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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

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.

2.2.2.2. Microcosm experiments with the NM-300 AgNPs. The experimental design of the microcosm, including sample collection and preservation, chemical analysis and the study of the fate of the NM-300 in the water column has been described previously by us (Echavarri-Bravo et al., 2015). Briefly, the microcosm was established with seawater and sediment samples collected from the Firth of Forth estuary (Scotland). Each tank contained 3 L of water (salinity 31‰, temperature 10 °C, pH 7.9, dissolved oxygen 7.8 mg L^{-1}, initial chemical oxygen demand (COD) 26. 55 mg L^{-1}). The initial concentration of NM-300 added to the water column was 1 mg L^{-1} (total silver concentration measured by AAS) based on previous studies (Bradford et al., 2009; Colman et al., 2014). The concentration of total silver decreased to 0.08 mg L^{-1} within 24 h and remained below the detection limit (0.03 mg L^{-1}) for the final next three days of the 5 day experiment (Echavarri-Bravo et al., 2015).

Three conditions were established: the NM-300 treatment containing dispersant (T1), the carrier control (T2) containing dispersant only (polyoxyethylene glycerol trioleate and Tween 20 (all at 4% w/w) and 7% NH_4NO_3), and the negative control (TC). Further details can be found in our previous work (Echavarri-Bravo et al., 2015).

Total microbial abundance in the water column was quantified at different time points (0, 24, 72 and 120 h) for all treatments by direct counts using epifluorescence microscopy and DAPI (4′, 6-diamidino-2-phenylindole) staining as described previously (Pernthaler et al., 2001). The viability of heterotrophic bacteria was monitored with the plate count method in slightly modified Marine Agar (MA, Difco, Detroit, MI, USA) from Lebaron (Lebaron et al., 2000). In short, the media composition of the MA was 0.25% peptone, 0.05%, yeast extract 17.5% sand-filtered natural seawater, 1.4% Bacteriological Agar. Plates were incubated at 15 °C and CFU counted after 8 days of incubation.

2.2.2.3. Respirometry assay. This assay was applied to study the physiological status (by means of O_2 uptake) of single bacterial species and also mixed populations present in the water samples collected from the microcosm. One of the advantages of the respirometry assay performed in the present study was that it provided information about the bacterial immediate response to AgNPs exposure as measurable differences in O_2 were observed within 0.5–1 h. The instrument used was the Strathtox™ respirometer (Strathkelvin Instruments, North Lanarkshire, UK) equipped with six microcathode oxygen electrodes to measure dissolved O_2 (mg $L^{-1} \times$ h) and continuously monitor respiration in individual glass tubes (Aspray et al., 2007).

2.2.2.4. Exposures with single bacterial strains. The bacterial cultures were prepared in the same way as the flasks (section 2.2.2.1) to analyse the effects of the NM-300 on bacterial growth, but in a final volume of 20 ml (according to the capacity of the vessel). The O_2 consumption in the pure bacterial cultures was measured over 1 h following a 1 h incubation period.

2.2.2.5. Microcosm exposures. A respirometry assay was applied to measure the O_2 uptake of the whole microbial community inhabiting the water column, providing an insight into the overall physiological status of the community, including non-culturable aerobic bacterial groups. Water samples (20 ml) were collected from the microcosm tanks at different time points during the course of the microcosm exposures and the O_2 consumption in each sample was registered for 0.5–1 h.

2.3. Nanoparticle characterization and persistence in relevant media

Nanoparticle size in bacterial broth media (ZM/10) was analysed at two different time points (0 and 24 h) by Transmission Electron Microscopy (TEM, Jeol 1200 Microscope) and Atomic Force Microscopy (XE-100 Microscope) at the Facility for Environmental Nanoscience Analysis and Characterization (FENAC, University of Birmingham). The hydrodynamic diameter and the surface charge or zeta potential were monitored for up to 48 h in ZM/10 (pH ranged between 7.5 and 8) and 0 and 20 g L^{-1} NaCl (equivalent concentration of chloride in the ZM/10 containing 75% seawater, pH 7.5, 20 mM HEPES) using Zetasizer Nano-ZS (Malvern Instruments). The effects of NaCl alone, (0, 10 and 20 g L^{-1}) to investigate the effects of chloride on the fate and persistence of NM-300 in the microcosm, have been covered in previous work (Echavarri-Bravo et al., 2015).

