Comparative Biochemistry and Physiology, Part C 172–173 (2015) 36–44

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part C

journal homepage: www.elsevier.com/locate/cbpc

# The influence of zinc chloride and zinc oxide nanoparticles on air-time survival in freshwater mussels

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ARTICLE INFO

*Article history:*
Received 21 October 2014
Received in revised form 22 April 2015
Accepted 29 April 2015
Available online 7 May 2015

*Keywords:*
*Elliptio complanata*
Air-time survival
inflammation
Oxidative stress

ABSTRACT

The purpose of this study was to determine the cumulative effects of exposure to either dissolved zinc or nanozinc oxide (nanoZnO) and air-time survival in freshwater mussels. Mussels were exposed to each forms of zinc for 96 h then placed in air to determine survival time. A sub-group of mussels before and after 7 days of exposure to air were kept aside for the determination of the following biomarkers: arachidonate-dependent cyclooxygenase (COX) and peroxidase (inflammation and oxidative stress), lipid metabolism (total lipids, esterases activity, HO-glycerol, acetyl CoA and phospholipase A2) and lipid damage (lipid peroxidation [LPO]). The results showed that air-time survival was decreased from a mean value of 18.5 days to a mean value of 12 days in mussels exposed to 2.5 mg/L of nanoZnO although it was not lethal based on shell opening at concentrations below 50 mg/L after 96 h. In mussels exposed to zinc only, the median lethal concentration was estimated at 16 mg/L (10–25 95% CI). The air-time survival did not significantly change in mussels exposed to the same concentration of dissolved Zn. Significant weight losses were observed at 0.5 mg/L of nanoZnO and at 2.5 mg/L for dissolved zinc chloride, and were also significantly correlated with air-time survival ($r = 0.53$; $p < 0.01$). Air exposure significantly increased COX activity in control mussels and in mussels exposed to 0.5 mg/L of nanoZnO and zinc chloride. The data also suggested fatty acid breakdown and β-oxidation. Mussels exposed to contaminants are more susceptible to prolonged exposure to air during low water levels.



## 1. Introduction

Nanotechnologies are becoming a major consumer product. For example, they are found in optical devices (e.g. solar panels), microelectronic circuits, drug delivery systems, and personal care products such as textiles, creams and sunscreens (Lee et al., 2008). The commercial interests in nanoproducts reside in their high ratio of surface area to volume, which brings about new and enhanced quantum properties. Consequently, consumer products and cosmetics represent a major area of economic growth for nanotechnology in most countries (NCI, 2006). However, legitimate concerns have been raised by the public and regulatory community about nanotechnology's safety for the human population and environment. For example, in an effort to decrease the risk of developing skin cancers from sun exposure, cream-based products composed of zinc oxide show strong UV-absorbing properties compared to other metal oxides, and have been promoted as a broad spectrum sunscreen over the last decade (Mitchnick et al., 1999). Nanoparticles of zinc oxide (nanoZnO) have the additional advantage of being transparent compared to zinc oxide-based creams, which appear as a white paste. NanoZnO also has anti-microbial properties which is considered an additional advantage for consumers. Hence, the increasing use of these sunscreens could result in the release of nanoZnO and dissolved zinc if they degrade to the water column during swimming or via wastewaters. Currently, the impacts of nanoparticles on aquatic biota are not well understood; the low solubility of zinc oxide suggests that it will partition in the benthic environment at the sediment/water interface.

Most freshwater mussels live in the benthic environment, where, typically, they are partly buried in sediment. They are sessile, live for long periods, and feed on suspended material in the water column. The breathing/feeding behavior of mussels makes them species vulnerable to contaminants absorbed to fine particles or associated to fine suspended matter such as nanoparticles (Canesi et al., 2012). Indeed, the gills possess ciliary activity and act as a primordial mouth where trapped particles are directed in the digestive gland. In a recent study, exposure of marine mussels *Mytilus galloprovincialis* to copper nanoparticles led to the accumulation of copper in the digestive gland and produced toxicity such as lipid oxidation (Gomes et al., 2012). In another study, exposure of the oyster *Crassostrea gigas* to nanoZnO led to zinc accumulation in gills within the first 24 h followed by the digestive gland (24 to 48 h), which is consistent with the feeding behavior of mussels. Ultrastructural electron microscopy and biochemical analyses revealed signs of mitochondrial disruption and oxidative stress (decreased thiols and increased lipid peroxidation [LPO]) in both tissues (Trevisan et al., 2014). In a recent study,

