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Elemental profiles of freshwater mussels treated with silver nanoparticles: A metallomic approach

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ABSTRACT

Nanoparticles released into the environment could pose a risk to resident organisms that feed on suspended particles in aquatic ecosystems. The purpose of this study was to examine the effects of silver nanoparticles (nanoAg) of different sizes in freshwater mussels using a multi-elemental (metallomic) approach in order to determine signature effects of nanoparticulate and ionic Ag. Mussels were exposed to three concentrations (0.8, 4 and 20 μg/L) of 20-nm and 80-nm nanoAg and $AgNO_3$ for 48 h at 15 °C. After the exposure period, mussels were placed in clean, aerated water for a depuration step and analyzed for the following total elements in gill, digestive gland and gonad tissues: Al, Ag, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Pb, Na, Ni, Se, Sr, Th, U, V and Zn. Metallothioneins (MT; digestive gland only) and lipid peroxidation (LPO) were also determined in gills, digestive glands and gonads. The 20-nm-diameter nanoAg was detected in all three tissues at 20 μg/L, while the 80-nm nanoAg was detected more strongly in the digestive gland. Ionic Ag was found at higher levels in gills than in other tissues. Correlation analysis revealed that gonad Ag levels were significantly correlated with Al ($r = 0.28$), V ($r = 0.28$), Cr ($r = 0.31$), Co ($r = 0.32$), Se ($r = 0.34$) and MT levels ($r = 0.28$). Indeed, the MT levels in the digestive gland were significantly increased by 20-nm nanoAg (20 μg/L) and 80-nm nanoAg (4 μg/L) and $AgNO_3$ (<0.8 μg/L). LPO was observed in gills, digestive glands and even gonads for all Ag forms. Discriminant function analysis revealed that all forms of Ag differed from each other and from unexposed mussels, where ionic Ag was more closely related to the 80-nm-diameter nanoAg. Factorial analysis revealed that Ba, Ca, Co, Mn, Sr, U and Zn had consistently high factorial weights in all tissues; that explained 80% of the total variance. Moreover, the following elements showed strong correlations ($r > 0.7$) with each other: Sr, Ba, Zn, Ca, Mg Cr, Mn and U. Comparisons of these elements with other elements showing low or no correlations (e.g., transition elements) revealed that these elements had significantly lower standard reduction potential and electronegativity, suggesting that stronger reducing elements were most influenced by the oxidizing effects of nanoAg and ionic Ag in tissues. Indeed, tissues with oxidative stress (LPO) had decreased levels for most of these reducing elements. We conclude that exposure to Ag nanoparticles produces a characteristic change in the elemental composition of gills, digestive gland and gonad tissues in freshwater mussels. Elements most responsive to oxidative stress were more influenced by both nanoAg and ionic Ag. Sr and Ba were readily decreased by Ag and appeared to respond more sensitively to nanoAg than to ionic Ag. The metallomic approach could contribute in the understanding of fundamental mode of action of nanoparticles in mussels.

1. Introduction

The increasing commercial interest in silver (Ag) arises not only from its use in jewellery but also from its antimicrobial properties (Whitehouse, 2015). Ag is a contaminant commonly found in the environment near urban areas, and its recent use as nanoparticles (nanoAg) has contributed to its increasing presence. From the environmental perspective, aggregation of nanoparticles is common and arises when the surface charges are neutralized by salts at concentrations usually found in freshwater (Louis et al., 2010). In a recent survey of nine British sewage effluents, the mean concentration of colloidal Ag in wastewaters (size range between 2 nm and 450 nm) was 12 ng/L before treatment and 6 ng/L after treatment (Johnson et al., 2014). However, particulate Ag (>450 nm) was detected at much higher concentrations reaching 3.3 and 0.08 μg/L before and after treatment (effluent). NanoAg is currently used as an embedding agent in clothing and other consumer products such as hand sanitizers and soaps for its lasting biocidal properties (Farkas et al., 2011). NanoAg and dissolved Ag from clothes washing could find their way into aquatic environments. For example, some municipal effluents contained in the order of 150 ng/L of colloidal Ag (in the size range of nanoAg), representing 23% to 75% of total Ag in