The chemical speciation of AgNPs in ZM/10 medium and in the microcosm water samples was calculated with Visual MINTEQ (version 3.1).

2.4. Statistical analysis

The statistical analysis was performed with SigmaPlot® 13.0 (Systat Software). Normality of the data was assessed using a Kolmogorov-Smirnov test. The differences in nanoparticle size between samples were analysed with a Mann-Whitney test for independent samples when data did not follow a normal distribution. Data relating to bacterial abundance obtained with the bacterial plate counts and epifluorescence microscopy counts were log10-transformed prior to ANOVA analysis followed by a Bonferroni Post-Hoc test. The differences between treatments relating to the O_2 uptake rate were analysed with an independent *t*-test.

3. Results

3.1. Physicochemical properties of NM-300 and Mesosilver AgNPs

3.1.1. Characterization of AgNPs in Milli- Q water and bacterial broth medium (ZM/10)

The set of techniques developed in the present study confirmed the presence of AgNPs in the Mesosilver product. NM-300 and Mesosilver AgNPs were characterised in Milli-Q water and a summary of their physicochemical characteristics is provided in Table 1.

Both AgNP types were negatively charged (negative zeta potential) and showed a similar hydrodynamic diameter (Z-average). The Mesosilver nanoparticle size distribution in Milli-Q water, analysed by AFM and TEM, is available in the supplementary material, Fig. S1 A-B. NM-300 size distribution, analysed by AFM, is depicted in Fig. S1 C (TEM nanoparticle distribution for NM-300 is provided in our previous work (Echavarri-Bravo et al., 2015)). TEM images of NM-300 and Mesosilver AgNPs and their absorbance spectrum in the UV-vis range are depicted in Figs. S2 and S3, respectively. The concentration of ionic silver measured using an ISE was greater in Mesosilver (11.63%) than NM-300 (4.93%). The total concentration of silver in the Mesosilver product measured by AAS was 116 mg L^{-1}, 42% lower than the concentration reported by the manufacturer.

The NM-300 and Mesosilver AgNPs particle size in bacterial media (ZM/10) was characterised by AFM at two time points, 0 and 24 h (Table 2).

The nanoparticle size analysed by AFM was not statistically significantly different (Mann-Whitney *U* test, p > 0.05) between both time points (0 and 24 h) (size distribution available in the supplementary information, Figs. S4 and S5). AFM images show

Table 1
Physicochemical properties of the NM-300 and Mesosilver AgNPs in Milli-Q.

AgNP type	Z-average (nm)*	zeta potential (mV)*	Size (TEM) (nm)	Size (AFM) (nm)	% ionic silver	UV-vis peak absorbance (nm)
NM-300	58.2 ± 2.7	−22.3 ± 3.3	18.2 ± 7.3[a]	8.9 ± 8.6	4.93	~ 412
Mesosilver Hot tub	51.0 ± 18.8	−28.9 ± 4.8	14.0 ± 6.9	10.5 ± 8.9	11.63	~ 394

*Values expressed as Mean ± SD (n = 3).
[a] (Echavarri-Bravo et al., 2015).

Table 2
Size and surface charge of NM-300 and Mesosilver AgNPs in ZM/10 media.

