Bull Environ Contam Toxicol (2016) 97:456–462
DOI 10.1007/s00128-016-1883-7

Toxicological Effects Induced by Silver Nanoparticles in Zebra Fish (*Danio Rerio*) and in the Bacteria Communities Living at Their Surface

Carla Bacchetta[1] · Gerardo López[2,3] · Gisela Pagano[2,3] · Diana Tomazi Muratt[4] · Leandro Machado de Carvalho[4] · José María Monserrat[5,6,7]

Received: 31 March 2016 / Accepted: 4 July 2016 / Published online: 8 July 2016

Abstract The antimicrobial activity of silver nanoparticles (AgNP) makes them useful in a wide range of products although their environmental impact is still uncertain. The main goal of this study was to evaluate short-term effects induced by AgNP on gills oxidative status and bacterial communities living at the skin mucus of zebrafish. Both the number of bacteria colony forming units and bacteria growth obtained from skin mucus were lower in all concentrations tested (25, 50 and 100 µg nAg/L). Besides, AgNP exposure caused a significant decrease in bacteria growth in zebrafish exposed to 100 µg nAg/L. AgNP accumulated in zebrafish gills at both highest concentrations tested, but this accumulation did not appear to result in oxidative stress. Overall the results indicated toxicological effects of AgNP on bacteria communities living at the zebrafish mucus surface. Although silver accumulation was verified in gills, no evidence of toxicity in terms of oxidative stress was found.

Keywords Nanomaterials · Nanosilver · Bioaccumulation · Oxidative stress · Antibacterial activity · Mucus

There are many consumer products and applications utilizing silver nanoparticles (AgNP). The Woodrow Wilson database (http://www.nanotechproject.org) has listed 1600 consumer products presently on the market incorporating nanoparticles, with 438 containing AgNP (February 2015). The several AgNP applications are mainly related to the exceptional broad spectrum bactericidal activity of silver (Kim et al. 2007; Marambio-Jones and Hoek 2010). The relatively low cost of manufacturing AgNP has also made them the largest and fastest growing class of nanomaterials in product applications such as plastics, metals, textiles, and in medical and veterinary devices (Ahamed et al. 2010; Messaoud et al. 2010; Rather et al. 2011; Rhim et al. 2013). However, the potential environmental impact of AgNP have yet to be fully understood (Massarsky et al. 2014a), deserving carefully analysis particularly in virtue of their toxic properties.

AgNP may be discharged to the environment by several routes, including during synthesis, manufacturing and incorporation of the nanoparticles into goods, during the use of goods containing nanoparticles, and while recycling or disposal of goods and AgNP (Fabrega et al. 2009). Recent studies reported that AgNP may be released from biocidal plastics, textiles, paints and other home products (Geranio et al. 2009; Benn et al. 2010; Lorenz et al. 2012; Künniger et al. 2014) leaching into the aquatic environment. Although the environmental concentrations of

✉ Carla Bacchetta
carlabacchetta@yahoo.com.ar

1 Instituto Nacional de Limnología (INALI-CONICET-UNL), Paraje El Pozo, Ciudad Universitaria UNL, 3000 Santa Fe, Argentina

2 Nanotek S.A., Santa Fe, Argentina

3 Universidad Tecnológica Nacional, Santa Fe, Argentina

4 Departamento de Química, Universidade Federal de Santa María (UFSM), Santa María, RS, Brazil

5 Instituto de Ciências Biológicas (ICB), Universidade Federal do Rio Grande - FURG, Rio Grande, Brazil

6 Programa de Pós-Graduação Em Ciências Fisiológicas, Universidade Federal de Rio Grande - FURG, Rio Grande, Brazil

7 Programa de Pós-graduação em Aquicultura, Universidade Federal de Rio Grande - FURG, Rio Grande, Brazil

engineered nanoparticles have yet to be measured, Blaser et al. (2008) predicted the surface water concentrations of AgNP to range between 40 and 320 ng/L, what could mean a potential risk to aquatic species.

Some evidence is emerging that metal nanoparticles can affect fish by association with their external surfaces. Attention was thus given to epithelial surfaces such as the gills and the gut. Bilberg et al. (2010) studied the effect of AgNP on oxygen consumption in *Perca fluviatilis* and found that AgNP impaired the tolerance to hypoxia. Scown et al. (2010) showed that AgNP uptake by the gills of rainbow trout induced the expression of oxidative stress related genes. In a recent study, Katuli et al. (2014) demonstrated the potential of AgNP to disrupt the ionoregulation in zebrafish gills.

