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RESEARCH REPORT

The impact of zinc oxide nanoparticles in freshwater mussels exposed to municipal effluents

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Abstract

Zinc oxide nanoparticles (nano-ZnO) are used in the production of transparent sunscreens and cosmetics, which are released into the environment through municipal effluents. The purpose of this study was to examine the toxicity of nano-ZnO to freshwater mussels (*Elliptio complanata*) in the presence of municipal effluents. Mussels were exposed for 21 days at 15 ^{o}C to 1 and 10 µg/L nano-ZnO, and $ZnCl_2$ in the presence of a physico-chemically treated municipal effluent (3 and 10 % v/v). After the exposure period and a 24 h depuration step, mussels were analyzed for free Zn in gills, metallothioneins (MT), oxidative stress (production of malondialdehyde (MDA) during lipid peroxidation), gonad alkali-labile phosphate (ALP) levels and genotoxicity. Gill MT levels were increased at 10 µg/L nano-ZnO and $ZnCl_2$ and in the presence of the municipal effluent. MT levels were positively correlated with free Zn in gills and negatively correlated with MDA levels, indicating its involvement in the prevention of oxidative stress. However, MDA levels were significantly related to DNA damage in gills, indicating that MT induction did not prevent oxidative-mediated damage in cells. Gonad ALP levels were increased by exposure to $ZnCl_2$ and to the highest concentration of municipal effluent. DNA strand breaks were increased in mussels treated to nano-ZnO indepentely of municipal effluent. Multivariate discriminant function analysis revealed that control mussels differed from mussels exposed to the municipal effluent and from those exposed to nano-ZnO or $ZnCl_2$ alone. When the municipal effluent was added, changes in MDA, MT and labile Zn were produced and formed another cluster, suggesting a change in the toxicity of the municipal effluent in the presence of nano-ZnO.

Key Words: zinc oxide nanoparticles; municipal effluent; freshwater mussels; oxidative stress; DNA damage; metallothioneins; alkali-labile phosphates

Introduction

Nanotechnology is an area of intense commercial interest and development. Products derived from nanotechnology are used in the production of many consumer products, such as cosmetics, textiles and sunscreens (Contado *et al*., 2015). With the growing presence of nanoproducts in personal care products, municipal effluents and urban runoffs, there are concerns that these compounds with emerging properties at the nanoscale could alter the toxic properties of complex mixtures such as effluents. Indeed, legitimate concerns have also been raised by the public and regulatory agencies about their safety to human health and ecosystems. Given their strong UV absorptive capacity, sunscreens composed of zinc oxide nanoparticles (nano-ZnO) are currently used worldwide as effective protection against sunlight (Cole *et al.,* 2016). Sunscreen lotions usually contain bulk suspensions of ZnO and titanium dioxide, which form a white film on the skin. However, lotions composed of nano-ZnO are transparent, while retaining their UV light absorption properties. Nano-ZnO also possesses antimicrobial properties, an additional benefit. Notwithstanding this, little is known about the release, fate and toxicity of these sunscreens at the present time. The toxic behaviour of nano-ZnO in complex matrices such as municipal wastewaters is not well understood. In addition, the Zn content of municipal effluents was shown to be in the order of 10 -50

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ug/L (Gagnon *et al*., 2006) and a recent study on the acute toxicity of ZnO nanoparticles revealed that nano-ZnO could pose a risk to aquatic organisms (Adam et al., 2015). The 5 % hazard concentration for nano-ZnO was 60 µg/L and 30 µg/L for $ZnCl_2$ which suggests that municipal effluents enriched in Zn nanoparicles could represent a risk to aquatic life.

Bivalves (clams and mussels) are considered at risk of contamination by suspended solids and colloids (Canesi *et al.,* 2012). They are sessile organisms which feed on suspended matter, which leads to the bioaccumulation of large quantities of particles, including nanoparticles and their aggregates. For example, a study on *Mytilus galloprovincialis* has shown that copper oxide nanoparticles accumulate in the digestive gland, leading to lipid peroxidation (Gomes *et al.,* 2012). In another study, mussels exposed to cerium oxide nanoparticles and nano-ZnO accumulated large quantities of Ce and Zn at mg/g levels (Montes *et al*., 2012). The toxicity of nanoparticles is due to more than the release of their components (*i.e*., leaching of Zn^{2+} from nano-ZnO) (Gagné *et al*., 2008a). Indeed, toxic interactions could arise from the size, reactive surface properties and coatings of nanoparticles, in addition to the leaching of their components. Recent studies have shown that nanoparticles can lead to oxidative stress and genotoxicity, which cannot be explained solely by the release of their components. Oxidative stress involves the mobilization of iron(III), copper(II) and Zn(II), which are sequestered by metallothioneins (MT) (Formigari *et al.,* 2007; Gagné *et al*., 2008b). The mobilization of ionic metals could lead to the production of reactive oxygen radicals, which could result in lipid peroxidation and DNA damage through the formation of 8-oxoguanine (Valko *et al.,* 2006; Rocha *et al.,* 2015). In snails, a 3-week exposure to 7 mg/L of nano-ZnO lead to increased malondialdehyde and nitric oxide with decreased glutathione S-transferase activity in both the hemolymph and tissues (Fahmy *et al.,* 2014). Exposure to nano-ZnO also increased total lipids and cholesterol levels in snails which suggest decreased mobilization of energy reserves in snails. However, in clams exposed to a more realistic concentration (10 ug/L) for 7 days of either nano-ZnO or $ZnCl_2$, DNA damage in hemocytes were higher in the former form of zinc (Marisa *et al.,* 2016). Significant increases in superoxide dismutase and catalase activities suggested that oxidative stress was at play in clams exposed to nano-ZnO but not for $ZnCl_2$. Recent studies have also shown that both nano-ZnO and municipal effluents could induce oxidative stress in aquatic organisms (Gillis *et al.,* 2014; Gagné *et al*., 2015), but less is known about their combined effects in freshwater mussels downstream from municipal effluent discharge sites.

The purpose of this study was to examine the toxicity of nano-ZnO and municipal effluents in freshwater mussels. Mussels were exposed to increasing concentrations of municipal effluent and to nano-ZnO. Additional mussels were exposed to $ZnCl_2$ for comparison purposes. The levels of free Zn in gills were examined in conjunction with MT, oxidative stress (as determined by the malondialdehyde (MDA) assay for lipid peroxidation) and DNA damage endpoints. The interaction of nano-ZnO and municipal effluent exposures was examined based on both the effluent and Zn concentrations in freshwater mussels.