NP type	Time (h)	Z-average (nm)*	PDI	Zeta potential (mV)*	TEM(nm)	AFM (nm)
NM-300	0	52.1 ± 1.6	0.35	−5.1 ± 2.6	17.8 ± 6.5	13.6 ± 8.7
NM-300	24	595.6 ± 56.6	0.50	−4.3 ± 0.2	20.8 ± 9.6	13.1 ± 7.6
Mesosilver	0	776.7 ± 197.8	0.47	−12.1 ± 3.4	N/A	6.3 ± 3.0
Mesosilver	24	1558.6 ± 1094.6	0.83	−6.5 ± 1.3	N/A	6.6 ± 4.7

*Values expressed as Mean ± SD (n = 3).
N/A: not available.

that NM-300 particle density was higher at 0 h than after 24 h (SI Fig. S6 A-B, respectively) whereas this was not observed for the Mesosilver. The presence of some agglomerates, a few microns in size, was observed by AFM in the NM-300 sample (SI Fig. S6 B3) after 24 h. The analysis of NM-300 size measured by TEM showed similar size distribution although the statistical analysis of the nanoparticle size between 0 and 24 h indicated that mean particle size was significantly larger after 24 h in ZM/10 (Mann-Whitney U test, $p < 0.001$) (SI, Fig. S7, 17.8 nm at 0 h and 20.8 at 24 h).

At time 0 h, Mesosilver showed higher Z-average (hydrodynamic diameter size) in ZM/10 than in the absence of chloride (NaCl $0 \, g \, L^{-1}$) whereas for NM-300 this increase in size was observed only after 24 h in ZM/10 (Fig. S8). The hydrodynamic diameter (Z-average) of both AgNP types increased after 24 h in ZM/10 and $20 \, g \, L^{-1}$ NaCl indicating particle agglomeration. The hydrodynamic diameter increased more in broth ZM/10 (75% seawater) than in media containing only NaCl (Fig. S8). The absolute value of the nanoparticles surface charge, measured as zeta potential, decreased immediately upon introduction to media containing NaCl and in ZM/10 for both NM-300 and Mesosilver (Fig. S9). The polydispersity index (PDI) > 0.5 for Mesosilver after 24 h in ZM/10 (Table 2) indicated a broad nanoparticle size distribution. Thus the Z-average value analysed for Mesosilver at 24 h in ZM/10 was not reliable and the information provided by the other complementary techniques (e.g. electronic microscopy and zeta potential) was required to characterise the fate of Mesosilver in ZM/10.

3.1.2. Chemical speciation of AgNPs in ZM/10 media and seawater

The chemical speciation of silver dissolved from AgNPs in ZM/10 media (containing 75% seawater, equivalent to $20 \, g \, L^{-1}$ of NaCl) and seawater (the salinity measured in the water of the microcosm was $31 \, g \, L^{-1}$) was calculated with Visual MINTEQ (3.1, KTH, Sweden) (António et al., 2015). The simulation output data indicated that Na^+, Cl^- and Ag^+ ionic species dissolved completely in ZM/10 and in the microcosm, even at the highest concentration of AgNPs used during the toxicity tests in ZM/10, $1.5 \, mg \, L^{-1}$. The main silver chloro-complexes formed in ZM/10 (based on the speciation modelling analysis) were $AgCl_2^-$ (64.06%), followed by $AgCl_3^{2-}$ (33.78%) and AgCl (aqueous form, 2.16%) under the following conditions: $1.5 \, mg \, L^{-1}$ of AgNPs, pH 7.5 (the pH in the bacterial cultures remained above 7.5) and temperature 25 °C. The simulation output data in the water column of the microcosm (conditions: $1 \, mg \, L^{-1}$ AgNP, salinity $31 \, g \, L^{-1}$, pH 7.9 remained constant for the 120 h exposure, temperature 10 °C) indicated that the silver chloro-

complex $AgCl_3^{2-}$ was the most abundant chemical species formed (52.95%), followed by $AgCl_2^-$ (46.06%) and AgCl (aqueous form, 1.01%).

### 3.2. Effects of AgNPs on single bacterial strains

The effects of the NM-300 on bacterial growth inhibition, cell viability and bacterial respiration was investigated with pure cultures of two Gram positive and two Gram negative bacteria identified at species level based on a partial sequence of the 16S rRNA gene (the partial sequence and the accession number of the closest relative is available in the SI). The effects of the Mesosilver on bacterial growth was also examined.

