Toxic effects and bioaccumulation of nano-, micron- and aqueous-Ag in the estuarine polychaete, *Nereis (Hediste) diversicolor*

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Abstract

Rapidly expanding growth in the field of nanotechnology has led to the development of numerous applications of nanomaterials in industrial and consumer products. Nanosilver is one of the most commonly used nanomaterials due to its effective antibacterial properties. However, there is increasing concern about the fate and potential risks of nanosilver for the aquatic environment after its eventual release via wastewater discharges.

In this thesis, dispersion and stability tests of commercially available nano (<100 nm)- and micron (2-3.5 μm and <45 μm)-Ag particles in two media (deionized water vs. filtered natural seawater) were firstly performed with the purpose to investigate the behavior of Ag particles in aqueous environments. A sediment exposure pathway was selected for the following toxicity experiments (I and II) as both Ag particles tended to precipitate in the water phase over time.

Due to the importance of fully characterizing nanoparticles for interpretation of toxicity results, the crystal structure, particle size and morphology of commercial nano (<100 nm, 20 and 80 nm)- and micron (2-3.5 μm)-Ag particles were determined in parallel to the toxicity studies. The characterization of hydrodynamic diameter and zeta potential was only carried out on nano-Ag in stock suspension (deionized water). However, we observed a clear difference of particle sizes between the manufacturer's information and what we measured for both nano- and micron-Ag samples.

In toxicity experiment I, toxic effects of sediment-spiked nano-Ag_{100}, micron$_{2\text{-}3.5}$ or aqueous-Ag ($AgNO_3$) on the sediment-dwelling polychaete, *Nereis* (*Hediste) diversicolor* were compared after 10 d of exposure, using mortality, growth, bioaccumulation and DNA damage (comet assay) as endpoints, with the purpose to decipher if effects were driven by the small size of particles, or other characteristics (i.e., ion release). The nominal concentrations used in all exposure scenarios were 0, 1, 5, 10, 25 and 50 μg Ag/g dry weight (dw) sediment.

In toxicity experiment II, toxic effects of sediment-spiked nano-Ag_{20}, nano-Ag_{80} or aqueous-Ag ($AgNO_3$) on *N. diversicolor* were investigated. Mortality, burrowing

behavior, bioaccumulation, lysosomal membrane stability (neutral red assay) and DNA damage were used as endpoints as a result of 10 d of exposure, with the purpose to decipher if toxic effects were different between the two nano-Ag particle sizes. The nominal concentrations used in all exposure scenarios were 0, 5, 10, 25, 50 and 100 μg Ag/g dw sediment.

The overall results demonstrated that there was no significant growth in any of the Ag treatments or concentrations during 10 days. Burrowing behavior, which was assessed by burrowing time, was significantly affected in nano-Ag_{20} treatment compared to the aqueous-Ag, indicating that worm mobility was impaired at exposure concentrations used. Ag was bioavailable and accumulated in *N. diversicolor* regardless of the form added to sediment in both toxicity experiments. Ag body burden increased in a concentration-dependant manner but there was no form-related difference in bioaccumulation of Ag in either experiment. There was a monotonic increase of Ag body burdens with increasing exposure concentrations in experiment I, which pattern however, was lack in experiment II. This was likely in part because of the wider size range and larger sizes of worms used in experiment II. Worm size (expressed as dry weight) significantly affected Ag body burden, such that smaller worms accumulated more Ag per body weight than larger worms. Lysosomal membrane stability of worm coelomocytes, which was measured by neutral red retention time (NRRT), decreased in a concentration-dependent manner in both nano- and aqueous-Ag treatments in toxicity experiment II, indicating increased permeability of lysosomal membranes. Worms exposed to sediment-spiked nano-Ag_{20} had significantly shorter NRRT than worms exposed to the aqueous-Ag. However, no significant difference was observed between nano-Ag_{20} and nano-Ag_{80} for either burrowing behavior or NRRT, which is likely attributed to their overlap in particle sizes. Tail moment and tail intensity (%), which are two commonly used indicators of DNA damage, increased significantly with increasing exposure concentrations after all three (nano-, micron-, and aqueous-) Ag treatments. Nano-Ag_{100} had significantly greater genotoxicity than micron-$Ag_{2\text{-}3.5}$ and aqueous-Ag in experiment I and nano-Ag_{80} had significantly greater genotoxicity than aqueous-Ag in experiment II. Furthermore, the levels of DNA damage were comparable in nano-Ag_{100}, nano-Ag_{20} and nano-Ag_{80} treated worms at the same exposure concentrations for both experiments, which were consistent with the evidence that they had a similar size distribution as indicated by TEM and DLS characterization. Overall, our study showed that nano-Ag treatments tended to be more toxic than the micron- and aqueous-Ag for tested endpoints

even though bioaccumulation was similar among forms. Such enhanced nano-size specific effects warrant further investigation and attention.

Keywords: Silver nanoparticles; Micrometer Ag; $AgNO_3$; Fate; Dispersion and stability; Uptake; Characterization; Marine invertebrate; Sediment exposure; Survival; Ag body burden; Growth; Burrowing behavior; Lysosomal membrane stability; DNA damage

Danish Abstract

Hurtigt voksende vækst inden for nanoteknologi har ført til udviklingen af talrige anvendelser af nanomaterialer i industri-og forbrugerprodukter. Nanosølv er en af de mest almindeligt anvendte nanomaterialer på grund af dens effektive antibakterielle egenskaber. Den potentielle udledning af nanosølv til det akvatiske miljø har skabt stigende bekymring omkring nanosølvs skæbne og potentielle risici for vandmiljøet.

I denne afhandling undersøgte jeg først spredning og stabilitet af kommercielt tilgængelige nano (<100 nm)- og micron (2-3.5 μm og <45 μm)-Ag partikler i to medier (deioniseret vand vs filtreret naturligt havvand) med det formål at undersøge Ag partiklers adfærd i det vandige miljø. Sediment blev valgt som eksponeringsvej til følgende toksicitets eksperimenter (I og II) hvor Ag partiklers tendens til at udfældes i vandfasen over tid blev undersøgt.

På grund af betydningen af at karakterisere nanopartikler fuld ud førtolkning af toksicitet resultater blev krystalstrukturen, partikelstørrelse og morfologi af kommercielle nano (<100 nm, 20 og 80 nm)- og micron (2-3.5 μm)-Ag partikler bestemt parallelt med toksicitetsundersøgelser. Karakterisering af hydrodynamiske diameter og zeta potentiale blev kun udført på nano-Ag på stamopløsningen (demineraliseret vand). Vi observerede en tydelig forskel i partikelstørrelse mellem fabrikantens oplysninger, og hvad vi målte for både nano-og Micron-Ag prøver. I toksicitet eksperiment I undersøgte jeg de toksiske effekter af sediment hvortil nano-Ag_{100}, micron$_{2\text{-}3.5}$ eller vandig-Ag ($AgNO_3$) var tilsat på den sedimentlevende polychaete *Nereis (Hediste) diversicolor* efter 10 d eksponering. Dødelighed, vækst, bioakkumulering og DNA-skader (comet assay) blev anvendt som endpoints, med det formål at undersøge om potentielle effekter blev drevet af partiklernes ringe størrelse eller af andre egenskaber (f.eks. ion-release). Den nominelle koncentration der blev anvendt i alle eksponeringsscenarier var 0, 1, 5, 10, 25 og 50 μg Ag/g tørstof (TS) sediment.

I toksicitet eksperiment II, blev de toksiske effekter af sediment tilsat nano-Ag_{20}, nano-Ag_{80} eller vandige-Ag ($AgNO_3$) på *N. diversicolor* blev undersøgt. I dette forsøg blev dødelighed, nedgravning, bioakkumulering, lysosomal membran

stabilitet (neutral rød assay) og DNA-skader anvendt som endpoints efter 10 d eksponering, med det formål at bestemme om de toksiske effekter var afhængig afnano-Ag partikelstørrelse. Den nominelle koncentration i alle eksponeringsscenarier var 0, 5, 10, 25, 50 og 100 µg Ag/g biopsi-tørvægt sediment.

De overordnede resultater viste, at der ikke var nogen betydelig vækst hos *N. diversicolor* i nogen af de Ag behandlinger eller koncentrationer i løbet af de 10 dage. Ormenes nedgravnings hastighed var signifikant påvirket hos nano-Ag_{20} behandling i forhold til det vandige-Ag, hvilket indikerer at N. diversicolor undgår sedimentet efter Ag eksponering. AG var biotilgængeligt og akkumuleredes i *N. diversicolor* uanset hvilken form Ag form der blev tilsat til sedimentet i begge toksicitet eksperimenter. Ag kropsbyrden steg på en koncentrations-afhængig måde, men der var ingen form-relaterede forskel i bioakkumulering af Ag i nogen af eksperimenterne. Der var en monoton forøgelse af Ag kropsbyrden med stigende eksponerings koncentration i eksperiment I, hvilket ikke blev observeret i eksperiment II. Dette skyldes sandsynligvis til dels på grund af størrevariation i ormenes størrelse og anvendelsen af større orme, i eksperiment II. Ormenes størrelse (udtrykt som tør vægt) havde en signifikant indflydelse på Ag kropsbyrden, således at mindre orme akkumulerede mere Ag pr kropsvægt end større orme. Lysosomale membran stabilitet i ormenes coelomocytter, som blev målt som neutrale rød opholdstid (NRRT), faldt på en koncentrations-afhængig måde i både nano-og vandige-Ag behandlinger i toksicitet eksperiment II, hvilket indikerer at der er tale om en øget permeabilitet af de lysosomale membraner. Orme der blev udsat for sediment tilsat nano-Ag_{20} havde betydeligt kortere NRRT end orme udsat for sediment tilsat vandig-Ag. Dog var der ingen signifikant forskel mellem nano-Ag_{20} og nano-Ag_{80} hverken for nedgravning eller NRRT, hvilket sandsynligvis tilskrives overlap i partikelstørrelser. Tail-moment og tail-intensity (%), som er to almindeligt anvendte indikatorer for DNA-skader, steg markant med stigende eksponeringskoncentration i alle tre (nano-, micron- og vandige-) Ag behandlinger. Nano-Ag_{100} havde signifikant større genotoksicitet end micron-$Ag_{2\text{-}3.5}$ og vandige-Ag i eksperiment I. Nano-Ag_{80} havde signifikant større genotoksicitet end vandig-Ag i eksperiment II. Desuden var niveauet af DNA-skader sammenlignelige for nano-Ag_{100}, nano-Ag_{20} og nano-Ag_{80} behandlet orme ved samme eksponeringskoncentrationer for begge eksperimenter, hvilket er i overensstemmelse med at de havde en tilsvarende partikelstørrelses fordeling (angivet af TEM og DLS karakterisering). Samlet set viser undersøgelsen, at nano-Ag har en tendens til at være mere giftig end micron-og vandige-Ag for de testede endpoints

med undtagelse af bioakkumulering. Sådanne forstærkede effekter af Ag i nano-størrelse understøtter behovet for yderligere undersøgelse og opmærksomhed.

Nøgleord: Sølv nanopartikler; Micrometer Ag; $AgNO_3$; Skæbne; Spredning og stabilitet; Optagelse; karakterisering, Marine invertebrater; Sediment eksponering, Overlevelse; Ag kropsbyrde; Vækst; Nedgravning; lysosomale membran stabilitet; DNA-skader

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List of Papers

The thesis is based on the following manuscripts:

I. **Cong, Y., Pang, C., Dai, L., Banta, G.T., Selck, H., Forbes, V.E., 2011.** Importance of characterizing nanoparticles before conducting toxicity tests. *Integr. Environ. Assess. Manag.* 7(3), 502-503.

II. **Cong, Y., Banta, T.G., Selck, H., Berhanu, D., Valsami-Jones, E., Forbes, E.V., 2011.** Toxic effects and bioaccumulation of nano-, micron- and ionic-Ag in the polychaete, *Nereis diversicolor*. *Aquat. Toxicol.* 105, 403-411.

III. **Cong, Y., Banta, T.G., Selck, H., Berhanu, D., Valsami-Jones, E., Forbes, E.V.** Toxic effects and bioaccumulation of sediment-associated silver nanoparticles in the estuarine polychaete, *Nereis (Hediste) diversicolor*. *In preparation.*

IV. **Cong, Y., Banta, T.G., Selck, H., Forbes, E.V.** A review of ecotoxicological effects and possible toxic mechanisms of silver nanoparticles in aquatic organisms. *In preparation.*

1. Introduction

Nanoparticles (NPs) are defined as having two or three dimensions between 1 nm and 100 nm (ASTM International, 2006). NPs in the environment mainly have three sources, i.e., natural (e.g., volcano eruption, combustion), anthropogenic (e.g., industry emissions, diesel exhaust) and engineered (applied in consumer products, e.g., clothes, cosmetics). Rapidly expanding growth in the field of nanotechnology has led to the development of numerous applications of engineered NPs in industrial and consumer products, of which, silver is one of the most frequently used nanomaterials. Nanosilver has distinctive physical-chemical properties, including a high electrical and thermal conductivity, surface-enhanced Raman scattering, chemical stability, catalytic activity and non-linear optical behavior (Capek, 2004; Frattini et al., 2005; Morones et al., 2005; Pal et al., 2007). Accordingly, these properties make them of potential value in inks (Perelaer et al., 2009; Tay and Edirisinghe, 2002), microelectronics (Wu et al., 2006) and medical imaging (Jain et al., 2008). Furthermore, due to the effective antibacterial activity, nanosilver has been increasingly incorporated into many commercial products, such as food packaging, odor resistant textiles and household appliances (Cohen et al., 2007; Sondi and Salopek-Sondi, 2004; Vermeiren et al., 2002). The application of nanosilver has also become more widespread in biochemical and medical sciences like bio-sensing, catalysts, transport, water purificants, bandages, and surgical instruments (Lansdown, 2006; Savage and Diallo, 2005).

Silver itself is classified as an environmental hazard because it is toxic, persistent and bioaccumulative under at least some circumstances (Luoma, 2008). However, most of our knowledge about the behavior and toxicity of Ag is related to its aqueous form, i.e., Ag^{+}, which was especially a historical problem related to the photo industry. There is an increasing concern about toxicities and potential risks, both still poorly understood, of nanosilver for the environment and human health after its eventual release, as it is indicated as one of the fastest growing applications in nanotechnology industries and consumer products. Nevertheless, the fate of nanosilver released from consumer products during their production

and use remains unclear, but the wide occurrence of consumer product related chemicals in the environment suggests that release into the environment is highly likely (Farkas et al., 2011).

1.1 Sources and fates of silver in the environment

1.1.1 Sources

Silver is not common naturally in the environment, so its elevated presence in water, sediment or biological tissues is usually indicative of anthropogenic influences (Irwin et al., 1998; Wang et al., 2003). Silver production from mining and smelting increased steadily with industrial and manufacturing development during the last century, and recycling of silver from such products is a major source of the metal (Luoma, 2008). In 1990, the estimated world production of silver was 14.6 million kilograms (kg) (Eisler, 1996). In 2007, approximately 20.5 million kg of silver were mined worldwide (USGS, 2008).

The production of nanosilver was estimated at about 500 tons per year in 2008 (Mueller and Nowack, 2008), and a steady increase in the volume manufactured is predicted for the next few years (Boxall et al., 2008). The Woodrow Wilson Database (http://www.nanotechproject.org), although not exhaustive, has listed 1015 consumer products presently on the market incorporating NPs, with 259 containing silver (May 2010). As indicated in the report of Luoma et al. (2008), the most prevalent use is in products coated with a polymer containing NPs, such as nanosilver embedded handrails, medical devices, food storage containers, dressings for wounds and female-hygiene products. The second most prevalent use is as "colloidal silver," which refers to nano-sized Ag particles (0.6-25 nm in this case) in a water suspension (e.g., suspensions recommended for daily ingestion). Spun and powdered Ag (e.g., cotton or synthetic fabrics, impregnating sheets, clothing, sportswear, socks) are two other prevalent applications of nanosilver. In addition, silver ions are intentionally generated by some nanosilver incorporated products (e.g., washing machines and dishwashers). All of the above mentioned applications of nanosilver will likely add to the total burden of silver discharged to the environment.

1.1.2 Environmental fate

Silver metals (Ag^0) or ions (Ag^+) introduced into the environment by human activities may be transported by air, remain in the soil or wastewater sludge at the emission site, be kept in solution by binding to a variety of inorganic and organic ligands such as chloride, metal sulfides, and thiols, then be transported downstream and enter lakes, estuaries and the sea, or typically be incorporated into sediments after speciation and precipitation where they are immobilized and to a great extent retained (Luoma et al., 1995; Ratte, 1999).

Nanosilver-containing products that end up in the terrestrial environment or as solid waste, such as silver polymers incorporated in handrails and catheter linings, will be found in landfills or in soils but it is unlikely that much of this silver will be released to the surrounding environment. The greatest risk for environmental release is expected from nanosilver-containing products used by millions of people in ways that release silver particles or ions directly to wastewaters (Luoma, 2008). The amount of silver (particles, ions or compounds) reaching natural waters depends on the fraction of wastewater that is effectively treated. However, the presence of nanosilver in wastewater can reduce the efficiency of wastewater treatment process by nitrifying bacteria (Choi et al., 2008; Choi and Hu, 2008), with a great potential of nanosilver being released into aquatic environments via wastewater charge. As a result, aquatic organisms are likely to be exposed to these metal NPs (Fig.1).

Environmental concentrations of nanosilver are estimated to range between 0.03 and 500 ng/L in nature waters (Luoma, 2008), and will possibly increase with increasing applications of nanosilver. Nanosilver released into the aquatic environment may complex with ligands and then agglomerate, react with natural materials like dissolved organic matter (DOC) and natural particulates, release Ag^+ ions, or still be present as nanoparticles (Benn and Westerhoff, 2008; Blaser et al., 2008; Luoma, 2008). The coexistence of the particle and aqueous forms, which may exhibit different fate and transport characteristics, and may have independent or synergistic toxicity pathways, can complicate the understanding of environmental effects of nanosilver (Liu and Hurt, 2010; Lubick, 2008). In addition, the formation of aggregates in water presents the opportunity for other organic materials, including toxicants, to become associated with the aggregates, which can change the bioavailability of these NPs and create additional toxicological concerns (Farré et al., 2009). In a general way, the novel physical-chemical properties of nanosilver (e.g., size, shape, surface reactivity, functional groups), as well as environmental

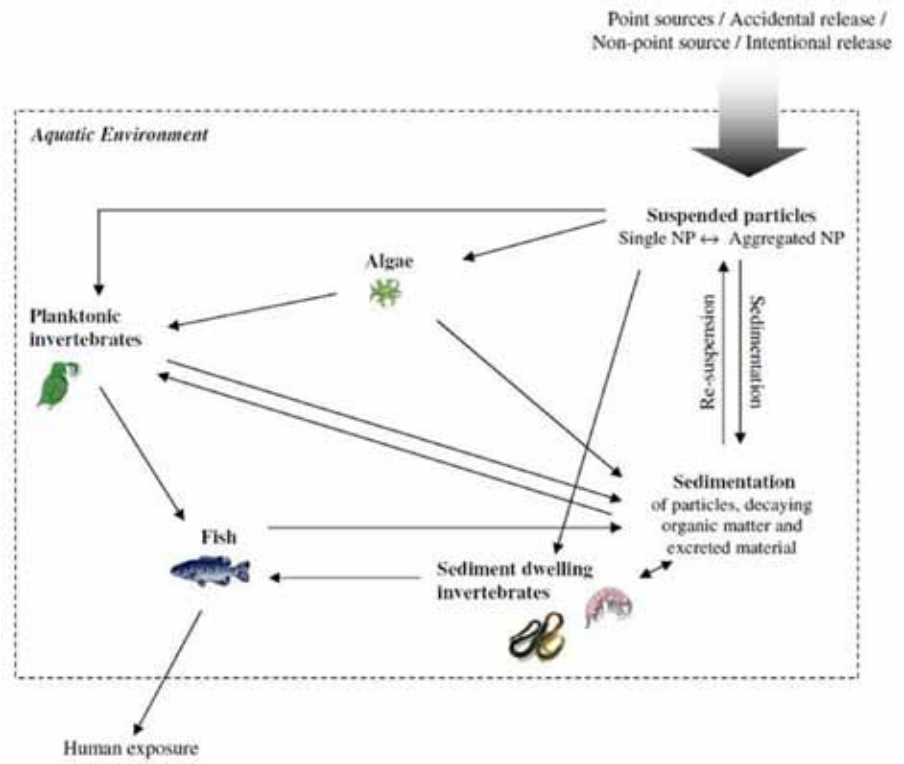

Fig.1 The fate of NPs after release into the aquatic environment. After entry of engineered nanoparticles (ENPs) into the aquatic environment the suspended particles will be taken up by planktonic or sediment-dwelling invertebrates through different exposure routes (i.e. direct uptake from the water phase, food, or through sediment ingestion) (reproduced from Baun et al. with permission).

conditions (e.g., hydrology, water chemistry, sediment characteristics), which vary widely from year-to-year in estuaries, will likely influence and determine the persistence, dispersal pathways, compartment in which nanosilver is retained and toxic consequences in aquatic environments. However, the environmental determining factors, which we know relatively little about, should be included in further ecotoxicological studies of nanosilver.

1.2 Nanosilver: Bioavailability, uptake and bioaccumulation

Key challenges in environmental risk assessment and management are understanding the bioavailability, uptake, bioaccumulation and trophic transfer of aquatic contaminants, including NPs. Biological transport of nanosilver, which most likely

occurs in the water phase or at the sediment-water interface, contributes greatly to its bioavailability to aquatic and benthic dwellers.

Laboratory studies have shown that nanosilver is bioavailable under different exposure scenarios (e.g., water, dietary and sediment) and can be taken up in various compartments inside aquatic organisms. For instance, nanosilver can be taken up through water exposure pathway by the aquatic vertebrates such as carp (*Cyprius carpio*), zebrafish (*Danio rerio*) and fathead minnow (*Pimephales promelas*) embryos, and that the NPs existed within the body in both single and agglomerated forms (Asharani et al., 2008; Choi et al., 2010; Farkas et al., 2011; Gaiser et al., 2009; Laban et al., 2010; Lee et al., 2007a, b). Zhao and Wang (2010) demonstrated the uptake of nanosilver in *Daphnia magna* from both water and dietary routes, and found that more than 70 % of nanosilver accumulated in the daphnids was through ingestion of algae, indicating the important role of dietary uptake. Sediment exposure is an important route of uptake for nanosilver because of its propensity to aggregate and settle in the higher salinity waters typical of estuaries. García-Alonso et al. (2011) observed that Ag particles accumulated in the lumen and epithelium of gut of sediment-dwelling polychaete, *Nereis diversicolor*, after exposure to sediment-associated nanosilver for 10 d, and subcellular fractionation revealed differing cellular internalization processes between nano- and aqueous-Ag in this species, with nanosilver being predominantly associated with inorganic granules, organelles and the heat-denatured proteins; whereas dissolved Ag was localized to the metallothionein fraction.

Body surface contact and oral ingestion are two main individual uptake routes of NPs entering aquatic organisms. For instance, Gaiser et al. (2009) demonstrated that much of the uptake of nanosilver into carp (*C. carpio*) after 21 d exposure possibly occurred as a consequence of the fish eating agglomerated NPs, rather than uptake via surface contact of the gills with water, indicating the importance of oral uptake route during exposure, and possibly subsequent trophic transfer through predation by other organisms. Oral ingestion of sediment-spiked nanosilver was observed in *N. diversicolor* with the evidence that most of Ag particles accumulated in their gut epithelial cells (García-Alonso et al., 2011). Furthermore, Griffitt et al. (2009) indicated that nanosilver itself contributed to the Ag body burden in zebrafish, as exposure to nanosilver produced significantly higher levels of silver associated with the gills than did exposure only to the aqueous fraction. Observed internal nanosilver particles were also demonstrated in the uterine of

Caenorhabditis elegans (Roh et al., 2009), as well as mammalian cell lines with the particles distributing throughout the cytoplasm, nuclei, lysosomes, endosomes and depositing in mitochondria (Asharani et al., 2009; Hsin et al., 2008; Kim et al., 2009).

The potential of nanosilver to be taken up and internalized within different compartments of cells largely depends on NP properties, especially size. For small single nanosilver, they can be taken up by cells via diffusion through chorion pore canals (CPCs), which has been demonstrated in zebrafish embryo (Lee et al., 2007a, b). Endocytosis (i.e., pinocytosis and phagocytosis) was also a possible way for nanosilver to enter cells, which have been shown for *N. diversicolor* (García-Alonso et al., 2011) and mammalian cells (Asharani et al., 2009; Park et al., 2010). Furthermore, it has been suggested that nanosilver acts as Trojan horse-type carriers that deliver free silver ions inside the cells or across the membranes into cells of organisms (Lubick, 2008; Luoma, 2008; Park et al., 2010). All of these pathways will contribute to the bioavailability and bioaccumulation of nanosilver. However, it is still not clear to what degree nanosilver can be eliminated by organisms once it is taken up. Detoxification mechanisms such as the formation of metal-containing extracellular granules and mineralized lysosomes, excretion of metals by cell exocytosis, and synthesis and turnover of metal binding proteins (e.g., metallothioneins, MTs), which have been observed after exposure to aqueous Ag (Bryan and Hummerstone, 1977; Fernandez and Jones, 1989; Mouneyrac et al., 2003; Ng et al., 2008; Poirier et al., 2006), still receive few investigations for nanosilver.

1.3 Nanosilver: *In vivo* and *in vitro* toxicities

The impact and potential toxicity of nanosilver within the aquatic environments are of concern. There is emerging literature reporting ecotoxicological data regarding nanosilver in aquatic ecosystems. Below, recently published studies of nanosilver toxicities were reviewed and compared. However, these studies have been initiated focusing on a water exposure pathway and using vertebrates or mammalian *in vitro* cell lines as models. This highlights the importance and necessity of sediment exposure studies as the NP physicochemistry and impacts on sediment inhabitants may be different from those in freshwater, seawater or *in vitro* cells. Clearly, nanoexotoxicological studies of Ag in the aquatic environment are still

in an initial phase and therefore, there is a pressing need to collect data on the aquatic invertebrates, especially sediment-dwelling species.

1.3.1 Individual effects

1.3.1.1 Acute toxicity

Recently, acute toxicity studies of nanosilver to aquatic organisms have been carried out under laboratory conditions and compared to its aqueous form (Ag^+). For instance, Griffitt et al. (2008) studied the toxicities of silver and other metals as both nanoparticles and aqueous salts to several representative freshwater organisms of various trophic levels, including zebrafish, daphnids, and algal species. The corresponding LC50s of aqueous Ag (as $AgNO_3$) for these animals were almost all lower than for nanosilver, indicating a higher toxic effect of the aqueous Ag than of nano-Ag on the basis of mass of metal added. The exception was *D. rerio* fry that were found to be more sensitive to the nano-Ag and nano-Cu than to the aqueous forms of the same metals. Furthermore, daphnids and algae were markedly more susceptible to toxicity from these nanometals compared with either stage of zebrafish, suggesting different likelihood for different taxonomic organisms to be affected in real aquatic environments. Particle size was found to be critical for nanosilver toxicity in the study of Bar-Ilan et al. (2009), which demonstrated a size-dependent mortality in zebrafish embryos with smaller NPs (3 nm) being more toxic than larger sizes (10, 50 and 100 nm).

Laban et al. (2010) included environmental relevant factors into their experimental design to compare toxic effects of commercial nanosilver prepared by two methods, sonication (controlled laboratory settings) and stirring (reproducing environmental condition, i.e., water current), on fathead minnow (*P. promelas*), and demonstrated that LC50 values for 35 nm and <100 nm Ag by sonication were much lower than LC50 values of stirring groups, indicating the relevant role of environmental factors in modifying nanosilver toxicities. However, the lower estimated LC50 for aqueous Ag ($AgNO_3$) than nano-Ag indicated its higher toxicity. Furthermore, silver ions were observed to be released from nanosilver in both sonicated and stirred treatments during exposure. However, the toxicity caused by dissolved Ag released from nanosilver (slower release rate) was likely different from Ag^+ ions released from $AgNO_3$ (dissociates completely with a log K value = -0.3), and both particulate and soluble silver contributed to nanosilver toxicity. Nevertheless, it is not always the case that toxicity of nanosilver is derived from both particles and ions. Asharani et al. (2008) found concentration-dependent

toxicity of nanosilver (5-20 nm) on zebrafish embryos, whereas aqueous Ag showed no significant effects on developing embryos, suggesting that nanosilver-mediated toxicity was not due to the presence of Ag^+ ions in the medium.
The considerable differences in acute toxicity of nanosilver reported in the literature are likely due to the size-dependent effects of nanoparticles and interspecific differences of organisms (Wu et al., 2010), as well as different exposure conditions. Furthermore, it has been recommended that the concentration of NPs be expressed as number or surface area per volume as opposed to mass per volume in ecotoxicity tests (Crane et al., 2008) as this may better reflect the dose and effects of NPs.

1.3.1.2 Chronic toxicity

Nanosilver can induce several kinds of sublethal phenotypic abnormalities and cause physiological dysfunction of zebrafish and fathead minnow embryos during development, with effects such as hatching delay, embryonic morphological malformations, abnormal body axes, twisted notochord, slow blood flow, pericardial emeda, heart malformation, eye defects (Asharani et al., 2008, 2009; Laban et al., 2010; Lee et al., 2007a, b; Wu et al., 2010). Passive diffusion via chorion pore canals and accumulation of nanosilver were assumed to be responsible for these dose-dependent abnormalities (Lee et al., 2007a, b). These various abnormalities observed in embryos are of concern as most of the affected fry would likely not develop normally. For the marine oyster *Crassostrea virginica*, exposure to nanosilver (15±6 nm) for 48 h significantly decreased the percent of normally developing embryos (Ringwood et al., 2010). Such a retardation of growth would serve to prolong the pelagic life of the larvae and thus increase their chances of loss through predation and disease, possibly reducing recruitment to the population (Calabrese et al., 1973). For the marine diatom, *Thalassiosira weissflogii,* cell growth was also suppressed as a function of increased Ag^+ ions released from nanosilver (60-70 nm) with increasing exposure concentrations (Miao et al., 2009), suggesting the contribution of dissolution of nanosilver to its toxicity.

In addition to developmental abnormalities and growth inhibition, a few studies have also reported toxic impacts of nanosilver on other physiological parameters and different toxicity modes were suggested to exist between nano- and aqueous-Ag. For instance, zebrafish exposed to aqueous silver (as $AgNO_3$) exhibited significant thickening of the gill filament while no change in filament width was observed in zebrafish exposed to nanosilver (26.6±8.8 nm) (Griffitt et al., 2009). Navarro et al. (2008) found that both nano (10-200 nm)- and aqueous ($AgNO_3$)-

Ag inhibited photosynthesis of freshwater algae, *Chlamydomonas reinhardtii,* in a dose- and time-dependent manner, and based on total Ag concentration, toxicity was 18 times higher for aqueous-Ag than for nano-Ag. If, however, the comparison was based on the measured Ag^+ concentration, the toxicity of nanosilver appeared to be much higher than that of $AgNO_3$ (in terms of EC50). Moreover, free ions were observed to be released from nanosilver during exposure and contributed to observed NP toxicity. Inhibition of photosynthetic activity and a reduction in the production of cellular chlorophyll were also observed in the marine diatom *T. weissflogii* as a function of increased Ag^+ ions released from nanosilver (60-70 nm) (Miao et al., 2009). Nanosilver (81 nm) was also shown be able to affect oxygen consumption of fish (*Perca fluviatilis*) gills, with effects including a significant increase in basal metabolic rate, which then led to reduced ability to extract oxygen from water and increased vulnerability to hypoxia (Bilberg et al., 2010).

1.3.2 Cellular effects

Although cellular responses of organisms to NPs are not the direct goal of ecotoxicological testing and environmental risk assessment, insights into such effects can be helpful for developing rapid tests. Sometimes they can be used as inexpensive and conservative screens for potential adverse effects of NPs on more ecologically relevant demographic endpoints, e.g., survival, growth and reproduction (Crane et al., 2008). Recent *in vitro* and *in vivo* tests performed with various cell types to evaluate the toxicities and mechanisms of nanosilver indicated that oxidative, cytotoxic, genotoxic and inflammatory consequences were all associated with silver particulate exposure and linked to each other. Below, the recent emerging cellular toxicity data in aquatic organisms for nanosilver are reviewed, and compared with the results of available mammalian *in vitro* cell lines, as well as the data of aqueous Ag.

1.3.2.1 Biomembrane damage and cell viability

One main toxic effect of NPs is interaction with biomembranes causing cell permeability changes as well as decreasing viability, which has been shown for both metal and non-metal NPs (Yang et al., 2008). Dose-dependent increase of lysosomal membrane permeability was observed in oysters (*C. virginica*) when they were exposed to nanosilver for 48 h. Biomembrane damage was also observed in rainbow trout (*Oncorhynchus mykiss*). The integrity of their hepatocytes was significantly reduced after 48 h exposure to nanosilver (1-10 nm, 6.35 mg/L) (Farkas et al., 2010). Recent *in vitro* studies of mammalian cell lines also demon-

strated various cell morphological changes by exposure to nanosilver, such as cell shrinkage, polyhedric and irregular cell shape, restricted spreading patterns, cell metabolic arrest, as well as changes in membrane integrity and cell viability (Arora et al., 2008, 2009; Carlson et al., 2008; Foldbjerg et al., 2009; Hussain et al., 2005; Kim et al., 2009). Biomembrane damage was also argued to be an important mechanism for nanosilver toxicity in bacteria (Lok et al., 2006).

1.3.2.2 Mitochondrial dysfunction and ROS-dependent oxidative stress

NPs have been shown to cause mitochondrial dysfunction by disrupting the electron transport chain, resulting in high concentrations of reactive oxygen species (ROS), low ATP production and alterations in various antioxidant enzyme systems (Asharani et al., 2009; Foley et al., 2002; Li et al., 2003). Reactive oxygen species (ROS)-dependent oxidative stress induced by NPs is among the possible toxic mechanisms of greatest concern (Nel et al., 2006; Xia et al., 2006) and has been shown for titanium dioxide (Long et al., 2006; Park et al., 2008), zinc oxide, silica dioxide (Yang et al., 2008) and carbonaceous NPs (Oberdörster, 2004; Pickering and Wiesner, 2005; Zhu et al., 2007).

An increasing amount of evidence shows that nanosilver is capable of localizing in mitochondria (Asharani et al., 2009) as well, triggering mitochondrial dysfunction such as membrane potential alternation (Carlson et al., 2008) and ROS dependent oxidative stress (Carlson et al., 2008; Kim et al., 2009). Various oxidative damages induced by nanosilver, such as decreased GSH and SOD contents, reduced mRNA levels of catalase and glutathione peroxidase, increased expression of oxidative-related genes, increased lipid peroxidation and total glutathione, were observed in zebrafish (Choi et al., 2010), rainbow trout (Scown et al., 2010), Japanese medaka (Chae et al., 2009) and some mammalian cell lines (Arora et al., 2008, 2009; Carlson et al., 2008; Park et al., 2010). These oxidative stresses demonstrated in aquatic organisms are suggested possibly due to the ROS generated by NPs (Chae et al., 2009). The evidence that pretreatment of human HepG2 cells with antioxidant prevented nanosilver-induced oxidative stress and alleviated mitochondrial damage (Kim et al., 2009), further validates that the intrinsic toxicity of nanosilver was associated with oxidative damage-dependent pathways. Furthermore, the expression of oxidative stress-related mRNA species of human hepatoma cells (HepG2) was regulated differentially as a function of exposure to nano (<10 nm)- and aqueous-Ag (Kim et al., 2009), suggesting that the mechanism of nanosilver-induced toxicity was possibly different from that

of aqueous Ag. However, not all cells treated with nanosilver exhibit deleterious oxidative damage due to the presence of effective anti-oxidative systems in some cases (Asharani et al., 2009).

1.3.2.3 Chromosomal aberrations and DNA damage

Tendencies for nanosilver to accumulate in cell nuclei have been described in the zebrafish (*D. rerio*) embryo, indicating that it may lead directly to genomic damage and chromosomal aberrations (Asharani et al., 2008). Treatments of medaka (*Oryzias latipes*) cell lines with nanosilver (~30 nm in diameter) induced DNA damage and aberrations in metaphases (Wise et al., 2010). Among various types of DNA damage, double-strand breakages are considered to be the most biologically significant lesions in cells (Rothkamm and Lobrich, 2003). Choi et al. (2010) detected a high level of γ-H2AX, which is an indication of DNA double-strand breaks after treatment of zebrafish liver tissue with nanosilver (5-20 nm in diameter) for 24 h, indicating nanosilver induced DNA damage, which is consistent with the study of Kim et al. (2009) using human hepatoma cells (HepG2) as a model system. Nanosilver has also been shown to cause chromosomal breaks and cell cycle arrest in the G2/M phase in human glioblastoma cells, which were possibly due to DNA damage and repair (Asharani et al., 2009). Furthermore, ROS are considered to be the major source of spontaneous damage to DNA. This evidence indicates that nanosilver possibly behaves differently than aqueous Ag to cause DNA damage, with the former mainly through a ROS-dependent mechanism and the latter mainly covalently binding with DNA (Hossain et al., 2002) and inhibiting DNA synthesis (Hidalgo and Dominguez, 1998).