Materials and Methods

Mussel handling and exposure to municipal effluents and zinc forms

Mussels (*Elliptio sp.*) were collected by hand in a pristine lake in the Laurentians under a provincial permit in June 2012. The mussels were transported dry at 4 ^{o}C and transferred to 300-L tanks filled with UV-treated dechlorinated City of Montreal tap water. The mussels were held in the tanks at 15 ^{o}C under constant aeration for at least 6 weeks before initiating exposure. The mussels were fed three times a week with commercial coral reef feed enriched with *Pseudokirchneriella subcapitata* algal suspensions ($100x10^6$ algae/mL). For the exposure experiments, n = 20 mussels were placed in 60-L tanks receiving a continuous flow (0.15 L/h) of physico-chemically treated municipal effluent from a city of 1.5 million people. The physical/chemical treatment consisted in reducing suspended matter down to the mg/L range by means of grid traps, flocculation (surfactants) and sieving. The exposure concentrations were 0, 3 and 10 % diluted in dechlorinated UV-treated City of Montreal tap water (Qc, Canada). Mussels were exposed to 1 and 10 µg/L Zn as nano-ZnO or $ZnCl_2$ using an “instillation” technique. To ensure contact of the Zn solutions with the mussels, 60 and 600 µg of either nano-ZnO or $ZnCl_2$ were dissolved in 20 mL of aquarium water and placed directly over the mussel during active filtratrion (each mussel received 1 mL over the siphons). Control mussels received only the aquarium water. The mussels were held under static conditions for one hour prior to continuous exposure to the municipal effluent. This process was repeated every 3 days for 21 days. At the end of the exposure period, mussels were allowed to depurate in clean aquarium water overnight (12 h). Morphological characteristics were determined for mussel weight and shell length. Soft tissues, gills and gonad were dissected on ice and weighed. Sex was determined by examination of gonad smears on glass slides under a binocular microscope at 200x magnification. The gills and gonad were homogenized on ice using a Teflon pestle tissue grinder in 145 mM NaCl containing 10 mM Hepes-NaOH, pH 7.4, 10 µg/mL aprotinin and 1 mM dithiothreitol. Part of the homogenate was centrifuged at 15,000x*g* for 30 min at 4 ^{o}C and the supernatant (S15 fraction) was removed and stored at -85 ^{o}C until biomarker analyses. Total proteins were determined using the protein-dye binding principle with standard solutions of bovine serum albumin for calibration (Bradford, 1976).

Metal metabolism

Metal metabolism was characterized by monitoring changes in labile Zn and metallothioneins (MT) in gill tissues. Labile Zn levels in tissues were determined using a fluorescent

probe method (Gagné *et al*., 2008b). Fluorescent probe TSQ (N-(6-methoxy-8-quinolyl)-p-toluenesulfonamide) was prepared in 20 % dimethyl sulfoxide (in 5 mM KH_2PO_4, pH 7.4, containing 125 mM NaCl) at 50 µM concentration. A volume of 150 µL of the probe was mixed with 25 µL of the gill S15 fraction for 10 min and fluorescence readings were taken at 400 nm excitation and 485 nm emission (Biotek Instruments, USA). Standard solutions of zinc sulfate were prepared for calibration. The data were expressed as ng zinc/mg protein. Metallothionein (MT) levels in gills were determined using a modified spectrophotometric assay (Viarengo *et al.*, 1995; Gagné *et al*., 2010). Briefly, total MT levels were determined by the addition of a strong reducing agent, phosphine, in the S15 fraction for 15 min prior to the addition of ethanol-chloroform solvent. The data were expressed as µmole of glutathione (GSH) per mg protein.

Oxidative stress and DNA damage

Malondialdehyde (MDA) levels were determined for lipid peroxidation in gill homogenates using the thiobarbituric acid method (Wills, 1987). A volume of 50 µL of the homogenate was mixed with 200 µL of 10 % trichloroacetic acid containing 1 mM $FeSO_4$ and 50 µL of 0.67 % thiobarbituric acid and heated at 70^{o}C for 10 min. The mixture was cooled and centrifuged at 10,000x*g* for 5 min. A 200-µl sample of the supernatant was transferred to a 96-well dark microplate, and fluorescence readings were taken at 520 nm excitation and 600 nm emission. Standard solutions of tetramethoxypropane (stabilized form of malondialdehyde) were prepared for calibration in the blank (homogenization buffer). Results were expressed as µmole thiobarbituric acid reactants (TBARS) per mg total protein in the homogenate. DNA damage was determined using the alkaline precipitation assay (Olive, 1988), which is based on the potassium detergent precipitation of DNA proteins. Protein-free DNA strand breaks which remain in the supernatant were determined using fluorescence spectroscopy (at 360 nm excitation and 450 nm emission) in the presence of a special buffer to control for the interference of detergent traces (Bester *et al.,* 1994). The diluent consisted of 0.4 M NaCl, 0.1 M Tris-acetate, pH 8.2, 4 mM sodium cholate and 10 µM SYBR® Green dye. Standard solutions of salmon sperm DNA were prepared for calibration. The data were expressed as µg DNA strand/mg total protein in the homogenate. The levels of vitellogenin-like proteins were determined in the gonad of both male and female mussels as described elsewhere (Gagné, 2014). Briefly, the S15 fraction was treated with 35 % acetone at 4 ^{o}C and the high molecular weight proteins were precipitated at 10,000x*g* for 5 min at 4 ^{o}C. The protein pellet was washed in 50 % acetone and treated with 1 M NaOH for 30 min at 40 ^{o}C. The released phosphates were determined using the phosphomolybdate methodology at 640 nm absorbance (Stanton, 1968). The data were expressed as µg of inorganic phosphates/g gonad.

Data analysis

The exposure experiment consisted of 20 mussels per treatment aquarium. Tissue biomarkers were performed on all mussels (N = 10 mussels) using 2-way factorial analysis of variance [municipal effluent concentration, zinc concentration (for dissolved and nanoparticulate) and their interaction] after verifying for homogeneity of variance and normality using Levene's test and the Shapiro-Wilks test, respectively. Post-hoc analysis was performed using Fisher's Least Square Difference test. Correlation analysis was also performed using the Pearson product-moment method. The physiological changes induced by exposure to municipal effluent, nano-ZnO and $ZnCl_2$ were determined using discriminant function and factor analysis methods. All statistical tests were performed using Statistica software (version 8). Significance was set at $\alpha = 0.05$.

