).

Oxidative stress was determined in the mussels by following changes in COX activity and LPO levels (Figure 3 A–F). For COX activity, two-way factorial ANOVA revealed a significant interaction between surface water types and Ce form ($p < 0.001$). In the mussels exposed to surface waters only (Figure 4A), COX activity was significantly lower in the mussels placed in green water compared with the mussels placed in brown water ($p < 0.01$) and aquarium water ($p < 0.05$), and marginally lower in the diluted municipal effluent ($0.1 < p < 0.05$). In the mussels exposed to Ce(IV), COX activity was significantly lower ($p < 0.01$) than the activity in the mussels exposed to aquarium water (Figure 4B). In the mussels exposed to Ce(IV) in brown water, COX activity was significantly higher than in the mussels exposed to Ce(IV) in green water ($p < 0.05$), diluted municipal effluent ($p < 0.01$) and aquarium water ($p < 0.01$). COX activity was significantly lower in the mussels exposed to Ce(IV) and the municipal effluent compared with the mussels exposed to the municipal effluent alone ($p < 0.01$). In the mussels exposed to nCeO (Figure 4C), COX activity was significantly increased in the mussels exposed to nCeO in green water compared with the mussels in green water only ($p < 0.001$). COX activity was significantly decreased in the mussels exposed to nCeO in brown water compared with those in brown water only ($p < 0.01$) and the mussels exposed to nCeO in green water ($p < 0.001$) and in aquarium water ($p = 0.05$). COX activity in the mussels exposed to aquarium (control) water and nCeO was significantly higher than COX activity in the mussels exposed to Ce(IV) in aquarium water ($p < 0.01$). The same was observed for the mussels placed in green water and the diluted municipal effluent. However, the reverse situation was observed when the mussels were exposed to nCeO in brown water. Correlation analysis revealed that COX activity was marginally correlated (r = 0.2; $0.1 < p < 0.05$) with weight loss and Ce tissue loadings (r = -0.2; $0.1 < p < 0.05$), which suggests that COX activity

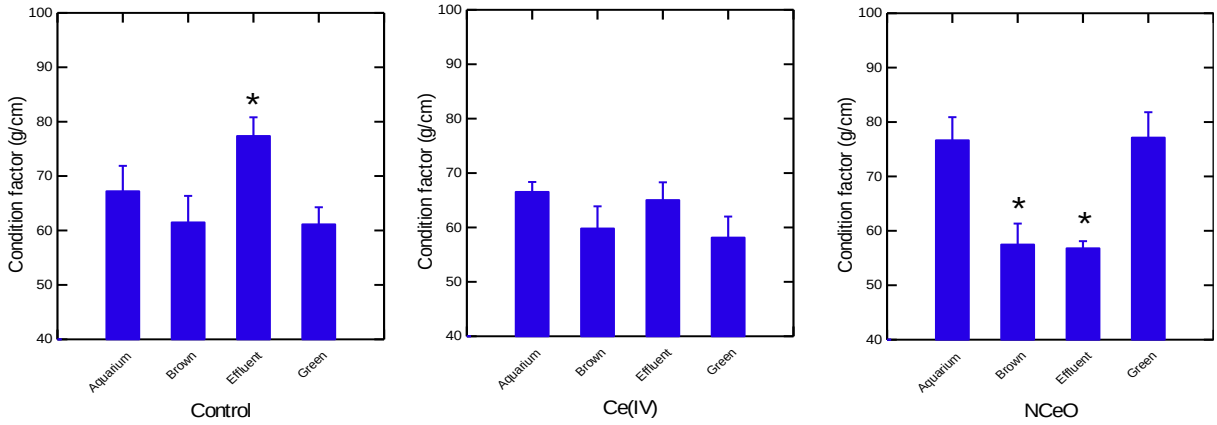

Fig. 2 Influence of surface water and cerium on the condition factor of mussels. The mussels were exposed to Ce(IV) and nCeO in the 4 types of water for 96 h at 15 ^{o}C. After the exposure period, the weight-to-shell-length ratio was determined in the mussels. *Indicates significance from aquarium water