1.3.2.4 Apoptosis

Apoptosis (programmed cell death) is a biologically essential, complex cellular process that is a regulated sequence of events occurring in response to a variety of stress stimuli including metal exposure (Wang et al., 2008; Yang et al., 2007) and oxidative stress (Ott et al., 2007; Sharma et al., 2007).

An *in vivo* study with zebrafish liver tissue demonstrated that p53-related pro-apoptotic genes Bax, Noxa, and p21 were upregulated after treatment with nanosilver (5-20 nm in diameter) for 24 h, indicating nanosilver induced apoptosis (Choi et al., 2010). Apoptosis was also observed in nanosilver-treated (5-20 nm) zebrafish embryos for 72 h, as well as in Japanese medaka (*O. latipes*), which were

exposed to nanosilver (49.6 nm) for 1 d with the induction of p53 gene whereas aqueous Ag ($AgNO_3$) induced this gene on the second day with lower amount than nanosilver, indicating different toxic fingerprints between these two Ag forms (Chae et al., 2009). The study of Hsin et al. (2008) and Foldbjerg et al. (2009) provided evidence that the molecular mechanism by which nanosilver induced apoptosis was mitochondria-dependent and ROS-mediated.

1.3.2.5 Inflammation

Inflammation is an early protective homeostatic immune response to tissue trauma. It is a complicated process with numerous cytokines and pathways involved. In medaka (*O. latipes*), the mRNA level of transferrin (TF) gene, which can be enhanced by inflammation, was increased after a 2-day exposure to $AgNO_3$, whereas its level in nanosilver treated fish was dramatically lower, suggesting aqueous Ag caused inflammation and different toxic modes by these two Ag forms (Chae et al., 2009). However, there are conflicting conclusions about inflammation (Carlson et al., 2008; Cha et al., 2008; Park et al., 2010) or anti-inflammation (Wong et al., 2009) effects of nanosilver on mammalian cells.

1.3.2.6 Metallothionein induction

Metallothioneins (MTs) are low weight and cysteine-rich proteins, which can bind both physiological and xenobiotic heavy metals, and play an important role in metal detoxification in organisms. MT expression levels increased significantly in both adult and embryo oysters (*C. virginica*) after 48 h of exposure to nanosilver (15±6 nm) (Ringwood et al., 2010), indicating that this detoxification processes was induced by nanosilver. Similarly, MT2 mRNA expression in zebrafish liver tissue was also found to increase in a dose-dependent manner after nanosilver (5-30 nm in diameter) water exposure for 24 h (Choi et al., 2010). In another freshwater fish, Japanese medaka (*O. latipes*), a statistically significant induction of MT expression (6.0-fold increase) was observed 1 day after the fish were exposed to nanosilver (49.6 nm), which was much higher than the response seen with an equivalent mass of silver from $AgNO_3$. Furthermore, this induction of the MT mRNA levels in the liver was abrupt but not prolonged, showing an initial increase during the first couple of days and then a decrease back to a basal expression level with longer exposure times (>4 days) (Chae et al., 2009). However, whether the induction of MTs is due to metal-responsive pathways, or whether the nanosilver exposures caused an increase in oxyradicals so that MT induction is associated with its role as an antioxidant remains unclear.

1.3.3 Bactericidal effects

Silver has for centuries been recognized as a strong antibacterial agent against a wide range of microorganisms, and given its special properties, silver ions and silver-based compounds have been used extensively in many bactericidal applications (Fung and Bowen, 1996; Gotjamanos, 1997; Hermans, 2006; Pal et al., 2007; Vermeiren et al., 2002). The mechanisms of the inhibitory effects of aqueous Ag include the electrostatic attraction between the negatively charged cell membrane of microorganisms and the positive charged Ag^+ ions (Dibrov et al., 2002) and affecting the function of membrane-bound enzymes (Bragg and Rainnie, 1974; McDonnell and Russell, 1999).

From the existing studies, nanosilver appears to exhibit bactericidal effects through the following mechanisms: (1) ROS related oxidative stress. Free radicals may form on the surface of nanosilver (Nover et al., 1983) which cause intracellular oxidative stress and interfere with energy metabolism of bacteria; (2) Nanosilver attaches to and accumulates in the microbial surface and causes cell wall pitting, leading to an increased membrane permeability which then induces a progressive release of Lipopolysaccharide (LPS) molecules and membrane proteins (Amro et al., 2000; Choi et al., 2008; Sondi and Salopek-Sondi, 2004) as well as the dissipation of the ATP pool and proton motive force (Lok et al., 2006); (3) Nanosilver is susceptible to oxidation by oxygen to form partially oxidized particles which appear to be more toxic to organisms than the freshly prepared NPs (Lok et al., 2007); (4) Ag^+ ions can be released from nanosilver leading to antibacterial effects in the same way as aqueous Ag (Lok et al., 2006; Pal et al., 2007); (5) Nanosilver with smaller size is able to penetrate inside the cell and cause further damage by possibly interacting with sulfur- and phosphorus-containing compounds such as DNA and proteins (Morones et al., 2005), inhibiting replication (Feng et al., 2000), interacting with the bacterial ribosomes (Yamanaka et al., 2005), and possibly accumulating to toxic levels that may cause death. Furthermore, electrostatic attraction and repulsion in nanosilver-bacteria interactions may also be important in regulating the collision and attachment of these particles of different sizes, i.e., nanometer (NPs) and micrometer (bacterial cells) (Dror-Ehre et al., 2009; Stoimenov et al., 2002). Therefore, in addition to its similarities with aqueous Ag, nanosilver also has its own special modes to exert bactercidal effects, and these effects are related to particle size, shape, the release of Ag^+ ions by oxidation, as well as the generation of ROS, as a result of its unique physical-chemical properties.

1.4 Aims of the thesis

Most existing research on nanosilver to date focuses on comparison of toxicities among different sizes of nanosilver or between the particulate and aqueous form of Ag, and few studies have directly compared nano-sized versus micron-sized Ag. These studies have also primarily included exposure from the water phase, despite the fact that most nanoparticulate metals released to the aquatic environment will accumulate in the sediment compartment. Therefore, sediment exposure route has received little attention. Furthermore, despite an increasing number of *in vitro* studies concerning the toxic effects of nanosilver on vertebrates (particularly freshwater fish and mammalian cell lines), there is a serious lack of information about its *in vivo* toxicities and toxicities to aquatic invertebrates. It is essential to include aquatic invertebrates as test organisms under relevant exposure conditions for environmental pollution studies because they represent key species in the transformation, detoxification and cycling of environmentally relevant contaminants discharged into aqueous environments (Cattaneo et al., 2009; Ratte, 1999). The overall aim of this thesis was two-fold (Fig.2). Firstly, the pilot dispersion and stability tests of both nano- and micron-Ag in deionized water versus filtered natural seawater were conducted to investigate the behavior of Ag particles in aqueous environments. Secondly, nanosilver has properties that are different from micron- and aqueous-Ag. The inclusion of both 'bulk' and aqueous forms of Ag in comparison with nanosilver is of importance to decipher if toxic effects are driven by the small size of NPs, or other characteristics (e.g., ion release) of these particles under a sediment exposure senario. Therefore, we examined toxic effects (mortality, growth, DNA damage) and bioaccumulation of sediment-spiked nano-Ag (<100 nm) in the estuarine polychaete, *Nereis* (*Hediste) diversicolor*, as a comparison with those spiked with micron (2-3.5 μm)- and aqueous ($AgNO_3$)-Ag after 10 d of exposure in toxicity experiment I. The toxicity experiment II was a continuation of experiment I that focused on sediment-spiked small sized nano-Ag (20 and 80 nm) as a comparison with the aqueous-Ag ($AgNO_3$) after 10 d of exposure. Mortality, burrowing behavior, bioaccumulation, lysosomal membrane stability and DNA damage were used as endpoints. It is likely that changes in speciation of silver may occur upon mixing with sediment particles but for the sake of simplicity we refer to the different Ag treatments below according to the initial silver form added to the sediment namely nano-Ag_{100}, micron-$Ag_{2\text{-}3.5}$ and aqueous-Ag in toxicity experiment I and nano-Ag_{20}, nano-Ag_{80} and aqueous-Ag in toxicity experiment II, respectively, throughout the text.

Due to the importance of fully characterizing NPs for interpretation and comparison of toxicological studies, the basic physical-chemical properties of both nano- and micron-Ag particles were identified in parallel with both toxicity experiments. Based on the present toxicity data and published studies, the potential toxic mechanisms of nano-Ag are discussed and compared to the micron- and aqueous-Ag. Finally, knowledge gaps and challenges of nanosilver in aquatic environmental risk assessment are addressed.

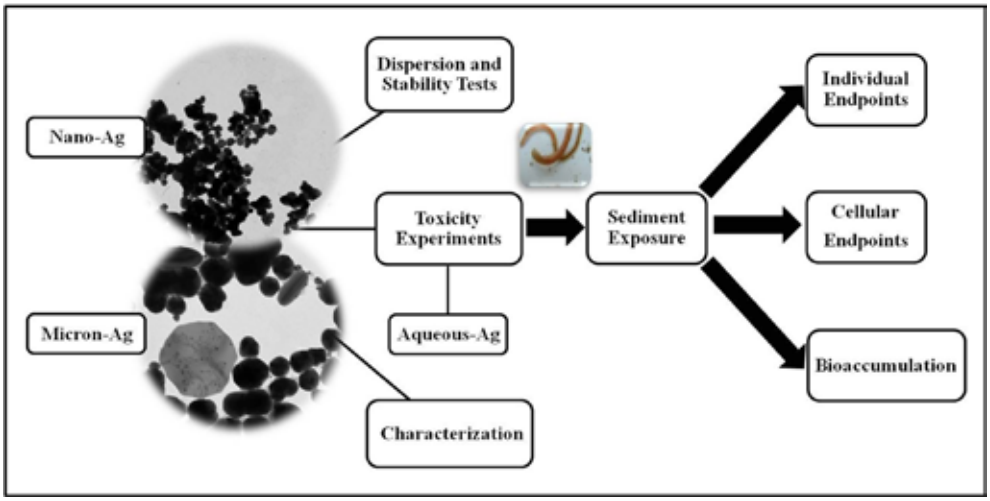

Fig.2 Overall aims of the PhD thesis.

2. Materials & Methods

2.1 Research overview

2.1.1 Paper I (Learned discourse)

There is a growing consensus on the necessity of proper and accurate characterization of NPs in environmental media and biological systems to ensure that reliable and reproducible toxicity studies are performed. Therefore, the importance of fully characterizing NPs before conducting toxicological studies was addressed in this short learned discourse.

2.1.2 Paper II (Toxicity experiment I)

In this study, toxic effects of sediment-spiked nano-Ag_{100}, micron$_{2\text{-}3.5}$ or aqueous-Ag ($AgNO_3$) on *N. diversicolor* were assessed and compared, using mortality, individual growth and cellular DNA damage as endpoints, as well as to measure Ag bioaccumulation in worm tissue as a result of 10 d of exposure. The nominal concentrations used in exposure scenarios were 0, 1, 5, 10, 25 and 50 μg Ag/g dry weight (dw) sediment.

2.1.3 Paper III (Toxicity experiment II)

In this study, toxic effects of sediment-spiked nano-Ag_{20}, nano-Ag_{80} or aqueous-Ag ($AgNO_3$) on *N. diversicolor* were assessed and compared, using mortality, burrowing behavior, lysosomal membrane stability and DNA damage as endpoints, as well as to measure Ag bioaccumulation in worm tissue as a result of 10 d of sediment exposure. The nominal concentrations used in exposure scenarios were 0, 5, 10, 25, 50 and 100 μg Ag/g dw sediment.

2.1.4 Paper IV (Review paper)

In this paper, basing on the available aquatic toxicological data of nanosilver, we summarized that how environmental-related factors affect nanosilver behaviors, the bioavailability and uptake (especially cellular uptake) routes, ecotoxicological effects (i.e., acute, chronic, cellular and bactericidal effects) and possible toxic

mechanisms of nanosilver in aquatic organisms compared to the aqueous Ag, as well as the knowledge gaps and challenges which should be addressed in future aquatic experimental designs and ecotoxicological studies.

2.2 Commercial Ag particles

Nano-Ag (<100 nm, cat.# 576832, 99.5 % trace metals basis, w/~0.2 % PVP coated), micron-Ag (2-3.5 μm, cat.# 32,708-5, ≥99.9 % trace metal basis; <45μm, cat.# 327107, ≥99.99 % trace metal basis) and aqueous-Ag ($AgNO_3$, cat.# S-6506) used in dispersion and stability tests and toxicity experiment I were purchased from Sigma-Aldrich (Steinheim, Germany). Nano-Ag (20 and 80 nm, stock # 0478HW and 0476HW, 99.9 %, w/~0.3 % PVP coated) used in toxicity experiment II were purchased from NanoAmor (Houston, USA).

2.3 Dispersion and stability tests

Prior to toxicity experiments, pilot studies were conducted to investigate the dispersion of different sized Ag particles, i.e., nano-Ag_{100}, micron-$Ag_{2\text{-}3.5}$ and micron-Ag_{45}, in two different media, i.e., deionized water (18.2 Ω, Millipore) vs. filtered natural seawater (<0.2 μm, 15 ‰, DMU, DK) with the same stock concentration (0.01 g/L) (dispersion test I), or in deionized water alone with different stock concentrations (0.01, 0.1 and 0.5 g/L) (dispersion test II), as well as to examine the concentration changes of three Ag particle suspensions in the above two media over time (stability test, 11d in deionized water and 10 d in seawater, respectively) with (to simulate environmentally realistic conditions, i.e., water current) and without stirring (to simulate controlled laboratory conditions), to test the behavior of nano- and micron-Ag in aqueous environments. Different dispersion methods have been used for the preparation of NP suspensions in ecotoxicity tests, such as stirring, shaking, solvents/dispersants and sonication. Due to the effectivity and convenience of sonication, we use this method to disperse Ag particles in either dispersion and stability tests or the following toxicity experiments.

Stock suspensions of three Ag particles were prepared in both deionized water and seawater by adding corresponding weights of particles into 1 L of each water type. Afterwards, the suspensions were ultrasonicated (100 W, 47±6 % kHz) in

a water bath (Struers KEBO Lab, DK) for 15 min followed by a 15 min pause. This process was repeated 4 times (total time: 2 h), and Ag concentrations in final suspensions were measured using Flame Atomic Absorption Spectrometry (FAAS, SpectrAA-220, Varian, Australia). In the stability test, the sampling of Ag suspensions was conducted at 0, 1, 24, 72, 120, 168, 264 h (nano-Ag_{100} and micron-Ag_{45}) and 0, 1, 48, 96, 120, 144, 264 h (micron-$Ag_{2\text{-}3.5}$) from deionized water and at 0, 1, 18, 24, 72, 120, 168, 240 h from filtered natural seawater. Afterwards, all of these samples were analyzed for Ag concentrations by FAAS.

2.4 Characterization of Ag particles

The identification of crystal structure of nano-Ag_{100}, nano-Ag_{20}, nano-Ag_{80} and micron-$Ag_{2\text{-}3.5}$ was performed on the powder, as received, using X-ray diffraction (XRD). Data were collected using a Nonius PSD 120 powder diffraction system equipped with a position sensitive detector. The characterization of hydrodynamic diameter and zeta potential (particle surface charge, an indication of suspension stability) was only carried out on nano-Ag. A stock nanosilver suspension was prepared in deionized water and ultrasonicated (80 W, 45 kHz) in a water bath (Ultrasonic Bath VWR, Lutterworth, UK) as described previously. The final suspension was analyzed using a Zetasiser Nano ZS (Malvern Zetasiser Nano ZS, Malvern, UK). Original particle size and morphology of both nano- and micron-Ag particles were visually assessed with a Hitachi H-7100 transmission electron microscope (TEM) operating at 100 kV. The micron-$Ag_{2\text{-}3.5}$ suspension in deionized water was unstable, as expected due to the large size of the microparticles. However, the sample was composed of microparticles as well as nanoparticles. Further analysis was therefore carried out in toluene. Micron-Ag was suspended in toluene, ultrasonicated at 100 W and 30 kHz with a probe sonicator (Hielscher UP100H, Teltow, Germany) for 30 s before imaging. Afterwards, a drop of Ag suspension was left to dry at room temperature on a carbon coated copper grid for later TEM imaging.

2.5 Sediment exposure of *N. diversicolor*

2.5.1 N. diversicolor

The sediment-dwelling ragworm, *N. diversicolor*, is known to play a crucial role

in the fate of chemicals in estuarine areas as a consequence of its relative tolerance and its influence on metal speciation and aromatic hydrocarbon transformation in sediment through bioturbation, particle mixing and irrigation (Banta and Andersen, 2003; Berthet et al., 2003; François et al., 2002; Mouneyrac et al., 2003) and thus is an appropriate test organism for examining the fate and effects of metal NPs in sediment systems.

N. diversicolor adults were collected from Roskilde Fjord near shore (55°40.710'N, 11°59.120'E) (Fig.3) during winter of 2009 (0-4 °C) and spring of 2010 (4-6 °C) (toxicity experiment I) and fall of 2010 (10-15 °C) (toxicity experiment II). After transferring to the laboratory, the worms were placed in natural sediment (collected from the same site, sieved <1 mm) to acclimatize for 5-8 days (toxicity experiment I) or 2-3 days (toxicity experiment II) with aerated natural seawater (collected from the same site, 15 ‰ salinity, pH around 8.0). Afterwards, a stepwise increase of temperature by 3-4 °C every 3 days from ambient temperature up to 15 °C was applied during the acclimatization period in toxicity experiment I. Eventually, the natural seawater was replaced with filtered natural seawater and worms were acclimatized for another two days. In toxicity experiment II, after acclimatization in natural seawater, worms were transferred directly to filtered natural seawater (15 °C) without temperature adjustment for another two weeks. One day before exposure, all worms were carefully picked out of the sediment and placed in clean filtered seawater to depurate their guts overnight. During the acclimation periods, worms were fed natural sieved sediment without additional food supply.

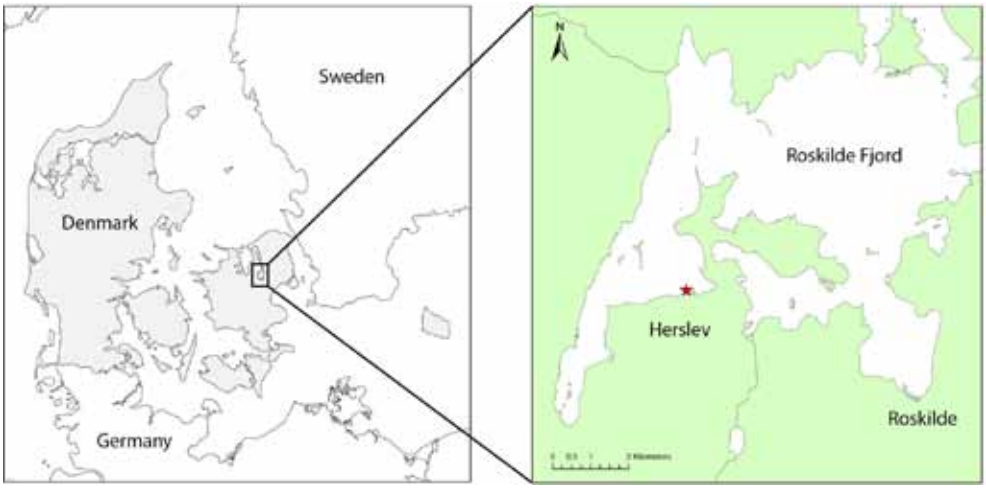

Fig.3 Collecting site (Herslev Harbor, Roskilde, Denmark) of N. diversicolor and sediment.

2.5.2 Sediment preparation

Sandy sediment for all experiments was collected from Roskilde Fjord at the same site as worms (Fig.3). The top few centimeters of the sediment surface were scraped off and sieved to less than 1 mm in the field with natural seawater. After transferring to the laboratory, the overlying seawater was removed, and the sediment was rinsed once by mixing with filtered natural seawater. After settling, the overlying water was carefully removed, and three to six aliquots of sediment (around 1 g) were taken out for detection of background Ag concentration by Graphite Furnace Atomic Absorption Spectrometry (GFAAS, GTA 120, Varian, Australia). Briefly, the sediment samples were lyophilized (Christ Alpha 1-2, Osterode, Germany) overnight at -50 °C. Afterwards, the sediments were transferred to Weflon tubes, weighed and digested with 65 % HNO_3 in a microwave oven (Milestone MLS-1200 Mega, Leutenkirch, Germany). The digestion program was 250 W, 400 W, 650 W and 250 W for 6 min, respectively. After cooling, the digested suspension was neutralized by adding 25 % ammonium solution (Merck, Darmstadt, Germany) and filtered before GFAAS measurement (performed within a few hours). Another six aliquots of sediment (around 5 g) were taken for dw/ww ratio and organic content measurements. All the remaining sediment was kept at 4 °C (toxicity experiment I, in the winter of 2009) for no more than one week before spiking and no more than two weeks before the start of exposure or at -20 °C (toxicity experiment II, in the fall of 2010) no more than one month before use.

The stock sediments (nominal concentration of 100 and 200 μg Ag/g dw sed. for toxicity experiment I and II, respectively) for all Ag treatments were prepared as follows. In toxicity experiment I, for nano-Ag_{100}, two bottles of stock suspension were prepared by adding 0.5 g powder into 1 L deionized water, and the suspensions were sonicated as described previously. Afterwards, 1.6 L suspension was added to sediment and homogenized. For aqueous-Ag, 0.944 g $AgNO_3$ was dissolved in a small amount of 65 % HNO_3 (Merck, Darmstadt, Germany), and deionized water was added to a final volume of 2 L. After mixing, 1.6 L of solution was added to sediment and homogenized as for nano-Ag. Due to the poor dispersion of micron-$Ag_{2\text{-}3.5}$ in deionized water by sonication (pilot study), its stock suspension was prepared by adding 0.48 g particles to 1.6 L deionized water which was stirred with a magnetic stirrer for 15 min, and all the suspension was added immediately into sediment and homogenized. Similarly, in toxicity experiment II, for nano-Ag_{20} and nano-Ag_{80}, 0.64 g powder was added to 1 L deionized water and sonicated as described previously. Afterwards, all the suspension was added to

sediment and homogenized. For aqueous-Ag, 1.007 g $AgNO_3$ was dissolved in 1 L deionized water and then added to sediment and homogenized as for nano-Ag. Exposure concentrations (nominal concentrations of 0, 1, 5, 10, 25, 50 μg Ag/g dw sed. and 0, 5, 10, 25, 50, 100 μg Ag/g dw sed. in toxicity experiment I and II, respectively) of all Ag treatments were obtained by homogeneously mixing stock sediment with clean sediment. The measured concentrations of stock and exposure sediment were analyzed using GFAAS and FAAS. In addition, PVP-controls were prepared by homogeneously mixing a solution of PVP into sediment in the same way as for the Ag treatments. The PVP concentrations were 0.1 and 0.3 μg/g dw in toxicity experiment I and II, respectively, corresponding to those in the highest nano-Ag exposure groups (50 and 100 μg Ag/g dw sed., respectively), and these controls were used to determine whether there was a genotoxic effect from the PVP coating alone.

2.5.3 Experimental setup

All experimental plastic beakers were acid washed (20 % HNO_3) and rinsed once with seawater before use. Following overnight depuration in clean filtered natural seawater, individual worms were transferred to beakers (one worm per beaker) containing around 320 g wet sediment of the appropriate Ag concentration and form and 600 ml filtered natural seawater (the beakers were kept still for six hours to allow the settling of suspended sediment before adding the worms). In toxicity experiment I, each of five replicates for the three Ag forms (6 concentrations for each Ag form) were set up during a period from the end of December, 2009 to the middle of March, 2010. In toxicity experiment II, six Ag concentrations were tested for each Ag form with five replicate worms in a randomized block design. In detail, each replicate block which included all the three Ag treatments was set up on the same day of the week over the course of one month. Sediment exposure (Fig.4) was conducted in the dark at 15 ºC for 10 d. The overlying seawater was aerated and changed once or twice during the 10 d of exposure to refresh the water and remove any metabolic waste products. We measured the Ag concentration in the sediment after exposure in a similarly designed pilot study and found that <0.1 % of Ag was lost to the water in our exposure systems. Beakers and air supply were inspected daily. During exposure periods, worms were fed natural sieved sediment without additional food supply. Mortality and growth in all treatments were recorded after 10 d of sediment exposure.

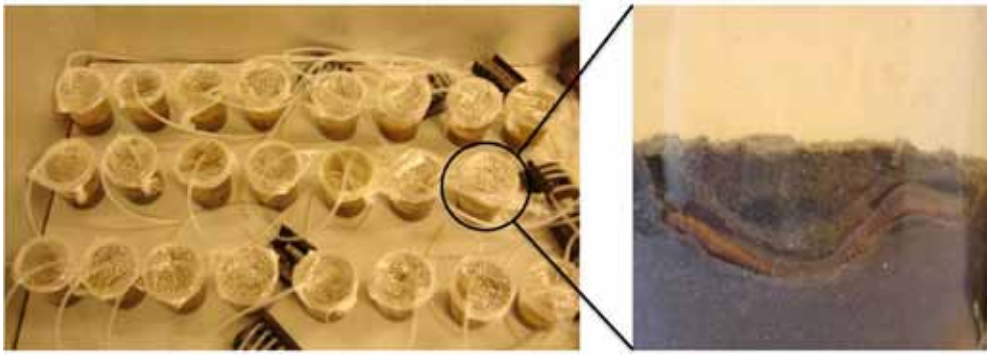

Fig.4 Experimental setup of Ag sediment exposure.

In toxicity experiment I, five to seven replicates (one individual is one replicate), three to five replicates (one individual is one replicate) and three replicates (a pool of 1-2 individuals per replicate) were used for each Ag concentration in growth, comet assay and bioaccumulation, respectively. In toxicity experiment II, three to five replicates (one individual is one replicate) for each Ag concentration were used for each of the tested endpoints.

2.6 Endpoints and measurements

2.6.1 Mortality

The number of dead worms in all Ag treated groups was recorded after 10 d of sediment exposure in both toxicity experiments.

2.6.2 Growth

After gut depuration overnight but prior to exposure, worms were gently blotted dry, and their wet weights were recorded using a balance (Mettler AE 163, Switzerland). After 10 d of exposure, worms were carefully picked out of the sediment, allowed to depurate in natural filtered seawater overnight and subsequently weighed as described above. The growth of worms was expressed as the difference in wet weight (ΔWeight) before and after 10 d of exposure.

2.6.3 Burrowing behavior

The burrowing behavior of *N. diversicolor* was investigated according to Bonnard et al. (2009), with some modifications. After 10 d of sediment exposure, worms were carefully picked out and placed individually in beakers filled with 5 cm clean natural sandy sediment (≤1 mm) and 100 mL natural filtered seawater.

The positions of worms during burrowing were recorded every 2 min during 30 min and scored as follows: 1: organism was totally un-burrowed; 3/4, 1/2, 1/4: organism was partially burrowed to various degrees (from least burrowed to mostly burrowed); 0: organism was totally burrowed.

2.6.4 Lysosomal membrane stability-Neutral red assay

The neutral red assay was conducted to measure lysosomal membrane stability after Ag exposure based on the protocol of Weeks and Svendsen (1996), with some modifications. In addition, PVP-controls were used to observe if there was a cytotoxic effect from the PVP coating alone. After the burrowing test, worms were carefully picked out of the sediment and allowed to purge their gut contents in natural filtered seawater overnight. Afterwards, a neutral red (toluene red, $C_{15}H_{17}N_4Cl$, cat.# N7005, Sigma-Aldrich, Steinheim, Germany) stock solution was freshly prepared by dissolving 20 mg neutral red powder in 1 mL of dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany). The neutral red working solution was obtained by dissolving 10 µL of stock solution in 2.5 mL of worm physiological solution (452.4 mM NaCl, 10.8 mM KCl, 58 mM $MgCl_2 \cdot 6H_2O$, 30.25 mM Na_2SO_4, 11.2 mM $CaCl_2 \cdot 2H_2O$, pH 7.8, adapted for marine annelids) (Wells and Ledingham, 1940), which was also freshly prepared and changed every hour. Afterwards, 30-50 µL coelomic fluid of *N. diversicolor* was gently extracted into physiological solution for each assay by carefully inserting a 1 ml syringe fitted with a 0.4 mm × 20 mm needle (chilled prior to use) into the posterior region of the worm (avoiding the gut). Afterwards, 20 µL of this mixture was placed on a slide where it was mixed with an equal volume of neutral red working solution and covered with a cover slip.

The number of coelomocytes with fully stained cytosol (i.e., exhibiting dye leakage from lysosomes to the cytosol) and that with unstained cytosol (no leakage) were counted under light microscopy (Laborlux S, Leitz, Portugal) with 100× magnification, and the ratio of the two cell types was determined. For each slide the ratio was assessed during 2 min of counting every 4-10 min. The observation was stopped when the ratio of coelomocytes with fully stained cytosol was greater than 50 % of the total number of cells or the observation time was more than 50 min. This interval was recorded as the neutral red retention time (NRRT).

2.6.5 DNA damage-Comet assay

The comet assay was based on the protocol of Singh et al. (1998) and Rank and

Jensen (2003), and the procedure for extraction of Nereid coelomocytes described by De Boeck and Kirsch-Volders (1997) and Lewis and Galloway (2008), with some modifications. Three to five replicates (one individual is one replicate) for each exposure concentration were used in the comet assay. A positive control (worms from 0 µg Ag/g dw, but extracted cells exposed to ultraviolet light) was established as a comparison with the control (0 µg Ag/g dw) in each Ag-form treatment. PVP-controls were used to determine whether there was a genotoxic effect from the PVP coating alone.

After 10 d of exposure, worms were carefully picked out of the sediment and allowed to depurate their guts in natural filtered seawater overnight. Afterwards, 30-50 µL coelomic fluid of *N. diversicolor* was gently extracted into 50 µL PBS (137 mM NaCl, 2.68 mM KCl, 1.75 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) for each assay by carefully inserting a 1 ml syringe fitted with a 0.4 mm×20 mm needle (chilled prior to use) into the posterior region of the worm (avoiding the gut) and stored on ice briefly before use. Coelomic fluid mixed with PBS was then gently centrifuged (Allegra X-15R centrifuge, Beckman Coulter, Inc., USA) at 300×*g*, 4 °C for 5 min, and the supernatant was removed. The cell pellet (i.e., the concentrated coelomic cells) was gently mixed with 125 µL of 0.65 % low melting point (LMP) agarose (Invitrogen, Paisley, UK), and 100 µL of the mixture was added to a fully frosted slide (one individual per slide) precoated with a 100 µL layer of 0.8 % normal melting point (NMP) agarose (Invitrogen, Paisley, UK). Another 100 µL of 0.65 % LMP agarose was then coated as the third layer. The agarose solutions used for the coelomocytes were dissolved in Kenny's salt solution (200 mM NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$, pH 8.0) and melted in a microwave oven for 1 min. For each layer, a cover slip was placed upon the gel, and the slides were cooled on ice for 15 min to solidify the gel. Afterwards, all work was carried out under yellow light to avoid UV damage to the DNA.

After having removed the cover slips, the slides used as positive controls were exposed to UV light from a Ren UV C lamp (253.7 nm, 15 W, Sylvania, Japan) for 20 sec. Then all the slides were placed in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1 % Triton X-100, pH 10.0) for at least 1.5 h in the refrigerator. Excess salts were removed by rinsing the slides for 10 s in electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH>13). The slides were then placed in the electrophoresis chamber filled with cold electrophoresis solution. Alkaline

unwinding was carried out for 40 min followed by 20 min of electrophoresis at 300 mA and 25 V. Finally, the slides were neutralized twice for 5 min in 0.4 M Tris solution (pH 7.5).

The slides were stained with 80 µL ethidium bromide (20 µg/mL) for at least 10 min and examined with a fluorescence microscope (50×oil immersion objective, Dialux 22EB, Leica, Wetzlar, Germany) with 625×magnification. On each slide, 50 randomly chosen cell nuclei (obviously apoptotic and necrotic DNA excluded) were examined automatically using software from Kinetic Imaging (Comet assay III). The levels of DNA damage were measured as tail moment, defined as the product of the tail length and the fraction of total DNA in the tail, and tail DNA intensity (%), expressed as the DNA intensity of the tail compared with the intensity of the whole comet (cell) (Dhawan and Anderson, 2009).

2.6.6 Enzyme activity (superoxide dismutase)

Superoxide dismutase (SOD), which catalyzes the dismutation of the superoxide anion ($O_2^{\cdot-}$) into hydrogen peroxide and molecular oxygen, is one of the most important antioxidative enzymes. In a pilot study, the SOD enzyme activity in worm tissue was measured using an SOD assay kit (#19160, Sigma-Aldrich, Switzerland) after exposure to sediment-spiked nano-Ag_{100} and micron-$Ag_{2\text{-}3.5}$ for 11 d and 10 d, respectively. The SOD Assay Kit allows very convenient SOD assaying by utilizing highly water-soluble tetrazolium salt, WST-1 (2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2H-tetrazolium, monosodium salt, Fig.5b), which produces a water-soluble formazan dye upon reduction with a superoxide anion. The rate of the reduction with O_2 is linearly related to the xanthine oxidase (XO) activity, and is inhibited by SOD (Fig.5a).

Nominal exposure concentrations of 0, 2, 20 and 200 µg Ag/g dw sed. were used for both Ag forms. After sediment exposure, worms were picked out and lyophilized overnight. Afterwards, individual worms were ground into powder using a mortar and pestle. Around 0.001 g tissue powder was transferred into a 1.5 mL eppendorf tube. Two hundred microliter of dilution buffer was added into the eppendorf tube and mixed with the sample for 30 sec in a vortex mixer. The mixed sample was centrifuged at 15,000× rpm, 4 ºC for 15 min. The resulting supernatant was transferred into a new eppendorf and 20 µL of above supernatant was mixed with 200 µL WST-1 solution and 20 µL enzyme working solution in a cuvette. The SOD enzyme activity was measured at 450 nm for 120 sec by

a UV-Vis spectrophotometer (UV-1650 PC, Shimadzu). Three blanks (Table 1) were used to calculate the inhibition percent (%) of SOD activity using the following formula:

SOD activity (Inhibition %)=[($Slope_{Blank1}$- $Slope_{Blank3}$) - ($Slope_{Sample}$-$Slope_{Blank2}$)]/($Slope_{Blank1}$- $Slope_{Blank3}$) × 100 %

Table 1. Constituents of the three blanks in the SOD assay.

	µL				
	Sample supernatant	WST-1 solution	Dilution buffer	Milli-Q water	Enzyme solution
Blank 1	-	200	-	20	20
Blank 2	20	200	20	-	-
Blank 3	-	200	20	20	-

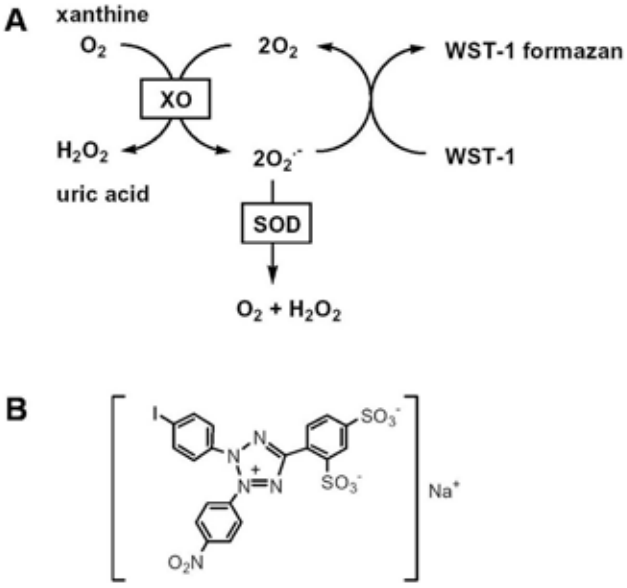

Fig.5 Principle of SOD Assay Kit (A) and chemical structure of WST-1 (B).

2.6.7 Bioaccumulation

After the above assays, worms were frozen individually at -20 ºC until use. The Ag concentrations in worm tissues were determined using GFAAS. Before digestion of worm tissues, all worms were lyophilized overnight at -50 ºC. Afterwards, a

pool of 1-2 worms per replicate (3 replicates for each concentration, toxicity experiment I) or each worm as one replicate (4-5 replicates for each concentration, toxicity experiment II) was ground into powder using a mortar and pestle. The resulting powder was digested and analyzed on GFAAS as described for sediments. We confirmed the validity of our calibration and analysis of tissue metal concentration by analyzing with a certified biological material (lobster hepatopancreas, LUTS-1, NRC Canada) with a known Ag concentration.