Results

The municipal effluent resulted from physico-chemical treatment with the following characteristics (pH 7.8; conductivity 350 µSxcm^{-1}; total ammonia 0.8 mg/L and dissolved organic carbon content 16 - 24 mg/L). Previous investigation of the zinc content in the municipal effluent indicated that total Zn content was in the range of 10 - 25 ug/L, *i.e.*, in the same range of each of the applied Zn forms. Nano-ZnO were diluted in bidistilled water and the particles exhibited an initial size range of 50 ± 10 nm determined using dynamic light scattering, but the mean size increased to 440 ± 70 nm when diluted in aquarium water. This suggests aggregation of nano-ZnO in the presence of tap water. No change in the Zeta potential was observed (-35 mvolt). The general condition of the mussels was assessed based on the mussel's weight/shell length (condition factor or CF) and the gonado-somatic index (GSI). In mussels exposed to nano-ZnO, the condition factor was affected only by Zn exposure concentrations (Fig. 1A). CF decreased at 10 µg/L for both forms of Zn, while municipal effluent concentrations were not significant. However, the presence of municipal effluent removed the significance at 10 ug/L nano-ZnO. In mussels exposed to $ZnCl_2$, a two-way factorial ANOVA revealed that Zn concentration only was significant ($p < 0.05$) (Fig. 1B). However, only a small decrease in CF was found in mussels exposed to 10 µg/L Zn and to the 3 % municipal effluent concentration. In mussels exposed to nano-ZnO, the GSI was significantly affected by both the municipal effluent and by both forms Zn (Fig. 1C). The GSI was increased by 1 µg/L Zn in the presence of 3 % and 10 % municipal effluent concentrations. In mussels exposed to $ZnCl_2$ and municipal effluent, no significant change in the GSI was observed. There was no significant correlation between CF and GSI.

Metal metabolism was assessed by measuring changes in labile Zn and MT expression in gills (Figs 2A-D). In mussels exposed to nano-ZnO and the municipal effluent, a significant interaction between municipal effluent and Zn was obtained (Fig. 2A). Labile Zn was increased at the 3 % effluent concentration and returned to control levels

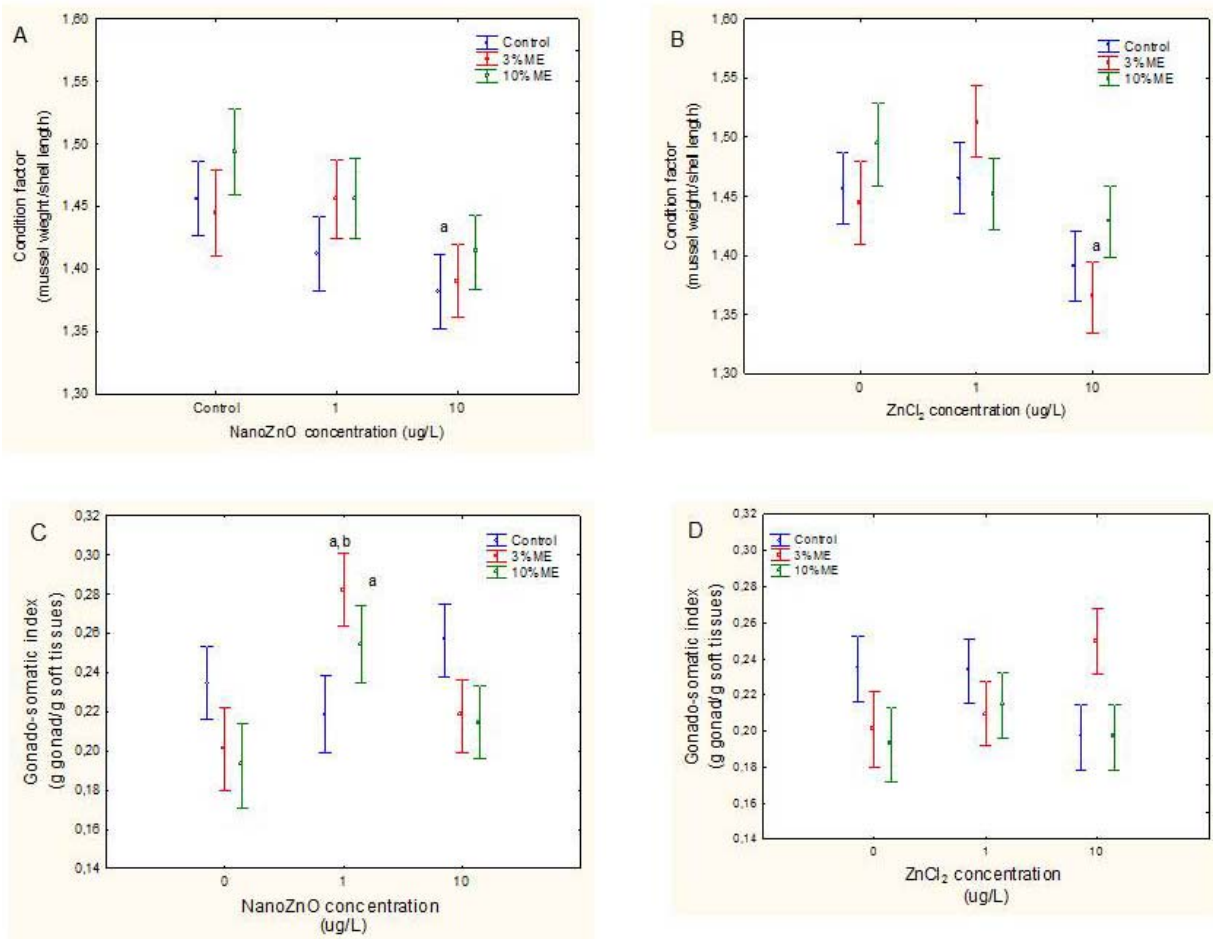

Fig. 1 Morphological characteristics of mussels co-exposed to municipal effluent and both forms of Zn. The condition factor was determined by the mussel total weight / shell length ratio for nano-ZnO (A) and $ZnCl_2$ (B) and municipal effluent. The gonado-somatic index is reported in mussels treated with nano-ZnO (C) and $ZnCl_2$ (D) and municipal effluent. The letter "a" indicates significant difference from controls while the letter "b" indicates significance from municipal effluent concentrations. The data are expressed as the mean with standard deviation from N = 8 mussels.

at the 10 % municipal effluent concentration owing to the high organic charge of the municipal effluent at this concentration. No significant change in labile Zn was observed for either form of Zn. In mussels exposed to $ZnCl_2$ and the municipal effluent, a significant interaction between Zn and municipal effluent exposure concentrations was observed, with the municipal effluent concentration being the dominant factor. Labile Zn was significantly increased in mussels exposed to the 3 % municipal effluent concentration and to 1 µg/L and returned to control levels at 10 µg/L Zn and 10 % municipal effluent. Both forms of Zn reduced the increase observed with the 3 % municipal effluent concentration and produced no changes on their own. MT levels in gills were determined in mussels exposed to the municipal effluent and to the two forms of Zn. In mussels exposed to nano-ZnO and the municipal effluent, a significant interaction between the municipal effluent and Zn concentration was obtained, with the municipal effluent concentration being the dominant factor. MT levels were significantly increased with nano-ZnO at 1 and 10 µg/L Zn in the absence of municipal effluent. When municipal effluent was present, the increase resulting from exposure to nano-ZnO was eliminated owing to the complex nature of the effluent matrix. The municipal effluent alone was able to significantly increase gill MT in mussels. In mussels exposed to $ZnCl_2$ and the municipal effluent, a positive interaction between the exposure concentration of Zn and the municipal effluent concentration was obtained. In the absence of municipal effluent, MT levels were significantly increased at 10 µg/L, but this effect was lost when the municipal effluent was present. Correlation analysis (Table 1) revealed that MT levels were significantly correlated with labile Zn ($r = 0.36$; $p = 0.001$) and GSI ($r = -0.31$; $p < 0.01$).