was associated with weight loss and decreased tissue Ce loadings. Oxidative damage was also followed by LPO levels (Figure 3 D–F). Two-way factorial ANOVA revealed that only the water type was significant (p = 0.001). LPO levels were significantly increased in green water compared with either aquarium or brown waters ($p < 0.001$ and p = 0.01 for aquarium and brown waters respectively). LPO levels were also significantly increased in the diluted municipal effluent compared with the aquarium water ($p < 0.05$) and green water ($p < 0.05$). Interestingly, these responses were lost when Ce(IV) and nCeO were present, indicating that these compounds have antioxidant effects. The levels of DNA strand breaks were also determined in the mussels exposed to the different Ce forms and water types (Figure 4). Two-way factorial ANOVA revealed a significant interaction between the Ce forms and water samples. Among the mussels exposed to the 4 types of surface water, DNA strand breaks were significantly lower in those exposed to brown water compared with aquarium water ($p < 0.001$), diluted municipal effluent ($p < 0.001$) and, marginally, with green water ($0.1 < p < 0.05$). In the mussels exposed to Ce(IV), DNA strand breaks were increased in the mussels exposed to Ce(IV) in brown water compared with those in the brown water only. The mussels exposed to Ce(IV) and brown water also had elevated levels of DNA strands breaks compared with the mussels exposed to the lanthanide in green water. DNA strand breaks were lower in the mussels exposed to Ce(IV) in green water compared with those immersed in green water only ($p < 0.01$). The same was also true with the diluted municipal effluent. Among the mussels exposed to nCeO, DNA strand breaks were lower in the mussels placed in green water compared with those exposed to aquarium water only ($p < 0.05$) and in the mussels exposed to Ce(IV) in aquarium water (p = 0.05). DNA strand breaks were marginally correlated with COX activity (r = -0.20; $0.1 < p < 0.05$), suggesting that decreased DNA strand breaks are associated with susceptibility to oxidative stress as determined by COX activity.

The influence of surface waters and Ce forms on triglyceride levels was examined (Figure 5). Two-way factorial ANOVA revealed a significant interaction between surface waters and Ce forms ($p < 0.001$). In the mussels exposed to surface waters only, triglyceride levels were significantly decreased in brown water compared with those exposed to aquarium water ($p < 0.01$), diluted municipal effluent ($p < 0.01$) and green water ($p < 0.001$). Triglyceride levels were significantly higher in the mussels placed in green water than those placed in aquarium water (p = 0.001). In the mussels exposed to Ce(IV), triglyceride levels in the mussels in aquarium water were lower compared with those placed in aquarium water ($p < 0.05$) and green water ($p < 0.001$). The same was found with the mussels exposed to Ce(IV) and green water, where the increase in triglyceride levels observed in the mussels in green water was lost when Ce(IV) was added. The levels were marginally higher in the mussels exposed to Ce(IV) and brown water compared with the mussels placed in brown water alone ($0.1 < p < 0.05$). A marginal decrease in triglycerides was observed in the mussels exposed to Ce(IV) and the diluted municipal effluent compared with the mussels exposed to the diluted municipal effluent ($0.1 < p < 0.05$). In the mussels exposed to nCeO, the increase in triglyceride levels observed in the mussels exposed to green water was lost compared with those exposed to green water alone ($p < 0.001$). The same was found with the mussels exposed to the diluted municipal effluent and nCeO, but it was marginal ($0.1 < p < 0.05$). Triglyceride levels also decreased in the mussels exposed to nCeO in brown water ($p < 0.05$) and the diluted municipal effluent ($p < 0.05$) compared with those exposed to nCeO in aquarium water. Triglyceride levels were significantly correlated with weight loss during air-time survival (r = -0.35; p = 0.001) and time of death (r = 0.24; $p < 0.05$), which suggests that weight loss occurs when