Fish are in a very close contact with their environment, so the bacterial genera present in the skin mucus seems to be those present in their environment or diet (Benhamed et al. 2014). Nevertheless, the bacterial population present in farmed fish changes depends on the storage conditions (temperature, pH), the diet, and the concentration of pollutants in water (Bernadsky and Rosenberg, 1992). Recent evidence indicates that AgNP changed endogenous microbiota in zebrafish intestine (Merrifield et al. 2013) and decreased bacterial communities living at the surface of the marine polychaeta *Laeonereis acuta* (Marques et al. 2013; Cordeiro et al. 2014). Despite there have been reports of metal nanoparticles effects on fish skin (Zhao et al. 2011; McNeil et al. 2014), the potential damage caused by AgNP on skin bacterial communities have not been investigated. The main goal of this study was to evaluate changes induced by AgNP on gills oxidative status and bacterial communities living at the skin mucus secretion of adult zebrafish.

Materials and Methods

A colloidal suspension of 1 % w/v nanoparticulate silver was synthesized and provided by Nanotek S.A., manufacturing the product under the brand name nanArgen®. Further details about AgNP preparation and characterization are explained in Marques et al. (2013).

All procedures in the toxicological assay with fish were in accordance with the EU Directive 2010/63/EU for animal experiments. Adult zebrafish (*D. rerio*; n = 100; mean mass: 0.71 ± 0.24 g) were purchased from a local commercial source and were acclimated for at least one week and fed for twice a day. Fish were placed in 60 L tanks. The pH and water temperature were maintained at 7.5–8.0 and 28°C, respectively. Fish (25 per treatment) were individually (one per aquarium) exposed for 24 h to 25; 50; and 100 µg nAg/L, plus a control group that was run in parallel, employing water with the same characteristics cited above but without AgNP. No mortality was observed during the acclimation period or toxicological trial. After 24 h, all fish were euthanized and their gills were dissected. Fish were not fed 24 h prior or during the experiment.

Bacterial communities living in the mucus of zebrafish were isolated and analyzed according to Marques et al. (2013). Number of colony forming units (CFU) and bacteria growth were measured.

For antioxidant and oxidative damage measurements, gills were dissected and homogenized (1:10) in Tris–HCl (100 mM, pH 7.75) buffer plus EDTA (2 mM) and Mg^{2+} (5 mM) (Gallagher et al. 1992). Samples were centrifuged at 10,000×*g* for 20 min at 4°C and the supernatant was reserved for biochemical measurements as performed previously by Da Rocha et al. (2009). Total protein content was determined through the Biuret method (550 nm), in triplicate, using a microplate reader (BioTek LX 800). Total antioxidant competence against peroxyl radicals was evaluated through ROS determination in gills samples of fish treated or not with a peroxyl radical generator, 2,2′-azobis 2 methylpropionamidine dihydrochloride (ABAP; 4 mM; Aldrich), according the methodology proposed by Amado et al. (2009). The fluorescence was registered after excitation at 485 nm and emission of 530 nm. The relative difference between ROS area with and without ABAP is an estimate of total antioxidant capacity against peroxyl radicals, with high area difference meaning low antioxidant capacity. Further details can be found in da Rocha et al. (2009). Lipid peroxidation was measured through determination of thiobarbituric acid reactive substances (TBARS), following the methodology of Oakes and Van Der Kraak (2003) and adapted to a microplate reader by da Rocha et al. (2009). The fluorescence was registered after excitation at 520 nm and emission of 580 nm. The concentration of TBARS (nanomoles/mg of wet tissue) was calculated employing tetramethoxypropane as a standard.

Gill samples were digested and total silver concentration was measured by atomic absorption spectrophotometry using methods described by Marques et al. (2013). The recovery for Ag using this extraction procedure was 95.05 % (n = 5) for an Ag concentration of 25 µg/L added to samples submitted to the digestion procedure. Recovery experiments were carried out by using a NIST reference standard of Ag (SRM 3151). Determination of Ag in the digested samples was made by using an external calibration curve ranging from 5.0 to 30.0 µg/L of Ag. Here, Aa stock solution of Ag 1.0 g/L was diluted to 10 mg/L, so that it was used for use as a working intermediate solution for preparing the calibration curve. Measurements were performed in a Analytikjena-ZEEnit 600 atomic absorption spectrometer (Analytikjena, Germany), equipped with an auto-sampler MPE 60.