Gonadal vitellogenin-like proteins (VTG-like proteins) levels were determined as an indirect assay for based on ALP levels in high molecular

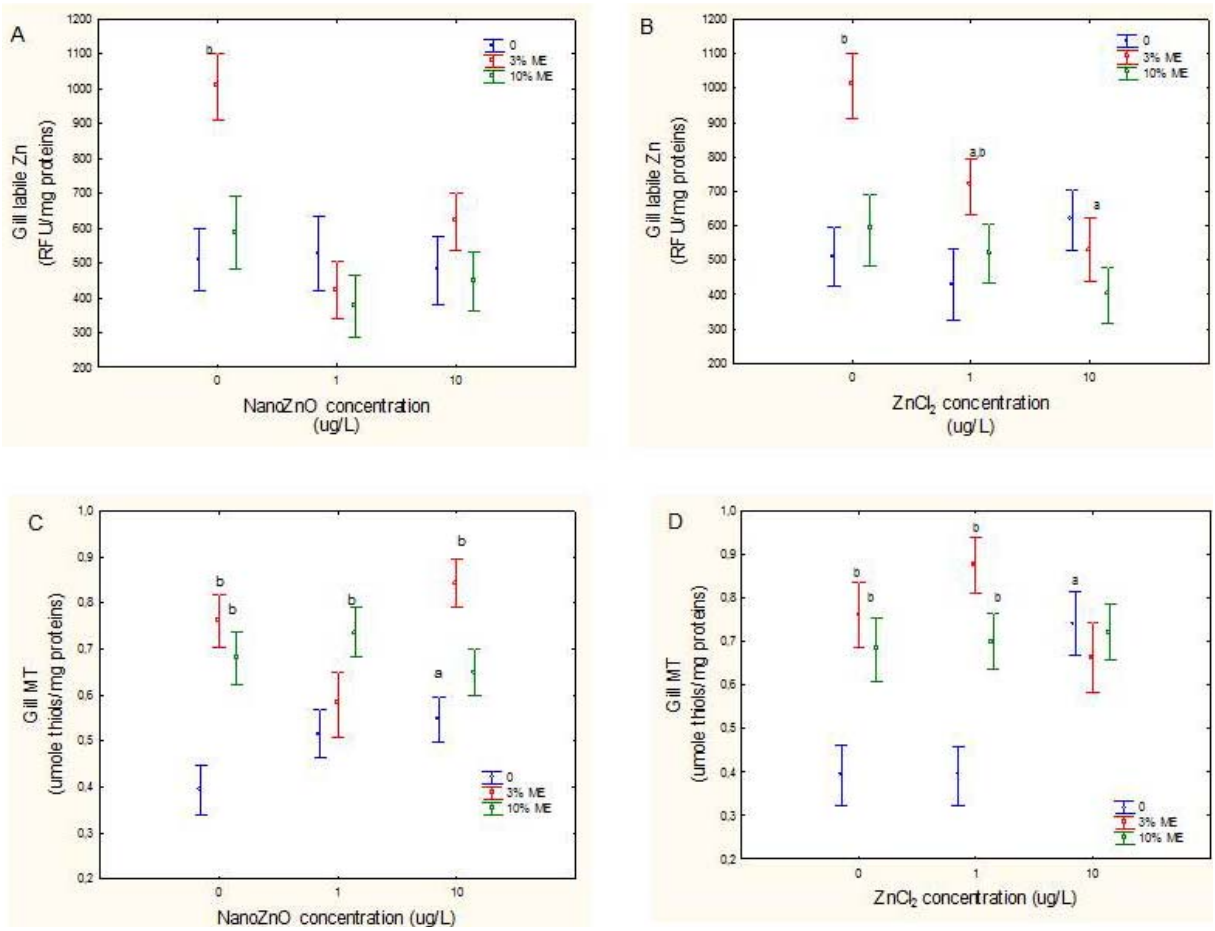

Fig. 2 Metal metabolisms in gills of mussels exposed to municipal effluent, nano-ZnO and $ZnCl_2$. Labile Zn was determined in gills for nano-ZnO (A) and $ZnCl_2$ (B) and municipal effluent. The gonado-somatic index is reported in mussels treated with nano-ZnO (C) and $ZnCl_2$ (D) and municipal effluent. The letter "a" indicates significant difference from Zn controls while the letter "b" indicates significance from municipal effluent concentrations. The data are expressed as the mean with standard deviation from N = 8 mussels.

weight proteins (Figs 3A-D). The data revealed that ALP levels marginally differ between males and females ($0.1<p<0.05$), with ALP levels somewhat higher in females than in males in control mussels. Mussels were at the post-spawning/resting phase, which explains the absence of differences between males and females. In addition, the presence of VTG-like proteins was also investigated by high resolution gradient gel electrophoresis and silver staining (results not shown). It corroborated the changes in ALP levels. ALP levels were significantly induced at the 10 % municipal effluent concentration in both males and females, whereas nano-ZnO had no significant effect on ALP levels in males. VTG-like proteins where increased by nano-ZnO and ZnCl2 but only for the lowest concentration of 1 µg/L Zn. However, a significant increase in VTG-like proteins levels was observed in male and female mussels exposed to 1 µg/L $ZnCl_2$, but the levels returned to control values when the municipal effluent was present. Correlation analysis revealed that VTG-like proteins were not significantly correlated with any of the parameters tested, which suggests that the estrogenic nature of the municipal effluent does not explain the changes observed with the other endpoints.