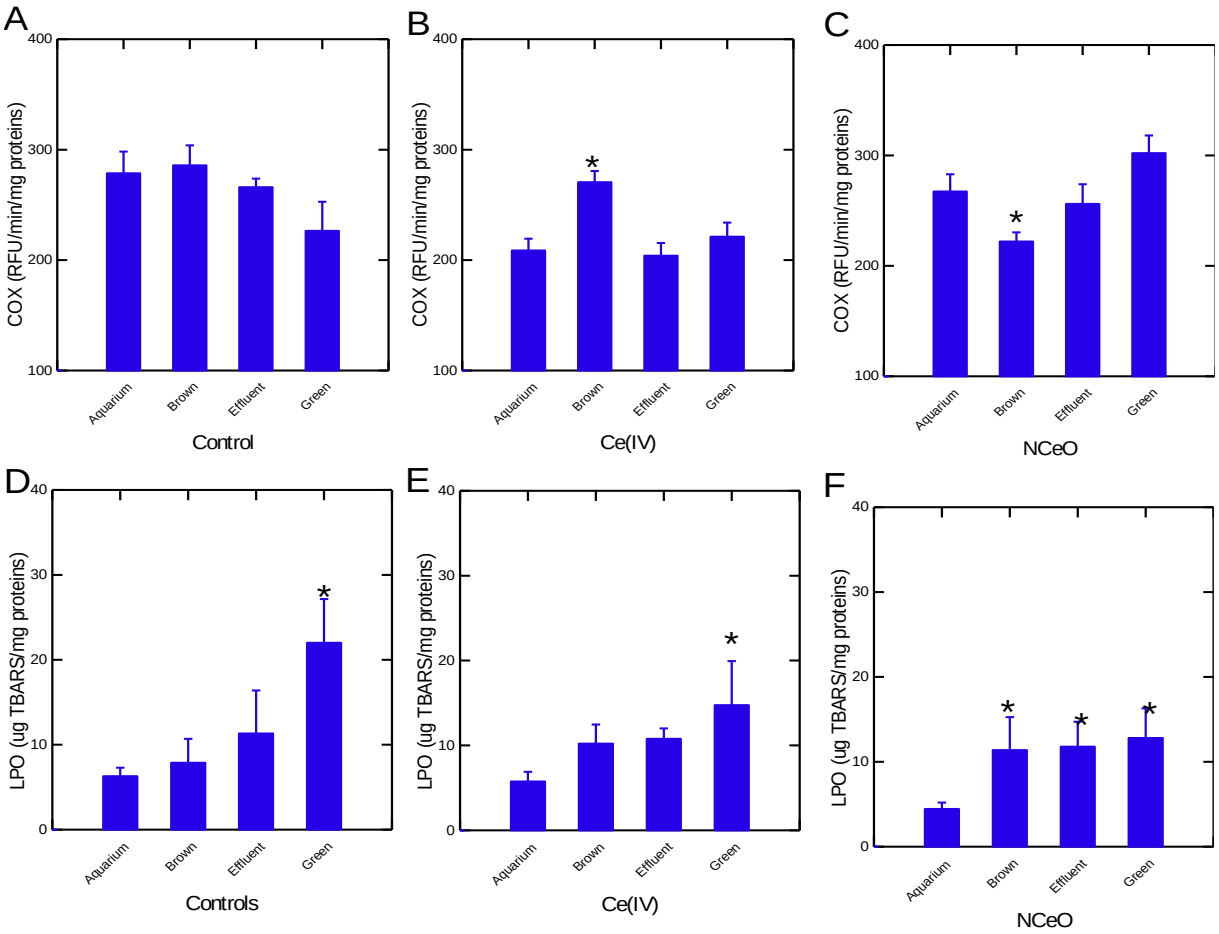

Fig. 3 Change in oxidative stress in mussels exposed to ceria forms and surface waters. The mussels were exposed to Ce(IV) and nCeO in the 4 types of water for 96 h at 15 ºC. After the exposure period, oxidative stress was measured in the mussels by COX activity (A, B, C) and LPO (D, E, F). *Indicates significance from aquarium water

triglyceride levels are lowered. Triglyceride levels were significantly correlated with DNA strand breaks ($r = 0.56$; $p < 0.001$).

