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Natural marine bacteria as model organisms for the hazard-assessment of consumer products containing silver nanoparticles

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ABSTRACT

Scarce information is available regarding the fate and toxicology of engineered silver nanoparticles (AgNPs) in the marine environment, especially when compared to other environmental compartments. Hence, the antibacterial activity of the NM-300 AgNPs (OECD programme) and a household product containing colloidal AgNPs (Mesosilver) was investigated using marine bacteria, pure cultures and natural mixed populations (microcosm approach). Bacterial susceptibility to AgNPs was species-specific, with Gram negative bacteria being more resistant than the Gram positive species (NM-300 concentration used ranged between 0.062 and 1.5 mg L^{-1}), and the Mesosilver product was more toxic than the NM-300. Bacterial viability and the physiological status (O_2 uptake measured by respirometry) of the microbial community in the microcosm was negatively affected at an initial concentration of 1 mg L^{-1} NM-300. The high chloride concentrations in the media/seawater led to the formation of silver-chloro complexes thus enhancing AgNP toxicity. We recommend the use of natural marine bacteria as models when assessing the environmental relevant antibacterial properties of products containing nanosilver.

1. Introduction

The usefulness of silver as an antimicrobial agent, especially silver nitrate salt ($AgNO_3$), has been known for centuries (Klasen, 2000). Increasingly, the use of silver nitrate is being replaced by nanosilver as the antimicrobial agent in a wide range of applications (e.g. wound dressings and antimicrobial surface coatings). As a result, the incorporation of nanosilver has increased during the last decade (Grand View Research, 2015) not only in medical and industrial products, but also in items of domestic use such as clothing, cosmetics and cleaning agents. In the European market for instance up to 379 products incorporating nanosilver have been identified[2] (*The Nanodatabase, accessed 29/07/2017*) (Foss Hansen et al., 2016).

The antibacterial activity of AgNPs depends on their physico-chemical properties which are largely determined by the respective environmental conditions. Aerobic conditions enhance AgNPs dissolution due to nanoparticle oxidation (Liu and Hurt, 2010; Molleman and Hiemstra, 2015). The dissolution phenomena increases the antibacterial activity of AgNPs due to the release of ionic silver (Xiu et al., 2012) and the formation of reactive oxygen species (ROS) (Joshi et al., 2015). Other nanoparticle-effects that can enhance the antibacterial properties of AgNPs have been proposed, such as membrane disruption due to nanoparticle-membrane interaction (Taglietti et al., 2012), a process that may also enhance the uptake of silver ions (Bondarenko et al., 2013). AgNPs contained in products marketed as cleaners and antimicrobials cannot be disposed of safely, because they are directly released into the sewage system (Brar et al., 2010). As a result, silver could reach the natural aquatic environment, including estuarine and coastal

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waters, if it is not efficiently removed during water treatment (McGillicuddy et al., 2017; Sun et al., 2016).

Previous work has shown that marine organisms in different trophic levels were susceptible to AgNPs in a dose dependent manner (Gambardella et al., 2015) and negatively affected the bacterial community function, crucial for nutrient cycling and bioremediation, in estuarine sediments (Echavarri-Bravo et al., 2015). In the marine environment the high concentration of chloride favours AgNPs dissolution, and the formation of bioavailable silver-chloro complexes (which can be accumulated in aquatic invertebrates (Kalman et al., 2010)) are also toxic for bacteria (Gupta et al., 1998). As the antibacterial activity of AgNPs is species-specific (Morones et al., 2005; Tamboli and Lee, 2013) the present work investigates the effect of a well characterised AgNP type, the NM-300 (OECD programme) on the growth of Gram positive and Gram negative benthic marine bacterial species. The effect of the NM-300 AgNPs on planktonic marine bacteria was also analysed with a microcosm approach to study the response of mixed populations of natural bacteria under more environmentally relevant conditions. In addition, the physicochemical properties of the AgNPs contained in a household product (Mesosilver Hot tub ™) were characterised as well as its antibacterial properties. Our hypothesis was that growth inhibitory concentrations (IC) of AgNPs would be lower for marine bacterial species than for non-marine bacteria due to the chemical conditions in the marine environment. For instance the high concentration of chloride in the marine environment is known to enhance the antibacterial activity of silver due to the formation of bioavailable silver chloro complexes (Gupta et al., 1998; Levard et al., 2013; Luoma et al., 1995). This hypothesis was tested by comparing the IC of the NM-300 AgNPs for marine bacteria reported in the present work with previous ICs and/or ECs (effective concentrations) of the NM-300/NM-300K series reported by other authors who used different bacterial strains or mixed bacterial populations from very different environments, such as intestines and waste water treatment plants (WWTPs).