3.2.1. Growth inhibition

The growth of bacterial cultures under exposure to a range of concentrations of NM-300 and Mesosilver was monitored by measuring OD_{600}. $AgNO_3$ and NM-300 toxicity tests, performed in small test tubes, showed that ionic silver (supplied as $AgNO_3$) was always more toxic than the NM-300. The IC_{50} of the $AgNO_3$ for each species was as follows: *A. agilis* $IC_{50} < 0.01 \, mg \, L^{-1}$, *C. fucicola* $IC_{50} < 0.25 \, mg \, L^{-1}$ and *P. aliena* $IC_{50} < 0.05 \, mg \, L^{-1}$. Overall, it was observed that at increasing concentrations of the NM-300 the lag phase was extended. In addition, the toxicity of NM-300 was species-specific: the Gram positive strain *A. agilis* was the most susceptible strain, and the Gram negative *P. aliena* the most resistant. The IC_{50} (concentration that inhibited 50% growth) of NM-300, based on the OD_{600} measurements, was $0.145 \, mg \, L^{-1}$ for *A. agilis* (Fig. 1A) (after 21 h incubation) and $0.544 \, mg \, L^{-1}$ for *C. fucicola* (Fig. 1B) (after 13 h incubation), and above $1 \, mg \, L^{-1}$ for *P. aliena* (Fig. 1C) after 12 h incubation. The exposure times differed between the bacterial strains, *P. aliena* had the shortest generation time. The Mesosilver product inhibited the growth of the three strains at lower concentrations than the NM-300, as low as $0.072 \, mg \, L^{-1}$ in the exposures with the bacterium *A. agilis*. A non-linear concentration-dependent effect of the NM-300 on the growth of *S. koyangensis* (growth analysed as total Nitrogen μg ml^{-1} of cell culture, Fig. 1D) was observed, but the results obtained showed that the growth of this Gram positive strain was reduced 52% after exposure to $0.50 \, mg \, L^{-1}$ of NM-300 and was statistically significantly lower than the control (*t*-test, p-value = 0.04).