2.7 Statistical analyses

Measured sediment concentrations of Ag at experimental start were analyzed by one-way analysis of variance (One-way ANOVA) to test for differences among Ag forms in both toxicity experiments. In toxicity experiment I, data of bioaccumulation were log transformed to fulfill homogeneity and normality requests prior to analysis. All data (except PVP control) were analyzed using two-way analysis of variance (two-way ANOVA) followed by planned comparisons using a Bonferroni correction to compare the effects of three Ag treatments in each concentration and to compare exposure concentrations (1, 5, 10, 25 and 50 μg Ag/g dw sed.) to the controls (one for each Ag form in each replicate) for each Ag form. A two sample *t*-test was performed to compare the effects of PVP on growth and DNA damage with that of nano-Ag control (0 μg Ag/g dw sed.). In toxicity experiment II, for burrowing time, lysosomal membrane stability and DNA damage, data from exposure treatments (5, 10, 25, 50 and 100 μg Ag/g dw sed.) were analyzed using two-way ANOVA to assess and compare the effects of Ag form and concentration on tested endpoints. For Ag bioaccumulation, data from exposure treatments (5, 10, 25, 50 and 100 μg Ag/g dw sed.) were analyzed using analysis of covariance (ANCOVA) with worm size (expressed as dry weight) as the covariate. Differences in average worm size among treatments were analyzed by two-way ANOVA with Ag form and concentration as factors. Due to the weekly sampling of worms for the above assays (i.e., one replicate per week), replicate was taken into consideration as a blocking factor during ANOVA and ANCOVA analyses to reduce variation in response parameters related to weekly variations. One-way ANOVA followed by planned comparisons with a Bonferroni correction were used to assess the effects of exposure concentration, PVP controls and positive controls (one PVP or positive control in each replicate for all three Ag forms) compared to the controls (0 μg Ag/g dw sed., one in each replicate for

all three Ag forms) for all experimental endpoints in three forms together or in each Ag form. All experimental data were tested for homogeneity and normality through examination of residuals resulting from the above statistical analyses. In all cases, data were log-transformed to achieve acceptable homogeneity and normality. Differences were considered statistically significant at $p \leq 0.05$ and marginally significant at $0.1 \geq p > 0.05$. All statistical analyses were performed using SYSTAT 13.0 software (Chicago, IL, USA).

3. Main Results and Discussions

3.1 The behavior of nanosilver in aqueous environments

3.1.1 Dispersion and stability

In dispersion test I, both nano- and micron-Ag had a better dispersion in deionized water than in filtered natural seawater, as shown by a dispersion rate (%) in Table 2. Furthermore, nano-Ag demonstrated a better dispersion than micron-Ag in both media due to its smaller particle size. In dispersion test II (Table 3), both nano- and micron-Ag stock suspensions were prepared only in deionized water with different nominal stock concentrations of 0.01, 0.1 and 0.5 g/L. For nano-Ag_{100}, it had a consistent dispersion rate (%) of around 60 regardless of the nominal stock concentrations used. However, for micron-$Ag_{2\text{-}3.5}$ and micron-Ag_{45}, the dispersion rate (%) decreased from 30 to 0.68 and from 4.45 to 1.74, respectively, when the stock concentrations increased from 0.01 to 0.5 g/L. All three Ag particles had poorer dispersion in seawater than in deionized water possibly due to the precipitation of Ag particles after combination with cations and compounds existing in the seawater.

Table 2. Comparison of dispersion of nano-Ag_{100}, micron-$Ag_{2\text{-}3.5}$ and micron-Ag_{45} in deionized water vs. filtered natural seawater after sonication with nominal stock concentration of around 0.01 g/L (pilot study, n=1)

Ag form and size	Source	Media	Nominal stock suspension-concentrations (mg/L)	Concentrations after sonication (mg/L)	Dispersion rate (%)*
Nano-Ag_{100}	Sigma-Aldrich	Deionized water	12.8	6.472	50.5
Micron-$Ag_{2\text{-}3.5}$			10.1	3.257	32.2
Micron-Ag_{45}			10.5	0.508	4.8
Nano-Ag_{100}		Seawater	11.5	0.399	3.5
Micron-$Ag_{2\text{-}3.5}$			10.7	0.031	0.3
Micron-Ag_{45}			10.7	0.072	0.7

** Calculated from the Ag concentrations after sonication divided by the stock suspension concentrations.*

Table 3. Comparison of dispersion of nano-Ag_{100}, micron-$Ag_{2-3.5}$ and micron-Ag_{45} in deionized water after sonication with different nominal stock concentrations ranging from 0.01 to 0.5 g/L (pilot study, n=1-3)

Ag form and size	Source	Media	Nominal stock suspension-concentrations (mg/L)	Concentra-tions after sonication (mg/L)	Dispersion rate (%)*
Nano-Ag_{100}	Sigma-Aldrich	Deionized water	11.4[b]	6.225	55
			100[a]	69.21	70
			502.5[a]	310-330	60
Micron-$Ag_{2-3.5}$			10.1[b]	3.012	30
			105[a]	4.199	4
			766.2[a]	0.520	0.68
Micron-Ag_{45}			10.3[c]	0.457	4.45
			109.5[b]	5.104	4.50
			501.7[a]	8.71	1.74

[a] one replicate; [b] two replicates; [c] three replicates.
** Calculated from the Ag concentrations after sonication divided by the stock suspension concentrations.*

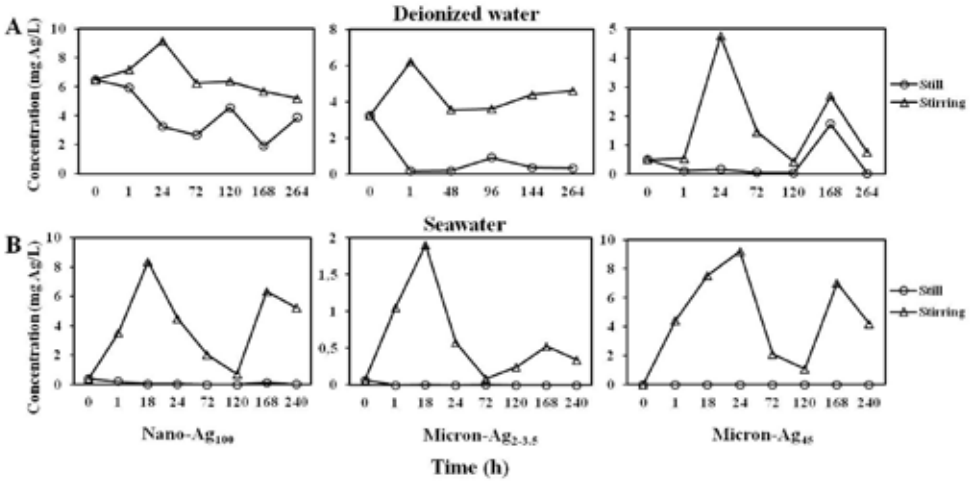

Fig.6 Concentrations changes of nano-Ag_{100}, micron-$Ag_{2-3.5}$ and micron-Ag_{45} in (A) deionized water (11 d) and (B) filtered natural seawater (10 d) with and without stirring (Pilot study, n=1).

The initial stability study explored whether it was possible to maintain constant exposure concentrations of nano- and micron-Ag in the water phase (deionized water vs. filtered natural seawater) over time, with and without stirring. The results (Fig.6) highlighted that settling of both Ag particles in both media is likely to occur over time without stirring and is even worse in seawater which contains organic matter and metal cations. These results, which demonstrated especially poor dispersion and stability of Ag particles in seawater overtime, supported the relevance of a sediment exposure pathway.

3.1.2 Affecting factors of stability

3.1.2.1 Effects of physical-chemical properties of NPs

One important property of NPs which is responsible for their behavior and stability in aqueous environments is the size. It has been shown that the stability of NPs is inversely proportional to their tendency to aggregate (Mackay et al., 2006). Aggregation is particle size dependent and results in efficient removal of small particles in environmental systems. In addition, adsorption processes (Fukushi and Sato, 2005), especially surface charge (Kallay and Zalac, 2002) can modify the nature and state of NPs in the environment.

Another property of particular relevance is the release of ions from the NPs. Although we did not actually measure the release of Ag^+ ions in dispersion and stability tests, the study of Ho et al. (2010) and Liu and Hurt (2010) demonstrated that nanosilver is likely to dissolve or degrade under oxidative conditions, even with the presence of PVP coatings (Ho et al., 2010). Furthermore, Liu and Hurt (2010) quantitatively tracked the appearance of dissolved silver in synthesized ion-free citrate-stabilized nanosilver (2-8 nm in diameter) suspensions over times ranging from 10 min to 125 days. Silver ions were observed to be released from nanosilver over time resulting from an oxidation process involving dissolved O_2 and H^+, as well as peroxide intermediates. This dissolution was complete leading to the disappearance of the particle phase. Therefore, even a simple nanosilver colloid may consist of three silver forms: Ag^0 nanoparticles, free Ag^+ (including any soluble complexes) and surface adsorbed Ag^+. This study suggests that Ag^0 nanoparticles will likely not be stable and persistent in realistic environmental compartments containing dissolved oxygen. Understanding the environmental fate of nanosilver should therefore take both particulate and aqueous forms into consideration.

3.1.2.2 Effects of environmentally relevant factors

Our pilot studies have shown that the dispersion media and the water current (stirring) have important effects on dispersion and stability of nanosilver. In addition, some other environmental relevant factors have also been demonstrated to influence the state and behavior of nanosilver in aqueous environments. For instance, the presence of electrolyte ions in the medium can screen the effective nanoparticle surface potential, and as a result of this surface charge reduction, aggregation is expected, which may then result in modified uptake, bioaccumulation and toxicity of nanosilver (Wise et al., 2010). Furthermore, the release rates of Ag^{+} ions from nanosilver have been demonstrated to increase with temperature in the range 0 to 37 °C, and decrease with increasing pH or addition of humic or fulvic acids. Sea salts have only a minor effect on dissolved silver release (Liu and Hurt, 2010). Cumberland and Lead (2009) studied the stability and size change of citrate-stabilized nanosilver (13.7±6.2 nm) in relation to solution conditions (i.e., pH, natural organic macromolecules and ionic strength), and found at low ionic strength that nano-Ag aggregate size increased as pH increased from 5 to 8. The presence or increase of ionic strength (Na^{+} and Ca^{2+}) also enhanced the instability and aggregation of nanosilver. However, the presence of humic substances (HS) improved stability of nanosilver under these conditions, most likely due to a reduction in the diffuse layer or by forming a surface coating resulting in both steric and charge stabilization. The authors concluded that immediate and irreversible aggregation and subsequent loss from the water to the sediment may in fact not occur so readily due to stabilization with HS. Therefore, in natural media, the behavior and stability of nanosilver will depend on specific realistic environmental conditions, which may be complex to predict and interpret.

3.2 The importance of characterizing NPs before conducting toxicity tests

Given that the behavior of NPs can be affected by many factors as discussed above, there is a growing consensus on the necessity of proper and accurate characterization of NPs in environmental media and biological systems to ensure that reliable and reproducible toxicity tests are performed. Without such characterization, nanotoxicity experiments will have limited value due to unknown variability in experimental conditions of the NPs (Warheit 2008). Some of the current divergent or conflicting results from nanotoxicological tests could also be better

explained if there had been adequate characterization in all studies. Exhaustive characterization of NPs is costly and time-consuming, however, and therefore a sufficient but practical approach is needed. Some principal characteristics of NPs which have been considered to deserve quantification before conducting toxicity tests are size, shape, state of dispersion, physical and chemical properties (e.g., electronic and optical properties, chemical composition and reactivity), surface area and surface chemistry (Powers et al., 2006).

We have characterized commercially available nano-Ag_{100} and micron-$Ag_{2\text{-}3.5}$ (Cong et al., 2011b, Paper II), as well as nano-Ag_{20} and nano-Ag_{80} (Paper III) in parallel with both toxicity experiments and found a clear difference between the manufacturer's information on the original particle size (<100 nm and 2-3.5 μm, 20 and 80 nm , respectively) and what we measured for all Ag particles. For nano-Ag_{100}, the TEM image exhibited polyhedral Ag particles with a size distribution ranging from approximately 20 to 200 nm. However, micron-$Ag_{2\text{-}3.5}$ was found containing both micron- and nano-sized particles. Preparation of micron-$Ag_{2\text{-}3.5}$ suspension in toluene further demonstrated polydispersed Ag particles with different shapes and a wide size distribution ranging from 8 nm to 3 μm (the major particle size being within the micron-size range). TEM images for nano-Ag_{20} and nano-Ag_{80} were similar, with polyhedral shape particles often observed. Complex particles displaying branching structures were commonly found in the nano-Ag_{20} sample, which were probably produced from the aggregation of smaller particles by collision upon sonication (such particles were not found when the powder was suspended with a shorter sonication time of 5 min). The most common particle size for the nano-Ag_{20} sample was approximately 50 nm but these could be particles that fused during treatment. Smaller sized particles of ~10 nm were also found in the nano-Ag_{20} sample, which might represent the primary components of the larger particles; these smaller particles could have formed agglomerates as the suspension droplet was dried on the TEM grid. For the nano-Ag_{80} sample, actual particle size ranged from approximately 2 nm to 100 nm with most particle sizes also being ~50 nm.

This difference in size between that reported by the manufacturer and that measured in the laboratory was also observed by Scown et al. (2010). The reasons for the differences may be related to the industrial production process, which often results in polydispersion, or to changes in material properties between synthesis and initial characterization and the particular conditions when utilized (e.g.,

storage time and conditions, pH, ionic strength, temperature and external force). In addition, approximately 5-10 % of non-crystalline material was observed in micron-$Ag_{2\text{-}3.5}$ sample, suggesting that it was not as pure as described by the manufacturer (≥99.9 % trace metal basis). Both problems highlight the importance of fully characterizing commercially obtained NPs before performing toxicity experiments, at the very least in the stock suspensions used to prepare exposure treatments (Cong et al., 2011a, Paper I). Given this sensitivity to experimental conditions, it is also important to characterize the NPs for each experiment conducted. Furthermore, the hydrodynamic size distributions of three nano-Ag forms were also quite similar, being 162±4, 187±4 and 144±2 nm for nano-Ag_{100}, nano-Ag_{20} and nano-Ag_{80}, respectively, which indicated a similar dispersion pattern and agglomeration state in deionized water among them. Zeta-potential measurements of nano-Ag_{100} (-49±0.75 mV), nano-Ag_{20} (-39.4±0.5 mV) and nano-Ag_{80} (-42.3±0.3 mV) suspensions suggested that the particles were stable (Derjaguin and Landau, 1941) in the dispersion medium in both experiments.

Environmental factors such as water pH, salinity and temperature, dissolved organic material and natural competing cations, are likely to play important roles in determining the dispersion, toxic consequences and compartment in which NPs are retained in the environment. Few, if any, studies of aquatic nanotoxicity have provided a full characterization of e.g., the size distribution (especially hydrodynamic size), dispersion state (especially in biological media) or surface chemistry (like surface charge) of NPs in the actual test media. Ideally, characterization of NPs should be performed under conditions as close as possible to the relevant exposure medium (e.g., sediment in our case).

In our toxicity experiments, we know little about the state of sediment-associated nanosilver due to the limitation of techniques and methods for characterizing NPs in such complex and "dirty" media. However, nanosilver is likely to undergo size or surface chemistry changes when it is transferred between media during experiments, such as from dispersion media (deionized water) to test media (sediment), as a result of interaction with organic compounds. Such changes may alter bioavailability or toxicity in ways that are not entirely understood. Because the reactivity and toxicity of NPs are believed to be influenced by such features as their size, shape, surface coating and other properties, we conclude that both the physical and chemical properties of NPs must be systematically and adequately defined prior to toxicological studies and risk assessment. Furthermore, there is a

pressing need for the development of techniques that permit examination of the state and behavior of NPs in complex compartments, such as sediment, so that a better understanding and interpretation of the effects of NPs on organisms can be achieved.

3.3 Ag bioaccumulation and detoxification

Sediment is likely to act as a sink for nanoparticles intentionally or accidentally released into the aquatic environment, as it does for many chemicals. Although some studies have demonstrated the uptake and localization of nanosilver inside cells, few of these have quantified the amount of Ag accumulated in organisms over time. Our bioaccumulation results from both experiments demonstrated that Ag was bioavailable to *N. diversicolor* to similar degrees regardless of the form in which it was added to sediment, possibly through the ingestion of Ag-associated particles in sediment during feeding, or through body surface contact during burrowing (Cong et al., 2011b, Paper II; Paper III). The lack of difference in Ag body burden among Ag forms despite detected differences in cellular indicators, which were consistent in both experiments, was also in agreement with García-Alonso et al. (2011), in which the calculated total Ag body burden of *N. diversicolor* after 10 d of exposure to sediment spiked with nano-Ag (30±5 nm) and aqueous-Ag ($AgNO_3$) were similar, being 218.7±14.8 and 226.7±11.2 ng Ag/g fresh weight, respectively.

In toxicity experiment I, Ag body burden increased significantly and monotonically with increasing exposure concentration, and the highest bioaccumulations were found at the highest exposure concentrations for worms in all three Ag treatments (Cong et al., 2011b, Paper II). Ag body burden in toxicity experiment II was also concentration-related but however, there was no monotonic increase with increasing exposure concentration. In addition, we found that worm size significantly affected Ag body burden with smaller worms accumulating more Ag per body weight than larger worms. The highest Ag body burdens were observed at different concentrations for the different Ag forms, but not in any systematic way. The lack of a monotonic bioaccumulation with increasing exposure concentration in experiment II was surprising given we observed monotonic relationships in the first bioaccumulation study and with the other endpoints (NRRT and DNA damage). This was likely in part because of the wider size range and larger sizes of

worms used in different exposure concentrations for each Ag form in experiment II. In experiment I, all worms had a similar average size of around 0.03 g dw (corresponding to the smallest size we used in experiment II). As a comparison, the largest sizes in this study (0.08-0.1 g dw) were found in the 25 and 100 μg Ag/g dw sed. exposure groups and had corresponding Ag body burdens of 1.3-3.1 and 3.0-6.2 μg/g dw, respectively, which were much lower than the accumulation by the smaller worms in experiment I (3.6-7.0 and 6.9-9.9 μg/g dw in 25 and 50 μg/g dw sed. groups, respectively). Despite efforts to allocate similar sizes of worms to forms, the significant differences among concentrations influenced Ag uptake and bioaccumulation. We also corrected for worm size by using the covariate effect to normalize Ag body burden and found that the monotonic relationship was more apparent than before, especially in the aqueous-Ag group (Paper III). Smaller worms have a higher surface area to volume ratio and a higher metabolic rate than larger worms. As demonstrated by Heip and Herman (1979), the weight-specific growth rate per day of *N. diversicolor* decreased from 0.0415 to 0.0008 when the length of worms increased from 0.25 cm to 7.25 cm (corresponding to ~0.001-0.1 g dw). This could indicate that smaller worms eat more per body weight and as a result, are more susceptible to accumulate Ag than larger worms.

If we assume that worms were in "steady state" with their exposure media (i.e., sediment), the corresponding BAF factors in experiment II would be 0.03-0.63, 0.06-1.45 and 0.05-0.30 for nano-$Ag_{20,}$ nano-Ag_{80} and aqueous-Ag, respectively, which were lower than previous published field and laboratory studies with calculated BAF values (also assuming that worms were in "steady state") ranging from 0.15 to 2.52 (Langston and Burt, 1994; Mouneyrac et al., 2003; Rainbow et al., 2009). While the assumption of steady state uptake is undoubtedly not true, the approximate BAF values do indicate that, while the worms are exposed to and take up Ag; they do not accumulate it to any great degree. The lowest BAF factors in experiment II were also lower than the lowest values in experiment I (0.16, 0.14 and 0.15 for nano-Ag_{100}, micron-$Ag_{2\text{-}3.5}$ and aqueous-Ag, respectively). In addition to the difference of geographic origin and relative metal tolerance compared to the previous field and laboratory bioaccumulation studies, the lower BAF values in experiment II, which were from 25 and 100 μg Ag/g dw sed. groups, indicated that larger sizes of worms had a lower ability to accumulate Ag than smaller worms (per body weight). Similarly, it may take larger worms longer time to achieve a given level (i.e., "steady state") than smaller worms given their smaller surface to volume ratio and weight specific activities. All of these factors and the variability

in bioaccumulation data highlight the importance of correcting for or minimizing body size differences in toxicological studies. However, after accounting for differences in worm size, bioaccumulation for both experiments was still variable which made it difficult to detect differences in the bioavailability or uptake of the different forms of Ag. Future bioaccumulation studies should aim to use more replicates or 'pooled' individuals as one replicate to reduce biological variations for this species. Furthermore, our bioaccumulation study also demonstrated substantial variation in Ag body burden compared with the existing bioaccumulation data (Choi et al., 2010; Scown et al., 2010), which is likely not only due to difference in exposure pathways, concentrations media conditions and metal forms among studies, but also to differences within and among species.

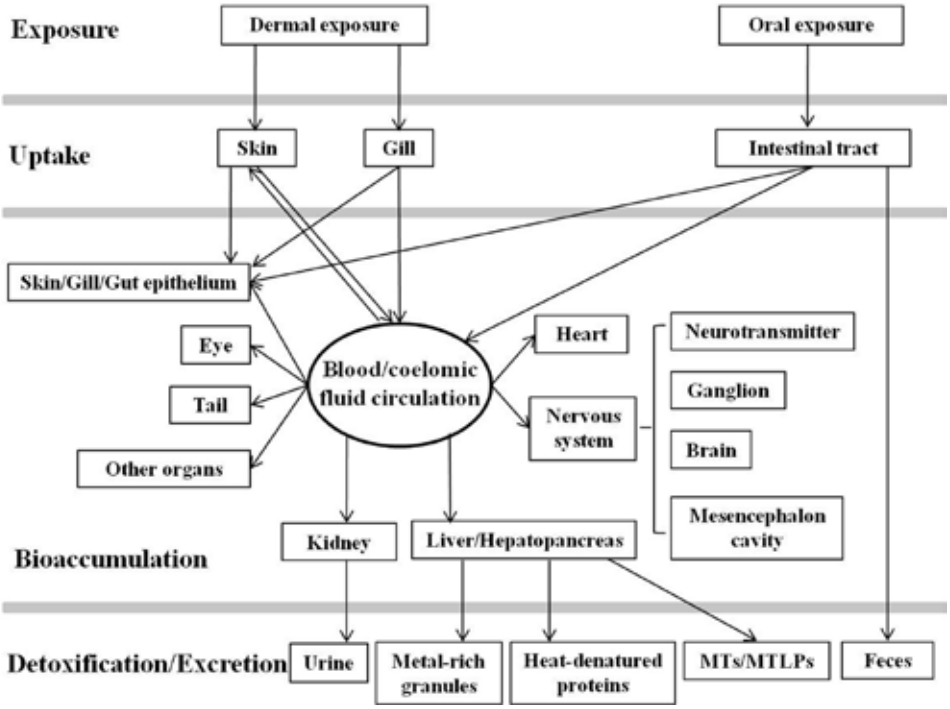

Fig.7 Individual uptake, bioaccumulation and detoxification/excretion processes of nanosilver.

Based on the existing studies, the potential individual uptake routes, accumulation locations and detoxification/excretion pathways of nanosilver for aquatic organisms are shown in Figure 7. Generally, *N. diversicolor* is tolerant of heavy metal exposure, especially Cu, Zn and Cd (Bryan and Hummerstone, 1971, 1973; Grant et al.,1989; Hateley et al., 1989; Mouneyrac et al., 2003), and is able to control its body concentration of certain metals by the induction of physiolo-

gical detoxification mechanisms, involving the formation of metal-containing extracellular granules, mineralized lysosomes, excretion of metals by exocytosis via coelomocytes, and synthesis and turnover of metal binding proteins, such as metallothioneins (MTs) (Amiard et al., 2006; Bryan and Hummerstone, 1977; Fernandez and Jones, 1989; Mouneyrac et al., 2003; Ng et al., 2008; Poirier et al., 2006). Potential different detoxification mechanisms involved between nano- and aqueous-Ag have been demonstrated by García-Alonso et al. (2011), in which Ag accumulated from nano-Ag was found predominantly associated with metal-rich granules and heat-denatured proteins, while Ag accumulated from aqueous-Ag mainly localized to the metallothionein-like proteins (MTLPs) fraction in *N. diversicolor* after 10 d of sediment exposure. The absence of MTLPs in the worms exposed to nano-Ag indicated differing detoxification pathways between the two Ag forms, and also raised the possibility that dissolution of the NPs, either externally or internally, was not important over the time scale (10 d) in this species. However, the specific detoxification mechanism(s) of micron-Ag remain unclear in *N. diversicolor*, which still need further investigation and to be compared with nano-Ag and aqueous-Ag.

3.4 Is nanosilver more toxic than its micron- and aqueous-forms?

3.4.1 Ag toxicities

Mortality was low and not affected by the Ag levels for all forms in both experiments after 10 d of sediment exposure.

3.4.1.1 Growth and burrowing behavior

Growth and burrowing behavior are two individual endpoints tested for Ag toxicities in our study. Growth is a commonly used indicator in many chronic toxicity tests. There have been some studies reporting growth inhibition by aqueous Ag in aquatic organisms (Calabrese et al., 1984; Galvez and Wood, 2002; Sanders et al., 1990). However, only a small number of publications have investigated the effects of nanosilver on growth of aquatic organisms and even fewer for micron-Ag. Cell growth of the marine diatom, *T. weissflogii,* was suppressed as a function of increased Ag^{+} ions released from nanosilver (Miao et al., 2009). However, in our study, there was no significant growth in any of the Ag forms or concentrations during 10 days. All worms were collected from an unpolluted site; therefore, they should not be conditioned or acclimated to metals. The lack of growth is possibly

due to the poor organic content (only 0.56 %) in sediment which was collected during winter; however, it is also likely that 10 days is too short a period over which to measure growth in *N. diversicolor*, since this species typically exhibits growth rates of 0.1-0.2 mm d^{-1} (Scaps, 2002) (Cong et al., 2011b, Paper II).

Burrowing behavior is another commonly used endpoint of invertebrate behavior response to sediment toxicity (Boyd et al., 2002). We recognize that the use of burrowing time with 4-5 replicates per treatment was probably not as sensitive as the commonly used 'percent of un-burrowed organisms', which typically uses 20 replicates (Bonnard et al., 2009) and a large variability did observed in our test. However, an increasing tendency of burrowing time for worms in the two nano-Ag treatments with increasing exposure concentration was observed, especially in the nano-Ag_{20} group, the time of which was significantly longer than the aqueous-Ag, indicating the potential greater effects of nanosilver exposure on worm burrowing activity than the aqueous Ag. Buffet et al. (2011) investigated the burrowing behavior of *N. diversicolor* after 7 d seawater exposure to aqueous Cu (as $CuNO_3$) and nano-CuO and found that only exposure to aqueous Cu led to a significant decrease of burrowing kinetics. The contradictory burrowing responses with our study were likely due to the different metal NPs used and exposure routes applied. As in seawater, nano-CuO tended to agglomerate and precipitate, which process reduced the effective concentration worms exposed to. The reduced burrowing speed in nano-Ag treatments in present study indicated the impairment of worm mobility at the exposure concentrations used (Paper III).

3.4.1.2 Cytotoxicity and genotoxicity

The SOD activity was not found to be affected after either nano- or micron-Ag exposure in a pilot study, with similar inhibition of around 90 % in control and exposure groups, indicating it was not a sensitive endpoint for Ag exposure in *N. diversicolor*. Therefore we did not include this assay in subsequent experiments. Lysosomal membrane stability after Ag (nano-Ag_{20}, nano-Ag_{20} and aqueous-Ag) treatments was assessed by neutral red retention time (NRRT). Lysosomes have been identified as a particular target site for toxic effects and play an important role in metabolism of various heavy metals (Moore, 1990). Lysosomal membrane stability is thought to be a general measure of stress, and it decreases in response to stress as permeability increases. The mechanism causing this alteration in membrane stability may involve direct effects of chemicals on the membrane or the increased frequency of secondary lysosomes in toxicant-stressed cells (Huggett

et al., 1992; Weeks and Svendsen, 1996). The neutral red assay, as a measure of lysosomal membrane damage, makes use of the fact that only lysosomes in healthy cells can retain the supravital dye after initial uptake (Weeks and Svendsen, 1996) (Fig.8).

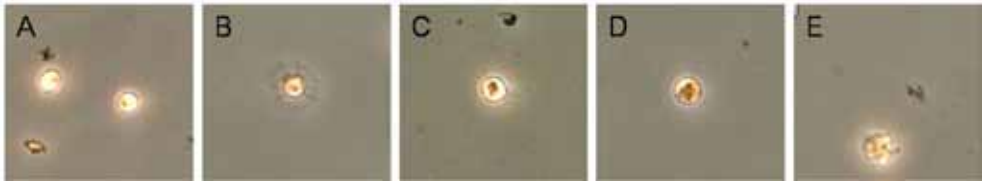

Fig.8 Coelomocytes of N. diversicolor with increasing lysosomal membrane permeability. (A) Normal cell (neutral red was retained in the lysosome); (B-D) Increasing permeability of lysosomal membranes with more and more neutral red leaking from lysosome to cytosol; (E) Dead cell.

The concentration-dependant reduction in NRRTs observed in the coelomocytes of *N. diversicolor* indicated stress resulting from exposure to Ag in sediment. Furthermore, nano-Ag_{20} had a significantly higher effect on the decrease of lysosomal membrane stability than the aqueous-Ag. However, there was no significant difference in NRRTs between nano-Ag_{20} and nano-Ag_{80} likely because of the lack of difference between the two particles' size as indicated by TEM and DLS characterization (Paper III).

Dose-dependent lysosomal destabilization was also observed in adult oysters (*C. virginica*) when they were exposed to nanosilver (15±6 nm) for 48 h (Ringwood et al., 2010). Our study confirms that lysosomes of worm coelomocytes are a subcellular target for the action of both particulate and aqueous Ag. This is consistent with published results showing that Ag can deposit in lysosomes. For instance, aqueous Ag is known to be accumulated as non-toxic deposits of silver-sulphur granules in lysosomes (Kristiansen et al., 2008; Lansdown, 2007). The specific transport system has been characterized by Havelaar et al. (1998) and involves a heavy metal ion transport protein in the lysosomal membrane which facilitates the uptake of silver through a typical carrier-mediated process. Recently, García-Alonso et al. (2011) characterized cellular internalization of nanosilver (30±5 nm) in gut epithelia of *N. diversicolor*, and found that clusters of electron dense particles resembling Ag nanoparticles deposited in the lysosomes. Furthermore, TEM images showed that the lysosomal membrane seemed to break down after 10 d of sediment exposure to nanosilver in *N. diversicolor*. The uptake of nanosilver

into lysosomes has also been observed in human cell lines (Asharani et al., 2009; Greulich et al., 2011; Kim et al., 2009), where uptake was suggested to be through clathrin-dependent endocytosis and macropinocytosis (Greulich et al., 2011).

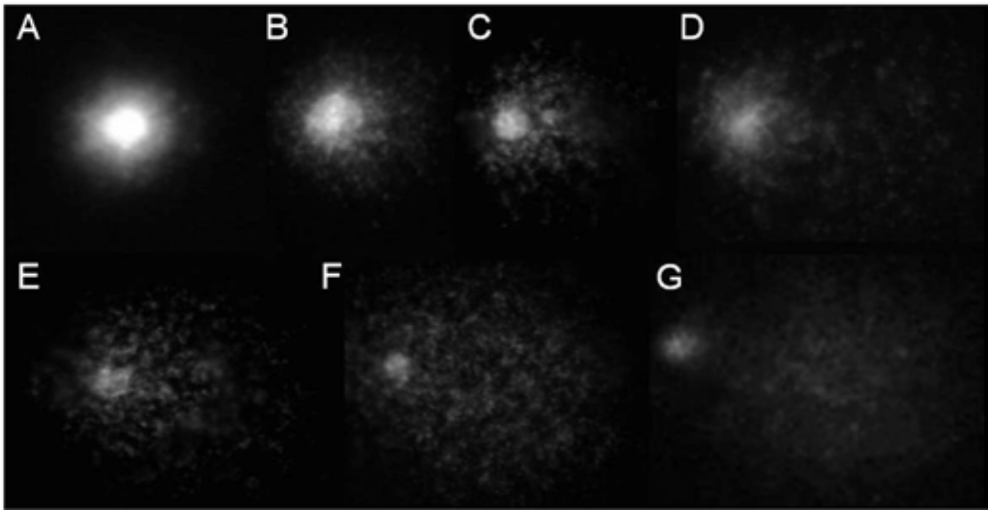

Fig.9 Image from comet assay showing different extents of DNA damage of N. diversicolor coelomocytes after Ag exposure (625× magnification). (A) Control (undamaged cell nucleus); (B-F) An increasing DNA damage of coelomocytes typical with less compactness and weaker brightness in the head and increasing DNA fractions around the head shown as a tail (more damaged DNA with increasing Ag exposure concentrations); (G) Positive control (worms from 0 μg Ag/g dw sed., but extracted cells exposed to UV) with tiny and obscure heads and a mass of DNA fractions accumulated in the tail (the tail also has a distance away from the head). Diameter of undamaged nucleus is approximately 5-8 μm.

Comet assay results from both toxicity experiments demonstrated that all three Ag treatments, i.e., nano-, micron- and aqueous-Ag, can cause DNA damage in *N. diversicolor* coelomocytes (Fig.9), and this effect was concentration-related. Tail moment and tail DNA intensity (%), which are two commonly used measures of DNA damage, showed comparable results in all controls and exposure treatments (5, 10, 25 and 50 μg Ag/g dw sed.) for nano-Ag_{100}, nano-Ag_{20} and nano-Ag_{80}, which was consistent with the fact of their similar size distributions (Cong et al., 2011b, Paper II; Paper III). Furthermore, our comet assay results are also consistent with previous studies regarding the genotoxicity of Ag observed in different cell systems (Ahamed et al., 2008; Asharani et al., 2009; Cha et al., 2008; Choi et al., 2010; Hidalgo and Dominguez, 1998). Both tail moment (2.1-2.3 and 2.8 in toxicity experiment I and II, respectively) and tail DNA intensity (13.5-15.9 % and 17.3 % in toxicity experiment I and II, respectively) of controls (0 μg Ag/g dw sed.)

demonstrated similar values with those of Asharani et al. (2009) (tail moment of ~0.5 to 2.5 in human lung fibroblast cells and glioblastoma cells) and Lewis and Galloway (2008) (% DNA in the tail of ~13 % in *N. diversicolor* coelomocytes), respectively. Furthermore, tail moment at concentrations of 25, 50 and 100 μg Ag/g dw sed. for all three nano-Ag treatments after 10 d of sediment exposure had comparable results with those of human glioblastoma cells after 48 h exposure to 25, 50 and 100 μg/mL nanosilver, respectively (Asharani et al., 2009).