The extent of tissue damage was assessed by MDA and DNA strand breaks in gills (Figs 4A-D). In mussels exposed to nano-ZnO and the effluent, both factors were significant for MDA levels in gills, with the Zn concentration being the dominant factor. MDA levels were decreased at the 10 % municipal effluent concentration, and this decrease was lost in the presence of nano-ZnO (Fig. 1A). In mussels exposed to $ZnCl_2$ and the municipal effluent, only the municipal effluent concentration was significant in respect of gill MDA levels (Fig. 1B). MDA levels were significantly reduced at the 10 % effluent concentration, but this decrease was lost in the presence of 1 and 10 µg/L $ZnCl_2$. Correlation analysis revealed that gill MDA was significantly correlated with MT in gills ($r = -0.27$; $p = 0.01$).

DNA strand breaks were also determined in mussel gill tissues. In mussels exposed to nano-ZnO and municipal effluent, only the Zn concentration was significant (Fig. 4C). DNA strand

Table 1 Correlation analysis of biomarker data

	Condition factor	GSI	MT gills	DNA damage gills	MDA gills	ALP gonad	Free Zn gills
Condition factor	1	-0.10 *p*>0.1	-0.02 *p*>0.1	0.02 *p*>0.1	0.02 *p*>0.1	0.16 *p*=0.1	-0.01 *p*>0.1
GSI		1	-0.32 *p*=0.001	-0.05 *p*>0.1	0.01 *p*>0.1	-0.12 *p*>0.1	-0.07 *p*>0.1
MT gills			1	-0.15 *p*>0.1	-0.27 *p*=0.01	-0.07 *p*>0.1	0.36 *p*<0.001
DNA damage gills				1	0.54 *p*<0.001	0.14 *p*>0.1	-0.08 *p*>0.1
MDA gills					1	*-0.11* *p>0.1*	*0.15* *p>0.1*
ALP						1	-0.11 *p*>0.1

breaks were increased in mussels exposed to 10 µg/L Zn only and to the 10 % municipal effluent concentration. In mussels exposed to $ZnCl_2$ and the municipal effluent, exposure to Zn was the significant factor (Fig. 4D). The levels of DNA strand breaks were increased in mussels exposed to 1 and 10 µg/L Zn in the presence of the 10 % municipal effluent concentration. Correlation analysis revealed that DNA strand breaks were significantly correlated with MDA in gills ($r = 0.53$; $p < 0.001$), indicating that oxidative stress contributed to DNA damage. This was further supported by analysis of covariance of DNA strand breaks against MDA levels, which showed that only MDA levels were significant and not the exposure concentrations of nano-Zno, $ZnCl_2$ and municipal effluent.

In the attempt to gain an overall picture of the effects of municipal effluent and each form of Zn, discriminant function and factor analysis was used (Fig. 5). The total variance of the biomarker data was explained at 86 % with a mean classification efficiency at 53 %, which suggests some similarities between the responses for nano-ZnO, $ZnCl_2$ and municipal effluent alone and in combination. The municipal effluent response patterns differ markedly from the controls and both forms of Zn and involved the following effects: free Zn, CF, DNA damage, MT, GSI and ALP (vitellogenin-like proteins). The response pattern for nano-ZnO and $ZnCl_2$ was more closely related, which suggests a common mechanism, perhaps the involvement of dissolved Zn^{2+} or low molecular size Zn. This holds true when the municipal effluent was present where the effects are associated with the first component on the x axis: MT, ALP and GSI. When each form of Zn was present with the municipal effluent, a different response pattern was observed, which was explained by the x axis with the following major biomarkers: MT, MDA and labile Zn. It is noteworthy that the combined exposure effects were more closely related to either nano-ZnO or $ZnCl_2$ than to the municipal effluent, indicating that Zn concentration-mediated effects were generally more predominant in the presence of municipal effluent.

Discussion

In the present study, mussels were exposed to two forms of Zn-nano-ZnO and $ZnCl_2$-both in the presence and absence of a primary physico-chemically treated effluent. Nanoparticles with low Zeta potential (between -40 to 40 mvolts) are expected to form aggregates at low salt concentrations found in freshwater (Gagné *et al.,* 2015). The surface charges of the nanoparticles could be canceled by salts in the media which results in aggregation. In mussels exposed to nano-ZnO, the following changes were observed: decreased CF and MDA levels with increased GSI, MT and DNA strand breaks. The induction of MT suggests the mobilization of Zn ions or reactive oxygen species given the dual role of MT in sequestration of divalent ions and reactive oxygen species (Gagné *et al.,* 2008b). Given that MT levels were negatively correlated with MDA levels and positively correlated with free Zn in gills, the data suggest that MT was involved in the prevention of oxidative stress and the mobilization of free Zn in gills. This was also observed in another recent study with the freshwater mussel *Unio tumidus* (Falfushynska *et al.,* 2015). The effects of nano-ZnO on MT levels and oxidative stress end points, such as protein carbonyl levels and superoxide dismutase, revealed that protein carbonyl levels were also decreased, suggesting the involvement of oxidative stress in addition to the release of Zn from the nanoparticles. In another study, exposure of Pacific oysters *Crassostrea gigas* to nano-ZnO led to oxidative stress and mitochondrial dysfunction (Trevisan *et al.,* 2014). The study revealed that gills were the primary target for nano-ZnO owing to its function in trapping fine particles and directing them to the digestive system. Increased MDA levels and glutathione-dependent peroxidase with lower glutathione reductase activities in addition to ultrastructural alterations of mitochondria were also observed in gills. Finally, in a previous study with *Elliptio complanata*, exposure to nano-ZnO also led to increased MT and MDA levels in the digestive