3.2.2. Bacterial cell viability after exposure to NM-300

To investigate the effects of the NM-300 on bacterial cell
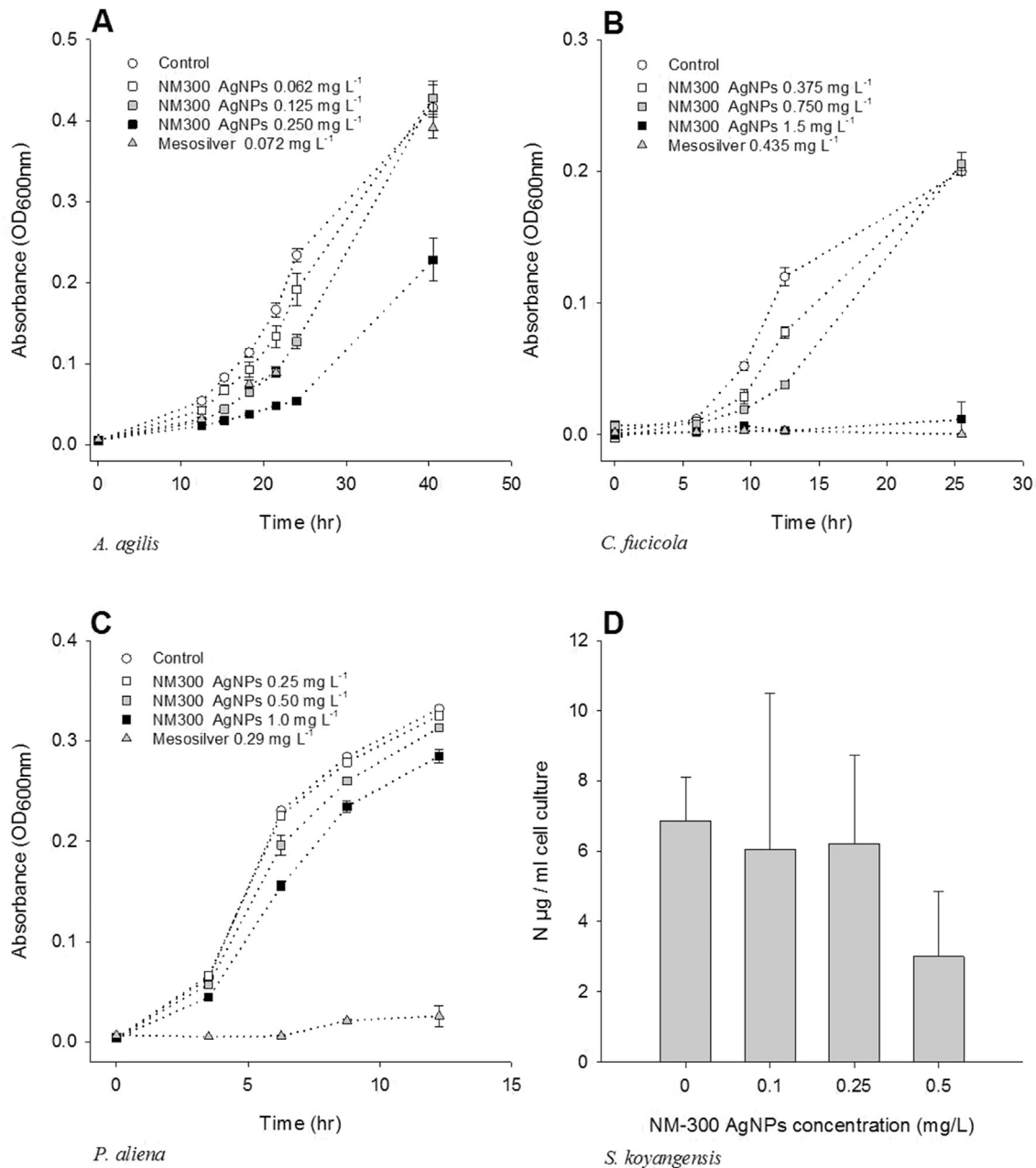
Fig. 1. Growth of the (A) *A. agilis*, (B) *F. fucicola* and (C) *P. aliena* under different concentrations of NM-300 and Mesosilver measured with OD_{600} expressed as the Mean (OD 600 nm) ± SD. *S. koyangensis* (D) growth measured as the concentration of total Nitrogen in cell culture, expressed as the Mean ± SD (n = 3), except treatment at 0.10 mg L^{-1} of NM-300 (n = 2).

viability, bacterial culture aliquots were collected from the experimental flasks, containing inhibitory concentrations of NM-300, and plated on solid medium free from AgNPs. Regarding the cell viability of *A. agilis* (Fig. 2A), no statistically significant differences were found at the beginning of the exposure (t = 0 h, immediately after having added the NM-300) between the control treatment and cell cultures exposed to 0.12 and 0.25 mg L^{-1} NM-300. However, cell viability decreased in the presence of NM-300 during the course of the exposures compared to the control. A similar trend was observed for *C. fucicola* (Fig. 2B).

The CFU values obtained in the treatment with 0.75 mg L^{-1} NM-300 were not significantly lower (p-value < 0.05) than the CFU in the control treatment at the beginning of the exposures (t = 0 h), although during the following hours significant differences in

bacterial viability were observed (p-value < 0.05). Cell viability of *C. fucicola* exposed to 0.75 mg L^{-1} of NM-300 recovered after 13 h of exposure. The cell viability of *P. aliena* was not negatively affected at 1 mg L^{-1} of NM-300.