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http://dx.doi.org/10.1016/j.cbpc.2015.04.005
1532-0456/

exposure to zinc nanoparticles (3 μM) to freshwater mussels Unio tumidus increased metallothionein (MT) levels and induced oxidative stress as with oysters. This response was also exacerbated by increase in water temperature at 25 °C (Falfushynska et al., 2015). Both marine and freshwater mussels have the capacity to survive for long periods in air, from days to weeks depending on conditions such as temperature and seasonal period. In stressful situations, mussels can close their shells for days or weeks, which represents an important survival mechanism for these organisms. It is also anticipated that, in the context of climate change, warmer temperatures could result in lower water levels (droughts) in some areas, increasing exposure to air and higher temperatures (Kim et al., 2014). Air-time survival is well-studied in marine organisms (Viarengo et al., 1995; Hellou and Law, 2003), but virtually no information exists for freshwater mussels. In marine organisms, exposure to air for long periods leads to anoxic conditions and restricted food intake. Moreover, long emersion times of blue mussels leads to increased gene expression involved in energetic metabolism, protein chaperoning and maintenance of the cytoskeleton in cells (Letendre et al., 2011). They also showed that the presence of some contaminants could impact the same genes, which could have cumulative deleterious effects on mussels. During prolonged exposure to air, mussels are known to adopt a gaping strategy, which is a behavioral compromise between air intake and of drying tissues. Air exposure also led to increased acid phosphatase in blue mussels' hemocytes, indicating the presence of bacterial activity and leading to the liberation of phosphates from proteins (Kuchel et al., 2010). During that time (air exposure), important physiological stresses are at play where mussels must endure for survival. Fasting will lead to depleted energy stores involving lipid mobilization, where anoxic/dry conditions could lead to loss of energy consumption and inflammation.

The purpose of this study, therefore, was to examine the cumulative toxicity of nanoZnO, dissolved zinc and aerial exposure to the freshwater unionid *Elliptio complanata*. We first determined the acute lethal concentration of zinc oxide nanoparticles and dissolved zinc chloride for comparison purposes at 15 °C for 96 h. Secondly, we exposed mussels to low doses of zinc oxide nanoparticles and zinc chloride for 96 h at 15 °C followed by exposure in air to determine the air time survival. A group of mussels were collected at day 7 to determine the physiological stress using a test battery of effect biomarkers known to respond to either zinc- or metal-based nanoparticles and air emersion (Gagné et al., 2008; Canesi et al., 2012). Special attention was given to lipid metabolism, oxidative stress and inflammation, which readily respond to air emersion. Lipid metabolism was determined by measuring total tissue lipids and markers of lipid mobilization, by following changes in general esterase activity (lipase), phospholipase 2 (PLA2), which is involved in the hydrolysis of carbon-2 fatty acids or inflammation precursors (arachidonic acid) on the glycerol backbone of phospholipids, and levels of glycerol from the hydrolysis of phospholipids. Our attention focused on the effects of nanoZnO and dissolved zinc on the coping mechanisms during air emersion.