In both toxicity experiments, a higher genotoxicity was observed in the nano-Ag treatments (nano-Ag_{100} and nano-Ag_{80}, respectively), while the aqueous-Ag tended to be less genotoxic (Cong et al., 2011b, Paper II; Paper III). Similarly with NRRT, the lack of genotoxic difference between nano-Ag_{20} and nano-Ag_{80} in experiment II was likely due to the overlap of their sizes (Paper III). Aqueous Ag has been shown to cause DNA damage by covalently binding with DNA (Hossain and Huq, 2002) and inhibiting DNA synthesis (Hidalgo and Dominguez, 1998) directly. In reality, most of the aqueous Ag is not present as free Ag^+ ions after addition into marine sediment, due to speciation and complexation with ligands, e.g., chloride and sulfide. The lower genotoxicity of aqueous Ag observed in our study may have been due to these complexed forms of Ag possibly reducing the amount of free Ag^+ ions entering the nuclei and interacting with DNA strands, as supported by the study of LeBlanc et al. (1984) which demonstrated less toxicity of complexed forms of Ag than free Ag^+ ions to fathead minnows. As for nanosilver, the mechanisms leading to genotoxicity have been indicated to include both direct and indirect aspects. The tendency for nanosilver to accumulate in the nuclei of zebrafish embryos and human cancer cells has been described, suggesting that it may lead to genomic damage and chromosomal aberrations directly (Asharani et al., 2008, 2009). On the other hand, it has been suggested that nanosilver entering cells disrupts the mitochondrial respiratory chain leading to the production of ROS and the interruption of ATP synthesis, which in turn causes DNA damage (Asharani et al., 2009). Nanosilver may also destroy the membrane integrity of organelles which are involved in metal detoxification, such as lysosomes, which was observed in our neutral red assay, and then cause genotoxicity. Additionally, the potential release of Ag^+ ions from particle surfaces is also a possible mechanism by which nanosilver exerts toxicity to nuclei. Although we did not measure Ag^+ ions released from nano-Ag during sediment exposure in our study, our results suggested that the cytotoxicity and genotoxicity of Ag NPs were not solely attributed to Ag^+ ion release (if any), as we observed a trend of

greater effect in nano-Ag treatments compared to the aqueous Ag, which suggests that either different mechanisms may be involved between Ag NP and aqueous Ag toxicity or that there are different degrees of reactivity of the different Ag forms in causing DNA damage. García-Alonso et al. (2011) found significantly larger fractions of particulate nanosilver existing in *Nereis* cell organelles than the aqueous-Ag, which suggested the potential higher cellular toxicities of nano-Ag than the aqueous-Ag. The authors also raised the possibility that dissolution of nanosilver particles, either externally or internally, was not important over a time scale of 10 d sediment exposure in *N. diversicolor*, as different Ag tissue distributions and detoxification mechanisms were observed between nano- and aqueous-Ag in their study. Clearly, further investigations are needed to determine the kinetics of oxidative dissolution of nanosilver under sediment exposure scenarios. For micron-sized Ag particles, few studies have reported their genotoxicity, and the mechanisms still remain unclear. The actual size of micron-$Ag_{2\text{-}3.5}$ used in our study covers a wide range from nano- to micron-size, with most particles falling outside of the nanometer range. Hence, although there was an overlap in size distribution between micron-$Ag_{2\text{-}3.5}$ and nano-Ag_{100}, different gentoxicities were observed in toxicity experiment I possibly due to the size effect as the majority of nano-Ag_{100} was within the nano size range. The smaller nano-sized particles contained in the micron-Ag may have entered nuclei and interacted with DNA strands. For larger Ag particles contained in the micron-Ag, phagocytosis and encapsulation (large particles are surrounded by large numbers of coelomocytes) by coelomocytes may have facilitated the uptake of bulk micron-Ag, as well as agglomerated nanoparticles, both of which processes may have contributed to the observed genotoxicity (Cong et al., 2011b, Paper II). However, cell internalization is not always essential for toxicity, as interactions of particles at the cell surface (even in agglomerated form) may also cause toxicity (Johnston et al., 2010). Additionally, the release of Ag^+ ions could be another contributor to micron-Ag toxicity but we would expect a lower release rate and smaller amount of ions due to a smaller fraction of surface atoms and less susceptibility to oxidation due to their higher redox potential compared to NPs (Ho et al., 2010; Pal et al., 1997). More work is needed to elucidate the mechanisms by which different forms of Ag cause genotoxicity to *N. diversicolor*.

3.4.2 Potential cellular uptake and toxic mechanisms of nanosilver

Overall, cellular uptake and toxicity of aqueous Ag is mainly via competition with Na^+ and ATP-dependent transport and disruption of membrane sodium

channels, as well as depletion of the cellular energy system (Bianchini and Wood, 2003; Bury and Wood, 1999; Bury et al., 1999; Galvez and Wood, 2002; Hidalgo and Dominguez, 1998; Pedroso et al., 2007) (Fig.10). In contrast, nanosilver is taken up by cells through diffusion (via unusually large pores) (Lee et al., 2007a, b), endocytosis (e.g., pinocytosis and phagocytosis) (García-Alonso et al., 2011; Luoma, 2008, Park et al., 2010), as well as by disturbance of membrane components through a Trojan-horse type mechanism (nanosilver acts as a carrier to deliver free silver ions inside the cells or across the membranes into cells) (Lubick, 2008; Luoma, 2008; Park et al., 2010) (Fig.10).

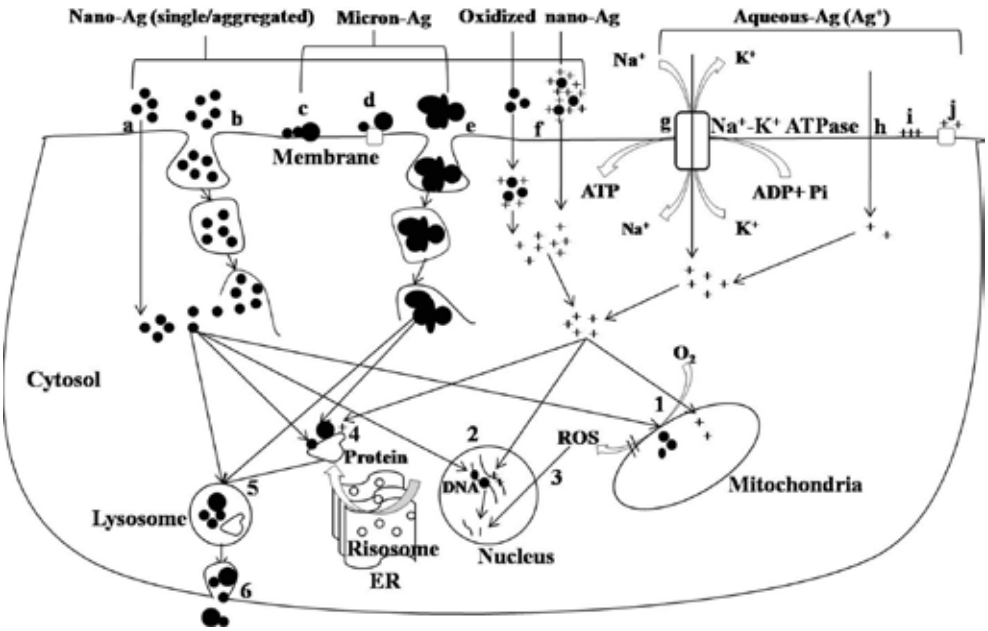

Fig.10 Potential cellular uptake routes, toxic mechanisms and detoxification processes of nano-, micron- and aqueous-Ag. A-j: Cellular uptake routes of (a-f) nano-, (c-e) micron- and (g-j) aqueous-Ag. (a) Diffusion via unusually large pores (chorion pore canals) and (b) pinocytosis of small and single Ag particles; Adsorption of nano-Ag (single or aggregated) and micron-Ag on the (c) cell membrane and (d) membrane protein; (e) Phagocytosis of big and aggregated Ag particles; (f) Trojan horse-type delivery. Nano-Ag acts as Trojan horse carrier to deliver free silver ions inside the cell or enables the transport of ions across cell membranes into cells; (g) Na^+-K^+ ATPase channel; (h) Facilitated transport (e.g., AgGSH and AgCys); Adsorption of Ag^+ ions on (i) cell membrane and (j) membrane protein. 1-6: Toxic mechanisms and detoxification processes of nano-, micron- and aqueous-Ag. 1: ROS-dependent oxidative stress produced by nano- and aqueous-Ag; 2: Small nano- and aqueous-Ag enter nucleus and cause DNA damage directly; 3: Indirect DNA damage cause by ROS; 4: Nano- and aqueous-Ag combine with newly-synthesized protein and dysfunction it, or trigger the expression of detoxification protein, like MT/MTLP and bind with it; 5: Ag and dysfunctioned proteins were enclosed within lysosome and digested; 6: Ag particles/compounds was excreted by cell exocytosis.

As far as cellular toxic mechanisms, ROS-dependent oxidative stress has been confirmed to be a critical mechanism of nanosilver toxicity in both aquatic organisms and mammals, which in turn causes DNA damage, functional protein inactivation, apoptosis and inflammation (Fig.10). The release of Ag^+ ions was also indicated as a contribution to nanosilver toxicity. Distinguishable toxic fingerprints of nano- and aqueous-Ag have been demonstrated in aquatic organisms, including Japanese medaka (*O. latipes*) (Chae et al., 2009), zebrafish (*D. rerio*) embryos (Asharani et al., 2008) and sediment-dwelling polychaete (*N. diversicolor*), indicating different toxic mechanisms involved between these two Ag forms. Actually, in *C. elegans*, the mode of action of nanosilver was also observed to be different from its aqueous counterpart, with the NPs affecting reproduction potential and inducing high levels of oxidative stress compared to the aqueous form (Roh et al., 2009). However, further studies are needed to elucidate the specific cellular toxic mechanisms of different Ag forms in aquatic invertebrates using a wider variety of cellular bio-indicators, such as membrane damage, subcellular distribution, reactive oxygen species (ROS) production and anti-oxidative enzyme activities, which have been indicated to be involved in toxicity, at least for nanosilver, in mammalian cell lines and some aquatic vertebrates.

3.5 Nanosilver in the aquatic environment: Knowledge gaps and challenges

The unique properties of nanosilver may result in different individual and cellular toxic effects and mechanisms compared to its metal, bulk and aqueous forms in aquatic organisms, especially benthic species, which have received much concern but with few investigations until now.

From a physical-chemical perspective, the extremely small size of NPs has been indicated by some studies as a principle factor in determining their toxicities. NPs have properties that are different from their 'bulk' particles, largely due to the relatively large surface area and related higher reactivity. They thereby have a much higher probability to collide with bio-substances than their larger counterparts. However, most existing aquatic (also mammalian) research focused on comparison of toxic consequences among different NP forms or between particle form and aqueous form, and few studies have directly compared nanoparticulate and microparticulate forms of metals, which should be taken into consideration in future

studies. Ag^+ ions have been shown to be released from the surface of nanosilver by chemical oxidation during exposure under laboratory conditions and contributed to the toxicity of nanosilver, which has been observed in algae. However, in some studies, the toxicities of nanosilver are only related to the NP properties but not the released ions. Our preliminary results suggested that the toxicity of nanosilver cannot be solely attributed the release of Ag^+ ions. The contradictory conclusions may result from differences in methodology when preparing NP suspensions, exposure conditions and test species, all of which need to be clarified in further study.

Furthermore, in most laboratory studies, when comparing the toxicities of nano-Ag to natural free aqueous Ag, the effects of surface modifications (e.g., coatings) of NPs, which may alter NP properties or toxic potentials, have been seldom taken into consideration. This is also one reason that the comparison among different treatments and exposure scenarios is difficult. Therefore, we expect that the effect of capping agents on NP behavior and toxicity needs to be accounted for more systematically when designing experiments.

From a molecular perspective, we conclude that different uptake routes and toxic mechanisms are likely involved among nano-, micron- and aqueous-Ag in aquatic organisms, as supported by the evidence from the present study (partially) and previously published aquatic and mammalian toxicological studies. However, the final toxicity of nanosilver within cells will be the result of the balance between NP effects versus cellular detoxification systems, such as induction of metallothioneins or formation of electron-dense granules, which have been observed after exposure to aqueous-Ag. Although increased heat-denatured proteins and metal-rich granules were observed after nano-Ag exposure in *N. diversicolor*, the detoxification processes for nano-Ag, as well as micron-Ag, still receive few investigations in other aquatic organisms.

From an environmental perspective, the toxicity of nanosilver will not only depend on its own particle characteristics, but also on ambient solution conditions like pH, hardness, ionic strength and the availability of various ligands, as these environmental parameters are likely to play important roles in determining the environmental partitioning and toxic consequences for NPs. Therefore, more environmentally realistic factors should be incorporated into future experimental designs to make laboratory studies more close to the real environmental conditions. Furthermore, the characterization of NPs when conducting toxicity studies

should also be carried out under conditions as close as to the real environment. However, at present, the preparation of stock NP suspensions in laboratory for characterization before toxicity experiments usually uses external mixing forces, like solvent dispersion, shaking, centrifugation, ultrafiltration, sonication as well as surface modification and coatings to make NPs disperse evenly. All of these processes and treatments may change the properties of the NPs and may therefore be environmentally unrealistic compared to NPs released to the environment. In addition, improvement of characterization techniques in environmental relevant media (e.g., freshwater, seawater, sediment and cell culture medium) could better explain and interpret divergent or conflicting nanotoxicological results from different studies.

From an ecotoxicological perspective, exposure pathway can influence metal biogeochemical cycling in aquatic systems, but this also receives few investigations for nanosilver. Comparison of experimental data among different exposure pathways or to a particular field situation is therefore difficult because toxicity tests of nanosilver rarely account for multiple pathways of exposure, nor do most designs consider nanoparticle behavior under realistic environmental conditions. For instance, a number of aquatic toxicological studies conducted until now focus on the water or dietary exposure pathway. If lost from suspension, however, nanosilver may accumulate in sediment, and we know very little about the bioavailability and effects of sediment-associated nanosilver. Sediment exposure routes are therefore highlighted because the sediment-associated nanosilver is likely to be ingested by benthic organisms and accumulated.

Furthermore, it has been frequently observed that unrealistically high concentrations of nanosilver have been administered to organisms in toxicity experiments that are unlikely to be encountered in the environment or by human consumers (Johnston et al., 2010). As such, we may expect the amounts of nanosilver to be lower in suspension in nature than has been the case in most laboratory studies. In addition, most studies until now have focused on aquatic vertebrates (i.e., fishes) and cellular indicators in exposure scenarios, and fewer studies have examined toxic consequences in aquatic and benthic invertebrates. Even fewer studies have examined effects of NPs at levels of individuals and populations in ecosystems, which are sorely needed in future studies. This thesis work is a small contribution in this regards.

4. Conclusions

A pilot behavior study of nano- and micron-Ag in two media (deionized water vs. filtered natural seawater) supports the relevance of the sediment exposure pathway of Ag for toxicity experiments since particles of both sizes were quickly lost from suspension in seawater.

The important role of aquatic and benthic invertebrates as test organisms is highlighted because they represent crucial species in the transformation, detoxification and cycling of environmentally relevant contaminants discharged into aqueous environments.

The physical-chemical properties of nano-Ag_{100}, nano-Ag_{20}, nano-Ag_{80} and micron-$Ag_{2\text{-}3.5}$ particles were characterized in parallel to both toxicity experiments. All tested particles had a polyhedral shape and polydispered in deionized water. There was no obvious difference in the size distributions for the three nanosilver particles, which suggested that our work was only based on the comparison of three Ag forms, i.e., nano-, micron- and aqueous-Ag. NP size effect, which was supposed to be investigated, cannot be achieved for nano-Ag_{20} and nano-Ag_{80} due to their overlap in size. There is a pressing need for the development of methods that allow adequate characterization of NPs in complex compartments, such as sediment.

Toxic effects and bioaccumulation of nano-, micron- and aqueous-Ag in the sediment-dwelling polychaete, *N. diversicolor* were assessed after 10 d of sediment exposure. The reduced burrowing speed after nano-Ag treatments indicated the impairment of worm mobility. Ag was bioavailable and accumulated in worms regardless of the form added, but there was no form-related difference in Ag body burden. Size of worms was found to affect the bioaccumulation of Ag; smaller worms accumulated more Ag per body weight than larger worms possibly due to their higher surface to volume ratio and higher metabolic activity. We confirmed that lysosomes were a subcellular target for either nano- or aqueous-Ag in worms. Lysosomal membrane permeability and DNA damage of *Nereis* coelomocytes

showed a concentration-dependant increase with increasing exposure concentration.

Nano-Ag tended to be more toxic than the micron- and aqueous-Ag for tested individual and cellular endpoints even though levels of bioaccumulation were similar. Although we might expect nanosilver to become highly aggregated upon contact with sediment, and therefore to lose its tendency to behave differently than its chemically identical counterparts, our preliminary results suggest that this is not necessarily the case. Furthermore, it seems that cytotoxicity and genotoxicity of nanosilver cannot be solely attributed to the release of Ag^+ ions.

Toxicological uncertainties related to the environmental chemistry of silver, differing biological sensitivities of species and the scarcity of toxicological studies make a conclusive risk assessment for nanosilver extremely difficult at the present time. Linking molecular changes which are readily measurable with relevant ecological responses would improve the predictive power and relevance of *in vivo* and *in vitro* tests based on molecular responses, but making this link in a realistic manner remains one of the great challenges in ecotoxicology. A more systematic analysis of the factors influencing nanosilver toxicity and of how effects at different levels of biological organization are linked is necessary to provide a robust risk assessment model for nanosilver.

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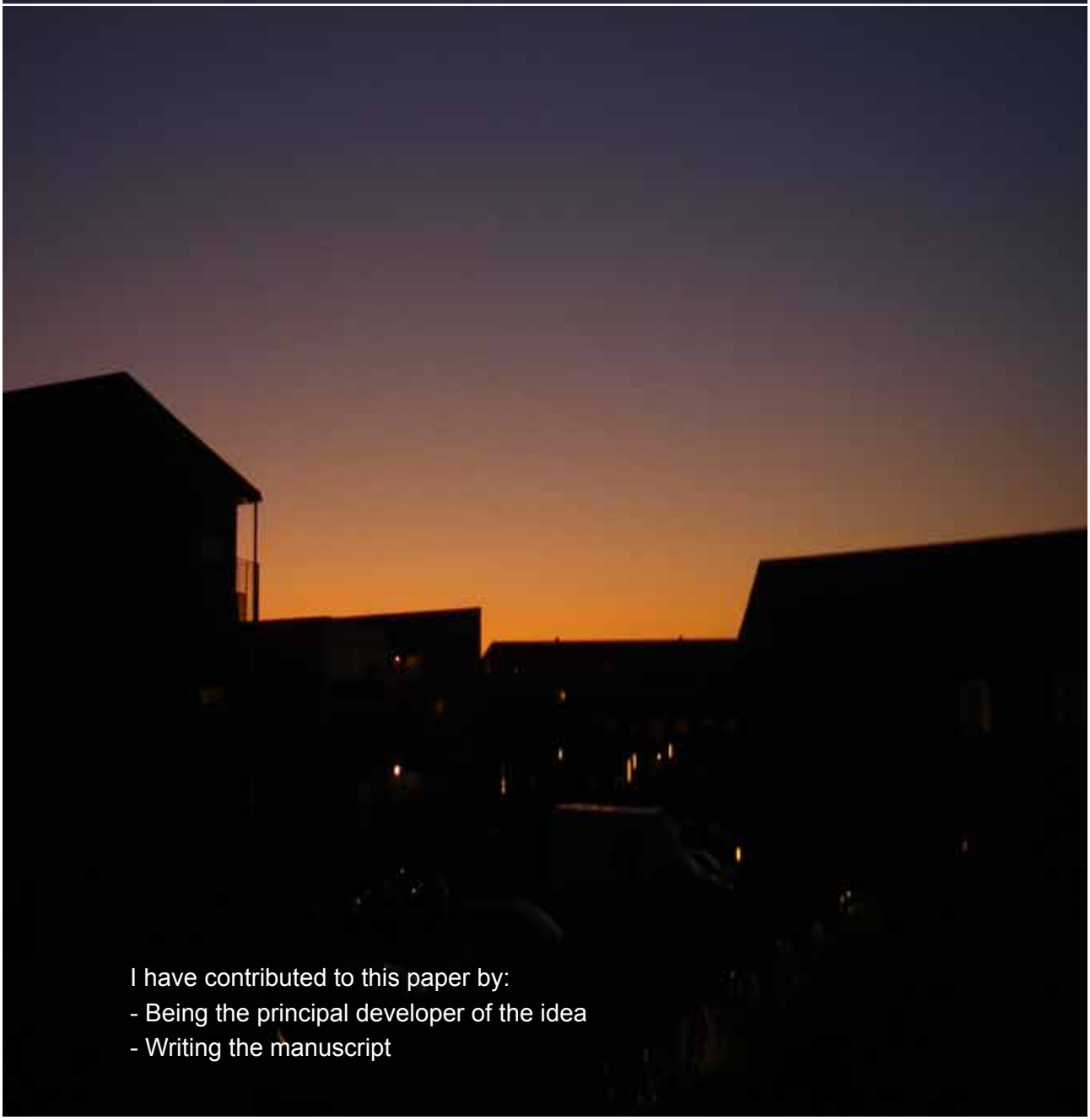
Paper I



I have contributed to this paper by:
- Being the principal developer of the idea
- Writing the manuscript

individuals have the same NEC'' and that ''all the different approaches implicitly assume that all individual organisms in a cohort have the same toxicological threshold'' (Baas et al. 2009). We acknowledge that these statements may have been intended to describe common practice in toxicokinetic and toxicodynamic modeling which, if true, only serves to reinforce our message that whatever the modeling framework, the *stochastic* component cannot be overlooked or assumed to be nonexistent. *Random* variation in concentration-response experiments is accounted for by an appropriate error term plus, in the case of the Bayesian framework, prior distributions on model parameters. In either case, the ''thing'' describing the scatter in concentration-response plot is a probability distribution which in turn is defined by its parameters.

Neglecting error models renders subsequent estimation and inference impossible. It is entirely possible to use ordinary least squares (OLS) to estimate model parameters in the absence of an error model (because OLS is a geometrical concept). However, an error model is necessary to compute confidence or prediction intervals or to test hypotheses about the true parameter values. That this type of inference can be performed even when there has been no explicit specification of any stochastic terms in the model (for example, Ashauer et al. 2010) is because the methods of OLS and maximum likelihood estimation (MLE) are equivalent under the assumption of normally distributed errors. Although this may not be a problem in some cases, there are situations where the assumption of a normally distributed error term is inappropriate—for example, modeling the number of surviving organisms in a concentration-response experiment when the sample sizes are very small (typically fewer than 10).

In our opinion, the stochastic part of modeling should be accorded as much attention as the deterministic part. Thinking about and identifying an appropriate error model underlies good statistical inference and may lead to additional insights and clarity that would otherwise remain undiscovered.

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IMPORTANCE OF CHARACTERIZING NANOPARTICLES BEFORE CONDUCTING TOXICITY TESTS

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Rapidly expanding growth in the field of nanotechnology has led to the development of numerous applications of nanomaterials in industrial (e.g., paints, electronics) and consumer (e.g., cosmetics, clothing treatments) products. These engineered nanoparticle (NP)-containing products have, however, the potential to release particles (single or aggregates) or ions by means of wastewater discharge into the aquatic environment. SCENIHR (2006) emphasized that the behavior of NPs is critically dependent on several particle characteristics, including size, surface area and surface reactivity, and that risk assessments for both human health and the environment have to be based on these characteristics. However, in practice, risks of NPs are in most cases assessed on the basis of their chemical composition alone and, to date, no widely accepted or well-defined risk assessment methods or test strategies exist explicitly designed for NPs.

There is a growing consensus on the necessity of proper and accurate characterization of NPs in environmental media and biological systems to ensure reliable and reproducible toxicity tests are performed. Without such characterization, nanotoxicity experiments will have limited value due to unknown variability in experimental conditions of the NPs (Warheit 2008). Some of the current divergent or conflicting results from nanotoxicological tests could also be better explained if there had been adequate characterization in all studies. However, exhaustive characterization of NPs is undoubtedly costly and time-consuming, and therefore, a sufficient but practical approach is needed. Some principal characteristics of NPs which have been considered to deserve quantification before conducting toxicity tests are size, shape, state of dispersion, physical and chemical properties (e.g., electronic and optical properties, chemical composition and reactivity), surface area, and surface chemistry (Powers et al. 2006). Whereas a significant number of papers list some of these characteristics for the powder or the initial dispersion media (usually in distilled water) few, if any, studies of aquatic nanotoxicity have provided a full characterization of the size distribution (especially hydrodynamic size), dispersion state (especially in biological media) or surface chemistry (like surface charge) of NPs in the actual test media. However, many NPs are likely to undergo significant size distribution or surface chemistry changes when they are transferred between media during experiments, such as from dispersion media (deionized water) to test media (e.g., sediment, freshwater, seawater, and cell culture media). Such changes may alter bioavailability or toxicity in ways that are not entirely understood.

We have characterized commercially available Ag NPs before conducting toxicity tests (Cong et al. unpublished data) and found a clear difference between the manufacturer's information ($<$ 100 nm and 2 to 3.5 μm, respectively) and what we measured (20 to 200 nm and 8 nm to 3 μm in deionized water, respectively) for 2 Ag forms. This difference in size between that reported by the manufacturer and that measured in the laboratory was also observed by Scown et al. (2010). The reasons are most likely due to batch-to-batch variation during production, changes in material properties between synthesis and initial characterization, and particular experimental conditions when used (e.g., pH, ionic strength, and temperature). This observed variability highlights the importance of fully characterizing commercially obtained NPs before performing toxicity experiments, at the very least in the stock solutions used to prepare exposure treatments.

Given this sensitivity to experimental conditions, it is also important to characterize the NPs for each experiment conducted.

The preparation of stock NP suspensions for characterization before conducting laboratory experiments usually uses external mixing forces, like solvent dispersion, shaking, centrifugation, ultrafiltration, sonication, as well as surface modification and coatings to make NPs disperse evenly. All of these processes and treatments may change the properties of the NPs and may, therefore, be environmentally unrealistic compared with NPs released to the environment. Environmental factors, such as water pH, salinity and temperature, dissolved organic material, and natural competing cations, are likely to play important roles in determining the dispersion, toxic consequences, and compartment in which NPs are retained in the environment. Ideally, characterization of NPs should be performed under conditions as close as possible to the relevant exposure medium. For NPs intentionally or accidentally introduced into the aquatic environment, sediment is likely to act as a potential sink as it does for many chemicals. However, we know little about the state of sediment-associated NPs due to the limitation of techniques and methods for characterizing NPs in such complex and ''dirty'' media. We can start our exposures with nicely dispersed and well characterized NPs in a deionized water stock solution, but as soon as we add the solution to sediment we are working literally with a black box. Although we might expect NPs to become highly aggregated upon contact with sediment and therefore lose their tendency to behave differently than their chemically identical counterparts, our preliminary results suggest that this is not necessarily the case. Because the reactivity and toxicity of NPs are believed to be influenced by such features as their size, shape, surface coating, and other properties, we conclude that both the physical and chemical properties of NPs must be systematically and adequately defined before toxicological studies and risk assessment. The publication of toxicity test results should require that a characterization be performed in stock solutions used for testing. At the same time, there is a pressing need for the development of better methods for effectively characterizing NPs in complex environmental media (e.g., seawater, sediment) and living tissue.

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INTEGRATED ASSESSMENT MODELING FOR CLIMATE CHANGE: WHY WE NEED IT

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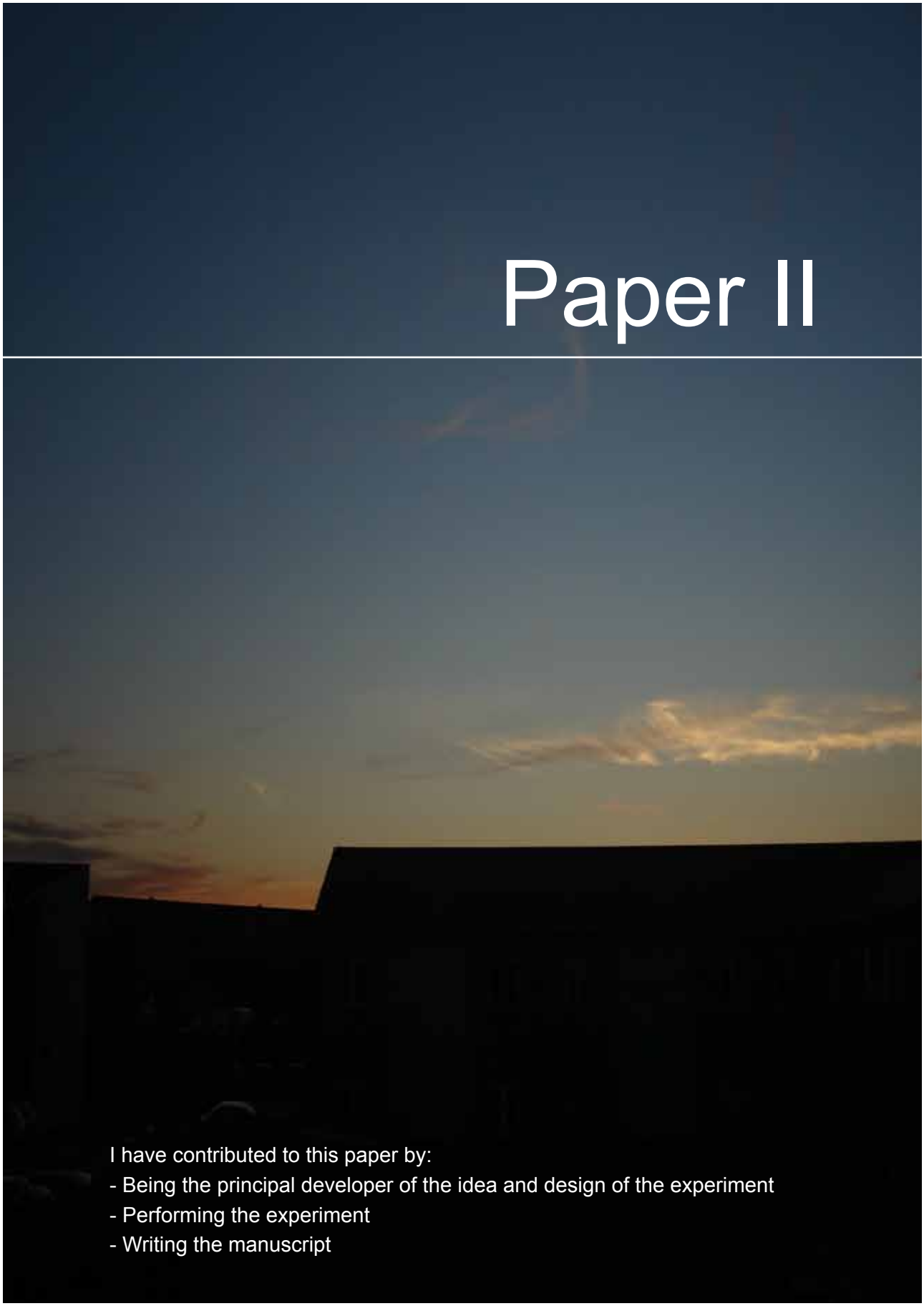
Several governments are presently grappling with the challenge of introducing policy that aims to decarbonize economies. However, in several developed nations such as Australia and the United States, public support for carbon reduction schemes is presently not strong. There are several reasons for this. For example, some members of the community simply choose to not believe that either the Earth is warming, or that any warming is not attributable to anthropogenic drivers. However, it can be argued that one of the major reasons for this lack of engagement and buy-in to carbon policy initiatives is because individual community members, industry sectors, and business owners in the private sector are unsure of the full implications of such a carbon reduction scheme. In other words, although they may be in general agreement that carbon emissions should be reduced, they are unsure whether proposed frameworks for implementing such policies are appropriate and are unsure of what it would mean for themselves, their business, and family, and the greater community. Such policy instruments by definition involve the reallocation and redistribution of wealth and access to resources; thus, there will be those who perceive that they may be winners or losers in any such reallocation scheme. Therefore, it is reasonable to expect that many community members and businesses will feel uncomfortable in the formative stages of such policy developments in the absence of robust information on the likely consequences such a reallocation policy will invariably lead to.

One of the principal reasons why it is so difficult to ascertain the full extent of reallocation policies such as carbon reduction frameworks is that national economies are increasingly complex. As a result of increased specialization, outsourcing, and globalization, and general increased connectivity as a result of ICT (Information and Communications Technology), manufacturing and supply lines in particular have dramatically increased in complexity. Furthermore, the widespread development of large transnational corporations also means that a policy instrument applied in one nation can have implications to other often-distant nations. This increased complexity means that it is increasingly difficult to predict the full consequences that a national carbon reduction policy will have on all community members within the nation in question, let alone interconnected nations.

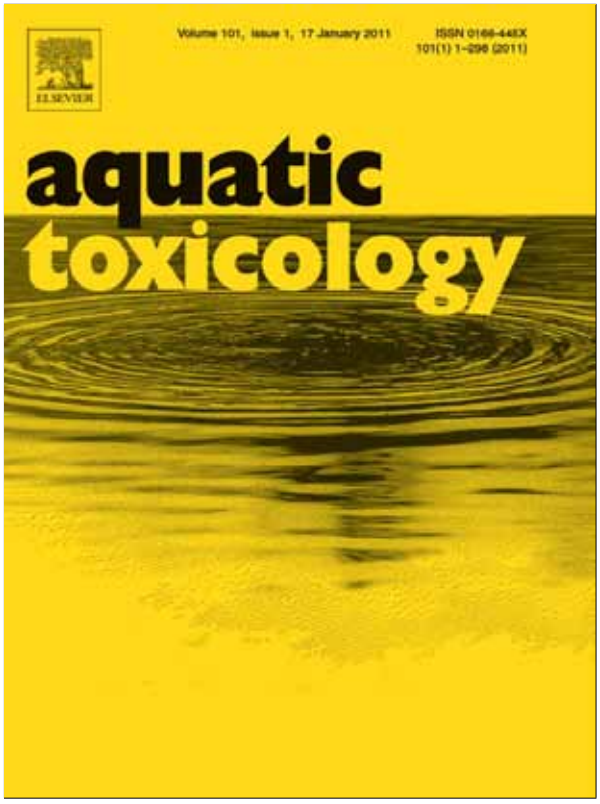
The obvious way to address this information gap is through the development of integrated assessment modeling frameworks that can encapsulate the key connectivity and feedbacks between different parts of national economies, but more importantly feedbacks between different national economies and between economies and natural (biophysical) processes in the Earth system. In the climate sciences, these approaches are broadly categorized as Integrated Assessment Models (IAMs; i.e., Mastrandrea and Schneider 2004). IAMs consist of economic models (mostly CGE; computational or computable general equilibrium models) that are either

Paper II



I have contributed to this paper by:

- Being the principal developer of the idea and design of the experiment
- Performing the experiment
- Writing the manuscript



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Toxic effects and bioaccumulation of nano-, micron- and ionic-Ag in the polychaete, *Nereis diversicolor*

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ABSTRACT

There is increasing concern about the toxicities and potential risks, both still poorly understood, of silver nanoparticles for the aquatic environment after their eventual release via wastewater discharges. In this study, the toxicities of sediment associated nano (<100 nm)-, micron (2–3.5 μm)- and ionic ($AgNO_3$)-Ag on the sediment-dwelling polychaete, *Nereis diversicolor*, were compared after 10 days of sediment exposure, using survival, DNA damage (comet assay) and bioaccumulation as endpoints. The nominal concentrations used in all exposure scenarios were 0, 1, 5, 10, 25, and 50 μg Ag/g dry weight (dw) sediment. Our results showed that Ag was able to cause DNA damage in *Nereis* coelomocytes, and that this effect was both concentration- and Ag form-related. There was significantly greater genotoxicity (higher tail moment and tail DNA intensities) at 25 and 50 μg/g dw in nano- and micron-Ag treatments and at 50 μg/g dw in the ionic-Ag treatment compared to the controls (0 μg/g dw). The nano-Ag treatment had the greatest genotoxic effect of the three tested Ag forms, and the ionic-Ag treatment was the least genotoxic. *N. diversicolor* did accumulate sediment-associated Ag from all three forms. Ag body burdens at the highest exposure concentration were 8.56 ± 6.63, 6.92 ± 5.86 and 9.86 ± 4.94 μg/g dw for worms in nano-, micron- and ionic-Ag treatments, respectively, but there was no significant difference in Ag bioaccumulation among the three treatments.

1. Introduction

Silver nanoparticles (Ag NPs) of size less than 100 nm are of concern due to their increasing use in consumer products which may pose risks for the environment and human health. Ag NPs have properties that are different from their bulk counterparts, exhibiting higher reactivity partly due to their relatively large surface area, but also potentially due to size-related quantum effects. Applications using Ag NPs have therefore become widespread in fields such as bio-sensing, the food industry, water purification, and medical sciences (Klaine et al., 2008). Ag NP-containing products, such as odor resistant textiles, food packaging, cosmetics, household appliances, and medical devices may release Ag particles (nanoparticles or aggregates) or Ag^+ ions via wastewater discharge into the aquatic environment (Benn and Westerhoff, 2008). Thus, aquatic organisms are particularly likely to be exposed to these metal contaminants, and aggregation and precipitation of Ag NPs in aquatic systems suggest that benthic species may be especially at risk.

Though still limited, an increasing number of studies concerning Ag NP effects on aquatic organisms under laboratory conditions have been published in recent years (reviewed by Fabrega et al., 2011), making more data available regarding their bioavailability, bioaccumulation and toxicities. Zhao and Wang (2010) demonstrated the uptake of Ag NPs in *Daphnia magna* from both water and dietary routes, and found that more than 70% of Ag NPs accumulated in the daphnids was through the ingestion of algae. Ag NPs were also shown to be taken up by aquatic vertebrates, such as zebrafish (*Danio rerio*) embryos and liver tissue, fathead minnow (*Pimephales promelas*) embryos, rainbow trout (*Oncorhynchus mykiss*) gill and carp (*Cyprius carpio*), and existed within the body in both single and agglomerated forms (Choi et al., 2010; Farkas et al., 2011; Gaiser et al., 2009; Laban et al., 2010; Lee et al., 2007a,b). Uptake of Ag NPs was also observed in mammalian cell lines, and the particles distributed throughout the cytoplasm, nuclei, lysosomes, endosomes, as well as deposited in mitochondria (Asharani et al., 2009; Hsin et al., 2008; Kim et al., 2009). Ag NPs are able to induce several kinds of sublethal phenotypic abnormalities and

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cause physiological dysfunction of aquatic vertebrate embryos during development (Asharani et al., 2008; Laban et al., 2010; Lee et al., 2007a,b; Ringwood et al., 2010; Wu et al., 2010). Recent studies have also demonstrated cytotoxic and genotoxic effects of Ag NPs, including biomembrane damage (Arora et al., 2008, 2009; Carlson et al., 2008; Farkas et al., 2010, 2011; Hussain et al., 2005), mitochondrial dysfunction (Farkas et al., 2010) and ROS-dependent oxidative stress (Carlson et al., 2008; Choi et al., 2010; Hussain et al., 2005; Kim et al., 2009), DNA damage and chromosomal aberrations (Asharani et al., 2009; Choi et al., 2010; Kim et al., 2009; Sr et al., 2010), apoptosis (Arora et al., 2008; Asharani et al., 2008; Choi et al., 2010; Park et al., 2010) and inflammation (Carlson et al., 2008; Cha et al., 2008; Park et al., 2010), which have been observed in different cell types. In addition, it was reported that Ag^+ ions could be released from Ag NPs by oxidation (Ho et al., 2010) and contributed to the observed toxicities (Navarro et al., 2008). Distinguishable toxic fingerprints of Ag NPs and $AgNO_3$ have been demonstrated in Japanese medaka (*Oryzias latipes*) liver (Chae et al., 2009) and zebrafish (*D. rerio*) embryos (Asharani et al., 2008), indicating different toxic mechanisms involved for these two Ag forms.