In an attempt to gain a comprehensive view of the interrelationships between the biomarkers, a principal component analysis was performed (Figure 6). The analysis revealed that most of the variance was explained (62%) by three factors. Among the biomarkers within each of the 3 components, the following responses had the highest factorial weights (> 0.5): DNA strand breaks, triglycerides, weight loss, time of death, condition factor, tissue Ce loadings and LPO. This suggests that Ce in different types of surface water will have an impact on lipid damage by oxidation, changes in triglyceride levels, DNA damage and general health status as determined by time of death in air, weight loss and condition factor.

Discussion

nCeO was fairly stable in the surface waters based on the total Ce measurement, the mean diameter and zeta potential after 1 h. Although the zeta potential was not affected after 48 h in the 4 types of surface water, the mean diameter of nCeO was significantly increased in the aquarium, brown and green waters, which suggests no degradation or major transformation within the (in)organic matrix of the water types. There was a great deal of variability in the mean diameter of nCeO in the diluted municipal effluent, so aggregation could not be confirmed statistically ($p < 0.05$). This is consistent with the observation that nCeO ingested by the marine mussel *Mytilus galloprovincialis* was not degraded in the gut and remained as nanoparticles (Montes *et al.*, 2012). Mussels exposed to 10 mg/L nCeO accumulated 62 µg/g of Ce and rejected 21 mg of Ce/g, which gives a bioavailability factor of 6.2 and suggests that mussels exposed to a continuous input of nCeO in the environment would tend to accumulate the nano-lanthanide. The bioavailability factor obtained for nCeO in this study was low with values at 0.13 (L/kg) in aquarium water but rose to 0.22 in brown water and diluted municipal effluent. The increase in

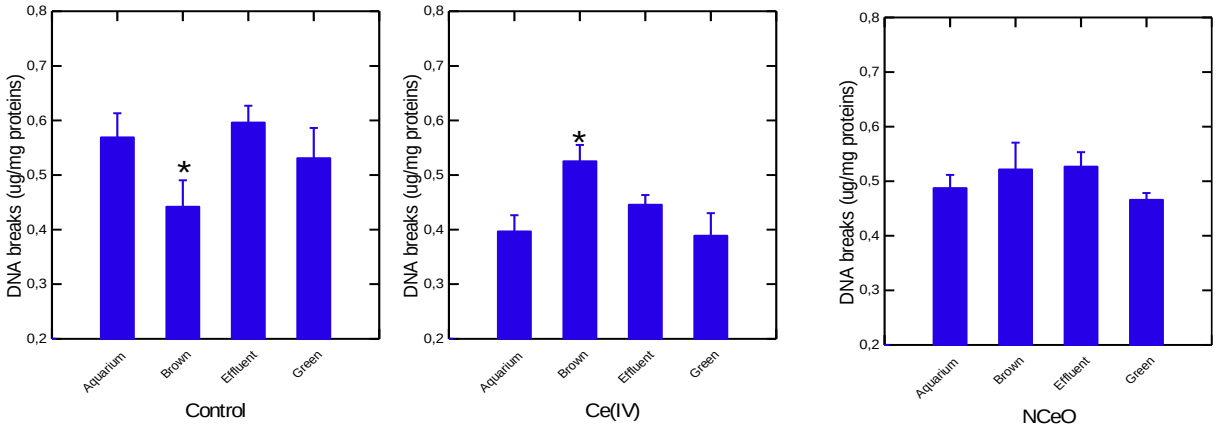

Fig 4 DNA strand breaks in mussels treated with cerium and surface waters. The mussels were exposed to Ce(IV) and nCeO in the 4 types of water for 96 h at 15 ºC. After the exposure period, oxidative stress was measured in the mussels by COX activity (A, B, C) and LPO (D, E, F). The star * indicates significance from aquarium water

Ce bioavailability with Ce(IV) was also observed by these organic-carbon rich waters. However, given that bioavaialbity could be tissue-specific and Ce analysis were done in the whole soft tissues, we cannot exclude the possibility that some specific target tissues might have accumulated the majority of Ce.