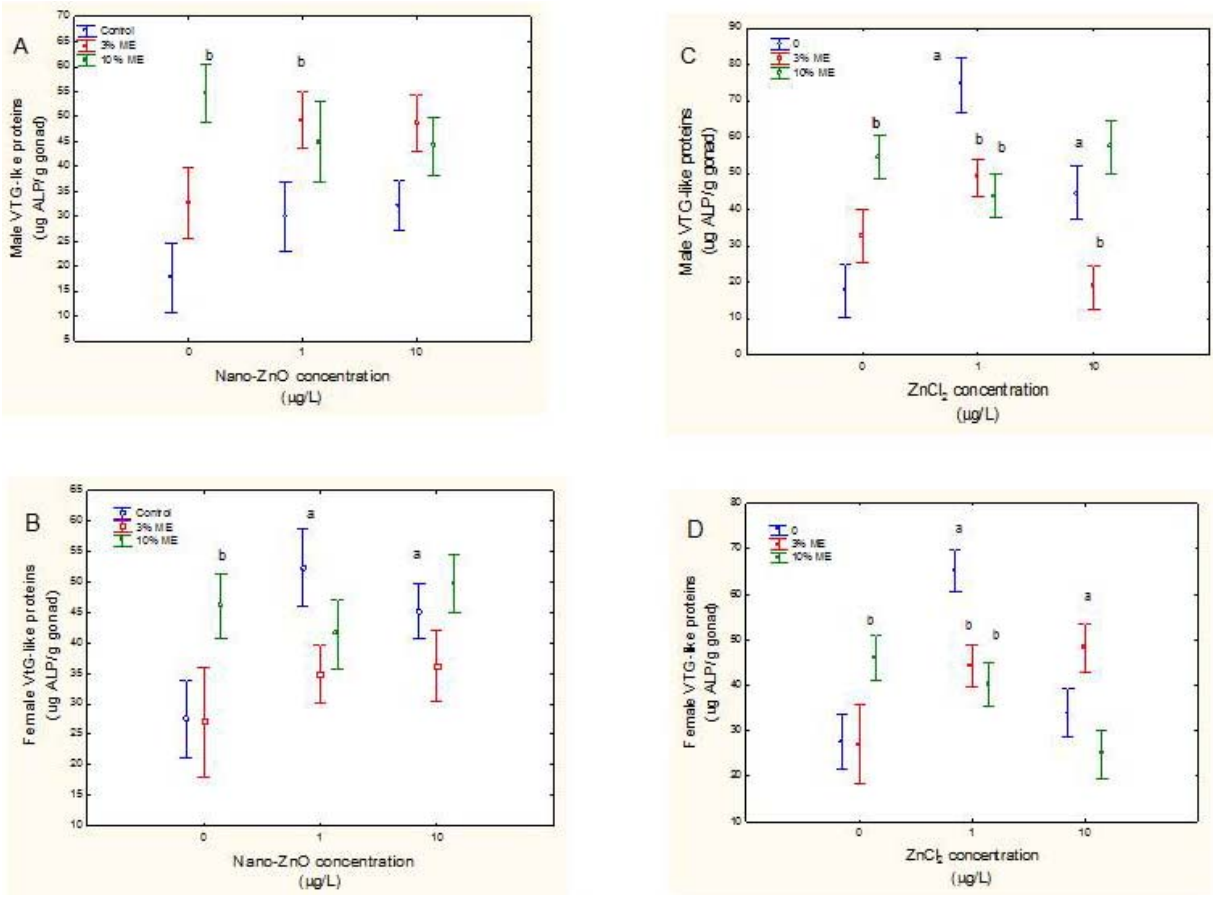

Fig. 3 Levels of vitellogenin-like proteins in male and female mussels exposed to municipal effluent and nano-ZnO. The levels of alkali-labile phosphates were determined in the gonad of mussels exposed to each form of Zn and to the municipal effluent. The letter "a" indicates significant difference from Zn controls. The letter "b" indicates significance from municipal effluent controls. The data are expressed as the mean with standard deviation from N = 4 males and females mussels.

gland (Gagné *et al.,* 2013). The study revealed that the metallome profile in the digestive gland of mussels exposed to nano-ZnO differed from that of mussels exposed to $ZnCl_2$. These studies support the hypothesis that nano-ZnO toxicity is not associated solely with the release of Zn^{2+} ions in tissues. Nano-ZnO, but not $ZnCl_2$, also caused genotoxic effects in mussel gills related to MDA levels in gills. Exposure to municipal effluent decreased gill DNA breaks which suggest reduced DNA repair activity. Exposure to both forms of Zn and to the municipal effluent led to an increase in DNA strand breaks, which suggests that increasing effects on DNA strand breaks in mussel exposed to either form of Zn were maintained in the presence of the municipal effluent. There are few studies in the literature on the genotoxicity of nano-ZnO in mussels. Nano-ZnO was found to cause genotoxic effects in the fruit fly *Drosophila melanogaster* (Carmona *et al*., 2015). In larvae hemocytes, a significant increase in both DNA damage and MDA levels was observed for nano-ZnO, which suggests genotoxicity and involves the production of reactive oxygen species. Nano-ZnO genotoxicity was shown to be size dependent, with small nanoparticles (26 nm diameter) not being genotoxic while larger nanoparticles (78 and 147 nm diameter) induced micronuclei formation in human lymphoblastoid cells (*Yin et al*., 2015). Mussels exposed to municipal effluents had elevated levels of ALP, an indirect assay for vitellogenin-like proteins in *E. complanata* mussels. ALP levels were induced for mussels exposed to 1 µg/L of nano-ZnO and $ZnCl_2$, but not to 10 µg/L. The increasing effect in ALP was lost when the municipal effluent was present, which suggests a dampening effect of the Zn-induced increase in ALP by the municipal effluent. An explanation for this increase is lacking at the present time. One possibility is that production of vitellogenin-like protein was somehow enhanced at the low Zn concentration given that vitellogenin synthesis involves the mobilization of zinc and binds Zn in oocytes (Thompson *et al.,* 2001). Increased vitellogenin gene expression was also observed in sexually-immature rainbow trout exposed to Cd-based quantum dots (Gagné *et al.,* 2010). Interestingly, gene expression of estradiol receptor