CFU, bacteria growth, total antioxidant capacity, lipid peroxidation and silver accumulation in gills were analyzed through analysis of variance (ANOVA), where the factor was AgNP concentration (control, 25, 50 and 100 µg nAg/L). ANOVA assumptions (normality and variance homogeneity) were checked and mathematical transformation applied if one of them was violated. Post-hoc comparisons between treatments were performed using Newman–Keuls method. In all cases a significance level of 5 % was employed.

Results and Discussion

Figure 1 shows the TEM images for AgNP. AgNP images showed spherical nanoparticles of an average size ranging between 20 and 25 nm.

The environmental chemistry and ecotoxicity of engineered nanoparticles has recently been reviewed (see Handy et al. 2008). The chemistry shares some superficial similarities with the metal in those abiotic factors such as pH, the presence of divalent ions, and ionic strength which can influence the colloidal behavior (aggregation) of nanoparticles. However, the reasons for these interactions are often fundamentally different to those for dissolved metals. Current metal speciation models are equilibrium models, whereas the behavior of nanoparticles is described in a very different way by a dynamic process where the system is dependent on the amount of energy added to the nanoparticles dispersion and the physico-chemical properties of the particles. These physico-chemical properties, which include particle size, shape, and surface charge, coating material, and silver ions release, are a key when nanometals toxicity is analyzed. Some studies have found that AgNP was less toxic than dissolved Ag to adult zebrafish (Griffitt et al. 2008; Bilberg et al. 2012). In contrast, in a study on juvenile Japanese medaka (*Oryzias latipes*), Chae et al. (2009) reported that nanosilver was more toxic than ionic silver after 24 h of exposure, but after 96 h their toxicity effects were similar.

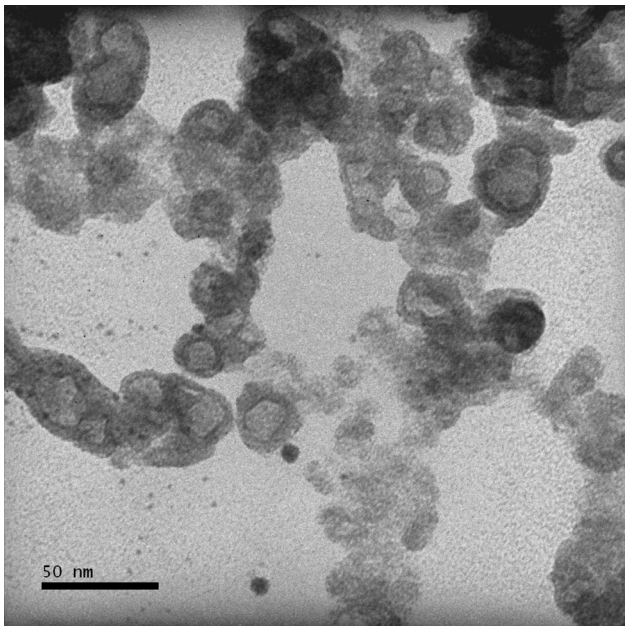

Fig. 1 Transmission electron microscopy image of the aqueous nanosilver (AgNP) suspensions employed in the bioassay

The first line of fish immune defensive mechanisms against invading pathogens and parasites is the mucus membrane layer of the gills, skin and intestines (Jovanović and Palić, 2012). Despite the presence of a number of antimicrobial factors within the epidermal mucus, fish are still colonized by bacteria, leading to the development of a biofilm and the formation of a microbial community (Wilson et al. 2008). Both the number of bacterial CFU and bacteria growth obtained from samples of the skin mucus of zebrafish exposed to AgNP during 24 h were lower in all concentrations tested when compared with the control group (Fig. 2). Soltani et al. (2009) demonstrated the inhibitory effects of AgNP fish pathogenic bacteria in vitro. Evidence suggests that AgNP may accumulate in fish skin and modulate the phosphotyrosine profile of putative bacterial peptides that could affect cellular signaling and therefore inhibiting bacteria growth (Shrivastava et al. 2007; Wijnhoven et al. 2009). Most engineering nanoparticles are colloidal and microorganisms lack of uptake mechanism for colloidal and complex particulate materials (Eduok et al. 2013). Pinocytosis, the uptake mechanism used by eukaryotic cells (see for example AshaRani et al. 2009; Sakhtianchi et al. 2013), cannot explain the nanoparticles absorption in the case of bacteria. One possible route for nanoparticle ingress is via various pores in the outer membrane (Neal 2008). Therefore nanoparticles are suspected to exert their toxic effect by solubilizing ions that enter the cell by oxidative disruption of the cell membrane. The mechanism of action attributed to release of Ag^{+} ions from AgNP was demonstrated in *Escherichia coli* and resulted to be dependent on concentration and contact time. Adverse effects such as membrane leakage of sugars and proteins, enzyme inhibition, cell disruption, and scattered vesicles which slowly dissolve are known to inhibit cellular respiration and cell growth (Li et al. 2010). Besides, some authors have recently explored mucus production in *Pimephales promelas* exposed to silver nitrate and a similar size of silver nanoparticles (20 nm). Garcia-Reyero et al. (2015) found that mucus production was increased by silver after 1–4 h, but significantly decreased by 25 h of exposure. Similar results were found by Hawkins et al. (2014). In spite of biocides properties on AgNP, the decrease in mucus production in the fish surface may be