According to the principal component analysis, changes to the oxidative status had a major effect in freshwater mussels. Exposure to Ce(IV) readily decreased COX activity in the mussels, and nCeO was less potent in decreasing COX activity in the control mussels placed in aquarium water. The antioxidant effect of nCeO was more evident only in the presence of organic-rich brown water. Moreover, the decrease in COX activity was also associated with lower levels of DNA strand breaks in the mussels placed in aquarium and green waters and in the diluted municipal effluent in the mussels exposed to Ce(IV), which suggests a decrease in oxidative-mediated DNA damage and repair activity. Nevertheless, Ce(IV) could interact with DNA in vitro, leading to its reduction to the fluorescent Ce(III) form in the presence of sodium triphosphate (Wu *et al.*, 2005). DNA scission was associated with the formation of Ce(III) from the reduction of Ce(IV) by DNA and sodium triphosphate. DNA strand break levels were significantly higher in the mussels exposed to nCeO than in the mussels exposed to Ce(IV), regardless of surface water type (t test, p = 0.01), which suggests that properties other than Ce(IV) of nCeO produced DNA breaks. A mixed-valence Ce (Ce^{+3}/Ce^{+4}) state at the surface of nCeO was observed for this nanoparticle (Bhargava *et al.*, 2016), which could account for the increase in DNA breaks. This was consistent with DNA damage in the nCeO2-exposed freshwater bivalve *Corbicula fluminea* (Koehlé-Divo *et al.*, 2018). DNA damage was associated with apoptosis (caspase) with the expression of anti-oxidative defense mechanisms and increased anaerobic activity (lactate dehydrogenase activity), which suggests that the removal of damaged cells was not affected.

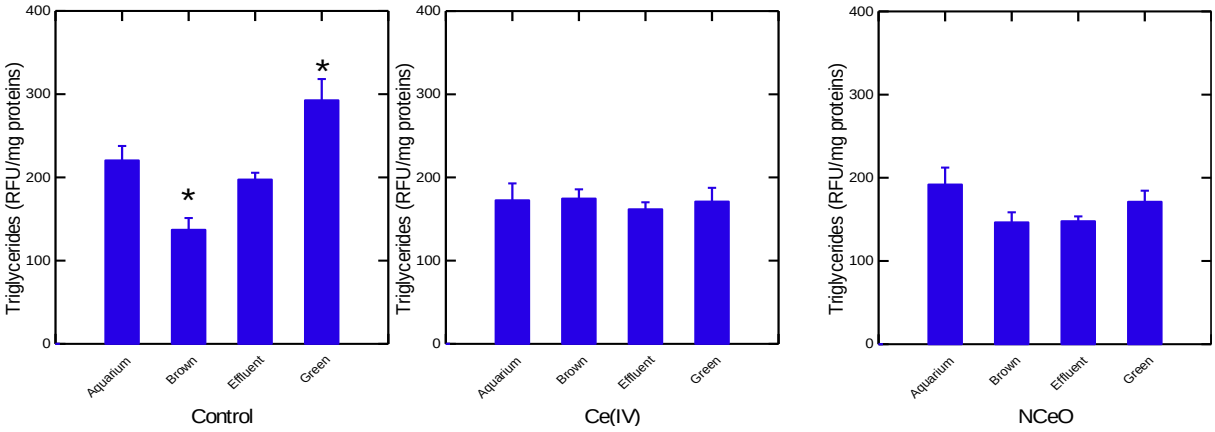

Fig. 5 Influence of cerium form and surface waters on triglyceride levels in mussels. The mussels were exposed to Ce(IV) and nCeO in the 4 types of water for 96 h at 15 ºC. After the exposure period, triglyceride levels were determined in soft tissues. The star * indicates significance from aquarium water