## 2. Materials and methods

### 2.1. Exposure of freshwater mussels to zinc chloride and zinc oxide nanoparticles

Freshwater mussels were collected in June at a pristine Laurentian Lake located north of the city of Montreal (QC, Canada). Mussels between 6–9 cm were collected and were transported back to the laboratory in iceboxes. Mussels were then transferred in 300-L tanks supplied with charcoal filter and UV-treated tap water of the City of Montréal maintained at 15 °C under constant aeration. They were fed with commercial coral reef solution enriched with 100 million/mL of *Pseudokirchneriella subcapitata* algae every 2 days. For determination of the acute lethality based on shell opening, *E. complanata* mussels were exposed to increasing concentrations of total zinc equivalents (0.5, 5, 25 and 50 mg/L), from either zinc chloride or nanoZnO preparations. Control mussels were exposed to aquarium water only. Twenty mussels per 20 L container lined with polyethylene bags were used in duplicates and exposed for 96 h at 15 °C. At the end of the exposure period, mortality (shell opening) was recorded. Because mussels have the capacity to close their shells in stressful conditions for many days if not weeks, the air-time survival was used to determine the capacity of mussels to resist emersion from water for long periods (Viarengo et al., 1995). The air-time survival was therefore determined for up to 28 days in air at 20 °C under humidified atmosphere (80%) with $N = 20$ mussels per treatment. Mortality was determined by sustained shell opening, recorded every day for 28 days. A subsample of surviving mussels ($N = 5$) were also removed after exposure to zinc for 96 h and following exposure to air for 7 days ($N = 5$) for stress biomarker analysis. The proportions of weight loss were determined by the following formula: % weight loss = ((mussel weight at the end of exposure to air / mussel weight before exposure to air) × 100) − 100.

### 2.2. Zinc oxide nanoparticle characterization

NanoZnO stock solution was purchased from Sigma Aldrich (# 721077) at 50% w/v at pH 7.1. The nanoZnO particles were capped with cationic 3-aminopropyl triethoxysilane to ensure stability in the stock solution. The nanoparticles were then diluted at 0.5, 2.5 and 10 mg/L in aquarium water, which was charcoal- and UV-treated tap water from the City of Montréal (pH 7.8, conductivity of 270 ± 10 $\mu S \times cm^{-1}$, and total organic carbon content of 1 mg/L). Particle size was determined by a dynamic light scattering (DLS) instrument with a gel electromobility option (Wyatt-Instrument Mobius, 532-nm laser). Zeta potential was determined from gel mobility data as described in Domingos et al., 2013.

### 2.3. Tissue preparation for biomarker analyses

Biomarkers of stress were determined in mussels exposed to increasing concentrations of either nanoZnO or zinc chloride for 96 h before and after air emersion for 7 days. Mussel weight and shell length were determined before and after exposure to each form of zinc, and after 7 days of exposure to air. At the end of the exposure to air, the mussels were placed on ice and the soft tissues were removed and weighted. The visceral mass homogenate was prepared by adding 5 volumes of ice-cold homogenization buffer, composed of 50 mM of NaCl, 25 mM of Tris–HCl, pH 7.5, 10 μg/mL of apoprotinin and 1 mM of dithiothreitol. A portion of the homogenate was centrifuged at 12,000 ×g for 20 min at 4 °C, and the supernatant (S12 fraction) was collected and stored at −85 °C. Total proteins were determined using the Coomassie Brillant blue dye binding assay (Bradford, 1976). Standard solutions of serum bovine albumin were used for calibration.

### 2.4. Lipid status and metabolism

The levels of total lipids were determined in soft tissue homogenate using the phosphovanillin method (Frings et al., 1972). Calibration was achieved with Triton X-100 instead of olive oil as the standard. The data were expressed as mg lipid equivalents/mg protein. Non-specific esterase (lipases) activity was determined using the carboxy-fluorescein diacetate methodology. Briefly, 50 uL of the S12 fraction of the visceral mass was mixed with 100 μL of 10 μM of carboxy-fluorescein diacetate in 50 mM of Tris–HCl, pH 7.4, containing 0.1% dimethyl sulfoxide. The release of fluorescein was measured at 485 nm excitation and 520 nm emission at 0, 10, 20 and 30 min at 30 °C. LPO was determined in the visceral mass homogenates by the thiobarbituric acid method (Wills, 1987). Thiobarbituric acid reactants (TBARS) were determined by fluorescence at 530 nm for excitation and 630 nm for emission using a fluorescent microplate reader. Standard solutions of tetramethoxypropane were used for calibration. The data were expressed as μg TBARS/mg proteins in the visceral mass.