2. Materials and methods

2.1. Isolation and identification of marine bacteria

Surface water (31‰ salinity) and intertidal sandy sediment samples (depth < 1 cm) were collected from the Firth of Forth estuary (Scotland, United Kingdom). Bacterial isolates were subsequently obtained from low nutrient agar medium consisting of ZM/10 agar (Green et al., 2004) (75% sand-filtered natural seawater, 0.05% Bacteriological Peptone (Oxoid), 0.01% yeast extract (Oxoid), 1.4% Bacteriological Agar (Oxoid)). Isolates were selected based on their colony morphology followed by other phenotypic analyses (Gram stain, motility) and enzyme production (catalase, oxidase and agarase activity). Bacterial isolates were identified to species level based on a partial sequence of the 16S rRNA gene. Two genus-specific sets of primers (set 1: 27F/685R; set 2: 341F/A976R) were used. The sequences of each primer are provided in the supplementary information (SI). DNA sequences were edited with the BioEdit Sequence Alignment Editor (v7.0.9) followed by the sequence analysis with Basic Local Alignment Search Tool (BLAST).

2.2. Toxicity tests

2.2.1. Preparation and characterization of AgNPs working suspensions

The NM-300 AgNPs, purchased as a suspension from LGC standard (composition: AgNPs 10% (w/w), polyoxyethylene glycerol trioleate and Tween 20 as stabilizing agents (all at 4% w/w) and 7% NH_4NO_3), were prepared and characterised in Milli-Q water as described by us in previous work (Echavarri-Bravo et al., 2015). The Mesosilver Hot tub™ cleaner was supplied in an aqueous suspension at a concentration of 200 mg L^{-1} colloidal silver (value provided by the supplier). However, using Atomic Absorption Spectroscopy (AAS) and a selective ion electrode (ISE, Nico, 2000 Ltd), in the present study the concentration of total silver in the Mesosilver suspension was found to be only 116 mg L^{-1}, 42% lower than the nominal concentration reported by the supplier.

2.2.2. Effects of AgNPs on marine bacteria

2.2.2.1. Effects of NM-300 and Mesosilver on single bacterial strains. Bacterial strains (*Pseudoalteromonas aliena, Cellulophaga fucicola, Arthrobacter agilis* and *Streptomyces koyangensis*) were chosen to develop toxicity tests with the well characterised NM-300 AgNPs based on the different characteristics of their respective bacterial envelopes (Gram positive/Gram negative), sizes and cell morphologies (information depicted in Table S1). The antibacterial activity of the Mesosilver suspension was assessed with three strains (*P. aliena, C. fucicola, A. agilis*) as these species represent potential model organisms to assess AgNPs toxicity due to their cell growth can be monitored in a cost-efficient way by measuring the absorbance of the cell culture. The growth of *S. koyangensis* could not be monitored by measuring the OD_{600} as this species formed clumps. The exposure of pure bacterial cultures to AgNPs was carried out in low nutrient liquid ZM/10 medium with the aim to minimise the concentration of compounds that exhibit high affinity by ionic silver such as thiol groups (-SH). Preliminary experiments were carried out in small glass test tubes (75 mm × 12 mm, 3 ml of broth) to screen rapidly for bacterial inhibitory concentration values of NM-300 AgNPs and $AgNO_3$ ($AgNO_3$ as a source of ionic silver). Thereafter the exposures were developed in 50 ml conical flasks containing 40 ml of ZM/10 (n = 3) and a range of concentrations of the NM-300 based on the inhibitory concentrations observed in our preliminary experiments. The concentrations tested ranged between 0.062 mg L^{-1} and 1.5 mg L^{-1} depending on the bacterial species. The antibacterial activity of Mesosilver was examined at a single concentration to compare it with the same concentration of the NM-300. The concentration was chosen based on the IC_{50} value observed for the NM-300 during our preliminary experiments. More detailed information about how the concentration of the Mesosilver was calculated is provided in section 3.1 of the SI (Table S2). Flasks were inoculated with pure cell cultures in stationary phase (dilution 1:100, in the order of 10^6 colony forming units (CFU) per ml), incubated at 25 °C in the dark and shaken continuously at 125 rpm on an orbital shaker. Growth inhibition was monitored by measuring OD_{600} with a Shimadzu 1650 UV-VIS Spectrophotometer. The IC_{50} (mg L^{-1}) values were calculated based on the OD_{600} measurements registered during the exponential growth phase using a graphical interpolation approach. The endpoint of the exposures was established when the bacterial growth in the control treatments (without AgNPs) reached the stationary phase. The growth of *S. koyangensis* was quantified by the production of nitrogen (N) analysed using the Kjeldahl method (Youmans, 1946) as an indicator of protein production. The endpoint for the exposures with *S. koyangensis* was established after 48 h of incubation.

The bacterial cell viability of the Gram positive strain *A. agilis* and the Gram negative strains *C. fucicola* and *P. aliena* was assessed by counting the CFU developed after exposure to NM-300. In short, bacterial aliquots collected from the experimental flasks at different time points were plated in solid medium (75% sand-filtered natural seawater, 0.5% Tryptone, 0.25% yeast extract, 1.4% Bacteriological Agar, free of AgNPs), and incubated at 20 °C for 3–4 days.