Ag NPs have properties that are different from micron-Ag and Ag^+ ions. The inclusion of both 'bulk' and ionic forms of Ag in comparison with Ag NPs is of importance to decipher if effects are driven by the small size of NPs, or other characteristics (e.g., ion release) of these particles. Nevertheless, most existing research on Ag NPs to date focuses on comparison of toxicities among different Ag NPs or between the particulate and ionic form of Ag, and few studies have directly compared nano-sized versus micron-sized Ag. These studies have also primarily included exposure from the water phase, despite the fact that most nanoparticulate metals released to the aquatic environment will accumulate in the sediment compartment. The sediment exposure route has received little attention. Furthermore, despite an increasing number of *in vitro* studies concerning the toxic effects of Ag NPs on vertebrates (particularly freshwater fish and mammalian cell lines), there is a serious lack of information about their *in vivo* toxicities and toxicities to aquatic invertebrates. It is essential to include aquatic invertebrates as test organisms under relevant exposure conditions for environmental pollution studies because they represent key species in the transformation, detoxification and cycling of environmentally relevant contaminants discharged into aqueous environments (Cattaneo et al., 2009; Ratte, 1999). The sediment-dwelling ragworm, *Nereis diversicolor*, is known to play a crucial role in the fate of chemicals in estuarine areas as a consequence of its relative tolerance and its influence on metal speciation and aromatic hydrocarbon transformation in sediment through bioturbation, particle mixing and irrigation (Banta and Andersen, 2003; Berthet et al., 2003; François et al., 2002; Mouneyrac et al., 2003). *N. diversicolor* is thus an appropriate test organism for examining the fate and effects of metal NPs in sediment systems.

The purpose of our study was to compare toxic effects of nano-, micron- and ionic-Ag mixed into sediment on *N. diversicolor* using survival and cellular DNA damage as endpoints, as well as to measure Ag bioaccumulation in worm tissue as a result of 10 days of sediment exposure.

2. Materials and methods

2.1. Animal collection and culturing

N. diversicolor adults, with an average length of 5–7 cm and an average wet weight of 0.3 g, were collected from Roskilde Fjord near shore (55°40.710′N, 11°59.120′E) during winter of 2009 (0–4 °C) and spring of 2010 (4–6 °C). After transferring to the laboratory, the worms were placed in natural sediment (collected from the same site, sieved <1 mm) to acclimatize for 5–8 days with aerated natural seawater (the same site, 15‰ salinity). A stepwise increase of temperature by 3–4 °C every 3 days from ambient temperature up to 15 °C was applied during the acclimatization period. Eventually, the natural seawater was replaced with filtered natural seawater (<0.2 μm, 15‰) and worms acclimatized for another two days. One day before exposure, all worms were carefully picked out of the sediment and placed in clean filtered seawater to depurate their guts overnight. During the acclimation and exposure periods, worms were fed natural sieved sediment without additional food supply.

2.2. Characterization of nano- and micron-Ag

Silver nanoparticles (<100 nm, cat.# 576832, 99.5% metals basis, coated by 0.2 wt% PVP) and micron-sized particles (2–3.5 μm, cat.# 32,708-5, ≥99.9% trace metal basis) were purchased from Sigma–Aldrich (Steinheim, Germany). The identification of crystal structure of both particles was performed on the powder, as received, using X-ray diffraction (XRD). Data were collected using a Nonius PSD 120 powder diffraction system equipped with a position sensitive detector. The characterization of hydrodynamic diameter (suspended particle size) and zeta potential (particle surface charge, an indication of suspension stability) was only carried out on nano-Ag. Stock Ag NP suspension (nominal concentration of 0.5 g/L) was prepared in deionized water (18.2 Ω, Millipore) and ultrasonicated (80 W, 45 kHz) in a water bath (Ultrasonic Bath VWR, Lutterworth, UK) for 15 min followed by a 15 min pause. This process was repeated 4 times (total time: 2 h), and the final suspension was analyzed using a Zetasizer Nano ZS (Malvern Zetasizer Nano ZS, Malvern, UK). Original particle size and morphology of both Ag particles were visually assessed by a Hitachi H-7100 transmission electron microscopy (TEM) operating at 100 kV. Micron-Ag suspension in deionized water was unstable as expected due to the large size of the microparticles. However, the sample was composed of microparticles as well as nanoparticles. Further analysis was therefore carried out in toluene. Micron-Ag was therefore suspended in toluene, ultrasonicated at 100 W and 30 kHz with a probe sonicator (Hielscher UP100H, Teltow, Germany) for 30 s before imaging. Afterwards, a drop of Ag suspension was left to dry at room temperature on a carbon coated copper grid for later TEM imaging.

2.3. Sediment exposure

Sandy sediment for all treatments was collected from Roskilde Fjord (Denmark) at the same site as worms. The top few centimeters of the sediment surface were scraped off and sieved to less than 1 mm in the field with natural seawater (15‰, pH around 8.0). After transferring to the laboratory, the overlying seawater was removed, and the sediment was rinsed once by mixing with filtered natural seawater. After settling, the overlying water was carefully removed, and six aliquots of sediment (around 1 g) were taken out for detection of background Ag concentration by Graphite Furnace Atomic Absorption Spectrometry (GFAAS, GTA 120, Varian, Australia). Briefly, the sediment samples were lyophilized (Christ Alpha 1–2, Osterode, Germany) at around −50 °C overnight. Afterwards, the sediments were transferred to Weflon tubes, weighed and digested with 65% HNO_3 in a microwave oven (Milestone MLS-1200 Mega, Leutenkirch, Germany). The digestion program was 250 W, 400 W, 650 W and 250 W for 6 min, respectively. After cooling, the digested suspension was neutralized by adding 25% ammonium solution (Merck, Darmstadt, Germany) and filtered before GFAAS measurement (performed within a few hours). Standard Ag solutions (Inorganic Ventures, Christiansburg, VA, USA) (0, 2, 4, 8, 16 and 32 μg/L) were used to calibrate Ag concentrations (μg/g dw). Another six aliquots of sediment (around 5 g) were taken for dw/ww ratio and organic content measurements. All the

remaining sediment was kept at 4 °C for no more than one week before spiking and no more than two weeks before the start of exposure.

The stock suspensions/solution and stock sediment (nominal concentration of 100 μg Ag/g dw) of the three Ag forms were prepared as follows. For Ag NPs, two bottles of stock suspension were prepared by adding 0.5 g powder to 1 L deionized water; the two bottles were sonicated as described previously. Afterwards, 1.6 L suspension was added to sediment and homogenized. For ionic-Ag, 0.944 g $AgNO_3$ (cat.# S-6506, Sigma–Aldrich, Steinheim, Germany) were dissolved in a small amount of 65% HNO_3 (Merck, Darmstadt, Germany), and deionized water was added to a final volume of 2 L. After mixing, 1.6 L solution was added to sediment and homogenized as for Ag NPs. Due to the poor dispersion of micron-Ag in deionized water by sonication (pilot study), its stock suspension was prepared by adding 0.48 g particles to 1.6 L deionized water which was stirred with a magnetic stirrer for 15 min, and all the suspension was added immediately into sediment and homogenized. It is likely that the changes in speciation of silver may occur upon mixing with sediment particles but for the sake of simplicity we refer to the different treatments according to the initial silver form added to the sediment namely nano-Ag, micron-Ag and ionic-Ag, respectively, throughout the text.

Exposure concentrations (nominal concentrations of 0, 1, 5, 10, 25, 50 μg/g dw) of the three Ag forms were obtained by homogeneously mixing stock sediment with clean sediment. The measured Ag concentrations of stock and exposure sediment were analyzed using Flame Atomic Absorption Spectrometry (FAAS, SpectrAA-220, Varian, Australia) and GFAAS (as before). In addition, PVP-controls (0.1 μg PVP/g dw sediment) were prepared by homogeneously mixing a solution of PVP into sediment in the same way as for the different Ag treatments. The PVP concentration corresponded to that in the highest Ag NP exposure group (50 μg Ag/g dw), and was used to observe if there was a genotoxic effect from the PVP coating alone.

All experimental plastic beakers were acid washed (20% HNO_3) and rinsed once with seawater before exposure. Following overnight depuration, individual worms were transferred to a beaker containing around 320 g wet sediment of the appropriate Ag concentration and form and 600 mL filtered natural seawater. Sediment exposure was conducted in the dark at 15 °C for 10 days. The overlying seawater was changed 2 times during the 10 days of exposure to get rid of worm waste products. We measured the Ag concentration in the sediment after exposure in a similarly designed pilot study and found that <0.1% of Ag was lost to the water in our exposure systems. Beakers and air supply were inspected daily. Mortality and growth in all treatments were recorded after 10 days of sediment exposure.

2.4. Comet assay procedure

The comet assay was based on the protocol of Singh et al. (1998) and Rank and Jensen (2003), and the procedure for extraction of Nereid coelomocytes described by De Boeck and Kirsch-Volders (1997) and Lewis and Galloway (2008), with some modifications. Three to five replicates (one individual is one replicate) for each exposure concentration were used in the comet assay. A positive control (worms from 0 μg Ag/g dw, but extracted cells exposed to ultraviolet light) was established as a comparison with the control (0 μg Ag/g dw) in each Ag-form treatment. PVP-controls were used to observe if there was a genotoxic effect from the PVP coating alone.

After 10 days of exposure, worms were carefully picked out of the sediment and allowed to depurate their guts in natural filtered seawater overnight. Afterwards, 30–50 μL coelomic fluid of *N. diversicolor* was gently extracted into 50 μL PBS (137 mM NaCl, 2.68 mM KCl, 1.75 mM KH_2PO_4, 8.1 mM Na_2HPO_4, pH 7.4) for each assay by carefully inserting a 1 mL syringe fitted with a 0.4 mm × 20 mm needle (chilled prior to use) into the posterior region of the worm (avoiding the gut) and stored on ice briefly before use. Coelomic fluid mixed with PBS was then gently centrifuged (Allegra X-15R centrifuge, Beckman Coulter, Inc., USA) at 300 × *g*, 4 °C for 5 min, and the supernatant was removed. The cell pellet (i.e., the concentrated coelomic cells) was gently mixed with 125 μL of 0.65% low melting point (LMP) agarose (Invitrogen, Paisley, UK), and 100 μL of the mixture was added to a fully frosted slide (one individual per slide) precoated with a 100 μL layer of 0.8% normal melting point (NMP) agarose (Invitrogen, Paisley, UK). Another 100 μL of 0.65% LMP agarose was then coated as the third layer. The agarose solutions used for the coelomocytes were dissolved in Kenny's salt solution (200 mM NaCl, 9 mM KCl, 0.7 mM K_2HPO_4, 2 mM $NaHCO_3$, pH 8.0) and melted in a microwave oven for 1 min. For each layer, a cover slip was placed upon the gel, and the slides were cooled on ice for 15 min to solidify the gel. Afterwards, all work was carried out under yellow light to avoid UV damage to the DNA.

After having removed the cover slips, the slides used as positive controls were exposed to UV light from a Ren UV C lamp (253.7 nm, 15 W, Sylvania, Japan) for 20 s. Then all the slides were placed in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris, 1% Triton X-100, pH 10.0) for at least 1.5 h in the refrigerator. Excess salts were removed by rinsing the slides for 10 s in electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH > 13). The slides were then placed in the electrophoresis chamber filled with cold electrophoresis solution. Alkaline unwinding was carried out for 40 min followed by 20 min of electrophoresis at 300 mA and 25 V. Finally, the slides were neutralized twice for 5 min in 0.4 M Tris solution (pH 7.5).

The slides were stained with 80 μL ethidium bromide (20 μg/mL) for at least 10 min and examined with a fluorescence microscope (50× oil immersion objective, Dialux 22EB, Leica, Wetzlar, Germany) with 625× magnification. On each slide, 50 randomly chosen cell nuclei (obviously apoptotic and necrotic DNA excluded) were examined automatically using software from Kinetic Imaging (Comet assay III). The levels of DNA damage were measured as tail moment, defined as the product of the tail length and the fraction of total DNA in the tail, and tail DNA intensity (%), expressed as the DNA intensity of the tail compared with the intensity of the whole comet (cell) (Dhawan and Anderson, 2009).

2.5. Bioaccumulation

After comet assay, the worms were frozen at −80 °C until use. The Ag concentrations in worm tissues were determined using GFAAS. Before digestion of worm tissues, all worms were lyophilized at around −50 °C overnight. A pool of 1–2 worms per replicate (three replicates for each concentration) was ground into powder using a mortar and pestle. The resulting powder was digested and analyzed on GFAAS as described for sediments. We confirmed the validity of our calibration and analysis of tissue metal concentration by analyzing with a certified biological material (lobster hepatopancreas, LUTS-1, NRC Canada) with a known Ag concentration.

2.6. Statistical analysis

All data (except PVP control) were analyzed using two-way analysis of variance (two-way ANOVA) followed by planned comparisons using a Bonferroni correction to compare the effects of the three Ag forms in each concentration and to compare exposure concentrations (1, 5, 10, 25, 50 μg/g dw) to the controls for each Ag form. A two sample *t*-test was performed to compare the effect of PVP on DNA damage with that of Ag NPs. Data were tested for

Table 1
Measured sediment exposure concentrations of three Ag treatments at experimental start. Nominal Ag concentrations of the three Ag treatments were 0, 1, 5, 10, 25 and 50 μg/g dw sed.

Form	Measured concentration (μg/g dw) (SD)
Nano-Ag (<100 nm)	0.025 (0.06), 0.64 (0.13), 2.98 (0.04), 7.04 (0.39), 25.96 (0.90), 46.87 (2.82)
Micron-Ag (2–3.5 μm)	0.025 (0.06), 0.26 (0.08), 1.68 (0.38), 8.63 (1.66), 22.73 (6.03), 47.34 (7.84)
$AgNO_3$ (Ag^+)	0.025 (0.06), 0.37 (0.04), 2.33 (0.23), 4.83 (0.12), 19.30 (1.60), 41.63 (1.94)

normality and homogeneity before analysis. Results are expressed as mean ± 1 standard deviation (SD) (n = 3–5 for comet assay, n = 3 for bioaccumulation). Differences were considered statistically significant at $p \leq 0.05$. All statistical analyses were performed using SYSTAT 13.0 software (Chicago, IL, USA).

3. Results

3.1. Characterization of nano- and micron-Ag

Crystal structure, original particle size and shape of nano- and micron-Ag were characterized by XRD and TEM. For Ag NPs, the XRD pattern corresponds to silver (Fig. 1A, 1), and no other material was detected. The presence of highly crystalline material was observed (Fig. 1A, arrow), suggesting the presence of large Ag particles (aggregates) in the primary manufacture. For micron-Ag, the XRD pattern also corresponds to Ag (Fig. 1A, 2). However, approximately 5–10% of non-crystalline material was observed, suggesting that it was not as pure as described by the manufacturer (≥99.9% trace metal basis). The TEM image exhibits polyhedral Ag NPs with a size distribution ranging from approximately 20 to 200 nm (Fig. 1B and C). However, micron-Ag was found containing both micron- and nano-sized particles as shown in Fig. 1D and E. Preparation of micron-Ag suspension in toluene further demonstrated polydispersed Ag particles with different shapes (Fig. 1F) and a wide size distribution ranging from 8 nm to 3 μm (Fig. 1G). Hydrodynamic diameter and zeta potential of nano-Ag were characterized by DLS. The average hydrodynamic diameter of Ag NPs in stock suspension prepared by ultrasonication was 162 ± 4 nm (Fig. 1H), and the obtained suspension was very stable, as confirmed by the zeta potential value of −49 ± 0.75 mV.

3.2. Sediment properties

The dw/ww ratio of natural sandy sediment was 0.78 (n = 6), the organic matter content (OMC) was 0.56% (n = 6) and the background Ag concentration was 0.025 μg/g dw (n = 6). Measured concentrations of stock sediment (n = 3) were 115, 80 and 75 μg Ag/g dw for nano-, micron- and ionic-Ag, respectively. Measured sediment exposure concentrations (n = 3) of the three Ag forms compared to nominal concentrations are shown in Table 1. At nominal concentrations of 10, 25 and 50 μg/g dw, the actual concentrations in ionic-Ag spiked sediment were slightly lower (12–38%) than in nano- and micron-Ag treatments. At nominal concentrations of 1 and 5 μg/g dw, the actual concentrations in micron-Ag spiked sediment were somewhat lower (37–49%) than in nano-and ionic-Ag treatments. Generally, the measured exposure concentrations at the start of the experiment were close to the nominal concentrations, and we did establish a concentration range spanning nearly 2 orders of magnitude.

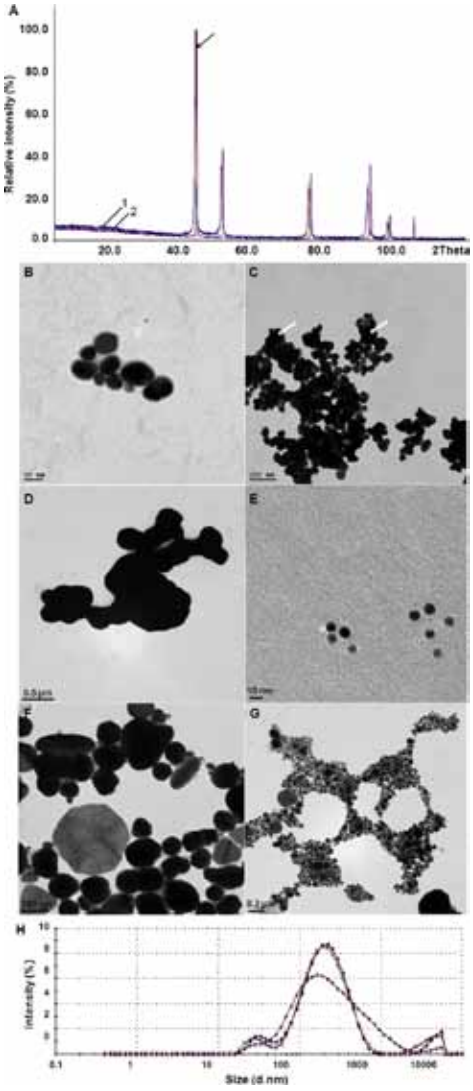

Fig. 1. Characterization of nano-and micron-Ag particles. (A) XRD pattern of nano-Ag (1) and micron-Ag (2) particles. The arrow represents the presence of highly crystalline material. TEM images of (B) high magnification and (C) low magnification of Ag NPs (white arrows refer to the particles with size up to 200 nm); (D and E) micron-Ag contains both micron- and nano-sized particles (prepared in deionized water); (F and G) further demonstrated particle types (size and shape) of micron-Ag by suspending samples in toluene and ultrasonicating with a probe for 30 s. (H) DLS of hydrodynamic size distribution of Ag NPs expressed by intensity (three replicate analyses are shown).

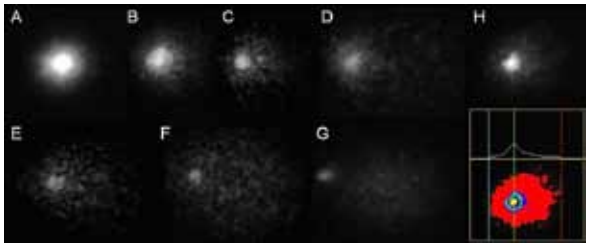

Fig. 2. Image from comet assay showing different extents of DNA damage of *N. diversicolor* coelomocytes after Ag exposure (625× magnification). (A) Control (undamaged cell nucleus); (B–F) an increasing DNA damage of coelomocytes typical with less compactness and weaker brightness in the head and increasing DNA fractions around the head shown as a tail (more damaged DNAs with increasing Ag exposure concentrations); (G) positive control (worms from 0 μg Ag/g dw, but extracted cells exposed to UV light) with tiny and obscure heads and a mass of DNA fractions accumulated in the tail (the tail also has a distance away from the head); (H) an example of analysis of a DNA image (upper panel) by the comet assay III software (lower panel). Blue line: the start of the comet; green line: the center of the head; red line: the end of the tail. Diameter of undamaged nucleus is approximately 5–8 μm.

3.3. Mortality and growth

Three worms were found dead after 10 days of sediment exposure, one in each Ag form-treated group, and there were no concentration-dependent mortality or growth effects. The worms did not differ in their growth or have any significant weight loss in any of the Ag treatments after exposure.

3.4. DNA damage

N. diversicolor coelomocytes exposed to Ag showed increased DNA damage with increasing exposure concentration (Figs. 2 and 3). The values of tail moment and tail DNA intensity (%), which are two commonly used measures of DNA damage, showed no significant difference among the three control treatments (0 μg Ag/g dw) ($p \leq 1.000$ and $p \leq 1.000$, respectively) or among the *in vitro* positive control groups ($p \leq 1.000$ and $p \leq 1.000$, respectively) (Fig. 3A and B). The *in vitro* positive controls exhibited significantly higher tail moment and tail DNA intensities compared to the control treatments as expected, however (Fig. 3A and B). In PVP-control treatments, no significant difference in tail moment ($p \leq 0.863$) or tail DNA intensity ($p \leq 0.974$) was observed compared to the nano-Ag control (i.e., 0 μg Ag/g dw) (data not shown).

The results of tail moment and tail DNA intensity both showed a form- and concentration-related genotoxicity of Ag (Table 2). Additionally, Ag form and concentration interacted significantly to affect tail moment whereas the interaction effect was marginal for tail DNA intensity (Table 2). There were significantly higher tail moment and tail DNA intensities (%) at 25 and 50 μg/g dw in nano- and micron-Ag treated groups and at 50 μg/g dw in the ionic-Ag treated group compared to the controls (0 μg/g dw) (Fig. 3A and B). At the highest exposure concentration of 50 μg/g dw, nano-Ag treated worms had significantly higher tail moment than micron-

Table 2
Effects of Ag form, concentration and their interaction on DNA damage and bioaccumulation by two-way ANOVA.

Endpoints	*p*-Value		
	Ag form	Concentration	Ag form*Concentration
Tail moment	0.003	<0.001	0.001
Tail DNA intensity	0.038	<0.001	0.095
Bioaccumulation	0.652	<0.001	0.901

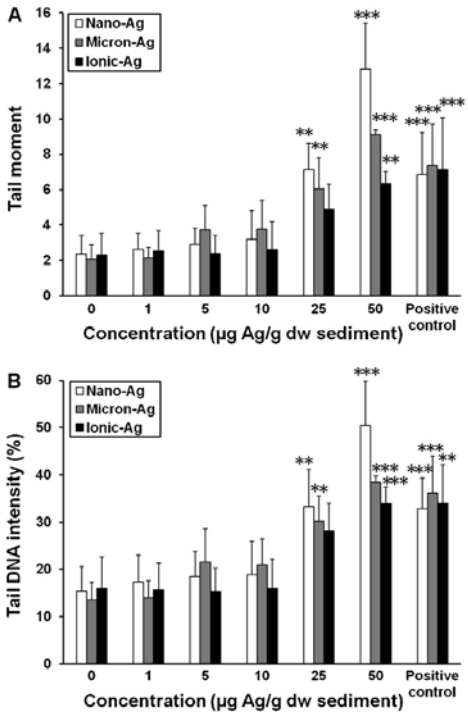

Fig. 3. DNA damage of *N. diversicolor* coelomocytes after 10 days of sediment exposure to nano (<100 nm)-, micron (2–3.5 μm)- and ionic ($AgNO_3$)-Ag treatments. (A) Tail moment, defined as the product of the tail length and the fraction of total DNA in the tail. (B) Tail DNA intensity (%), expressed as the DNA intensity of the tail compared with the intensity of the whole comet (cell). The positive control represents worms from 0 μg Ag/g dw, but extracted cells exposed to UV light. Bars represent the mean ± SD ($n = 3–5$). **,***Significantly different effects of concentrations on DNA damage ($p < 0.01$ and $p < 0.001$, respectively) in each Ag-treated group compared to the control (0 μg/g dw).

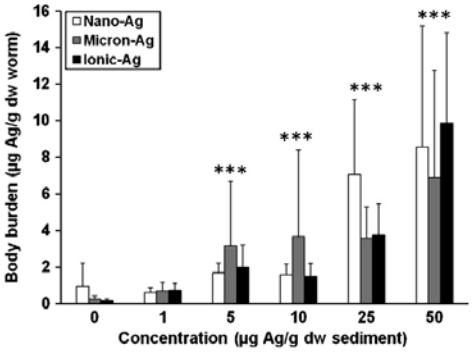

Fig. 4. Ag body burdens in *N. diversicolor* after 10 days of sediment exposure to nano (<100 nm)-, micron (2–3.5 μm)- and ionic ($AgNO_3$)-Ag treatments. Bars represent the mean ± SD ($n = 3$). ***Significant differences in body burdens of Ag ($p < 0.001$) for exposure concentrations compared to the control (0 μg/g dw). *Note*: There was no significant difference among Ag forms (Table 2); therefore the test of concentration effects was for all three forms together.

and ionic-Ag treatments ($p < 0.05$ and $p < 0.001$, respectively). Similarly, the tail DNA intensity also showed significantly higher tail DNA intensities (%) in nano-Ag treated worms than in ionic-Ag treated worms at 50 μg/g dw ($p < 0.01$). Despite a trend of higher tail DNA intensity in nano-Ag treated worms compared to the micron-Ag treated group, this effect was not statistically significant ($p \leq 0.173$).

3.5. Ag body burden

Silver was bioavailable in all three Ag treatments and was accumulated in *N. diversicolor*. Ag body burden increased significantly with increasing exposure concentration (Fig. 4). No significant difference in Ag body burden among the three Ag treatments at any concentration was observed, however, and there was no interaction effect between Ag form and concentration on bioaccumulation (Table 2). Ag body burdens in worms exposed to 5, 10, 25 and 50 μg/g dw of three Ag treatments were significantly greater than those of control worms (Fig. 4). Ag body burdens at the highest exposure concentration were 8.56 ± 6.63, 6.92 ± 5.86 and 9.86 ± 4.94 μg/g dw (mean ± SD) for worms in nano-, micron- and ionic-Ag treatments, respectively (Fig. 4).

4. Discussion

4.1. Ag characterization

To relate properties of nanoparticles to their toxicity potential and ensure that results are reproducible and meaningful, accurate characterization and storage of nanoparticles in dispersion media are essential (Jiang et al., 2009; Murdock et al., 2008; Oberdörster et al., 2005; Powers et al., 2006). In our study, zeta-potential measurement of Ag NP suspensions (−49 ± 0.75 mV) suggested that the particles were stable in the dispersion medium (Derjaguin and Landau, 1941). The better dispersion of micron-Ag observed in toluene compared to deionized water suggests that micron-Ag particles are possibly embedded in an organic matrix and behave in a hydrophobic manner. In contrast, the PVP coating of the Ag NPs provides a better suspension in polar solvents (including water). The TEM images demonstrate a clear difference between the manufacturer's information (<100 nm and 2–3.5 μm, respectively) and that observed by our investigation (20–200 nm and 8 nm–3 μm, respectively) for both Ag particle sizes. The reasons for the differences are most likely to be batch-to-batch variation or changes in material properties between synthesis and initial characterization and the particular conditions when utilized, which highlights the importance of fully characterizing commercially obtained NPs before performing toxicity tests or other experiments (Scown et al., 2010). However, the state of Ag NPs in sediment after spiking and during exposure still remains unknown due to limitations of existing characterization techniques. The examination of the state and behavior of NPs in a complex compartment, as the sediment, will provide a better understanding and interpretation of the effects of NPs on organisms.

4.2. Ag toxicity

Our comet assay results demonstrated that all silver cause DNA damage in *N. diversicolor* coelomocytes regardless of the Ag form added to the sediment, which is consistent with existing studies of the genotoxicity of Ag particles/ions in different cell systems (Ahamed et al., 2008; Asharani et al., 2009; Cha et al., 2008; Choi et al., 2010; Hidalgo and Dominguez, 1998). Both tail moment (2.1–2.3) and tail DNA intensity (13.5–15.9%) results of controls (0 μg Ag/g dw) demonstrated similar values with those reported by Asharani et al. (2009) (tail moment of around 0.5–2.5 in human lung fibroblast cells and glioblastoma cells) and Lewis and Galloway (2008) (% DNA in the tail of ∼13% in *N. diversicolor* coelomocytes), respectively. In addition, tail moments in 25 and 50 μg/g dw Ag NP treatments (7.2 and 12.8, respectively) after 10 days of sediment exposure had comparable results with those of human glioblastoma cells after 48 h exposure to 25 and 50 μg/mL (6 and 9, respectively) Ag NPs (6–20 nm in size). Furthermore, Lewis and Galloway (2008) found that 20–40% DNA was damaged in *N. diversicolor* coelomocytes after 1 h *in vitro* exposure to 52 mg/L genotoxicant methyl methanesulfonate (MMS), which is commonly used as a positive control in the comet assay (Hackenberg et al., 2011). In comparison, tail DNA intensities (%) of worm coelomocytes were 32.8–36.2 after *in vitro* exposure to UV in our study, suggesting a comparable genotoxic strength of different genotoxicants.

The nano-Ag treatment in our study had the highest genotoxicity of the three tested Ag forms, while ionic-Ag treatment tended to be the least genotoxic. This may suggest that there are different toxic mechanisms involved in the occurrence of DNA damage or that there are different degrees of reactivity of silver dependent on the Ag forms added. Ag^+ ions have been shown to cause DNA damage by covalently binding with DNA (Hossain and Huq, 2002) and inhibiting DNA synthesis (Hidalgo and Dominguez, 1998) directly. The lower genotoxicity of ionic-Ag in our study may have been due to Ag^+ ions ($AgNO_3$) being complexed by ligands such as chlorides and thiosulphates, which are abundant in sediment. These complexed forms of Ag possibly reduced the amount of free Ag^+ ions entering the nuclei and interacting with DNA strands, as supported by the study of LeBlanc et al. (1984) which demonstrated less toxicity of complexed forms of Ag than free Ag^+ ions to fathead minnows. As for Ag NPs, some studies have reported genotoxicity of Ag NPs to different cell types (Ahamed et al., 2008; Sr et al., 2010) by both direct and indirect mechanisms. The tendency for Ag NPs to accumulate in the nuclei of zebrafish embryos (5–20 nm) and human cancer cells (6–20 nm) has been described, suggesting that they may lead to genomic damage and chromosomal aberrations (Asharani et al., 2008, 2009) directly. On the other hand, it has been suggested that Ag NPs entering cells disrupt the mitochondrial respiratory chain leading to the production of ROS and

the interruption of ATP synthesis, which in turn cause DNA damage (Asharani et al., 2009). Additionally, the potential release of Ag^+ ions from particle surfaces is also a possible mechanism by which Ag NPs exert toxicity to nuclei. Ag NPs may dissolve or degrade under oxidative conditions, and this process is affected by pH, temperature, organic matter and surface coatings (Ho et al., 2010; Liu and Hurt, 2010; Luoma, 2008). Although the Ag NPs used in our study had a PVP coating, it has been demonstrated that the oxidative dissolution of Ag NPs in the presence of PVP can occur (Ho et al., 2010) and the released Ag^+ ions contribute to the NP toxicities (Navarro et al., 2008). However, the genotoxicity of Ag NPs observed in our study cannot be solely attributed to Ag^+ ion release, as a greater genotoxic effect was demonstrated in nano- than in ionic-Ag treatments. Further investigations are needed to determine the kinetics of oxidative dissolution of Ag NPs under sediment exposure scenarios. For micron-sized Ag particles, few studies have reported their genotoxicity, and the mechanisms still remain unclear. The actual size of micron-Ag used in our study covers a wide range from nano- to micron-size, with most particles falling outside of the nanometer range. Hence, although there was an overlap in size distribution between our micron-Ag and nano-Ag, different gentoxicities were observed possibly due to the size effect as the majority of Ag NPs were within the nano size range. The smaller nano-sized particles contained in the micron-Ag may have entered nuclei and interacted with DNA strands. For larger Ag particles contained in the micron-Ag, phagocytosis and encapsulation (large particles are surrounded by large numbers of coelomocytes) by coelomocytes may have facilitated the uptake of bulk micron-Ag, as well as agglomerated nanoparticles, both of which processes may have contributed to the observed genotoxicity. However, cell internalization is not always essential for toxicity, as interactions of particles at the cell surface (even in agglomerated form) may also cause toxicity (Johnston et al., 2010). Additionally, the release of Ag^+ ions could be another contributor to micron-Ag toxicity but we would expect a lower releasing rate and smaller amount of ions due to a smaller fraction of surface atoms and less susceptibility to oxidation due to their higher redox potential compared to NPs (Ho et al., 2010; Pal et al., 1997). Clearly, more work is needed to elucidate the mechanisms by which different forms of Ag (i.e., what metal species are present in the sediment after addition of the three Ag forms, respectively) cause genotoxicity to *N. diversicolor*.

Bioaccumulation of NPs in organisms is another issue which has received relatively little attention. Our bioaccumulation results demonstrated that silver is bioavailable to *N. diversicolor* regardless of the Ag form added, possibly through their ingestion of Ag-associated particles in sediment during feeding, or through body surface contact during burrowing. Ag body burdens were 8.56 ± 6.63, 6.92 ± 5.86 and 9.86 ± 4.94 μg/g dw at the highest exposure concentration for worms in nano-, micron- and ionic-Ag treatments, respectively. These correspond to BAF factors of 0.17, 0.14 and 0.20, respectively, if one assumes the worms were in "steady state" with their exposure media (i.e., sediment). While this assumption undoubtedly is not true, the approximate BAF values do indicate that, while the worms are exposed to and take up Ag; they do not accumulate it to any great degree. Furthermore, it is noteworthy that there were no significant differences in the degree to which *N. diversicolor* accumulated silver among treatments; despite expected differences in reactivity and chemical behavior of the three different forms of Ag and thus the potential uptake routes. In previous field studies, Ag body burdens observed in *N. diversicolor* were 0.1–36.4 μg/g dw when background Ag concentrations in sediment were 0.055–28.4 μg/g dw (Langston and Burt, 1994), and 2.90 ± 8.85 μg/g dw when background Ag concentrations were 1.15 ± 0.11 μg/g dw (Rainbow et al., 2009), respectively. In laboratory studies, Ag concentrations in *N. diversicolor* exposed to 3.4, 11 and 34 μg/L Ag were 2.5 ± 0.9, 3.4 ± 2.1, 5.0 ± 2.1 μg/g dw after 5 days of exposure (Mouneyrac et al., 2003), respectively, indicating similar levels of bioaccumulation (i.e., BAF = 0.15–2.52) as we observed at our highest concentrations. As for Ag NPs, a few studies have reported their bioaccumulation in freshwater fish after water exposure. Choi et al. (2010) demonstrated that Ag concentrations in liver tissue of zebrafish (*D. rerio*) after treatment with 30 and 120 mg/L Ag NPs (2–30 nm in diameter) for 24 h were 0.29 and 2.4 ng Ag/mg liver, respectively. In rainbow trout (*O. mykiss*), different sizes of Ag particles were found to be accumulated differently in tissues after 10 days of water exposure. Ag NPs (10 nm, 100 μg/L) were found to be highly concentrated within both gills (0.61 ± 0.07 μg/g tissue) and liver (1.50 ± 0.30 μg/g tissue), while micron-Ag particles (0.6–1.6 μm, 100 μg/L) were most highly concentrated in the liver (1.63 ± 0.18 μg/g tissue) (Scown et al., 2010). In summary, the existing bioaccumulation data suggest substantial variation in Ag body burden, which is likely not only due to differences in exposure pathways, concentrations and media conditions among studies, but also to differences within and among species.

Generally, *N. diversicolor* is tolerant of heavy metal exposure, especially Cu, Zn and Cd (Bryan and Hummerstone, 1971, 1973; Grant et al., 1989; Hateley et al., 1989; Mouneyrac et al., 2003), and is able to control its body concentration of certain metals by the induction of physiological detoxification mechanisms, involving the formation of metal-containing extracellular granules, mineralized lysosomes, excretion of metals by exocytosis via coelomocytes, and synthesis and turnover of metal binding proteins, such as metallothioneins (MTs) (Bryan and Hummerstone, 1977; Fernandez and Jones, 1989; Mouneyrac et al., 2003; Ng et al., 2008; Poirier et al., 2006). The lower approximate BAF values and the lack of difference in Ag body burden among Ag treatments observed in our study may suggest that detoxification mechanisms are triggered by the presence of Ag in all three treatments in *N. diversicolor* (i.e., regardless of Ag form added) which can have maintained Ag concentrations in the body at a similar and endurable level. However, the specific detoxification mechanism(s) used by *N. diversicolor* for Ag detoxification, whether the mechanisms differ for different Ag forms, how they are triggered, where and in what form Ag will distribute in cells and the extent to which different Ag forms are excreted all still need further investigation.