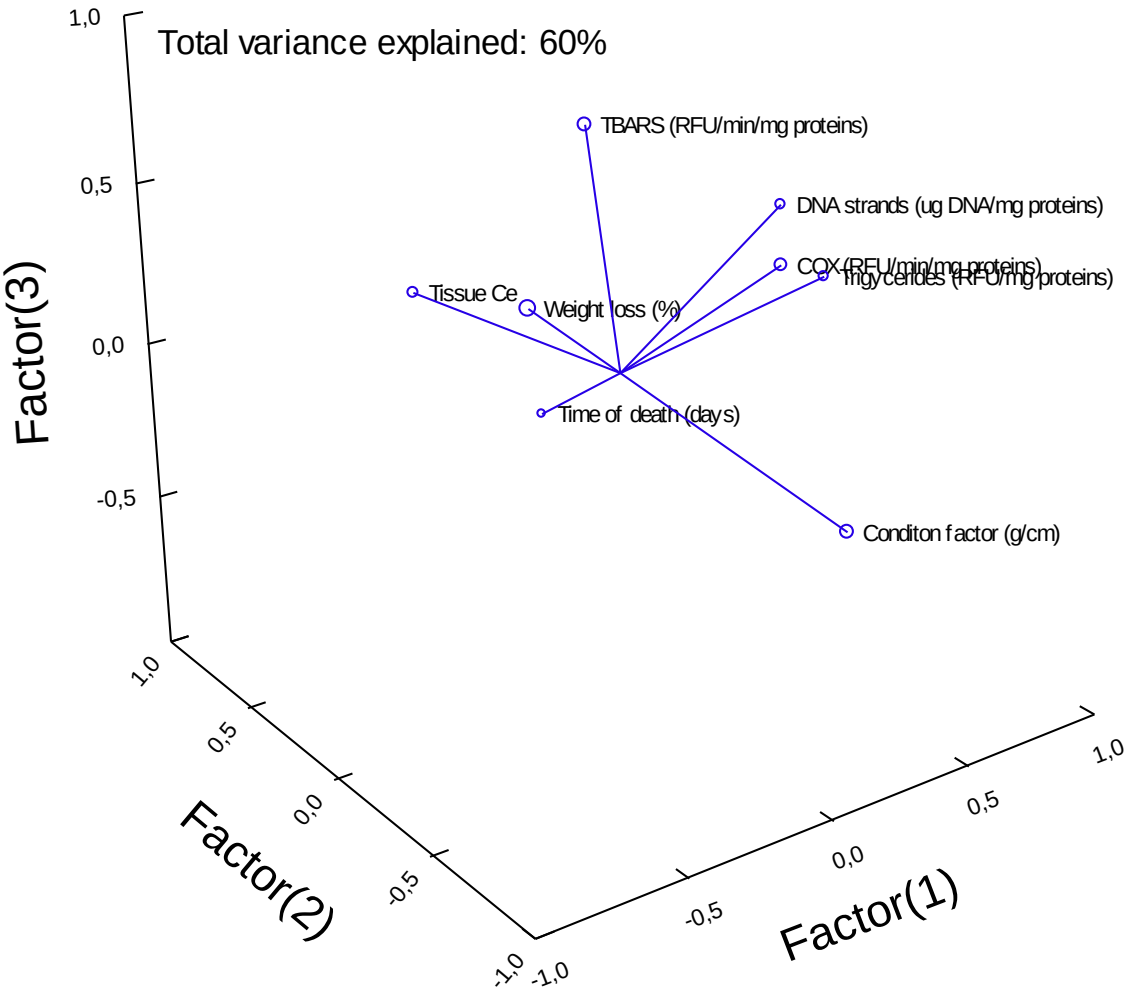

Fig. 6 Principal component analysis of biomarker responses in mussels exposed to cerium and surface waters