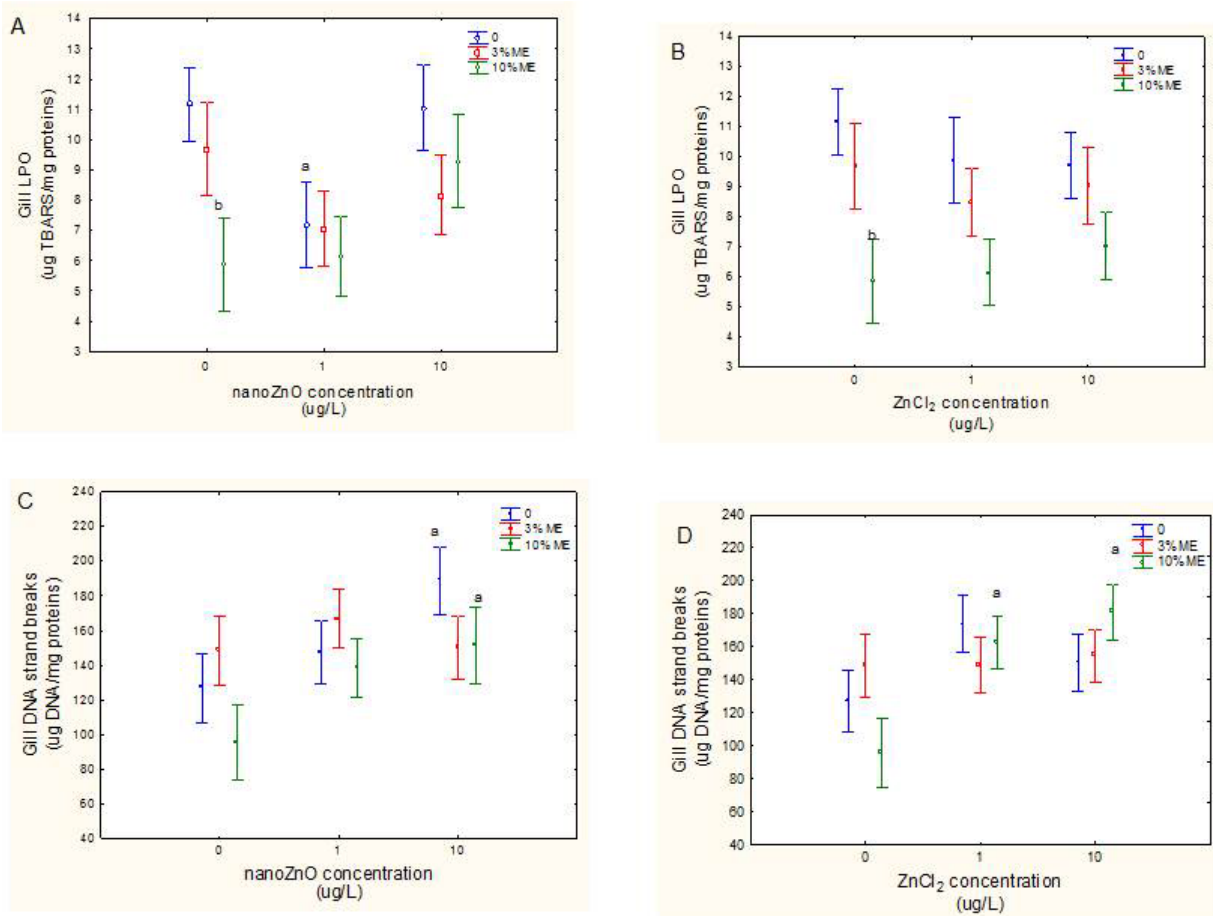

Fig. 4 Biomarker of tissue damage in mussels exposed to municipal effluent and Zn. Gill lipid peroxidation was determined in mussels exposed to nano-ZnO (A) and $ZnCl_2$ (B) and to municipal effluent. The levels of DNA strand breaks are also shown in mussels exposed to nano-ZnO (C) and $ZnCl_2$ (D) and to municipal effluent. The letter "a" indicates a significant difference from Zn controls. The letter "b" indicates significance from municipal effluent concentrations. The data are expressed as the mean with standard deviation from N = 8 mussels.

was also more strongly expressed which confers some endocrine-disrupting activity of Cd and Zn-based nanoparticles. Further research will be necessary to understand the mode of action of increased vitellogenin signaling and to confirm whether nano-ZnO or $ZnCl_2$ could induce vitellogenesis in mussels.

It is noteworthy that both the municipal effluent and nano-ZnO led to increased MT levels, but the effects were not additive. The maximum induction of MT for nanoZnO alone was about twice that in the controls, compared to 1.6 to 2.1 fold in mussels exposed to the municipal effluent (3 and 10 %) and 10 µg/L nano-ZnO. Induction of MT in the mussel *Meretrix meretrix* exposed to treated municipal effluent was observed (Wan *et al*., 2015). Signs of oxidative stress were also observed through increased superoxide dismutase and catalase activity and MDA levels. Increased MT levels and oxidative stress were associated with increased concentrations of Pb, Cr and Zn in *Lasmigona costata* mussels collected downstream of municipal discharges, which shows an impact directly in the receiving environment (Gillis *et al*., 2014). Hence, disruption of metal metabolism and oxidative stress is likely to occur in mussels exposed to nano-ZnO through municipal effluent. In conclusion, exposure to nano-ZnO for 21 days leads to a series of changes in freshwater mussels: decreased CF, increased MT, increased ALP and increased DNA strand breaks. For mussels exposed to municipal effluent for 21 days, the following biomarkers were significantly influenced: gill labile Zn (increase), MT (increase), ALP (increase), and MDA (decrease). The biomarkers MT and ALP (vitellogenin-like proteins) were therefore affected by both the municipal effluent and Zn, which could reach saturation in their response and overspills to other physiological targets such as labile Zn and MDA levels in gill tissues for MT. There is no evidence of overspill effects for ALP levels since no correlation with the other biomarkers was observed. The toxicity of municipal effluent to freshwater mussels could be modified by the addition of nano-ZnO. In conclusion, the effects of nano-ZnO could change in the presence of municipal effluents for freshwater mussels. When alone, the effects of ME differ from those of nano-ZnO, $ZnCl_2$ and controls. The effects of

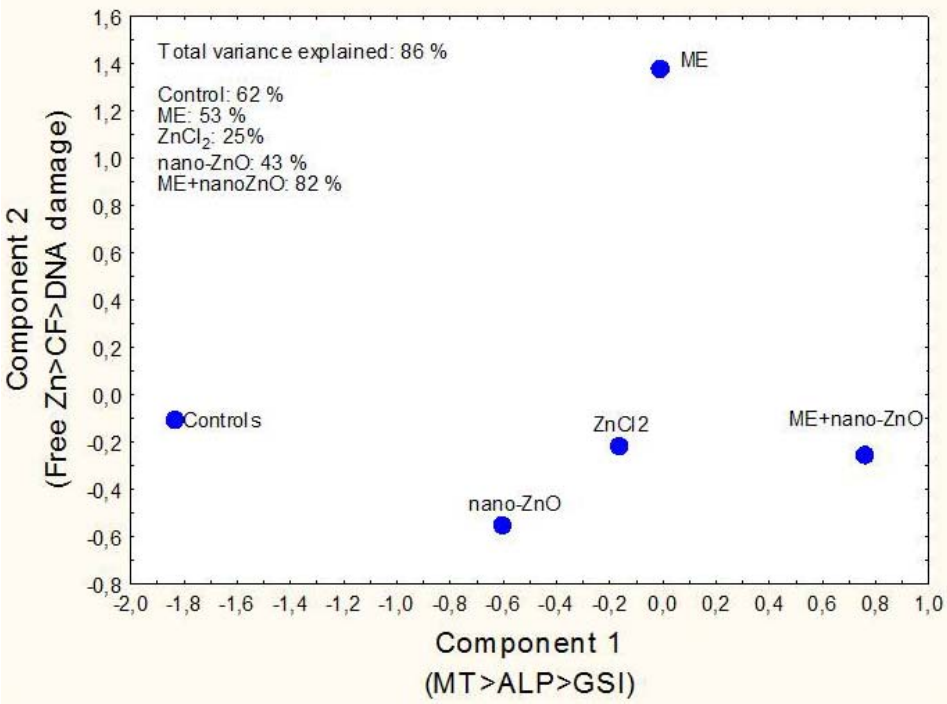

Fig. 5 Discriminant function of biomarker responses in mussels exposed to municipal effluents and Zn. The total variance was explained at 85 % with both components. The biomarkers in parentheses are the three most correlated biomarkers with each of the two components of the x and y axis.

nano-ZnO were more similar to those of ZnCl2 based on free Zn levels, MT levels and DNA damage. The levels of VTG-like proteins were influenced by both forms of Zn and ME and these could act in a cumulative fashion when mussels are exposed to the ME and nano-ZnO.