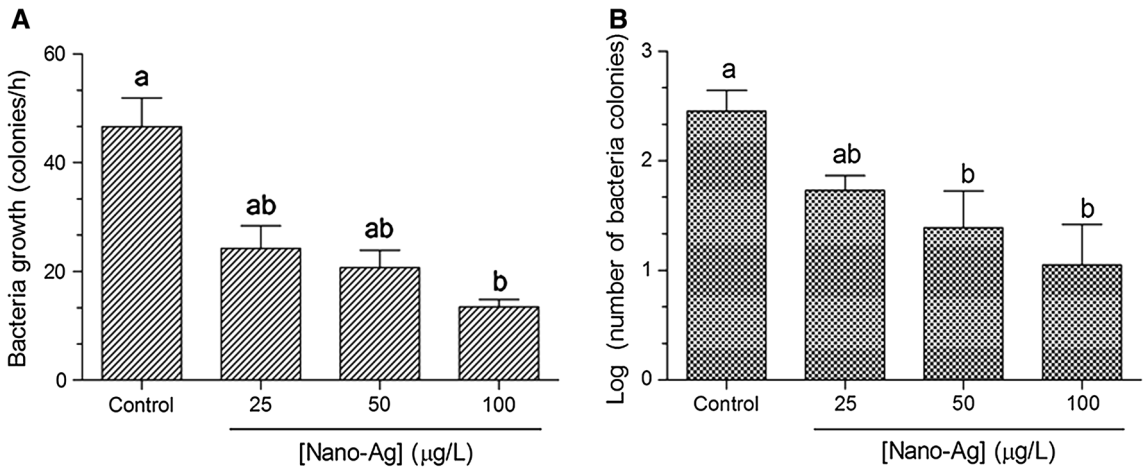

Fig. 2 Bacteria growth (CFU/hour) obtained from samples of the mucus of zebrafish exposed to AgNP (**a**). Colony forming units (CFU) obtained from samples of the mucus of zebrafish exposed to silver nanoparticles (AgNP) (**b**). Means not sharing the *same letter* (*a* or *b*) are significantly different at $p < 0.05$

directly connected with the decrease in bacteria growth found in the present study.

Bioaccumulation is an important process to quantify when evaluating hazard and risks from nanomaterials (Fabrega et al. 2011). In this study, adult zebrafish were exposed to sub-lethal concentrations of AgNP for 24 h. The organisms were examined for alterations in gills tissue burden, antioxidant competence against peroxyl radicals, and lipid peroxidation levels. Our results indicated that AgNP does accumulate in zebrafish gills at both highest concentrations tested (50 and 100 µg nAg/L) (Fig. 3). In comparison with the effects of nanometals with dissolved metals, Griffitt et al. (2009) found both dissolved and nanoparticulate forms of Ag increased metal levels in the gill tissue after 48 h. Branchial Ag levels were much greater in fish exposed to AgNP than those exposed to dissolved Ag, and this situation means

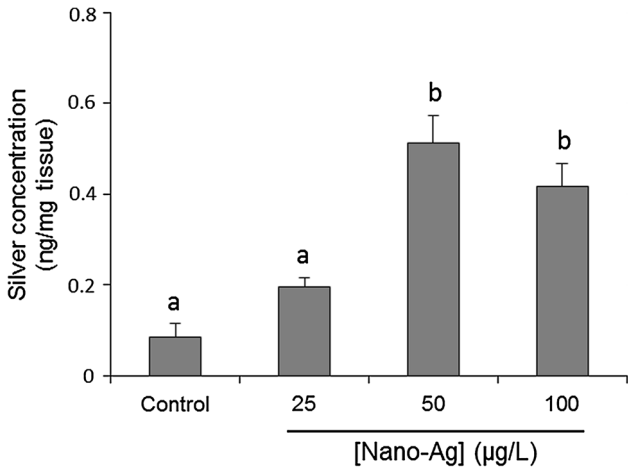

Fig. 3 Silver concentration (ng/mg of wet tissue) in gills of zebrafish exposed to silver nanoparticles (AgNP). Means not sharing the *same letter* (*a* or *b*) are significantly different at $p < 0.05$