It is currently considered that different mechanisms are involved in the uptake and toxicity of Ag^+ ions and Ag NPs. Uptake and toxicity of Ag^+ ions is mainly via competition with Na^+ and ATP-dependent transport and disruption of membrane sodium channels, as well as the depletion of the cellular energy system (Bianchini and Wood, 2003; Bury and Wood, 1999; Bury et al., 1999; Galvez and Wood, 2002; Hidalgo and Dominguez, 1998; Pedroso et al., 2007). In contrast, Ag NPs are taken up by cells through diffusion (Lee et al., 2007a,b), pinocytosis (Luoma, 2008) and phagocytosis (Park et al., 2010), as well as via disturbance of membrane components. Once taken up, Ag NPs can lead to toxicities via mechanisms such as ROS-dependant oxidative stress followed by cytotoxicity and genotoxicity, a Trojan-horse type mechanism (Ag NPs act as a carrier to deliver free silver ions into the cell) (Lubick, 2008; Luoma, 2008; Park et al., 2010), as well as synergistic effects of NPs and released Ag^+ ions. However, further studies are needed to elucidate the toxic mechanisms of different Ag forms in aquatic invertebrates using a wider variety of cellular bio-indicators, such as membrane damage, subcellular distribution, reactive oxygen species (ROS) production and anti-oxidative enzyme activities, which have been indicated to be involved in toxicity, at least for Ag NPs, in mammalian cell lines and some aquatic vertebrates. Our results suggest that there should be focus on detoxification mechanisms of different forms of Ag, especially given these known differences in uptake and internal effects.

5. Conclusions

Comet assay results demonstrated that all three Ag forms when added to sediment to which *N. diversicolor* were exposed can cause DNA damage and that, this effect is both Ag form- and concentration-related, with silver added as Ag NPs being most toxic and ionic-Ag being least toxic and also indicating that DNA damage is a sensitive endpoint in *N. diversicolor* exposed to Ag. Different reactivity or mechanisms are possibly involved in the DNA damage caused by the three Ag treatments (forms added), and genotoxicity of Ag NPs cannot be solely attributed to the release of Ag^+ ions. Ag body burden in worms increased with increasing exposure concentration. However, there was no significant difference in body burden among the three Ag forms after 10 days of exposure. Further studies should be directed to characterize Ag NPs in the sediment compartment (i.e., including Ag speciation) and investigate their uptake routes, mechanisms of toxicity and detoxification mechanisms in aquatic invertebrates.

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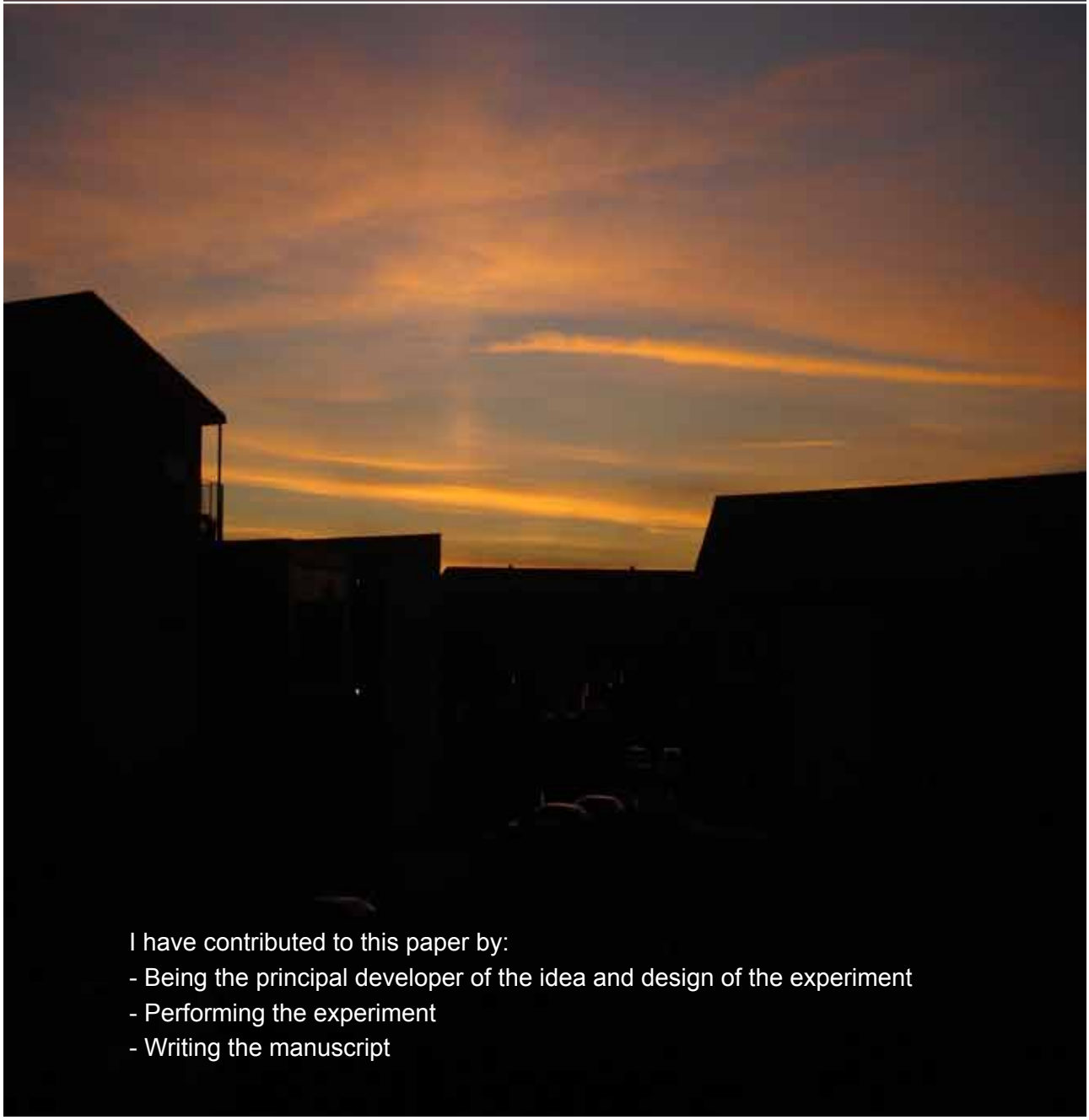
Paper III



I have contributed to this paper by:

- Being the principal developer of the idea and design of the experiment
- Performing the experiment
- Writing the manuscript

Toxic effects and bioaccumulation of sediment-associated silver nanoparticles in the estuarine polychaete, *Nereis (Hediste) diversicolor*

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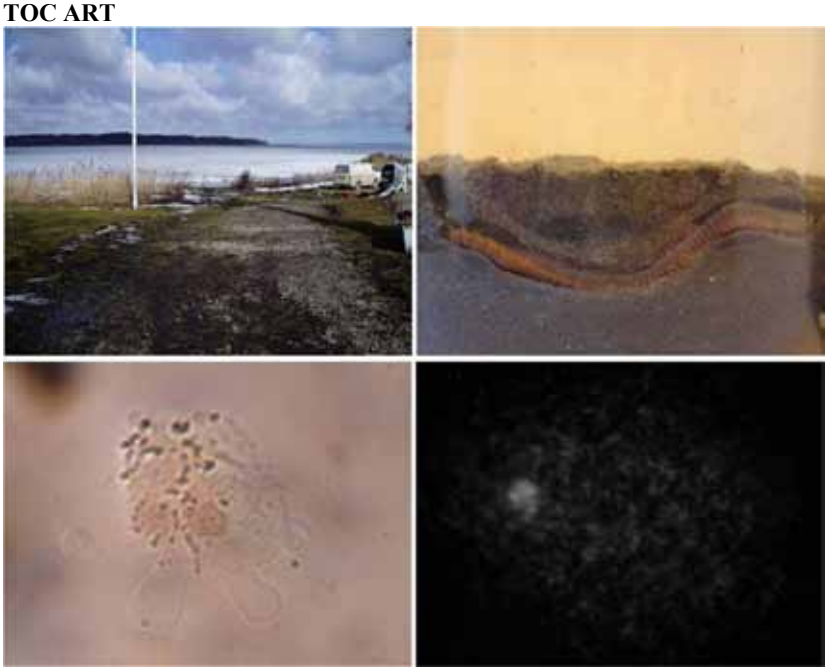

ABSTRACT: In this study, the toxicities of sediment-associated silver added as silver nanoparticles (Ag NPs, 20 and 80 nm) and aqueous Ag ($AgNO_3$) to the estuarine sediment-dwelling polychaete, *Nereis (Hediste) diversicolor*, were investigated after 10 d of exposure, using mortality, burrowing behavior, bioaccumulation, lysosomal membrane stability and DNA damage as endpoints. Both Ag NP types were characterized in parallel to the toxicity studies and found to be polydispersed and overlapping in size. Burrowing time in the Ag NP_{20} treatment significantly increased compared to the aqueous Ag treatment. All worms accumulated Ag regardless of forms added to sediment and worm size (expressed as dry weight) was found to significantly affect bioaccumulation, such that smaller worms accumulated more Ag per body weight than larger worms. Lysosomal membrane permeability (neutral red retention time, NRRT) and DNA damage (comet assay tail moment and tail DNA intensity) of *Nereis* coelomocytes increased in a concentration-dependent manner in all three Ag treatments. Ag NP treatments tended to be more toxic than the aqueous Ag for all tested endpoints except bioaccumulation. No significant difference in cytotoxicity and genotoxicity were observed between the two Ag NP treatments which was attributed to the overlap in particle sizes.

KEYWORDS: Silver nanoparticles, sediment exposure, burrowing behavior, Ag body burden, lysosomal membrane stability, DNA damage

INTRODUCTION

Rapidly expanding growth in the field of nanotechnology has led to the development of numerous applications of nanomaterials in industrial and consumer products. Silver nanoparticles (Ag NPs) are one of the most commonly used nanomaterials due to their enhanced antibacterial properties. They have been widely used in fields such as medical science, water purification, household appliances, bio-sensing, as well as in the food and textile industries.[1,2] These engineered Ag-containing products are likely to release Ag NPs, via wastewater discharge, into the aquatic environment, thus exposing aquatic organisms to metal contaminants.

However, to date, no accepted or well-defined risk assessment methods or test strategies for NP toxicity in aquatic environments exist,[3] which highlights the urgency of aquatic toxicity studies of NPs. Though still limited, an increasing number of studies concerning Ag NP toxicities to aquatic organisms under laboratory conditions have been conducted in recent years.[4] Ag NPs can cause sublethal phenotypic abnormalities of aquatic vertebrate embryos during development[5,6] and have been shown to be cytotoxic and genotoxic to different cell types, with effects such as cell biomembrane damage, lysosomal membrane permeability change, mitochondrial dysfunction and ROS-dependent oxidative stress, DNA damage and chromosomal aberrations, apoptosis and inflammation being observed.[7,8,9,10,11] However, most existing studies of Ag NPs were conducted on vertebrates (particularly freshwater fish or *in vitro* mammalian cell lines), and have also primarily included water exposure pathways, despite the fact that benthic species may be especially at risk as a result of agglomeration and precipitation of Ag NPs from the water phase into sediment.

The ragworm *Nereis (Hediste) diversicolor*, an estuarine, sediment-dwelling polychaete, is known to play a crucial role for the fate of chemicals in estuarine areas as a consequence of its

relative tolerance and influence on metal speciation and aromatic hydrocarbon transformation in sediment through bioturbation including particle mixing and irrigation.[12,13] Furthermore, Rainbow et al. have shown that a significant fraction (20-54 %) of the Ag taken up by *N. diversicolor* was from ingested sediment.[14] It is thus an appropriate test organism for examining the fate, bioavailability and effects of metal NPs in sediment systems.

The purpose of our study was to compare the toxic effects of commercially available silver added to sediment in three forms, namely Ag NP_{20}, Ag NP_{80} and aqueous Ag ($AgNO_3$) to *N. diversicolor*. This study is a continuation of our previous work on the bioavailability and toxicity of different Ag forms to *N. diversicolor*.[15] Mortality, burrowing behavior, bioaccumulation, lysosomal membrane stability (neutral red assay) and DNA damage (comet assay) were used as endpoints. The crystal structure, particle size, shape, hydrodynamic diameter and zeta potential were characterized for both Ag NP types in parallel to toxicity assessment.

EXPERIMENTAL SECTION

Animal Collection and Culturing. *N. diversicolor* adults were collected from Roskilde Fjord near shore (55°40.710'N, 11°59.120'E) during autumn 2010. After transfer to the laboratory, worms were placed in natural sandy sediment (collected from the same site, sieved ≤1 mm) to acclimatize for 2-3 days (15 °C) with aerated natural seawater (from the same site, 15 ‰ salinity). The natural seawater was replaced with filtered natural seawater (<0.2 µm, 15 ‰) to acclimatize worms for another two weeks. One day before exposure, all worms were carefully picked out of the sediment and placed in clean filtered seawater to depurate their guts overnight. During the acclimation and exposure periods, worms were fed natural sieved sediment without additional food supply.

Characterization of Ag NPs. Ag NP_{20} and Ag NP_{80} (stock # 0478HW and 0476HW, 99.9 %, w/~0.3 % PVP coated) were purchased from NanoAmor (Houston, TX, USA). The identification of crystal structure, particle size, hydrodynamic diameter and zeta potential of both particle types was performed as described in Cong et al.[15] Briefly, the crystal structure of Ag NPs was determined on the powder using X-ray diffraction (XRD). The particle size, hydrodynamic diameter (suspended particle size) and zeta potential (particle surface charge, an indication of suspension stability) were carried out on a stock Ag NP suspension which was prepared in deionized water (18.2Ω, Millipore) by sonication, using a Hitachi H-7100 transmission electron microscope (TEM, operating at 100 kV) and a Zetasizer Nano ZS (Malvern Zetasizer Nano ZS, Malvern, UK), respectively.

Sediment Preparation. Sandy sediment for all treatments was collected from Roskilde Fjord (Denmark) at the same site as worms. The top few centimeters of the sediment surface were scraped off and sieved to ≤1 mm in the field with natural seawater (15 ‰, pH≈8.0). After transferring to the laboratory, the sediment was rinsed with filtered natural seawater and the background Ag concentration was measured using graphite furnace atomic absorption spectrometry (GFAAS, GTA 120, Varian, Australia) as described in Cong et al.[15] Briefly, the sediment samples were lyophilized (Christ Alpha 1-2, Osterode, Germany) overnight at -50 °C followed by digestion with 65 % HNO_3, neutralization with 25 % ammonium solution and filtration before GFAAS measurement.

The stock sediment (nominal concentration of 200 µg Ag/g dw) and Ag exposure treatments (nominal concentrations of 5, 10, 25, 50, 100 µg Ag/g dw sed.) were prepared following the procedure in Cong et al.[15] Briefly, the stock sediment was obtained by mixing clean sediment with stock Ag suspension/solution. Different Ag exposure treatments were prepared by

homogeneously mixing clean sediment with the stock sediment according to different dilution factors. In addition, PVP-controls (0.3 µg PVP/g dw sed.) were prepared by homogeneously mixing a solution of PVP into sediment in the same way as for the Ag treatments. The PVP concentration corresponded to that in the highest Ag NP exposure group (100 µg Ag/g dw sed.) and was used to determine whether there was a toxic effect of the PVP coating alone.

Experimental Setup. All experimental plastic beakers were acid washed (20 % HNO_3) and rinsed once with seawater before use. Following overnight depuration in clean filtered natural seawater, individual worms were transferred to beakers (one worm per beaker) containing around 320 g wet sediment of the appropriate Ag concentration and form and 600 ml filtered natural seawater (the beakers were kept still for six hours to allow the settling of suspended sediment before adding the worms). Six Ag concentrations were tested for each Ag form with five replicate worms in a randomized block design. In detail, each replicate block which included all three Ag treatments was set up on the same day of the week over the course of one month. Sediment exposure was conducted in the dark at 15 ºC for 10 d. The overlying seawater was aerated and changed once during the 10 d of exposure to refresh the water and remove any metabolic waste products. Beakers were inspected daily and air supply adjusted if necessary. Replicate blocks were stopped in the same order that they were set up after the exposure time of 10 d. Mortality was recorded in all treatments. In the end, 4-5 worms for each Ag concentration were used for each of the tested endpoints.

Burrowing Tests. The burrowing behavior of *N. diversicolor* was investigated according to Bonnard et al.,[16] with some modifications. After 10 d of sediment exposure, worms were carefully picked out and placed individually in beakers filled with 5 cm clean natural sandy sediment (≤1 mm) and 100 mL natural filtered seawater. The positions of worms during

burrowing were recorded every 2 min during 30 min and scored as follows: 1: organism was totally un-burrowed; 3⁄4, 1⁄2, 1⁄4: organism was partially burrowed to various degrees (from least burrowed to mostly burrowed); 0: organism was totally burrowed.

Neutral Red Assay. The neutral red assay was conducted to measure lysosomal membrane stability after Ag exposure based on the protocol of Weeks and Svendsen,[17] with some modifications. In addition, PVP-controls were used to observe if there was a cytotoxic effect from the PVP coating alone. After the burrowing test, worms were carefully picked out of the sediment and allowed to purge their gut contents in natural filtered seawater overnight. Afterwards, a neutral red (toluene red, $C_{15}H_{17}N_4Cl$, cat.# N7005, Sigma-Aldrich, Steinheim, Germany) stock solution was freshly prepared by dissolving 20 mg neutral red powder in 1 mL of dimethyl sulfoxide (DMSO, Merck, Darmstadt, Germany). The neutral red working solution was obtained by dissolving 10 µL of stock solution in 2.5 mL of worm physiological solution (452.4 mM NaCl, 10.8 mM KCl, 58 mM $MgCl_2{\cdot}6H_2O$, 30.25 mM Na_2SO_4, 11.2 mM $CaCl_2{\cdot}2H_2O$, pH 7.8, adapted for marine annelids),[18] which was also freshly prepared and changed every hour. Afterwards, 30-50 µL coelomic fluid of *N. diversicolor* was gently extracted into physiological solution for each assay by carefully inserting a 1 ml syringe fitted with a 0.4 mm × 20 mm needle (chilled prior to use) into the posterior region of the worm (avoiding the gut). Afterwards, 20 µL of this mixture was placed on a slide where it was mixed with an equal volume of neutral red working solution and covered with a cover slip.

The number of coelomocytes with fully stained cytosol (i.e., exhibiting dye leakage from lysosomes to the cytosol) and the number of coelomocytes with unstained cytosol (no leakage) were counted under light microscopy (Laborlux S, Leitz, Portugal) with 100× magnification, and the ratio of the two cell types was determined. For each slide the ratio was assessed during 2 min

of counting every 4-10 min. The observation was stopped when the ratio of cells with fully stained cytosol was greater than 50 % of the total number of cells or the observation time was more than 50 min. This interval was recorded as the neutral red retention time (NRRT).

Comet Assay Procedure. The comet assay procedure was performed according to Cong et al.[15] Briefly, coelomic fluid of *N. diversicolor* was gently extracted, mixed with agarose and place on a fully frosted slide. The slides used as positive controls were exposed to UV light from a Ren UV C lamp (253.7 nm, 15 W, Sylvania, Japan) for 20 s. Afterwards, all slides were lysed, electrophoresed and neutralized before staining with ethidium bromide. After staining, the slides were examined under a fluorescence microscope (50× oil immersion objective, Dialux 22EB, Leica, Wetzlar, Germany) with 625× magnification using software from Kinetic Imaging (Comet assay III). The levels of DNA damage were measured as tail moment and tail DNA intensity (%).

Bioaccumulation. After the above assays, the worms were frozen at -20 ºC until use. The Ag concentrations in worm tissues were determined using GFAAS. Before digestion of worm tissues, all worms were lyophilized overnight at -50 ºC. Afterwards, each worm was ground into powder using a mortar and pestle. The resulting powder was digested and analyzed on GFAAS as described for sediments. We confirmed the validity of our calibration and analysis of tissue metal concentration by analyzing with a certified biological material (lobster hepatopancreas, LUTS-1, NRC Canada) with a known Ag concentration.

Statistical Analyses. Measured sediment concentrations of Ag at experimental start were analyzed by one-way analysis of variance (One-way ANOVA) to test for differences among Ag forms. For burrowing time, lysosomal membrane stability and DNA damage, data from exposure treatments (5, 10, 25, 50 and 100 µg Ag/g dw sed.) were analyzed using two-way ANOVA to assess and compare the effects of Ag form and concentration on tested endpoints. For Ag

bioaccumulation, data from exposure treatments (5, 10, 25, 50 and 100 µg Ag/g dw sed.) were analyzed using analysis of covariance (ANCOVA) with worm size (expressed as dry weight) as the covariate. Differences in average worm size among treatments were analyzed by two-way ANOVA with Ag form and concentration as factors. Due to the weekly sampling of worms for the above assays (i.e., one replicate per week), replicate was taken into consideration as a blocking factor during ANOVA and ANCOVA analyses to reduce variation in response parameters related to weekly variations. One-way ANOVA followed by planned comparisons with a Bonferroni correction were used to assess the effects of exposure concentration, PVP controls and positive controls (one PVP or positive control in each replicate for all three Ag forms) compared to the controls (0 µg Ag/g dw sed., one in each replicate for all three Ag forms) for all experimental endpoints in three Ag treatments together or in each Ag treatment. Differences were considered statistically significant at $p \leq 0.05$ and marginally significant at $0.1 \geq p > 0.05$. All experimental data were tested for homogeneity and normality through examination of residuals resulting from the above statistical analyses. In all cases, data were log-transformed to achieve acceptable homogeneity and normality. All statistical analyses were performed using SYSTAT 13.0 software (Chicago, IL, USA).

RESULTS

Characterization of Ag NPs. Both samples of Ag NPs were identified as silver (ICDD 4-783) with very similar crystal structure patterns based on powder XRD analysis (Figure S1, supporting information, SI). No other material was detected. The presence of highly crystalline material was observed (Figure S1, sharp peaks), suggesting the presence of large Ag particles in the primary material. A peak broadening was not observed between the Ag NP_{20} and Ag NP_{80} suggesting both samples were well crystallized and of a similar degree of crystallinity despite

their nominal difference in size. TEM images for Ag NP_{20} and Ag NP_{80} were also similar, with polyhedral shaped particles (Figure 1A, E) often observed. In addition, complex particles displaying branching structures were commonly found in the Ag NP_{20} sample (Figure 1C), which were probably produced from the aggregation of smaller particles by collision upon sonication (such particles were not found when the powder was suspended with a shorter sonication time of 5 min). The most common particle size for the Ag NP_{20} sample was approximately 50 nm (Figure 1A) but these could be particles that fused during treatment. Smaller sized particles of ~10 nm were also found in the Ag NP_{20} sample (Figure 1B), which might represent the primary components of the larger particles; these smaller particles could have formed agglomerates as the suspension droplet was dried on the TEM grid. For the Ag NP_{80} sample, actual particle size ranged from approximately 2 nm to 100 nm with most particle sizes also being ~50 nm (Figure 1D-F). The average hydrodynamic diameters, characterized by DLS and expressed in intensity, of the two samples in stock suspension prepared by sonication were 187±4 nm and 144±2 nm for Ag NP_{20} and Ag NP_{80} (Figure S2A, SI), respectively, suggesting a stable dispersion of the particles but much larger size than expected. The obtained suspension for both Ag NPs was visually very stable (assessed by degree of turbidity), and this was confirmed by zeta potential values of -39.4±0.5 mV and -42.3±0.3 mV for Ag NP_{20} and Ag NP_{80} (Figure S2B, SI), respectively.

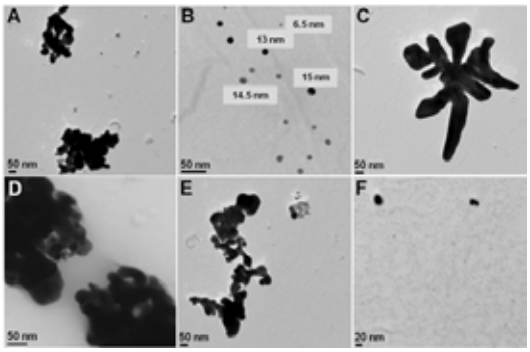

Figure 1. TEM images of (A-C) Ag NP_{20} and (D-F) Ag NP_{80}. Both Ag NP_{20} (A) and Ag NP_{80} (E) demonstrated a polyhedral shape. Complex branching shaped particles (C) were also found in the Ag NP_{20} sample. The major particle size of both Ag NP samples was approximately 50 nm (A and E). Some smaller sizes of ~10 nm (B) and 2-100 nm (D and F) were also found in Ag NP_{20} and Ag NP_{80} samples, respectively.

Sediment Properties. The dw/ww ratio of natural sandy sediment was 0.77 (n=6), the organic matter content (OMC) was 0.68 % (n=6) and the background Ag concentration was 0.027 µg Ag/g dw sed. (n=3). The mean measured concentrations of stock sediment (n=3) were 162.3±12.5, 164.2±15.3 and 171.0±6.2 µg/g dw for Ag NP_{20}, Ag NP_{80} and aqueous Ag, respectively. Measured sediment exposure concentrations of the three Ag forms were close to the nominal concentrations (Table 1) and we established a concentration range spanning two orders of magnitude. There were no significant differences in measured sediment Ag concentrations among the three forms (p>0.1).

Table 1. Measured (n=3) vs. nominal Ag concentrations (0, 5, 10, 25, 50, 100 µg Ag/g dw sed.) in sediment of three treatments at experimental start.

Form	Measured Concentration (µg/g dw) (SD)					
Ag NP_{20}		4.34 (0.29)	9.57 (1.88)	27.07 (2.77)	52.51 (6.71)	99.83 (6.16)
Ag NP_{80}	0.027 (0.01)	6.27 (0.28)	11.40 (0.29)	21.61 (1.10)	47.57 (5.84)	95.18 (3.85)
Aqueous Ag		5.49 (2.70)	10.98 (0.69)	23.34 (1.60)	47.52 (3.12)	93.18 (4.03)

Mortality. Six worms (out of a total of 105) were found dead after 10 d of sediment exposure, but there was no concentration-dependent mortality suggesting a low background level (6 %) of mortality.

Burrowing Behavior. All but 2 worms (one in each of Ag NP_{80}-10 and aqueous Ag-50) completely burrowed into the clean sediment (category 0) within 30 min upon transfer from the contaminated sediment. The lack of burrowing for these two worms was not related to Ag form or concentration indicating that they were probably in bad condition and they were therefore removed from further analysis. The time that individual worms took to completely burrow into clean sediment is shown in Figure 2. The burrowing time tended to increase in the two Ag NP treatments while it had comparable values in the aqueous Ag treatment compared to the control. Statistical analyses showed that Ag form and replicate had significant effects on burrowing time and concentration had a marginally significant effect. However, there was no interaction between Ag form and concentration on burrowing time (Table 2). Nor was there a significant effect of PVP on worm burrowing behavior compared to the control (0 µg Ag/g dw sed.) (p>0.1) (Figure 2).

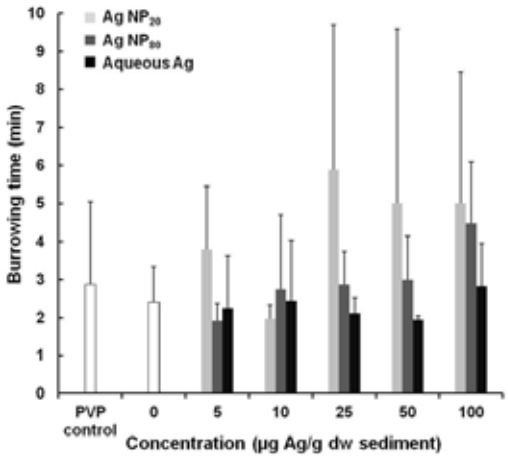

Figure 2. Time for *N. diversicolor* to completely burrow in clean natural sediment after 10 d of exposure to sediment-associated Ag NP_{20}, Ag NP_{80} and aqueous Ag ($AgNO_3$).

Table 2. Results of statistical tests. Effects of Ag form, concentration, Ag form*concentration and replicate (blocking factor) on burrowing time, NRRT and DNA damage analyzed by two-way ANOVA. Bioaccumulation was analyzed by two-way ANCOVA with replicate as a blocking factor and worm size (dw) as the covariate.

	P-Value				
Endpoints	Ag form	Concentration	Ag form*Concentration	Replicate	Worm size
Burrowing time	0.004	0.057	0.650	0.005	-
Bioaccumulation	0.724	0.021	0.939	0.322	<0.001
NRRT	0.017	<0.001	0.127	<0.001	-
Tail moment	0.033	<0.001	0.550	<0.001	-
Tail DNA intensity	0.040	<0.001	0.662	<0.001	-

Ag Body Burden. Silver was bioavailable in all three Ag forms and was accumulated by *N. diversicolor*. Ag body burden increased significantly as a function of exposure concentration but also was affected by worm size (Table 2, Figure 3). Worm sizes differed significantly (p<0.001, data not shown) among exposure concentrations making this bioaccumulation pattern less clear. Notably, worms used for 25 and 100 µg Ag/g dw sed. exposures were significantly larger (Figure S4, SI), with concomitant lower bioaccumulation, than those used at the other exposure levels. No significant difference in Ag body burden among the three Ag forms or replicates was observed, nor was there an interaction between Ag form and concentration on bioaccumulation (Table 2). Worms exposed to the PVP control had similar low Ag body burden levels as the worms in control sediments (p>0.1) (data not shown). The body burden for worms exposed to concentrations of 100 µg Ag/g dw sed. were significantly higher than the control worms for all three Ag forms (Figure 3).

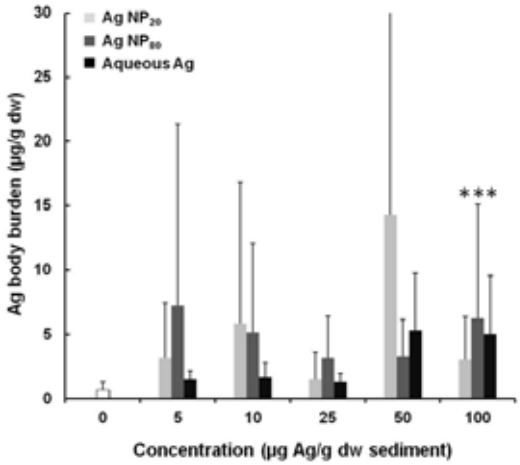

Figure 3. Ag body burden in *N. diversicolor* after 10 d of exposure to sediment-associated Ag NP_{20}, Ag NP_{80} and aqueous Ag ($AgNO_3$). *** represent significant differences in body burdens of Ag (p<0.001) for exposure concentrations compared to the control (0 µg Ag/g dw sed.) for all three Ag treatments. Note that there was no significant difference among Ag forms (Table 2); therefore the test of concentration effects was for all three forms together.

Lysosomal Membrane Stability. The control groups had an average NRRT of around 35 min. The NRRT showed a general decrease with increasing Ag concentration, indicating increased lysosomal membrane permeability (Figure S3, SI). Form, concentration and replicate significantly affected NRRT with the Ag NP_{20} treated groups having significantly lower (p<0.05) NRRT than the aqueous Ag. The NRRT significantly decreased compared to the control at concentrations of 25, 50 and 100 µg Ag/g dw sed. for Ag NP_{20} and aqueous Ag and at 50 and 100 µg Ag/g dw sed. for Ag NP_{80}, respectively (Figure 4). No interaction between Ag form and concentration on NRRT was observed (Table 2). PVP had no significant effect on NRRT compared to the control (p>0.1) (Figure 4).

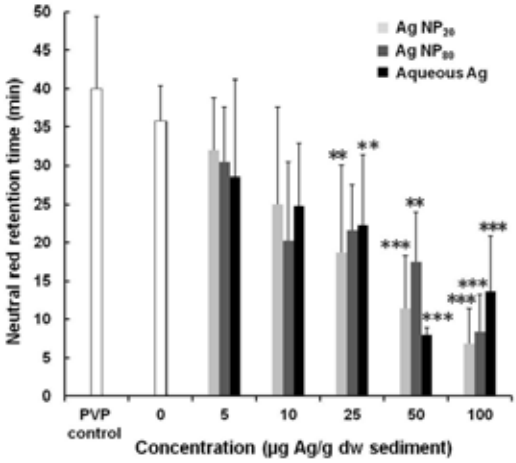

Figure 4. Neutral red retention time (NRRT) of *N. diversicolor* coelomocytes after 10 d of exposure to sediment-associated Ag NP_{20}, Ag NP_{80} and aqueous Ag ($AgNO_3$). ** and *** represent significant differences in NRRT (p<0.01 and p<0.001, respectively) for exposure concentrations compared to the control (0 µg Ag/g dw sed.) for each Ag form.

DNA Damage. Similar to NRRT, Ag form, concentration and replicate significantly affected tail moment and tail DNA intensity (%). There was no interaction between Ag form and concentration for either tail moment or tail DNA intensity (Table 2). Both parameters increased in a concentration-dependant manner with increasing exposure concentration (Figure 5). The Ag NP_{80} had significantly greater effects on tail moment (p<0.05) and tail DNA intensity (%) (p=0.05) compared to the aqueous Ag. In addition, Ag NP_{20} had a marginally significant effect on tail DNA intensity (%) (p=0.097) compared to the aqueous Ag. Tail moment was significantly higher at concentrations of 25, 50 and 100 µg Ag/g dw sed. for Ag NP_{80}, at 50 and 100 µg Ag/g dw sed. for Ag NP_{20} and at 100 µg Ag/g dw sed. for aqueous-Ag compared to the control (Figure 5A). Similarly, tail DNA intensity (%) was significantly higher at concentrations of 50 and 100 µg Ag/g dw sed. for Ag NP_{20} and Ag NP_{80} and at 100 µg Ag/g dw sed. for aqueous Ag compared

to the control (Figure 5B). There was no significant effect of PVP on either parameter compared to the control (p>0.1). The positive controls had significantly higher tail moment and tail DNA intensity compared to the control as expected (Figure 5).

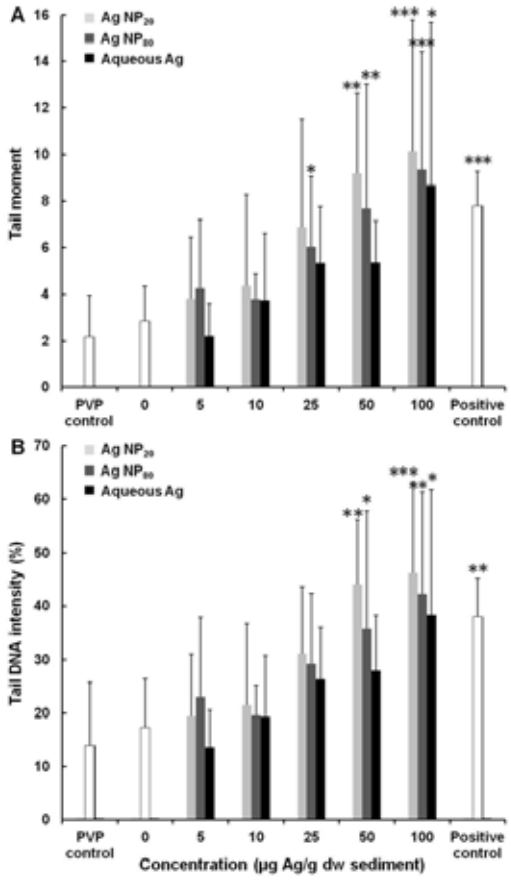

Figure 5. DNA damage of *N. diversicolor* coelomocytes after 10 d of exposure to sediment-associated Ag NP_{20}, Ag NP_{80} and aqueous Ag ($AgNO_3$). (A) Tail moment, defined as the product of the tail length and the fraction of total DNA in the tail. (B) Tail DNA intensity (%), expressed as the DNA intensity of the tail compared with the intensity of the whole comet (cell). *, ** and *** represent significantly different effects (p<0.05, p<0.01 and p<0.001, respectively) for exposure concentrations compared to the control (0 µg Ag/g dw sed.) for each Ag form.