The antioxidant properties of Ce(III) have also been reported in previous studies (Gonzalez *et al.*, 2014). For example, in vitro cultures of fish cone receptor cells exposed to mixed valence nCeO (Ce^{3+}/Ce^{+4}) led to decreased H_2O_2 levels and increased cell survival (Bhargava *et al.*, 2016). This highlights the ability of nCeO to scavenge H_2O_2 in cells. In another study with zebra fish embryos, exposure to albumin-coated nanoceria produced no oxidative stress and preserved the antioxidant system (Bhushan *et al.*, 2016). Zebra mussels exposed to nCeO showed decreased lysosome number, catalase activity and lipid peroxidation (Garaud *et al.*, 2015). It appears that the Ce(III) state of nCeO is responsible for superoxide dismutase mimetic activity, which could act as a primary radical scavenger (Hecker *et al.*, 2008). Hence, the antioxidant effect of Ce(IV) and nCeO involves the formation of intermediate Ce(III). It is reasonable to propose that the surface water properties (organic matter?) could favor the formation of Ce(III) given that the antioxidant effect of nCeO was observed in brown water. Ce could also protect against lead-induced hepatotoxicity in crucian carp (Ling and Hong, 2010). The fish were exposed via intraperitoneal injection (10–40 mg/kg) for 14 days, after which a subgroup of animals was injected with 1.5 mg/kg of Ce(III). This treatment prevented ROS accumulation and improved the hepatosomatic index of the fish. The surface properties of nCeO, such as negative charge (*i.e.*, less cationic form of Ce as Ce^{+4}), protein corona formation, shape and zeta potential but not aggregation state, were associated with a loss of viability of hemocytes of *M. galloprovincialis* (Sendra *et al.*, 2018). Indeed, the negative charge and the rounded shape of nCeO favored the formation of Cu,Zn-SOD activity in the hemolymph serum and favored more changes in lysosome membrane stability and phagocytosis activity. A decrease in oxidized DNA bases was observed in Caco-2 cells exposed to nCeO *in vitro,* which further supports its antioxidant activity although the COMET assay did not observe changes in tail length (Vila *et al.*, 2018). This corroborates our finding here, where decreased DNA strand breaks were obtained in the mussels exposed to the nanoparticle in aquarium water compared with the control mussels.

Based on air-time survival and condition factor assessments, the present study integrated impacts

at different levels of biological organization (biochemical changes to change in health status and resilience to air stress). Thus, the observed changes at the biochemical/cellular levels could be compared with changes at higher levels of organization to determine whether more severe impacts (toxicity) are manifest at the organism level. Based on the data on air-time survival, nCeO and Ce(IV) were more toxic (mussels survived less in air) in green water compared with the mussels in green water alone. This was not accompanied by weight loss, although triglycerides were lower in the mussels exposed to nCeO in green water. This was similar to the results of a previous study where the filtering activity of zebra mussels exposed to either bare or citrate-coated nCeO was not affected, although some changes were found at the molecular level *i.e.*, glutathione S-transferase and catalase gene expression (Garaud *et al.*, 2016). This highlights the need to measure changes not only at the biochemical level, but also in organism health (morphological changes, resistance to applied stress such as air exposure). The toxicity of nCeO could also be modulated by the surface water characteristics in fish (Gagnon *et al.*, 2018). Exposure to 10 µg/L of nCeO was lethal in trout juveniles in both the aquarium and green waters, but not in the brown water, suggesting that the natural organic matter in brown water might have prevented toxicity, perhaps through the formation of the antioxidant Ce(III). However, this could be due to increased bioavailability in aquarium and green waters, since Ce contents in gills were higher in fish exposed to nCeO in the aquarium and green waters compared with those of fish in the brown water. Some evidence exists that nCeO could alter ionic homeostasis in fish gills, which could be another pathway leading to toxicity (Xia *et al.*, 2013). Brain acetylcholinesterase was also decreased, suggesting downregulation of neuromuscular activity in fish. It also appears that co-occurrence of copper oxide with nCeO in automobile catalyst systems could lead to toxicity in daphnids (Jemec *et al.*, 2012). While nCeO was not toxic to daphnids, the presence of copper-nCeO mixed oxides (at 20% CuO) reduced survival at concentrations of 80 mg/L or more, while copper-nCeO at 10% and 15% did not produce any effects. This suggests that a mixture of metal oxides could have potentiated the toxicity of nCeO. More research will be required to further study the toxicity of mixed metal oxide nanoparticle formulation in aquatic organisms.

In conclusion, the influence of 4 different types of surface water on the biochemical effects of nCeO and Ce(IV) was examined in mussels. The data revealed that both forms of cerium decreased triglyceride levels in aquarium and green waters, but this was prevented by organic-matter rich surface waters (brown and diluted municipal effluent). The mussels exposed to nCeO survived less in air when exposed to green water compared with aquarium water. The biochemical effects of nCeO and Ce(IV) could be modulated by the surface water properties involving natural and anthropogenic organic matter in addition to conductivity and the presence of suspended matter.

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