that the nanoparticles themselves were contributing to the branchial Ag burden and they are more easily incorporated. According to Wu and Zhou (2013), there are several mechanisms that enhance gill AgNP content. For example, nanoparticles can be trapped in the mucus layer or been taken up by the gill epithelial cells. In this study, no differences were found between silver content measured at 50 and 100 µg nAg/L treatments. Nel et al. (2006) showed that lower nanomaterial concentrations should have smaller aggregates that could be more available for fish uptake. In the same way, Tiede et al. (2009) suggest that it is possible to observe higher toxicity at lower test concentrations because the extent of aggregation can be likely reduced, leaving free particles in un-aggregated form. The biological uptake and unstirred layer chemistry of nanoparticles in fish gills has been recently reviewed in detail and the main differences between metal ions and nanoparticles were summarized by Shaw and Handy (2011). The central issue is that nanoparticles are too big to use ion transporters, or paracellular diffusion pathways, and that the most likely route of uptake is by endocytosis. Choi et al. (2010) reported silver accumulation on 0.29 and 2.4 ng/mg of wet tissue in liver of zebrafish exposed to AgNP concentration of 30 and 120 mg nAg/L, respectively. On the other hand, Griffitt et al. (2013) actually measured accumulation in gills of zebrafish exposed to 50 µg nAg/L, but the time of exposure was longer than 24 h (7 days), reporting silver concentrations of 5 ng/mg of wet tissue. To the best of our knowledge, this is the first report of bioaccumulation in zebrafish gills after a short-term (24 h) exposure to low concentrations of AgNP.

Despite the silver content levels detected in the gills, no oxidative stress was observed (Table 1). Several authors registered ROS formation and increased lipid peroxidation in

Table 1 Total antioxidant capacity against peroxyl radicals (relative area difference) and concentration of thiobarbituric acid reactive substances (TBARS: nmoles/mg of wet tissue) in gills of zebrafish exposed to silver nanoparticles (AgNP)

	Control	AgNp concentration		
		25 μg/L	50 μg/L	100 μg/L
Total antioxidant capacity (relative area difference)	3.70 ± 1.18[a]	2.51 ± 0.68[a]	2.74 ± 0.55[a]	3.65 ± 1.10[a]
TBARS concentration (nmoles/mg of wet tissue)	0.71 ± 0.14[a]	0.90 ± 0.20[a]	0.86 ± 0.11[a]	0.72 ± 0.14[a]

The values are expected as mean ± SE

Means not sharing the same superscript (a or b) in each column are significantly at $p < 0.05$

fish exposed to AgNP in both in vitro and in vivo studies (Choi et al. 2010; Gagné et al. 2012; Wu and Zhou 2012; Massarsky et al. 2013, 2014b; Taju et al. 2014). The absence of lipid peroxidation occurrence observed in this study might be due to the short exposure duration or to differences in particles and cell types used. Recently, Wu and Zhou (2013) reported epithelial hyperplasia and increased mucus generation in medaka exposed to AgNP, which resulted in an alteration of nanosilver uptake by the gills. Mucus hypersecretion, increased ventilation rate, and increased surface respiration have also been observed in zebrafish exposed to PVP-AgNP (Bilberg et al. 2012). In this context, mucus layer covering zebrafish gill epithelia may act as a barrier, preventing nanoparticles uptake by the gills. However, the obtained results indicated that AgNP exposure had not a clear response in the assayed parameters but probably other gill parameters could be affected.

In conclusion, the rapid advances in the understanding and manipulation of AgNP undoubtedly will continue to sustain the explosive growth of products incorporating them. Thus, it is expected a corresponding increase in AgNP release into the environment. Without quantitative measures of both exposure and effects, ecological risk assessment cannot be conducted, and regulators will not have the tools to adequately manage AgNP burdens. Our results showed that AgNP exposure may affect fish skin and the bacteria communities living at their surface and compromise fish immunological capacities. Another short term effects was the significant accumulation of silver in the fish gills, despite it could not be associated with alterations in oxidative stress parameters.

Acknowledgments L. Machado de Carvalho and J.M.Monserrat are research fellows from Brazilian CNPq. The work was supported by Nanotoxicology Network (MCTI/CNPq Process Number 552131/2011-3).

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