DISCUSSION

Ag Characterization. There is a growing consensus on the necessity of proper and accurate characterization of NPs in environmental media and biological systems, or at the very least in the stock suspensions used to prepare exposure treatments, to ensure that reliable and reproducible toxicity tests are performed.[19]

We characterized commercially available Ag NPs and found a clear difference between the manufacturer's product specification (20 and 80 nm, respectively) and what we measured for the two Ag NP samples (both were polydispersed with wide nano-size ranges). In addition, there was no noticeable difference in the size distribution between the two Ag NP forms. This difference in size between that reported by the manufacturer and that measured in the laboratory was also observed by Scown et al. and by Cong et al.[15,20] The reasons for the differences may be related to the industrial production process, which often results in polydispersion, or to changes in material properties between synthesis and initial characterization and the particular conditions when utilized (e.g., storage time and conditions, pH, ionic strength, temperature and external force). Both problems highlight the importance of fully characterizing commercially obtained NPs before performing toxicity tests or other experiments. Ideally, characterization of NPs should be performed under conditions as close as possible to the relevant exposure medium (e.g., sediment in our case). Unfortunately, we know little about the state of sediment-associated Ag NPs due to the lack of available methods for characterizing NPs in such complex media. However, Ag NPs are likely to undergo size or surface chemistry changes when they are transferred between media during experiments, such as from dispersion media (deionized water) to test media (wet sediment). Such changes may alter bioavailability or toxicity in ways that are not entirely understood. Therefore, there is a pressing need for the development of techniques that permit

examination of the state and behavior of NPs in complex compartments, such as sediment, so that a better understanding and interpretation of the effects of NPs on organisms can be achieved. Based on our present analysis, we assume that there were no major differences in the two Ag NP forms used in this study and that they covered a wide range. Still, we assume that some fraction of the Ag particles to which worms were exposed for both Ag NP treatments possessed the unique characteristics associated with nanoparticle size ranges.

Burrowing Behavior. *N. diversicolor* is a sediment-dwelling polychaete that lives in U-shaped or J-shaped burrows in coastal and estuarine areas.[21] Burrowing activity is a commonly used indicator of invertebrate behavior response to sediment toxicity.[22] In this study, we use burrowing time to assess burrowing behavior of worms after sediment Ag exposure. We recognize that the use of burrowing time with 4-5 replicates per treatment was probably not as sensitive as the commonly used 'percent of un-burrowed organisms', which typically uses 20 replicates,[16] and a large variability was observed in our test. However, we observed an increasing tendency of burrowing time in Ag NP treated worms with increasing exposure concentration, especially in the Ag NP_{20} group, the time of which was significantly longer than the aqueous Ag, indicating the potential greater effects of Ag NP exposure on worm burrowing activity than the aqueous Ag. Buffet et al. investigated the burrowing behavior of *N. diversicolor* after 7 d seawater exposure to 10 μg/L aqueous Cu (as $CuNO_3$) and CuO NPs and found that only exposure to aqueous Cu led to a significant decrease of burrowing kinetics.[23] The contradictory burrowing responses with our study were likely due to the different metal NPs used and exposure routes applied. As in seawater, CuO NP tended to agglomerate and precipitate, which process reduced the effective concentration worms exposed to. The reduced burrowing speed in Ag NP treatments in present study indicated that worm mobility was impaired at these concentrations.

Ag Bioaccumulation. Although some studies have demonstrated the uptake and localization of Ag NPs inside cells, few of these have quantified the amount of Ag accumulated in whole organisms over time. Our bioaccumulation results demonstrated that Ag was bioavailable to *N. diversicolor* to similar degrees regardless of the form in which it was added to sediment. The lack of difference in Ag body burden among Ag treatments, despite detected differences in cellular indicators, is consistent with our previous study which also showed no difference in bioaccumulation between Ag NPs and aqueous Ag in this species.[15] These results are also in agreement with García-Alonso et al., in which the calculated total Ag body burden of *N. diversicolor* after 10 d of exposure to 250 ng/g dw sediment spiked with Ag NPs (30±5 nm) and aqueous Ag ($AgNO_3$) were similar, being 218.7±14.8 and 226.7±11.2 ng Ag/g fresh weight, respectively.[24]

In addition, we found that worm size significantly affect Ag body burden in the present study with smaller worms accumulating more Ag per body weight than larger worms (Table 2, Figure 7A). The highest Ag body burdens were observed at different concentrations for the different Ag forms, but not in any systematic way. The lack of a monotonic bioaccumulation with increasing exposure concentration was surprising given we observed monotonic relationships in our previous bioaccumulation study[15] and with the other endpoints (NRRT and DNA damage) in the present study. This was likely in part because of the wider size range and larger sizes of worms used in different exposure concentrations for each Ag form in this study. In our previous study, all worms had a similar average size of around 0.03 g dw (corresponding to the smallest size we used in the present study).[15] As a comparison, the largest sizes in this study (0.08-0.1 g dw) were found in the 25 and 100 μg Ag/g dw sed. exposure groups and had corresponding Ag body burdens of 1.3-3.1 and 3.0-6.2 μg/g dw, respectively, which were much lower than the

accumulation by the smaller worms in the previous study (3.6-7.0 and 6.9-9.9 μg/g dw in 25 and 50 μg Ag/g dw sed. groups, respectively). Despite efforts to allocate similar sizes of worms to forms, the significant differences among concentrations influenced Ag uptake and bioaccumulation. We also corrected for worm size by using the covariate effect (i.e., the regression equation in Figure 6A) to normalize Ag body burden and found that the monotonic relationship (Figure 6B) was more apparent than before (Figure 3), especially in the aqueous Ag group. Smaller worms have a higher surface area to volume ratio and a higher metabolic rate than larger worms. As demonstrated by Heip and Herman,[25] the weight-specific growth rate per day of *N. diversicolor* decreased from 0.0415 to 0.0008 when the length of worms increased from 0.25 cm to 7.25 cm (corresponding to ~0.001-0.1 g dw). This could indicate that smaller worms eat more per body weight and as a result, are more susceptible to accumulate Ag than larger worms. If we assume that worms were in "steady state" with their exposure media (i.e., sediment), the corresponding BAF factors would be 0.03-0.63, 0.06-1.45 and 0.05-0.30 for Ag $NP_{20,}$ Ag NP_{80} and aqueous Ag treatments, respectively, which are lower than published field and laboratory studies with calculated BAF values (also assuming that worms were in "steady state") ranging from 0.15 to 2.52.[13,26,27] In addition to the difference of their geographic origin and relative metal tolerance, the lower BAF values which are from 25 and 100 μg Ag/g dw sed. groups indicated that larger sizes of worms have a lower ability to accumulate Ag than smaller worms (per body weight). Similarly, it may take larger worms longer time to achieve a given level (i.e., "steady state") than smaller worms given their smaller surface to volume ratio and weight specific activities. All of these factors and the variability in bioaccumulation data highlight the importance of correcting for or minimizing body size differences in toxicological studies. However, after accounting for differences in worm size, bioaccumulation in this study

was still variable which made it difficult to detect differences in the bioavailability or uptake of the different forms of Ag. Future bioaccumulation studies should aim to use more replicates or 'pooled' individuals as one replicate to reduce biological variations for this species.

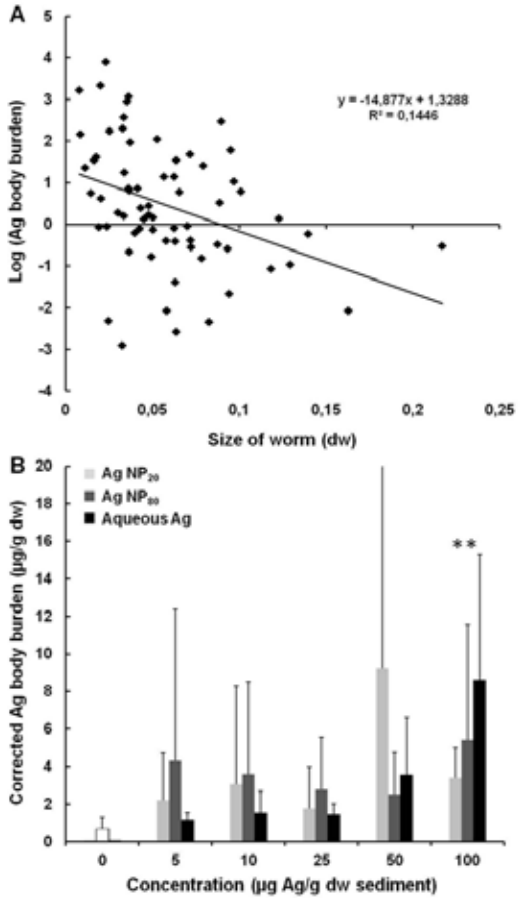

Figure 6. (A) Linear relationship between worm size (expressed as dw) and log Ag body burden in *N. diversicolor*. (B) Corrected Ag body burden in *N. diversicolor* according to the regression equation of (A) after 10 d exposure to sediment-associated Ag NP_{20}, Ag NP_{80} and aqueous Ag ($AgNO_3$). ** represent significant differences in body burdens of Ag (p<0.01) for exposure concentrations compared to the control (0 µg Ag/g dw sed.) in all three Ag treatments together.

Cytotoxicity and Genotoxicity. Lysosomes have been identified as a particular target site for toxic effects and play an important role in metabolism of various heavy metals.[28] Decreased lysosomal membrane stability (or increased permeability) is thought to be a general measure of stress. The mechanism causing this alteration in membrane stability may involve direct effects of chemicals on the membrane or an increased frequency of secondary lysosomes in toxicant-stressed cells.[17,29]

The concentration-dependant reduction in NRRTs observed in the coelomocytes of *N. diversicolor* in our study indicated stress resulting from exposure to Ag in sediment. Dose-dependent lysosomal destabilization was also observed in adult oysters (*Crassostrea virginica*) when they were exposed to Ag NP (15±6 nm) concentrations of 0.016 to 16 µg/L for 48 h.[9] Our study confirms that lysosomes of worm coelomocytes are a subcellular target for the action of both particulate and aqueous Ag. This is consistent with published results showing that Ag can deposit in lysosomes. For instance, aqueous Ag is known to be accumulated as non-toxic deposits of silver-sulphur granules in lysosomes.[30,31] The specific transport system has been characterized by Havelaar et al.[32] and involves a heavy metal ion transport protein in the lysosomal membrane which facilitates the uptake of silver through a typical carrier-mediated process. Recently, García-Alonso et al. have characterized cellular internalization of Ag NPs (30±5 nm) in gut epithelia of *N. diversicolor*, and found that clusters of electron dense particles resembling Ag nanoparticles deposited in the lysosomes.[24] Furthermore, TEM images in their study showed that the lysosomal membrane seemed to break down after 10 d of sediment exposure to 250 ng Ag/g dw sed. in *N. diversicolor*. The uptake of Ag NPs into lysosomes has also been observed in human cell lines,[33,34] where uptake was suggested to be through clathrin-dependent endocytosis and macropinocytosis.[34]

Comet assay results demonstrated that Ag caused DNA damage in *N. diversicolor* coelomocytes regardless of the Ag form added to the sediment, which is consistent with existing studies of the genotoxicity of Ag particles/ions in different cell systems.[10,33,35,36] Similar to our previous DNA damage data,[15] both tail moment (2.8) and tail DNA intensity (17.3 %) results of controls (0 µg/g dw) demonstrated similar values as those reported by Asharani et al. (tail moment of ~0.5 to 2.5 in human lung fibroblast cells and glioblastoma cells) and Lewis and Galloway (% DNA in the tail of ~13 % in *N. diversicolor* coelomocytes), respectively.[33,37] Worms in this study exposed to 5, 10, 25 and 50 µg Ag/g dw sed. had comparable results with our previous DNA study for both parameters.[15] In addition, tail moments in 25, 50 and 100 µg Ag/g dw sed. Ag NP exposure groups after 10 d of sediment exposure were also comparable to those of human glioblastoma cells after 48 h exposure to 25, 50 and 100 µg/mL Ag NPs (6-20 nm in size).[33]

The potential mechanisms leading to genotoxicity by Ag NPs have been discussed previously, and likely include binding with DNA strands directly, or ROS-induced damage and Ag^+ ion release.[15] Aqueous Ag (as Ag^+ ions) has been shown to cause DNA damage by covalently binding with DNA[38] and inhibiting DNA synthesis directly.[36] In reality, most of the aqueous Ag is not present as free Ag^+ ions after addition into marine sediment, due to speciation and complexation with ligands, e.g., chloride and sulfide. However, despite silver speciation or complexation in marine environments silver still maintains substantial bioavailability and toxicity.[39] Ho et al. demonstrated that the oxidative dissolution of Ag NPs in the presence of PVP can occur (the Ag NPs used in our study had a w/~0.3 % PVP coating).[40] Although we did not measure Ag^+ ions released from Ag NPs during sediment exposure in our study, our results suggested that the cytotoxicity and genotoxicity of Ag NPs were not solely attributed to Ag^+ ion release (if any), as we observed a trend of greater effect in Ag NP treatments compared to the

aqueous Ag treatment. Similarly, our previous study also found a significantly greater genotoxicity of Ag NPs (<100 nm) compared to aqueous Ag ($AgNO_3$),[15] which suggests that either different mechanisms may be involved between Ag NP and aqueous Ag toxicity or that there are different degrees of reactivity of the different Ag forms in causing DNA damage. Furthermore, García-Alonso et al. demonstrated separate routes of cellular internalization and differing *in vivo* fates of Ag delivered in NP and dissolved forms, and found significantly larger fractions of Ag NPs existing in the cell organelles of *N. diversicolor* than aqueous Ag, suggesting potentially higher cellular toxicities of Ag NPs than aqueous Ag.[24] In addition to the above mentioned possible mechanisms, Ag NPs may also destroy the membrane integrity of cellular organelles, such as lysosomes (which are involved in metal detoxification) and then cause genotoxicity. Also, Ag NPs may provide an internal source of Ag^+ ions in a Trojan-horse mechanism[2] that differs in bioavailability to externally available ionic or soluble Ag. However, García-Alonso et al. raised the possibility that dissolution of the Ag NPs, either externally or internally, was not important over a time scale of 10 d of sediment exposure in *N. diversicolor,* as metallothionein-like proteins (MTLPs) were found associated with aqueous Ag but were absent in worms after Ag NP treatment.[24] Clearly, further investigations are needed to determine the kinetics of oxidative dissolution of Ag NPs under sediment exposure (either externally or internally) and their mechanisms to cause DNA damage.

Overall, there is a pressing need for the development of methods that allow adequate characterization of NPs in complex compartments, such as sediment. The reduced burrowing speed after Ag NP treatments indicated the impairment of worm mobility at the exposure concentrations used. Ag was bioavailable and accumulated in worms regardless of the form added, but there was no form-related difference in Ag body burden after 10 days of exposure.

Size of worms was found to affect the bioaccumulation of Ag; smaller worms accumulated more Ag per body weight than larger worms possibly due to their higher surface to volume ratio and higher metabolic activity. We confirmed that lysosomes were a subcellular target for either Ag NP or aqueous Ag in worms. Lysosomal permeability and DNA damage of *Nereis* coelomocytes showed a concentration-dependant increase with increasing exposure concentration. Furthermore, Ag NP treatments tended to be more toxic than aqueous Ag for all tested endpoints even though levels of bioaccumulation were similar. The lack of difference between the two NP forms of Ag was attributed to their similar sizes as indicated by TEM and DLS characterization. Although we might expect nanosilver to become highly aggregated upon contact with sediment, and therefore to lose its tendency to behave differently than its chemically identical counterparts, our preliminary results suggest that this is not necessarily the case. Such enhanced nano-size specific effects warrant further investigation and attention.

ACKNOWLEDGEMENTS

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SUPPORTING INFORMATION AVAILABLE

Additional information about X-ray diffraction pattern, hydrodynamic size and zeta potential distributions of Ag NP_{20} and Ag NP_{80}, coelomocytes of *N. diversicolor* with increasing lysosomal membrane permeability, as well as average sizes of *N. diversicolor* in three Ag treatments is available free of charge via the Internet at http://pubs.acs.org.

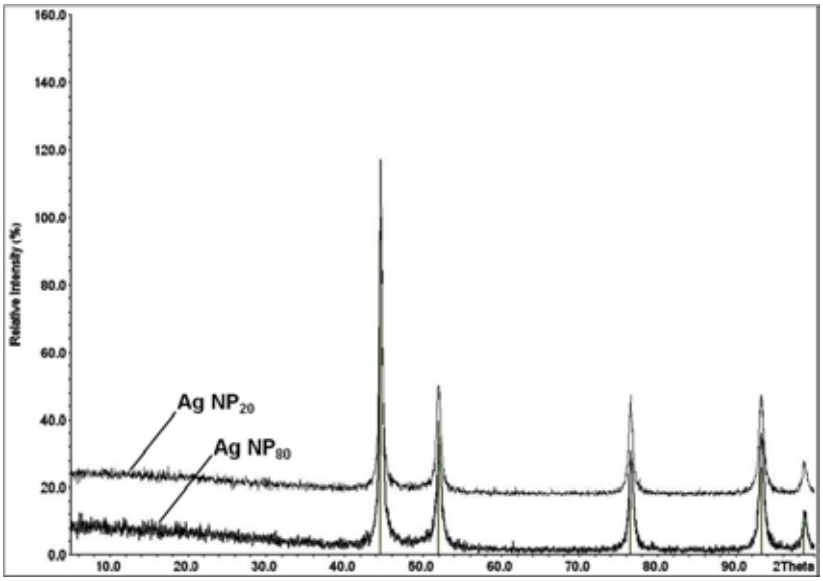

Figure S1. X-ray diffraction pattern of Ag NP_{20} and Ag NP_{80}.

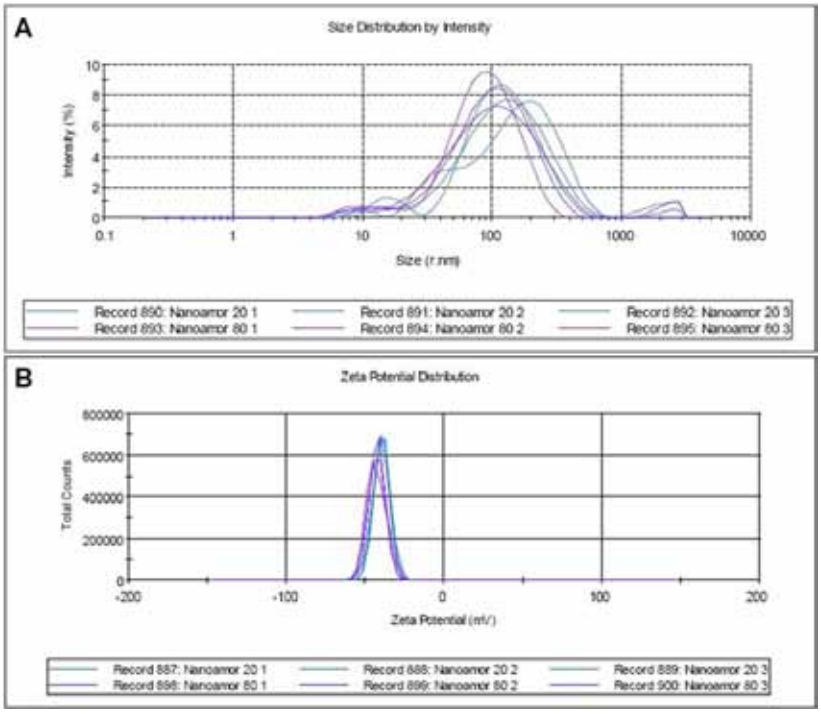

Figure S2. Hydrodynamic size distribution expressed by intensity (A) and zeta potential distribution (B) of Ag NP_{20} and Ag NP_{80} (three replicate analyses for each sample are shown).

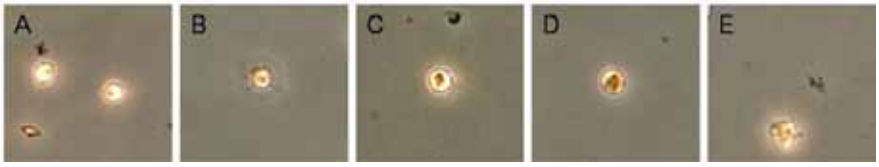

Figure S3. Coelomocytes of *N. diversicolor* with increasing lysosomal membrane permeability. (A) Normal cell (neutral red was retained in the lysosome); (B-D) Increasing permeability of lysosomal membranes with more and more neutral red leaking from lysosome to cytosol; (E) Dead cell.

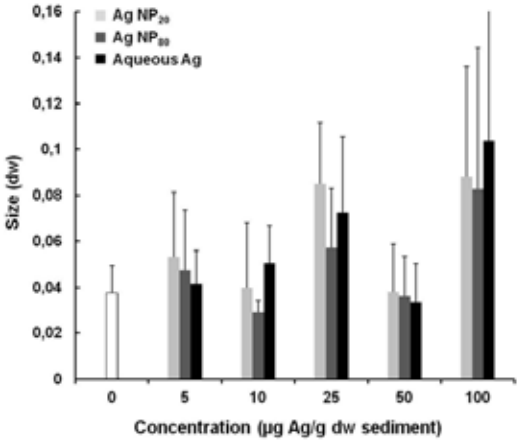

Figure S4. Initial size of *N. diversicolor* in three Ag treatments.

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Paper IV

I have contributed to this paper by:
- Being the principal developer of the idea
- Writing the manuscript

A review of ecotoxicological effects and possible toxic mechanisms of silver nanoparticles in aquatic organisms

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ABSTRACT

Although more and more toxicological studies of silver nanoparticles (Ag NPs) have been conducted with human and mammalian cell lines, there is still a poor understanding of their potential risks and toxicities for the aquatic environment and organisms after eventual release. How do the conditions of aquatic environment influence the behavior of Ag NPs, such as dispersion, stability and Ag^+ ions release? Are Ag NPs bioavailable and to what extent they can be accumulated in aquatic organisms? And in which body parts? What are the potential uptake routes and toxic mechanisms of Ag NPs in the aquatic organisms comparing to their ionic form? Will Ag NPs raise more attention for environmental risk assessment compared to the traditional ionic Ag form? With the purpose to find out the answers of the above questions, we summarize the available toxicological data of Ag NPs in aquatic organisms, as a comparison with aqueous Ag, as well as the knowledge gaps and challenges which should be addressed in future aquatic experimental designs and ecotoxicological studies.

Keywords: Silver nanoparticles, Aquatic organisms, Dispersion and stability, Bioavailability and uptake, Ecotoxicological effects, Toxic mechanisms

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1. Introduction

Silver is known to be a toxic contaminant in aquatic environments. The United States Environmental Protection Agency (USEPA) has set the recommended water quality criteria for Ag in fresh water and seawater at 3.2 and 1.9 μg/L, respectively. Most of our knowledge about Ag toxicity is related to the ionic form of silver, Ag^+, which was especially a historical problem related to the photo industry. There is an increasing concern about toxicities and potential risks, both still poorly understood, of silver nanoparticles (Ag NPs) for the environment and human health after their eventual release, as they are indicated as one of the fastest growing applications in nanotechnology industries and consumer products. Ag NPs have been incorporated into many commercial products, such as food packaging, odor resistant textiles, household appliances, and medical devices because of their notable antimicrobial properties (Cohen et al., 2007; Sondi and Salopek-Sondi, 2004). Nevertheless, besides the potential values in nano-products, the toxicities of Ag NPs in mammalian cells have been demonstrated, with the effects such as biomembrane damage (Arora et al., 2008, 2009), mitochondrial dysfunction (Carlson et al., 2008), apoptosis (Park et al., 2010), inflammation (Carlson et al., 2008), DNA damage (AshaRani et al., 2009) and chromosomal aberrations (Wise et al., 2010). ROS-dependent oxidative stress has been confirmed to be a critical mechanism of Ag NP toxicity in mammalian cells (Hussain et al., 2005; Kim et al., 2009). Furthermore, the release of Ag^+ ions from Ag NPs due to surface oxidation is believed to contribute to the observed NP toxicities in bacteria (Lok et al., 2007).

Ag NP-containing products likely release Ag^+ ions or Ag particles (nanoparticles or aggregates) via wastewater discharge into the aquatic environment (Benn and Westerhoff, 2008). Environmental concentrations of Ag NPs are estimated ranging between 0.03 and 500 ng/L in nature waters (Luoma, 2008), and possibly increased with increasing applications of Ag NPs. Thus, aquatic organisms are likely to be exposed to these metal NP contaminants. However, most nanotoxicological studies focus on atmospheric contamination and respiratory effects in mammals or *in vitro* assays with mammalian cells, few, if any, studies have conducted and little is known about the fate, bioavailability, bioaccumulation, potential toxic impacts of Ag NPs on organisms in aquatic environment and the specific mechanisms by which they cause toxicities. Furthermore, variable factors such as different physical-chemical properties (i.e., size, shape, electrostatic attraction potential, etc.), surface modification/coatings, the degree to which

particles agglomerate (become held together by relatively weak forces)/aggregate (become strongly bonded together) (http://www.astm.org/Standards/E2456.htm) and interact with other compounds in tested media make toxic effects of Ag NPs even more complex and difficult to compare under different exposure scenarios (i.e., freshwater, seawater, sediment). Unfortunately, in practice, risks of NPs are in most cases assessed on the basis of their chemical composition alone, and to date, no accepted or well-defined risk assessment methods or test strategies exist explicitly designed for NPs. Therefore, problems with regard to Ag NPs in aquatic environments that need to be addressed include:

1. How do the conditions of aquatic environment influence the behavior of Ag NPs, such as dispersion, stability and Ag^+ ions release?

2. Are Ag NPs bioavailable and to what extent they can be accumulated in aquatic organisms? And in which body parts?

3. What are the potential uptake routes and toxic mechanisms of Ag NPs in the aquatic organisms comparing to their ionic form?

4. Will Ag NPs raise more attention for environmental risk assessment compared to the traditional ionic Ag form?

With the purpose to find out the answers of the above questions and basing on the available aquatic toxicological data of Ag NPs, we summarize that how environmental related-factors affect NP behaviors, the bioavailability and uptake (especially cellular uptake) routes, ecotoxicological effects (i.e., acute, chronic, cellular and bactericidal effects) and possible toxic mechanisms of Ag NPs in aquatic organisms compared to aqueous Ag, as well as the knowledge gaps and challenges which should be addressed in future aquatic experimental designs and ecotoxicological studies.

2. The behavior of Ag NPs in aqueous environments: affecting factors

2.1 Ag NPs in aquatic environments

For Ag NPs intentionally or accidentally introduced into the environment, aquatic environment is likely to act as a potential sink as it does for many chemicals. Ag NPs released into the aquatic environment may complex with ligands and then agglomerate, react with natural materials like dissolved organic matter (DOC) and natural particulates, be converted into Ag^+ ions, or still be present as nanoparticles (Benn and Westerhoff, 2008; Blaser et al., 2008; Luoma, 2008). The

coexistence of the particle and ionic forms, which may exhibit different fate and transport characteristics, and may have independent or synergistic toxicity pathways, can complicate the understanding of Ag NPs' environmental effects (Liu and Hurt, 2010; Lubick, 2008). In addition, the formation of aggregates in water presents the opportunity for other organic materials, including toxicants, to become associated with the aggregates, which can change the bioavailability of these NPs and create additional toxicological concerns (Farré et al., 2009). Generally, the physical and chemical properties of Ag NPs themselves, such as size, surface area and reactivity, coating and functional group, combined with environmental factors such as water pH, salinity and temperature, dissolved organic material and natural competing cations, are likely to play important roles in determining their persistence and in which compartment Ag NPs are retained in the aquatic environments, as well as toxic consequences. However, due to the limitation of techniques and equipments for tracing the state of NPs in real aquatic environment, some studies have been conducted under laboratory conditions to investigate the effects of different NP properties and environmental realistic factors on dispersion and stability of NPs in aqueous media, with the purpose to get hints of the behavior of NPs under real complex environments.

2.2 Dispersion and stability of Ag NPs: effects of physical and chemical properties

To generate stable dispersion and toxicity data of Ag NPs for risk assessment, particle surface modification and coatings are often used. Table 1 listed dispersion tests of Ag NPs with different surface coatings in different test media. Another important property of Ag NPs of particular relevance which contributes to their instability and toxic effects is the release of Ag^+ ions from the particles. Ag NPs may dissolve or degrade under oxidative conditions, which process is affected by particle size, ionic strength, pH, organic matters, temperature, and surface coatings. Liu and Hurt (2010) have quantitatively tracked the appearance of dissolved silver in synthesized ion-free citrate-stabilized Ag nanoparticle suspensions (2-8 nm in diameter) over times ranging from 10 min to 125 days. Silver ions were observed to be released from Ag NPs over time resulting from an oxidation process involving dissolved O_2 and H^+, as well as peroxide intermediates. Finally this dissolution was complete leading to the disappearance of the particle phase. Thus even a simple Ag NP colloid may consist of three silver forms: Ag^0 nanoparticles, free Ag^+ (including any soluble complexes) and surface-adsorbed Ag^+. These studies suggest that

Ag^0 nanoparticles will not be stable and persistent in realistic environmental compartments containing dissolved oxygen. Understanding the environmental fate of Ag NPs is therefore complicated by the coexistence of the particle and ionic forms as described previously.

Table1. Dispersion of silver nanoparticles with different surface coatings

Size (nm)	Source	Surface coating	Test Species	Advantage	Dispersion test
69±3	C	Poly Vinyl Pyrrolidone (PVP)	Human acute monocytic leukemia cell line (THP-1)	Enable suspension and diminish aggregation	Free PVP coatings were detected in stock solution by DLS analysis (Foldbjerg et al., 2009).
15, 30, 55	S	Hydrocarbon	Rat alveolar macrophages cell lines	Prevent NP sintering during plasma synthesis, maintain a constant coating in aqueous solutions	Conduct in PBS (pH 7.4), deionized water, DMSO and ethanol. Ag NPs tended to agglomerate and settling. Final stock solution was prepared in deionized water using physical mixing (Carlson et al., 2008).
1-10	S	Sodium citrate	Rainbow trout hepatocyte primary cell cultures	Coordinate weakly with the silver surface, and stabilize the silver nanoparticle stock dispersions	Ag NPs in stock solution (prepared with Milli-Q water) were mainly present as 1-10 nm fractions. In media with higher ionic strength and DOC, particles tended to agglomerate (Farkas et al., 2010).
26.6±8.8	S	Metal oxide	Adult female zebrafish	NM	Ag NPs were suspended in Milli-Q water and sonicated. After 48 h exposure, only 5.1 % of initial dose presented in water column and the aggregation was significant (Griffitt et al., 2008).
81±2	C	0.2% PVP	Adult Eurasian perch	NM	Silver concentrations in tap water declined during exposure and all measured concentrations were lower than the nominal concentrations (Bilberg et al., 2010).
5-20	S	Starch and BSA	Zebrafish embryo	For stability and uniform dispersion	Ag NPs were suspended in ultrapure water and lyophilized. Stock solution was prepared from lyophilized pellet, sonicated and diluted using embryo water. No agglomeration or

					precipitation was observed (Asharani et al., 2008).
20-30	S	Metal oxide (~2-5 nm)	Zebrafish fry, daphnids, algae	NM	Ag NPs were suspended in moderately hard freshwater at 270 mg/L and dispersed with a probe sonicator. Less than 1% by mass of the original dose was present in the dissolved form after 48 h gently stirring (Griffitt et al., 2008).
10-200	C	Carbonate	Freshwater algae	Avoid aggregation and maintain Ag NPs in suspension	No aggregation under experimental conditions (MOPS, light and agitation), about 1 % of total Ag in Ag NP suspensions was present as Ag^+ (Navarro et al., 2008).
60-70	C	0.3 % polyvinyl-pyrrolidone (PVP)	Marine diatom *T. weissflogii*	Negligible effects on phytoplankton	Ag NPs were relatively well dispersed in deionized water, but aggregated quickly in ASW. No Ag NPs were found in the <0.22 mm filtrate when ASW only was used as the solvent (Miao et al., 2009).
14	S	Polyvinyl alcohol (PVA)	Nitrifying microorganism	To control the formation of nanocrystals and avoid agglomeration of NPs. No effect on bacteria growth.	The color of Ag NP suspensions changed from dark brown to yellow during a week of monitoring at room temperature due to oxidative dissolution of the Ag NPs (Choi et al., 2008).
1-100	S	NPs inside a carbon matrix	Gram-negative bacteria	Prevent coalescence during synthesis	Ag NPs were suspended in water and homogenized using ultrasonic cleaner. A significant number of Ag NPs was released from the carbon matrix which was considered as free surface particles, while the particles inside the carbon matrix tended to agglomerate (Morones et al., 2005).
6.7 7.2	S	MPA PL	Gram-negative bacteria, *E.coli*	As an anionic (MPA) and cationic (PL) stabilizing agent	PL was found to generate stable dispersions of isolated single or a few particles, indicating high positive charge density at the PL-coated particle surfaces (Dror-Ehre et al., 2009).

C=Commercial; S=Synthesis; NM=not measured

2.3 Dispersion and stability of Ag NPs: effects of environmental realistic factors

Some environmental factors have been shown able to influence the inherent properties of NPs. For instance, the presence of electrolyte ions in medium can screen the effective nanoparticle surface potential, and as a result of this surface charge reduction, aggregation is expected, which may then result in modified uptake, bioaccumulation and toxicity of Ag NPs (Wise et al., 2010). The release rates of Ag^+ ions from Ag NPs were demonstrated increasing with temperature in the range 0 to 37 °C, and decreasing with increasing pH or addition of humic or fulvic acids. Sea salts have only a minor effect on dissolved silver release (Liu and Hurt, 2010). Cumberland and Lead (2009) have studied the stability and size change of citrate-stabilized Ag NPs (13.7±6.2 nm) in relation to solution conditions (i.e., pH, natural organic macromolecules and ionic strength), and found at low ionic strength that Ag NP aggregate size increased as pH increased from 5 to 8. The presence or increase of ionic strength (Na^+ and Ca^{2+}) also enhanced the instability and aggregation of Ag NPs. However, the presence of humic substances (HS) improved stability of Ag NPs under these conditions, most likely due to a reduction in the diffuse layer or by forming a surface coating resulting in both steric and charge stabilization. The authors concluded that immediate and irreversible aggregation and subsequent loss from the water to the sediment may in fact not occur so readily due to stabilization with HS.

3. Ag NPs: Bioavailability, uptake and bioaccumulation

Key challenges in environmental risk assessment and management are understanding the bioavailability, uptake, bioaccumulation and trophic transfer of aquatic contaminants, including NPs.

The arisen studies have shown that Ag NPs can be taken up through many routes and accumulate in different compartments in aquatic organisms (Fig.1). Taking aquatic vertebrate as an example, Ag NPs were shown to be taken up by zebrafish (*Danio rerio*) embryos and liver tissue, fathead minnow (*Pimephales promelas*) embryos and carp (*Cyprius carpio*) in both single and agglomerated forms (Asharani et al., 2008; Choi et al., 2010; Gaiser et al., 2009; Laban et al., 2010; Lee et al., 2007a, b). Ag concentrations in zebrafish liver tissues treated with 30 and 120 mg/L Ag NPs for 24 h were 0.29 and 2.4 ng Ag/mg liver, respectively (Choi et al., 2010). The subcellular distribution of these Ag NPs was found in the region of chorion pore canals (CPCs), cytoplasm, plasma membrane and nucleus. Observations of Gaiser et al. (2009) demonstrated

that much of the uptake of Ag NPs (35 nm, 0.01 and 0.1 mg/L) into the carp (*Cyprius carpio*) after 21 d exposure possibly occurred as a consequence of the fish eating agglomerated NPs, rather than uptake via the water through the gills, indicating the importance of oral uptake routes during exposure, and the possible following trophic transfer through predation by other organism. Furthermore, Griffitt et al. (2009) indicated that Ag NPs themselves contributed to the silver burden in zebrafish gill rather than their dissolved ionic form, as exposure to Ag NPs produced significantly higher levels of silver associated with the gills than did exposure only to the soluble fraction.

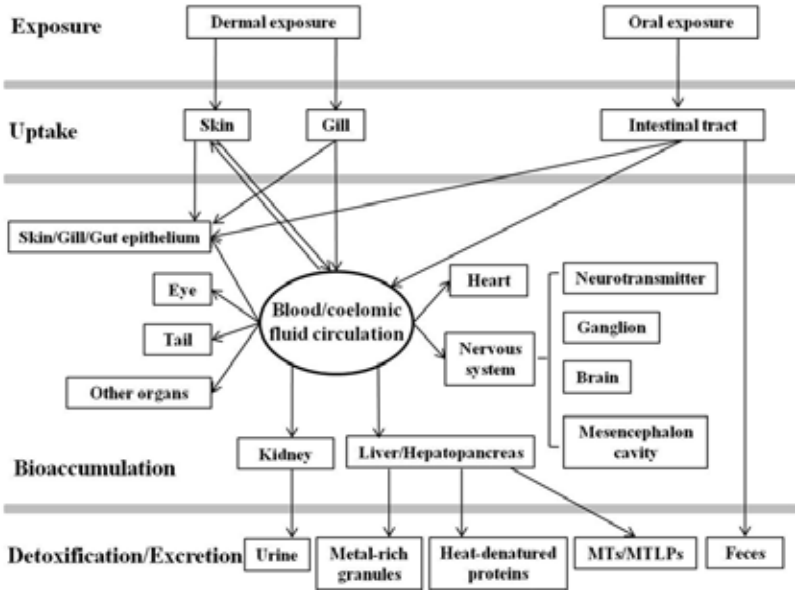

Fig.1. Individual uptake, bioaccumulation and detoxification/excretion processes of Ag NPs.