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municipal effluents (Mitrano et al., 2012), and it is released directly into the environment. NanoAg particles have relativity low surface charge (Zeta potential), making them susceptible to aggregation even in tap water, which leads to relatively large aggregates (>450 nm). Increasing ionic strength of nanoAg suspensions produced aggregates which were reduced by the addition of natural organic matter in suspension (Delay et al., 2011). Humic acids were better than fulvic acid at preventing aggregation in the organic matter content of natural water (Furman et al., 2013). Mussels are sessile organisms and feed on suspended particles (microorganisms), putting them at risk of environmental contamination by nanoparticles (Canesi et al., 2012; Gagné et al., 2008). This risk could also extend to other invertebrates that feed on suspended microorganisms, allowing aggregated nanoparticles to find their way into the intestinal tract (Cattaneo et al., 2010). The release of nanoAg from clothes in mesocosm experiments (for 90 days) led to the accumulation of Ag in hard clams, shrimps and gastropods (Cleveland et al., 2012). Hence, the pathways of exposure for nanoAg could differ from those for ionic Ag, depending on the ionic and organic matter composition of environmental water. It is expected that nanoparticles form aggregates in water, where the larger particles end-up in the digestive tract. However, nanoparticles could produce toxicity not only from the release of dissolved elements (i.e., ionic Ag from nanoAg) but also from size/form, surface reactivity and vectorization (i.e., transport of other elements at the surface of nanoparticles) (Gagné et al., 2008). For example, in rainbow trout liver, the toxicity of nanoAg could not be entirely explained by an equivalent concentration of ionic Ag using a toxicogenomic approach (Gagné et al., 2012). Indeed, exposure to nanoAg involved genes related to inflammation, whereas dissolved Ag involved genes related to oxidative stress and protein stability. At a more fundamental level, the occurrence of nanoAg could produce changes in the distribution of elements in tissues in ways characteristic of nanoAg and ionic Ag. The characteristic changes of the elemental profiles (alkali metals, alkaline earths, transition metals, actinides, etc.) could provide clues about the fundamental interactions (i.e., elemental fluxes in cells) of nanoparticles and other elements such as Ag in organisms. The release of ionic Ag could be followed by changes in metallothioneins (MTs), which are proteins involved in metal detoxification and oxidative stress. MTs are cysteine (thiol)-rich proteins that inactivate and remove dissolved monovalent and divalent metals such as Ag, Cd, Co, Cu and Zn (Ng and Wang, 2005). MTs could also sequester reactive oxygen species because of their high thiol content (30% of amino acids are cysteine residues), but in this process the production of disulfide bridges is accompanied by the release of metals attached to MTs. The occurrence of reactive oxygen species could lead to oxidative release of metals and, if uncontrolled by the normal antioxidant mechanisms in cells, could result in damage such as lipid peroxidation (LPO). In mussels exposed to 10 μg/L Ag as either nanoAg or ionic Ag for 15 days, oxidative stress was observed with both forms of Ag, but antioxidant enzymes showed a different pattern in gills in comparison with in the digestive gland (Gomes et al., 2014). MTs were induced in gills, increasing with Ag concentrations for each form of Ag, but only a small fraction of Ag was related to MT levels in the digestive gland, suggesting that MTs sequestered less Ag in this tissue. LPO was higher in gills exposed to nanoAg, but it was only induced by ionic Ag in the digestive gland, showing that nanoAg toxicity is not similar to that of ionic Ag. The surface properties of nanoAg could also lead to the formation of oxygen radicals at the surface; this in turn could lead to oxidative stress that is not only related to the oxidizing effects of ionic Ag. For example, exposure of fish to citrate-coated nanoAg resulted in increased catalase and glutathione S-transferase in gills and liver respectively, but the contribution of released ionic Ag was difficult to determine (Lee et al., 2012).