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References

Adam N, Schmitt C, De Bruyn L, Knapen D, Blust R. Aquatic acute species sensitivity distributions of ZnO and CuO nanoparticles. Sci. Total Environ. 526: 233-242, 2015.

Bradford MM. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72: 248-254, 1976.

Canesi L, Ciacci C, Fabbri R, Marcomini A, Pojana G, Gallo G. Bivalve molluscs as a unique target group for nanoparticle toxicity. Mar. Environ. Res. 76: 16-21, 2012.

Carmona ER, Inostroza-Blancheteau C, Rubio L, Marcos R. Genotoxic and oxidative stress potential of nanosized and bulk zinc oxide particles in Drosophila melanogaster. Toxicol. Ind. Health 2015 [in press].

Cole C, Shyr T, Ou-Yang H. Metal oxide sunscreens protect skin by absorption, not by reflection or scattering. Photodermatol. Photoimmunol. Photomed. 32: 5-10, 2016.

Contado C. Nanomaterials in consumer products: a challenging analytical problem. Front. Chem. 3: 48, 2015.

Fahmy SR, Abdel-Ghaffar F, Bakry FA, Sayed DA. Ecotoxicological effect of sublethal exposure to zinc oxide nanoparticles on freshwater snail *Biomphalaria alexandrina*. Arch. Environ. Contam. Toxicol. 67: 192-202, 2014.

Falfushynska H, Gnatyshyna L, Yurchak I, Sokolova I, Stoliar O. The effects of zinc nanooxide on cellular stress responses of the freshwater mussels *Unio tumidus* are modulated by elevated temperature and organic pollutants. Aquat. Toxicol. 162: 82-93, 2015.

Formigari A, Irato P, Santon A. Zinc, antioxidant systems and metallothionein in metal mediated-apoptosis: Biochemical and cytochemical aspects. Comp. Biochem. Physiol. 146C: 443-459, 2007.

Gagné F, Gagnon C, Blaise C. Aquatic Nanotoxicology: A review. Res. Trend. 4: 1-14, 2008a.

Gagné F, André C, Blaise C. The dual nature of metallothioneins in the metabolism of heavy metals and reactive oxygen species in aquatic organisms: implications of use as a biomarker of heavy-metal effects in field investigations. Biochem. Insights 1: 31-41, 2008b.

Gagné F, Fortier M, Yu L, Osachoff HL, Skirrow RC, van Aggelen G, *et al*. Immunocompetence and alterations in hepatic gene expression in rainbow trout exposed to CdS/CdTe quantum dots. J. Environ. Monit. 12: 1556-1565, 2010.

Gagné F, Turcotte P, Auclair J, Gagnon C. The effects of zinc oxide nanoparticles on the metallome in freshwater mussels. Comp. Biochem. Physiol. 158C: 22-28, 2013.

Gagné F. Neuroendocrine disruption. Chapter 9. In: Biochemical ecotoxicology: principle and methods, Elsevier Inc., 145-170, 2014.

Gagné F, Auclair J, Peyrot C, Wilkinson KJ. The influence of zinc chloride and zinc oxide nanoparticles on air-time survival in freshwater mussels. Comp. Biochem. Physiol. 172-173C: 36-44, 2015.

Gagnon C, Turcotte P, Trépanier S, Gagné F, Cejka PJ. Impacts of municipal wastewater oxidative treatments: Changes in metal physical speciation and bioavailability. Chemosphere 97: 86-91, 2014.

Gagnon C, Gagné F, Turcotte P, Saulnier I, Blaise C, Salazar MH, *et al*. Exposure of caged mussels to metals in a primary-treated municipal wastewater plume. Chemosphere 62: 998-1010, 2006.

Gillis PL, Higgins SK, Jorge MB. Evidence of oxidative stress in wild freshwater mussels (*Lasmigona costata*) exposed to urban-derived contaminants. Ecotoxicol. Environ. Saf. 102: 62-69, 2014.

Gomes T, Pereira CG, Cardoso C, Pinheiro JP, Cancio I, Bebianno MJ. Accumulation and toxicity of copper oxide nanoparticles in the digestive gland of *Mytilus galloprovincialis*. Aquat. Toxicol. 118-119: 72-79, 2012.

Marisa I, Matozzo V, Munari M, Binelli A, Parolini M, Martucci A, *et al*. In vivo exposure of the marine clam *Ruditapes philippinarum* to zinc oxide nanoparticles: responses in gills, digestive gland and haemolymph. Environ. Sci. Pollut. Res. Int. 23: 15275-15293, 2016.

Montes MO, Hanna SK, Lenihan HS, Keller AA. Uptake, accumulation, and biotransformation of metal oxide nanoparticles by a marine suspension-feeder. J. Hazard Mater. 225-226: 139-145, 2012.

Stanton MG. Colorimetric determination of inorganic phosphate in the presence of biological material and adenosine triphosphate. Anal. Biochem. 22: 27-34, 1968.

Thompson ED, Olsson PE, Mayer GD, Haux C, Walsh PJ, Burge E, *et al*. Effects of 17 beta-estradiol on levels and distribution of metallothionein and zinc in squirrelfish. Am. J. Physiol. Regul. Integr. Comp. Physiol. 280: 527-535, 2001.

Trevisan R, Delapedra G, Mello DF, Arl M, Schmidt ÉC, Meder F, *et al*. Gills are an initial target of zinc oxide nanoparticles in oysters *Crassostrea gigas*, leading to mitochondrial disruption and oxidative stress. Aquat. Toxicol. 153: 27-38, 2014.

Wan R, Meng F, Fu W, Wang Q, Su E. Biochemical responses in the gills of *Meretrix meretrix* after exposure to treated municipal effluent. Ecotoxicol. Environ. Saf.111: 78-85, 2015.

Yin H, Casey PS, McCall MJ, Fenech M. Size-dependent cytotoxicity and genotoxicity of ZnO particles to human lymphoblastoid (WIL2-NS) cells. Environ. Mol. Mutagen. 56: 767-766, 2015.

Yu LP, Fang T, Xiong DW, Zhu WT, Sima XF. Comparative toxicity of nano-ZnO and bulk ZnO suspensions to zebrafish and the effects of sedimentation, ˙OH production and particle dissolution in distilled waterJ. Environ. Monit. 13: 1975-1982, 2011.