The potential of Ag NPs to be taken up and internalized within different compartments of cells largely depends on NP properties, especially size. For small single Ag NPs, they can be taken up by cells through diffusion (Lee et al., 2007a, b). Endocytosis (i.e., pinocytosis and phagocytosis) is also a possible way for Ag NPs to enter cells (Asharani et al., 2009; Park et al., 2010). Furthermore, it has been suggested that Ag NPs act as Trojan horse-type carriers that deliver free silver ions to the membranes of organisms or enable the transport of ions into cells (Lubick, 2008;

Luoma, 2008; Park et al., 2010), both of which will contribute to the bioavailability and accumulation of Ag NPs. Therefore, different mechanisms are possibly involved in the uptake of aqueous Ag and Ag NPs, with the former mainly being via competition with Na^+ and ATP-dependent transport (Bury and Wood, 1999; Bury et al., 1999; Galvez and Wood, 2002; Bianchini and Wood, 2003; Pedroso et al., 2007) and facilitated transport across cell membranes (Bielmyer et al., 2002), whereas the latter are taken up through diffusion, endocytosis (i.e., pinocytosis and phagocytosis), as well as by disturbance of membrane components through a Trojan-horse type mechanism. However, it is still not clear to what degree these Ag NPs can be eliminated by organisms once they are taken up. The detoxification mechanisms such as the formation of metal-containing extracellular granules and mineralized lysosomes, excretion of metals by cell exocytosis, and synthesis and turnover of metal binding proteins (e.g., metallothioneins, MTs), which have been observed after exposure to aqueous Ag (Bryan and Hummerstone, 1977; Fernandez and Jones, 1989; Mouneyrac et al., 2003; Ng et al., 2008; Poirier et al., 2006), still receive few investigations for Ag NPs.

4. Ag NPs: Toxicities and potential toxic mechanisms in aquatic organisms

4.1 Individual effects

4.1.1 Acute toxicity

Recently, acute toxicity studies of Ag NPs on aquatic organisms have begun to be carried out under laboratory conditions and compared to their aqueous form (Ag^+). For instance, Griffitt et al. (2008) studied the toxicities of silver and other metals as both nanoparticles and soluble salts to several representative freshwater organisms of various trophic levels, including zebrafish, daphnids, and algal species. The corresponding LC50s of $AgNO_3$ for these animals were almost all lower than for Ag NPs (Table 2), indicating a higher toxic effect of the ionic form than of the nanoparticle on the basis of mass of metal added. The exception was *Danio rerio* fry that were found to be more sensitive to the nanoparticle forms of silver and copper than to the ionic forms of the same metals. Furthermore, daphnids and algae were markedly more susceptible to toxicity from these nanometals compared with either stage of zebrafish, suggesting a more opportunity for these lower trophic level organisms to be affected in real aquatic environments than that of higher levels. Particle size was found to be critical for Ag NP toxicity in the study of Bar-Ilan et

al. (2009), which demonstrated a size-dependent mortality in zebrafish embryos with smaller NPs (3 nm) being more toxic than larger sizes (10, 50 and 100 nm).

The study of Laban et al. (2010) took the environmental factors into experimental design to compared toxic effects of commercial Ag NPs prepared by two methods, sonication (laboratory methods) and stirring (mimic environmental condition), on fathead minnow (*Pimephales promelas*), and demonstrated that LC50 values for 35 nm and <100 nm Ag NPs by sonication were much lower than that of stirring groups (Table 2), indicating the important role of environmental factors in modifying Ag NP toxicities. However, the estimated LC50 of 0.015 μg/L for $AgNO_3$ indicated its higher toxicity than Ag NPs. Silver ions were observed to be released from Ag NPs in both sonicated and stirred treatments during exposure. However, the toxicity caused by dissolved Ag released from Ag NPs (slower release rate) was likely different from Ag^+ ions released from $AgNO_3$ (dissociates completely with a log K value = -0.3), and both particulate and ionic silver contributed to Ag NP toxicity. Nevertheless, it is not always the case that toxicity of Ag NPs is derived from both particulate and dissolved forms. Asharani et al. (2008) found concentration-dependent toxicity of Ag NPs (5-20 nm) on zebrafish embryos, whereas aqueous Ag showed no significant effects on developing embryos, suggesting that Ag NP-mediated toxicity was not due to the presence of Ag^+ ions in the medium.

The considerable difference in acute toxicity of Ag NPs is likely due to the size-dependent effects of nanoparticles and interspecific differences (Wu et al., 2010), as well as different exposure conditions. Furthermore, it has been recommended that the concentration of NPs be expressed as number or surface area per volume as opposed to mass per volume in ecotoxicity tests (Crane et al., 2008) as this may better reflect the effects of NPs.

Table 2. LC50s of silver nanoparticles and aqueous Ag (Ag^+) for some aquatic organisms

Species	Organisms	Exposure conditions	Silver form	LC50 (µg/L)	Ref.
Fishes	*Danio rerio* (zebrafish)				
	Adult	Filtered hard freshwater (0.45 µm), static renewal, 48 h	Ion ($AgNO_3$)	22.2	
			NP (20-30 nm)	7070	
	Fry (<24 h)	Filtered hard freshwater (0.45 µm), static renewal, 48 h	Ion ($AgNO_3$)	>10000	Griffitt et al., 2008
			NP (20-30 nm)	7200	
	Adult	Commercial available bottled water, static system, 24 h	Ion	NM	
			NP (diameter: 5-20 nm)	250000	Choi et al., 2010
	Embryo (64-128 cell stage)	Embryo water (60 mg sea salt/L ultrapure water), 72 h	Ion	NM	Asharani et al., 2008
			NP (5-20 nm)	25000-50000	
	Embryo	Egg water, 120 h	Colloidal NP (3 nm)	10070	
			(10 nm)	13550	
			(50 nm)	13700	Bar-llan et al., 2009
			(100 nm)	14810	
	Oryzias latipes (Japanese medaka)				
	Adult	Hard freshwater, static renewal, 48 h	Ion	NM	Wu et al., 2010
			NP (20-37 nm)	1030	
	4-5 month old	Fresh water, flow-through system, 96 h	Ion ($AgNO_3$)	36.5	Chae et al., 2009
			NP (49.6 nm)	34.6	
	Pimephales promelas (fathead minnow)				
	Adult	Unchlorinated well water, static system, 96 h	Ion ($AgNO_3$)	9.7	Nebeker et al., 1983
	Eggs, age < 24 h	Well water, static nonrenewal system, 96 h	Ion ($AgNO_3$)	15	
			NP (21-280 nm)	1360 (Sonicated) 10600 (Stirred)	
			NP (29-100 nm)	1250 (Sonicated) 9400 (Stirred)	Laban et al., 2010
Invertebrates	*Acartia tonsa* (copepod)	Filtered Southampton (NY, USA) surface seawater (0.2 µm), not fed, 48 h	Ion ($AgNO_3$)	43.2	Hook and Fisher, 2001
	Ampelisca abdita (amphipod)	Sand-filtered Narragansett Bay seawater, static renew system, not	Ion ($AgNO_3$)	20	Berry et al., 1999

	fed, 10 d			
Hyalella azteca (amphipod)	$AgNO_3$ spiked sediment. UMBFS Pond water was added as over-lying water, 10 d	Ion ($AgNO_3$)	1.62-379.7 (µg/g dw)	Rodgers et al., 1997
Mercenaria Mercenaria (bivalve)				
Embryo	Synthesis seawater, static system, 42-48 h	Ion ($AgNO_3$)	21	Calabrese and Nelson, 1974
Scrobicularia plana (bivalve)				
Adult	Filtered natural seawater, renewal system, fed, 96 h	Ion ($AgNO_3$)	200	Berthet et al., 1992
Ceriodaphnia dubia (daphnids)	Moderately hard water, 48 h	Ion ($AgNO_3$)	0.5	Bielmyer et al., 2002
	Filtered hard fresh water (0.45 µm), static renewal system, not fed, 48 h	Ion ($AgNO_3$) NP (20-30 nm)	160 67	Griffitt et al., 2008
	Filtered Suwannee river water (0.45 µm, SR-1, SR-2,SR-3) and deionized water, 48 h	NP (diameter: 20-30 nm)	6.18 (SR-1) 0.771 (SR-2) 0.696 (SR-3) 0.46 (DI- water)	Gao e tal., 2009
Daphnia pulex (daphnids)	Filtered hard fresh water (0.45 µm), static renewal system, fed, 48 h	Ion ($AgNO_3$) NP (20-30 nm)	8 40	Griffitt et al., 2008
Simocephalus sp. (daphnidae)	Deionized water added with WCL-1salts, not fed, 48 h	Ion ($AgNO_3$)	27	Hook and Fisher, 2001

NM = not measured

4.1.2 Chronic effects

4.1.2.1 *Growth and development*

Aqueous Ag (as $AgNO_3$) has been shown to delay hatching and cause dysmorphology of zebrafish embryos (Powers et al., 2010). Ag NPs also can induce several kinds of sublethal phenotypic abnormalities and cause physiological dysfunction of zebrafish and fathead minnow embryos during development, with effects such as hatching delay, embryonic morphological malformations, abnormal body axes, twisted notochord, slow blood flow, pericardial emeda,

heart malformation, eye defects, being observed (Asharani et al., 2008, 2009; Laban et al., 2010; Lee et al., 2007a, b; Wu et al., 2010). Rates of passive diffusion and accumulation of Ag NPs were assumed to be responsible for these dose-dependent abnormalities (Lee et al., 2007a,b). These various abnormalities observed in embryos are of concern as most of these affected fry would likely not develop normally. For the marine oyster *Crassostrea virginica*, exposure to 1.6 μg/L Ag NPs (average size: 15±6 nm) for 48 h significantly decreased the percent of normally developing embryos (Ringwood et al., 2010). Such a retardation of growth would serve to prolong the pelagic life of the larvae and thus increase their chances of loss through predation and disease, possibly reducing recruitment to the population (Calabrese et al., 1973). For marine diatom, *T. weissflogii,* cell growth was also suppressed as a function of increased Ag^+ ions released from Ag NPs (average size: 60-70 nm) with increased exposure concentrations (10.8-1080 μg/L) over 48 h (Miao et al., 2009), suggesting the contribution of dissolution of Ag NPs to their toxicities.

4.1.2.2 *Reproduction*

Silver ions have been shown to interfere with reproduction in a number of aquatic organisms, with effects such as the decrease in the larvae number, total protein content of eggs, and declined hatching rate being observed in the marine gastropod *Crepidula fornicata* and copepods *Acartia tonsa* and *A. hudsonica* (Nelson et al., 1983; Hook and Fisher, 2001). The mechanisms for Ag to depress egg production were suggested to be their binding to enzymes involving with vitellogenesis, and reducing yolk protein deposition and ovarian development (Hook and Fisher, 2001, 2002). However, in contrast to aqueous Ag, few data about toxic effects of Ag NPs on reproduction of aquatic organisms have been reported, which also highlights the urgency of relevant toxicological studies for risk assessment of NPs.

4.1.2.3 *Other physiological effects*

In addition to growth and reproduction, a few studies have also reported toxic impacts of Ag NPs to other physiological parameters and different toxicity modes were found possibly exist between Ag NPs and ions. For example, zebrafish exposed to soluble silver (as $AgNO_3$) exhibited significant thickening of the gill filament while no change in filament width was observed in zebrafish exposed to Ag NPs (26.6±8.8 nm) (Griffitt et al., 2009). In the study of Navarro et al. (2008), both Ag NPs (10-200 nm) and $AgNO_3$ inhibited photosynthesis of freshwater algae,

Chlamydomonas reinhardtii, in a dose- and time-dependent manner, and based on total Ag concentration, toxicity was 18 times higher for $AgNO_3$ than for Ag NPs. If, however, the comparison was based on the measured free Ag^+ concentration, the toxicity of Ag NPs appeared to be much higher than that of $AgNO_3$ (in terms of EC50). Moreover, the addition of cysteine, a strong Ag^+ ion ligand, abolished the inhibitory effects on photosynthesis of both Ag NPs and aqueous Ag. The author suggested that free ions were released from Ag NPs during exposure and contributed to observed Ag NP toxicities. The inhibition of photosynthetic activity and a reduction of the production of cellular chlorophyll was also observed in the marine diatom *T. weissflogii* as a function of increased Ag^+ ions released from Ag NPs (average size: 60-70 nm) with increased exposure concentrations (10.8-1080 µg/L) after 48 h exposure (Miao et al., 2009). Ag NPs (81 nm) were also shown be able to affect oxygen consumption of fish (*Perca fluviatilis*) gills, with effects including significant increase in basal metabolic rate, which then led to reduced ability to extract oxygen from water and increased vulnerability to hypoxia (Bilberg et al., 2010).

4.2 Cellular effects

Although cellular responses of organisms to NPs are not the direct goal of ecotoxicological testing and environmental risk assessment, insights into such effects can be helpful for developing rapid tests and can sometimes be used as inexpensive and conservative screens for potential adverse effects of NPs on more ecologically relevant demographic endpoints like survival, growth and reproduction (Crane et al., 2008). *In vitro* experimentation is required to minimise animal use, and thus provide relevant models that are predictive of the *in vivo* situation. Effects on *in vitro* cell lines could reflect toxic mechanisms and give insights into possible detrimental consequences at higher levels of biological organization (Johnston et al., 2010) although extrapolating from *in vitro* effects to whole organism or higher-level impacts involves substantial uncertainties.

Recent *in vitro* and *in vivo* tests performed with various cell types to evaluate the toxicities and mechanisms of Ag NPs indicated that oxidative, cytotoxic, genotoxic and inflammatory consequences were all associated with silver particulate exposure and linked to each other. Below, the recent emerging cellular toxicity data in aquatic organisms for Ag NPs are reviewed, and compared with the results of available mammalian *in vitro* cell lines, as well as the data of

aqueous Ag. Based on this, the mechanisms of cellular toxicities of Ag NPs were proposed and compared to aqueous Ag.

4.2.1 Cytotoxicity

4.2.1.1 *Biomembrane damage and cell viability*

One main toxic effect of NPs is interaction with biomembranes causing cell permeability changes as well as decreasing viability which has been shown for both metal and non-metal NPs (Yang et al., 2008). Dose-dependent increase of lysosomal membrane permeability was observed in oysters (*Crassostrea virginica*) when they were exposed to Ag NP (15±6 nm) concentration of 0.016 to 16 µg/L for 48 h. Biomembrane damage was also observed in rainbow trout (*Oncorhynchus mykiss*). The integrity of their hepatocytes was significantly reduced after 48 h exposure to Ag NPs (1-10 nm, 6.35 mg/L) (Farkas et al., 2010). Recent *in vitro* studies of mammalian cell lines also demonstrated various cell morphological changes by exposure to Ag NPs, such as cell shrinkage, polyhedric and irregular cell shape, restricted spreading patterns, cell metabolic arrest, as well as changes in membrane integrity and cell viability (Arora et al., 2008, 2009; Carlson et al., 2008; Foldbjerg et al., 2009; Hussain et al., 2005; Kim et al., 2009). Biomembrane damage was also argued as an important mechanism for Ag NP toxicity in bacteria (Lok et al., 2006).

4.2.1.2 *Mitochondrial dysfunction and ROS-dependent oxidative stress*

Mitochondria are the cellular organelles with the most active redox reactions, and NPs have shown to be able to localize at these active redox centers. NPs cause dysfunction by disrupting the electron transport chain, resulting in high concentrations of reactive oxygen species (ROS), low ATP production and alterations in various antioxidant enzyme systems (Asharani et al., 2009; Foley et al., 2002; Li et al., 2003). Reactive oxygen species (ROS)-dependent oxidative stress induced by NPs is among the possible toxic mechanisms of greatest concern (Nel et al., 2006; Xia et al., 2006) and has been shown for titanium dioxide (Long et al., 2006; Park et al., 2008), zinc oxide, silica dioxide (Yang et al., 2008) and carbonaceous NPs (Oberdörster, 2004; Pickering and Wiesner, 2005; Zhu et al., 2007).

An increasing amount of evidence shows that Ag NPs are capable of localizing in mitochondria (Asharani et al., 2009) as well, triggering mitochondrial dysfunction such as membrane potential alternation (Carlson et al., 2008) and ROS dependent oxidative stress (Carlson et al., 2008; Kim

et al., 2009). Various oxidative damages induced by Ag NPs, such as decreased GSH and SOD contents, reduced mRNA levels of catalase and glutathione peroxidase, increased expression of oxidative-related genes, increased lipid peroxidation and total glutathione, were observed in zebrafish (Choi et al., 2010), rainbow trout (Scown et al., 2010), Japanese medaka (Chae et al., 2009) and some mammalian cell lines (Park et al., 2010; Arora et al., 2008, 2009; Carlson et al., 2008). These oxidative stresses demonstrated in aquatic organisms are suggested possibly due to the ROS generated by NPs (Chae et al., 2009). The evidence that pretreatment of human HepG2 cells with antioxidant prevented Ag NP-induced oxidative stress and alleviated mitochondrial damage (Kim et al., 2009), further validate that the intrinsic toxicity of Ag NPs was associated with oxidative damage-dependent pathways. Furthermore, the expression of oxidative stress-related mRNA species of human hepatoma cells (HepG2) was regulated differentially as a function of exposure to Ag NPs (<10 nm) and aqueous Ag (Ag^+) (Kim et al., 2009), suggesting that the mechanism of Ag NP-induced toxicity was possibly different from that of aqueous Ag. However, not all cells treated with Ag NPs exhibit deleterious oxidative damage due to the presence of effective anti-oxidative systems in some cases (Asharani et al., 2009).

4.2.1.3 *Apoptosis*

Apoptosis (programmed cell death) is a biologically essential, complex cellular process that is a regulated sequence of events occurring in response to a variety of stress stimuli including metal exposure (Wang et al., 2008; Yang et al., 2007) and oxidative stress (Ott et al., 2007; Sharma et al., 2007).

An *in vivo* study with zebrafish liver tissue demonstrated that p53-related pro-apoptotic genes Bax, Noxa, and p21 were upregulated after treatment with 120 mg/L Ag NPs (5-20 nm in diameter) for 24 h, indicating Ag NP induced apoptosis (Choi et al., 2010). Apoptosis was also observed in Ag NP-treated (50 μg/mL, 5-20 nm) zebrafish embryos for 72 h by acridine orange staining, as well as in Japanese medaka (*Oryzias latipes*), which was exposed to Ag NP (49.6 nm, 1 and 25 μg/L) for 1 d with the induction of p53 gene whereas aqueous Ag ($AgNO_3$) induced this gene on the second day with lower amount than Ag NPs, indicating different toxic fingerprints between these two Ag forms (Chae et al., 2009). The study of Hsin et al. (2008) and Foldbjerg et al. (2009) provided evidence that the molecular mechanism by which Ag NPs induced apoptosis was mitochondria-dependent and ROS-mediated.

4.2.1.4 *Inflammation*

Inflammation is an early protective homeostatic immune response to tissue trauma. It is a complicated process with numerous cytokines and pathways involved. In medaka (*Oryzias latipes*), the mRNA level of transferrin (TF) gene, which can be enhanced by inflammation, was increased after a 2-day exposure to $AgNO_3$, whereas its level in Ag NP treated fish was grammatically lower, suggesting aqueous Ag caused inflammation and different toxic modes by these two Ag forms (Chae et al., 2009). However, there are conflicting conclusions about inflammation (Carlson et al., 2008; Cha et al., 2008; Park et al., 2010) or anti-inflammation (Wong et al., 2009) effects of Ag NPs on mammalian cells.

4.2.1.5 *Metallothionein induction*

Metallothioneins (MTs) are low weight and cysteine-rich proteins, which can bind both physiological and xenobiotic heavy metals, and play an important role in metal detoxification in organisms. MT expression levels were observed increase significantly in both adult and embryo oysters (*Crassostrea virginica*) after 48 h of exposure to 0.16 µg/L Ag NPs (15±6 nm) (Ringwood et al., 2010), indicating the detoxification processes induced by Ag NPs. Similarly, MT2 mRNA expression in zebrafish liver tissue was also found increase in a dose-dependent manner after treatment with 30, 60, and 120 mg/L of Ag NPs (5-30 nm in diameter) for 24 h (Choi et al., 2010). In another freshwater fish, Japanese medaka (*Oryzias latipes*), a statistically significant induction of MT expression (6.0-fold increase) was observed 1 day after the fish were exposed to 25 µg/L Ag NPs (49.6 nm), which was much higher than the response seen with an equivalent mass of ionic silver from $AgNO_3$ (2.2-fold). Furthermore, this induction of the MT mRNA levels in the liver was abrupt but not prolonged, showing an initial increase during the first couple of days and then a decrease back to a basal expression level with longer exposure times (>4 days) (Chae et al., 2009). However, whether the induction of MTs is due to metal-responsive pathways, or whether the Ag NP exposures caused an increase in oxyradicals so that MT induction is associated with their role as an antioxidant remains unclear.

4.2.2 Genotoxicity

Tendencies for Ag NPs to accumulate in cell nuclei have been described in zebrafish (*Danio rerio*) embryo model, indicating that they may lead directly to genomic damage and chromosomal aberrations (Asharani et al., 2008). Treatments of medaka (*Oryzias latipes*) cell

lines with 0, 0.05, 0.1 and 0.3 μg/cm^2 of Ag NPs (around 30 nm in diameter) induced damage in 8, 10.8, 16 and 15.8 % of metaphases and 10.8, 15.6, 24 and 24 % aberrations in metaphases, respectively (Wise et al., 2010). Among various types of DNA damage, double-strand breakages are considered to be the most biologically significant lesions in cells (Rothkamm and Lobrich, 2003). Choi et al. (2010) detected a high level of γ-H2AX, which is an indication of DNA double-strand breaks after treatment of zebrafish liver tissue with 120 mg/L Ag NP (5-20 nm in diameter) for 24 h, suggesting that Ag NP induced DNA damage, which is consistent with the study of Kim et al. (2009) using human hepatoma cells (HepG2) as test model. Ag NPs also have been shown to cause chromosomal breaks and cell cycle arrest in the G2/M phase possibly due to DNA damage and repair in human glioblastoma cells (Asharani et al., 2009). These evidences indicate that Ag NPs possibly behave differently with aqueous Ag to cause DNA damage, with the latter mainly covalently binding with DNA (Hossain et al., 2002) and inhibiting DNA synthesis (Hidalgo and Dominguez, 1998).

4.3 Bactercidal effects

Silver has for centuries been recognized as a strong antibacterial agent against a wide range of microorganisms, and given its special properties, silver ions and silver-based compounds have been used extensively in many bactericidal applications (Gotjamanos, 1997; Fung and Bowen, 1996; Hermans, 2006; Pal et al., 2007; Vermeiren et al., 2002). The mechanisms of the inhibitory effects of aqueous Ag include the electrostatic attraction between the negatively charged cell membrane of microorganisms and the positive charged Ag^+ ions (Dibrov et al., 2002) and affecting the function of membrane-bound enzymes (Bragg and Rainnie, 1974; McDonnell and Russell, 1999), both of which will change membrane permeability, or interfering with intracellular components (e.g., inhibit DNA replication, disrupt expression of ribosomal subunit proteins, inactivate cellular proteins and enzymes essential to ATP production, inhibit cellular enzymes for the P, S, and N cycles of bacteria) (Ratte, 1999; Yamanaka et al., 2005).

From the existing studies, Ag NPs appear to exhibit bactericidal effects through the following mechanisms: (1) ROS related oxidative stress. Free radicals may form on the surface of Ag NPs (Nover et al., 1983) which cause intracellular oxidative stress and interfere with energy metabolism of bacteria; (2) Ag NPs attach to and accumulate in the microbial surface and cause cell wall pitting, leading to an increased membrane permeability which then induces a

progressive release of LPS molecules and membrane proteins (Amro et al., 2000; Choi et al., 2008; Sondi and Salopek-Sondi, 2004) as well as the dissipation of the ATP pool and proton motive force (Lok et al., 2006); (3) Ag NPs are susceptible to oxidation by oxygen to form partially oxidized particles which appear to be more toxic to organisms than the freshly prepared NPs (Lok et al., 2007); (4) Ag^+ ions can be released from Ag NPs leading to antibacterial effects in the same way as aqueous Ag alone (Lok et al., 2006; Pal et al., 2007); (5) Ag NPs with smaller size are able to penetrate inside the cell and cause further damage by possibly interacting with sulfur- and phosphorus-containing compounds such as DNA and proteins (Morones et al., 2005), inhibiting replication (Feng et al., 2000), interacting with the bacterial ribosomes (Yamanaka et al., 2005), and possibly accumulating to toxic levels that may cause death. Furthermore, electrostatic attraction and repulsion in Ag NP-bacteria interactions may also be important in regulating the collision and attachment of these particles of different sizes, i.e., nanometer (NPs) and micrometer (bacterial cells) (Dror-Ehre et al., 2009; Stoimenov et al., 2002).

Therefore, besides the ways that is similar with aqueous Ag, Ag NPs also have their own special modes to exert bactercidal effects, and these effects are related to particle size, shape, the release of Ag^+ ions by oxidation, as well as the generation of ROS (Choi and Hu, 2008; Inoue et al., 2002; Kim et al., 2007; Lok et al., 2007; Morones et al., 2005; Pal et al., 2007; Sondi and Salopek-Sondi, 2004) as a result of their unique physical-chemical properties.

4.4 Different mechanisms between Ag NPs and aqueous Ag

Overall, Ag NPs entering the organisms can be taken up by cells through many pathways (i.e., diffusion, pinocytosis, phagocytosis or as Trojan horse-type carriers) which are different from uptake pathways for aqueous Ag (i.e., ion transport channel and facilitated transport) (Fig.2). Ag NPs can deposit in cytoplasm, mitochondria and nuclei, and produce a chain of physiological responses in organisms, which have been well demonstrated in mammalian cell lines. ROS-dependent oxidative stress was confirmed to be a critical mechanism of Ag NP toxicity in both aquatic organisms and mammalians, which in turn causes oxidative stress, DNA damage, functional protein inactivation, apoptosis and inflammation. As for the aqueous Ag, the uptake and toxic mechanisms mainly involve disruption of membrane sodium channels and depletion of energy reserves and inhibition of DNA synthesis (Fig.2). Actually, in *Caenorhabditis elegans*, the mode of action of Ag NPs was also observed different from their soluble counterpart, with

the NPs affecting reproduction potential and inducing high levels of oxidative stress compared to ionic form (Roh et al., 2009).

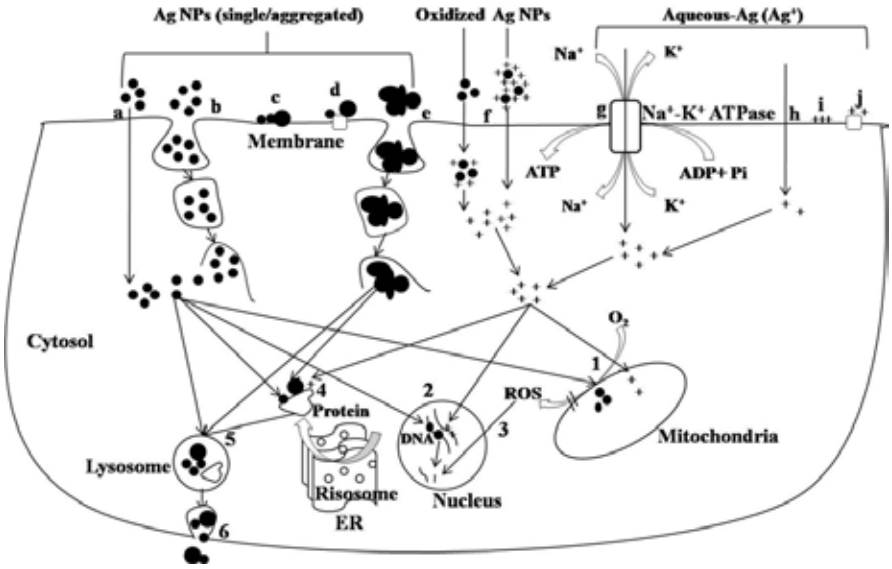

Fig 2. Potential cellular uptake routes, toxic mechanisms and detoxification processes of Ag NPs and aqueous Ag. A-j: Cellular uptake routes of (a-f) Ag NPs and (g-j) aqueous-Ag. (a) Diffusion via unusually large pores (chorion pore canals) and (b) pinocytosis of small and single Ag particles; Adsorption of Ag NPs (single or aggregated) on the (c) cell membrane and (d) membrane protein; (e) Phagocytosis of big and aggregated Ag particles; (f) Trojan horse-type delivery. Ag NPs act as Trojan horse carrier to deliver free silver ions inside the cell or enable the transport of ions across cell membranes into cells; (g) Na^{+}-K^{+} ATPase channel; (h) Facilitated transport (e.g., AgGSH and AgCys); Adsorption of Ag^{+} ions on (i) cell membrane and (j) membrane protein. 1-6: Toxic mechanisms and detoxification processes of Ag NPs and aqueous Ag. 1: ROS-dependent oxidative stress produced by Ag NPs and aqueous Ag; 2: Small Ag NP and aqueous Ag enter nucleus and cause DNA damage directly; 3: Indirect DNA damage cause by ROS; 4: Ag NPs and aqueous-Ag combine with newly-synthesized protein and dysfunction it, or trigger the expression of detoxification protein, like MT/MTLP and bind with it; 5: Ag and dysfunctional proteins were enclosed within lysosome and digested; 6: Ag particles/compounds was excreted by cell exocytosis.

5. Ag NPs in aquatic environment: Knowledge gaps and challenges

The unique physical-chemical properties of Ag NPs may result in different bioavailability,

cellular uptake routes, toxic effects and mechanisms compared to their metal or ionic forms in aquatic environments, which have been received much concern but with few investigations until now.

From a physical-chemical perspective, the extremely small size of NPs has been indicated by some studies as a principle factor in determining their toxicities. NPs have properties that are different from their bulk particles, largely due to the relatively large surface area and related higher reactivity. They thereby have a much higher probability to collide with bio-substances than their larger counterpart. However, most existing aquatic (also mammalian) research focuses on comparison of toxic consequences among different NP forms or between particle forms and ionic forms, and few studies have directly compared nanoparticulate and microparticulate forms of metals (Johnston et al., 2010), which should be taken into consideration in future studies.

Ag^+ ions have been shown to be released from the surface of Ag NPs by chemical oxidation during exposure under laboratory conditions. Furthermore, the released Ag^+ ions have been demonstrated to contribute to the toxicity of Ag NPs. However, it appears that the released Ag^+ ions do not seem to behave similarly to the natural ionic form of silver (Asharani et al., 2008; Kim et al., 2009; Roh et al., 2009; Laban et al., 2010) and the toxicities of Ag NPs cannot be solely attributed to the releases of Ag^+ ions. However, in some studies, the toxicities of Ag NPs are only related to the NP properties but not the released ions. The contrary conclusions may result from the differences of operations when preparing NP suspension, exposure conditions and test species, which need further studies designed followed standard procedures to verify it.

In most laboratory studies, when comparing the toxicity of natural free aqueous Ag and Ag NPs, the former appeared to be more toxic than the latter, indicating potential less environmental concern of Ag NP toxicities. However, surface coatings or modifications of some engineered Ag NPs possibly hinder the contact between Ag NPs and surfaces of organisms, reduce dissolved Ag released from particles or change the properties of Ag NPs leading to different toxic effects. These are also the reasons which make the comparison among different treatments and exposure scenarios hard under experimental condition. Therefore, we expect the synthesis of nanoparticles with a common surface chemistry to remove potential matrix effects from capping agents, or at least, taking the coating effects into consideration when designing experiments as they may change NP surface chemistry.

There is a growing consensus on the essential role of proper and accurate characterization of NPs in environmental media and biological systems to ensure reliable and reproducible toxicity tests performed. However, few, if any, studies of aquatic nanotoxicity have provided a full characterization of the size distribution (especially hydrodynamic size), dispersion state (especially in biological media) or surface chemistry (like surface charge) of Ag NPs in the actual test media. NPs are likely to undergo significant size distribution changes when they are transferred between media during experiments, such as from dispersion media (e.g., deionized water) to test media (i.e., sediment, freshwater, seawater and cell culture media). Such changes may alter bioavailability or toxicity in ways that are not entirely understood. Because the reactivity and toxicity of NPs are believed to be influenced by such features as their size, shape, surface coating and other properties, we conclude that both the physical and chemical properties of NPs must be systematically and adequately defined prior to toxicological studies and risk assessment. At a minimum, publication of toxicity test results should require that a characterization be performed in stock solutions used for testing. At the same time, there is a pressing need for the development of better methods for effectively characterizing NPs in complex environmental media (e.g., sediment) and living tissue, as we know little about the state of NPs in such complex targets due to the limitation of techniques and imaging equipments.

From a molecular perspective, we conclude that different uptake routes and toxic mechanisms are involved between Ag NPs and aqueous Ag in aquatic organisms, which were supported by evidences from the previous both aquatic and mammalian toxicological studies. However, the final toxicity exertion of Ag NPs within cells will be the result of the balance between NP effects versus cellular detoxification systems, such as induction of metallothioneins and formation of electron-dense granules, which have been observed after exposure to aqueous Ag but still receive few investigations for Ag NPs.

From an environmental perspective, the toxicity of Ag NPs will not only depend on their own particle characteristics, but also on ambient solution conditions like pH, hardness, ionic strength and the availability of various ligands, as these environmental parameters are likely to play important roles in determining the dispersion, toxic consequences and compartment in which NPs are retained in the environment. However, not all complexes or agglomerates can completely eliminate silver toxicity in aqueous surroundings; just as bioavailability is not

absolutely eliminated under some circumstances, such as chloro-complexation with Ag ($AgCl^0$) which favors the retention of at least some Ag in dissolved form (Luoma, 2008; Luoma et al., 1995) and these derived forms of Ag NPs are likely to be available to phytoplankton, fish, filter-feeding and benthic invertebrates through sedimentation and re-suspension. Therefore, more environmentally realistic factors should be incorporated into future experimental designs to make laboratory studies more close to the real environmental conditions.

From an ecotoxicological perspective, exposure pathways can influence metal biogeochemical cycling in aquatic systems, but this also receives few investigations for Ag NPs. Comparison of experimental data among different exposure pathways or to a particular field situation is therefore difficult because toxicity tests of Ag NPs rarely account for multiple pathways of exposure, nor do most designs consider nanoparticle behavior under realistic environmental conditions. For instance, a number of aquatic toxicological studies conducted until now focus on the water or food exposure pathway. If lost from suspension, however, Ag NPs may accumulate in sediment, and we know very little about the bioavailability and effects of sediment-associated Ag NPs. Sediment exposure routes are therefore highlighted because these sediment-associated Ag NPs are likely to be ingested by benthic organisms and accumulated.

The use of drastic experimental mixing methods like solvent dispersion, shaking, centrifugation, ultrafiltration and sonication in deionized water in the preparation of NP suspensions under laboratory conditions leads to greater dispersion of NPs probably differing from dispersion in natural waters (Gao et al., 2009). Furthermore, it has been frequently observed that excessively high concentrations of Ag NPs have been administered to organisms that are unlikely to be encountered in the environment or by human consumers (Johnston et al., 2010). As such, we may expect the amounts of Ag NP to be lower in suspension in nature than has been the case in most laboratory studies. Additionally, most studies until now have focused on aquatic vertebrates (i.e., fishes) and cellular indicators in exposure scenarios, and fewer studies have examined toxic consequences in aquatic and benthic invertebrate and levels of individuals and populations in ecosystems, which are sorely needed in future studies.

6. Conclusions

The unique physical-chemical properties of Ag NPs result in different cellular uptake routes, toxic effects and mechanisms involved in aquatic organisms compared to aqueous Ag. However,

the less toxicity of Ag NPs than aqueous Ag in most studies indicates the potential less environmental concern of NPs than ions. Further investigations incorporating the influences of environmental realistic factors and surface modifications/coatings are needed to confirm this. Both the physical and chemical properties of Ag NPs should be systematically and adequately defined in the actual test media, or at a minimum, in stock solutions used for testing prior to toxicological studies and risk assessment. The important role of aquatic and benthic invertebrates as sensitive test organisms is highlighted because they represent crucial species in the transformation, detoxification and cycling of environmentally relevant contaminants discharged into aqueous surroundings, not least of which for contaminants such as Ag NPs which are also likely to partition into sediment compartments. Toxicological uncertainties related to the environmental chemistry of silver, differing biological sensitivities of species and the scarcity of toxicological studies make a conclusive risk assessment for Ag NPs extremely difficult at the present time. Linking molecular changes which are readily measurable with relevant ecological responses would greatly improve the predictive power and relevance of *in vivo* and *in vitro* tests based on molecular responses, but making this link in a realistic manner remains one of the great challenges in ecotoxicology. A more systematic analysis of the factors influencing Ag NP toxicity and of how effects at different levels of biological organization are linked is necessary to provide a robust risk assessment model for Ag NPs.

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