The purpose of this study was to determine the bioavailability and toxicity of ionic Ag and 20-nm and 80-nm-diameter nanoAg in freshwater mussels (*Elliptio complanata*). In addition to Ag tissue levels, the elemental composition profiles of the gill, digestive gland and gonad tissues were evaluated in order to understand the fundamental changes in the elemental composition of tissues given different Ag exposure concentrations and forms (ionic Ag and 20-nm and 80-nm nanoAg). Metal sequestration and oxidative stress were also monitored by MT and LPO respectively, for the influence of ionic Ag and changes in redox status of tissues in mussels exposed to Ag forms. The null hypothesis states that changes in bioavailability, elemental profiles and toxicity effects are independent of Ag form and concentration.

2. Materials and methods

2.1. Mussel collection and exposure to Ag

Freshwater mussels (*E. complanata*) were collected by snorkeling in a pristine reference lake (Lake Achigan, Montreal, Quebec, Canada) during the third week of June 2011 and were placed in aerated aquaria at a temperature of 15 °C under 11 h/8 h dark cycles. Mussels were placed on sand beds and fed 3–4 times weekly with phytoplankton commercial feed (Phytoplex, Kent Marine, Franklin, WI) and *Pseudokirchneriella subcapitata* algal preparations. The mussels were maintained for one month in these conditions prior to running the Ag exposure experiments, and dead mussels (those whose shells were opened, which represented <1%) were removed. For the exposure experiments, mussels were sorted to select those between 6 cm and 8 cm in length, and $n = 12$ individuals (per treatment) were placed in 20-L containers lined with polyethylene bags under air bubbling. The mussels were exposed to 0.8 μg/L, 4 μg/L and 20 μg/L total Ag as 20-nm nanoAg, 80-nm nanoAg and $AgNO_3$ for 48 h at 15 °C. The exposure experiment was repeated once more. The nanoparticles were citrate-coated and obtained from the same manufacturer (Ted Pella, Inc.; Redding, CA). The exposure water consisted of tap water dechlorinated using carbon filters and UV irradiation; its pH was between 6.6 and 7. After the exposure period, mussels were placed for 24 h in dechlorinated tap water at the same temperature. This 24-h water depuration step is a common procedure for the removal of loosely bound metals at the surface (including gills and digestive system) of the mussels and emptying of gut content. The mussels were then placed on ice, weighed, and measured for shell length. Half of gills, digestive gland and gonad tissues were dissected with plastic/ceramic knives (acid washed in 0.5% HNO_3 to remove heavy metal contamination) and the remaining half for biomarkers as described below.

2.2. Nanosilver characterization and tissue elemental analysis

NanoAg of 20-nm and 80-nm mean diameter were dissolved in MilliQ water and aquarium dechlorinated tap water at a concentration of 10 μg/L for one hour. The Ag solutions were then filtered through 450-nm, 100-nm, 50-nm and 25-nm pore filters (cellulose acetate) and the filtrated material was analyzed for total Ag by ion coupled plasma (ICP) mass spectrometry (Thermal Ash, USA). Filtrates were treated first with 5% HNO_3 for 24 h at 4 °C before analysis. The relative particle sizes of the stock solutions (0.1 mg/mL) were determined by absorption spectroscopy (plasmon resonance) between 300 nm and 600 nm using the nanoDrop UV–visible spectrometer (Thermo Scientific, USA). To determine metal bioaccumulation in mussel tissues, the gills, digestive glands and gonads were first homogenized using a Teflon pestle tissue grinder at a ratio of 20% in ice-cold homogenization buffer composed of 25-mM Hepes-NaOH, pH 7.4. A portion (1 mL) of the homogenate was acid-digested with 8 mL of concentrated HNO_3, 2 mL of concentrated H_2O_2 (Seastar Baseline) and 1 mL of concentrated HCl. The tissues were treated at 170 °C for 2 h using a microwave bomb-digestion system (Ethos EZ, Milestone Scientific Inc., ON, Canada). The samples were completed to final volume of 12 mL with deionized water. The following 24 elements were determined using ion-coupled mass spectrometry: Al, Ag, As, Ba, Be, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Se, Sr, V, Zn, Th and U. Standard solutions of these elements