3.2.3. Respirometry assay with single bacterial strains

The respirometry assay was a quick technique to determine the range of the inhibitory concentrations of NM-300 to single bacterial species. The results obtained in the single culture experiments (see SI Fig. S10) for the Gram negative strain *P. aliena* showed a reduced O_2 uptake rate in a concentration dependent manner. On the other hand, for Gram positive strains (*A.agilis* and *S. koyangesis*) at low concentrations (<0.25 mg L^{-1}) the O_2 uptake rate was higher than the control, but at concentrations above 0.5 mg L^{-1} showed a
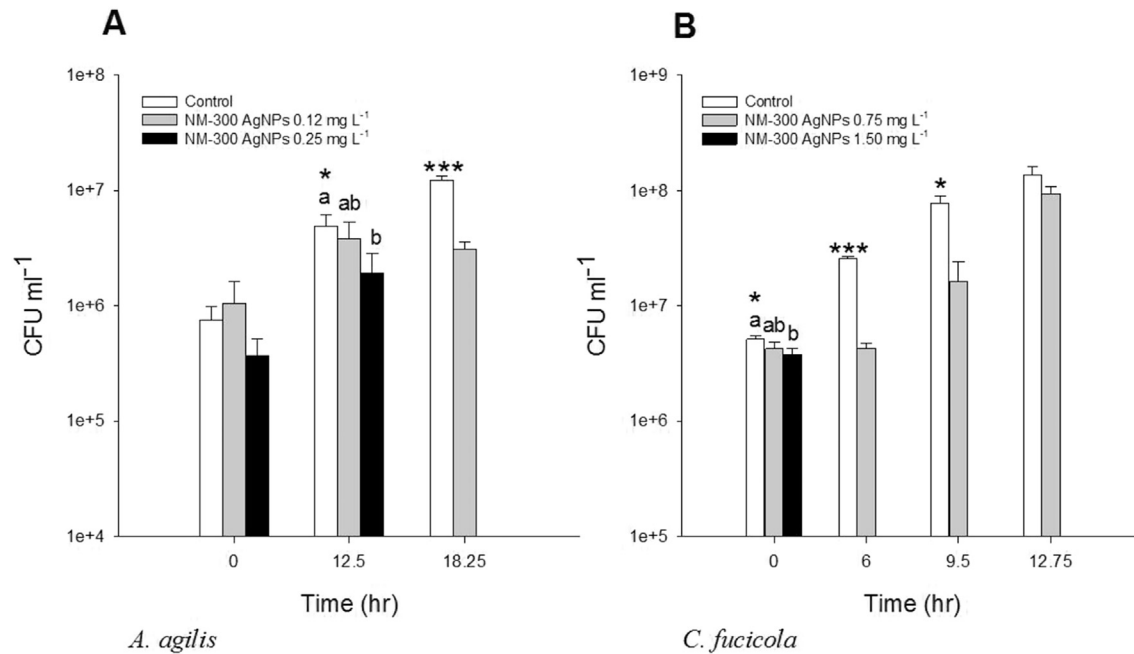
Fig. 2. Cell viability of (A) *A. agilis* and (B) *C. fucicola* expressed as the Mean ± SD of the CFU ml⁻¹ formed in agar plates under different concentrations of NM-300 (n = 3). Columns with different letters are significantly different, (One Way ANOVA, p < 0.05). *, *** symbolizes statistically significant differences ($p < 0.05$), ($p < 0.01$) $p < 0.001$), respectively.

similar response to the Gram negative species. However, owing to the lack of replication, statistical significance could not be assessed.

### 3.3. Microcosm exposures: effects of NM-300 on planktonic estuarine bacteria

3.3.1. Bacterial abundance

The viability and abundance of heterotrophic bacterial groups inhabiting the water column of the microcosm was monitored by the plate count method (Fig. 3A) and the total bacterial abundance was analysed by direct counts with epifluorescence microscopy (Fig. 3B).

Both data sets show that there was a reduction of the bacterial abundance in the presence of NM-300 (One Way ANOVA, p-value <0.001) 24 h after the beginning of the AgNP exposures in the microcosm. The abundance of heterotrophic groups, culturable in marine agar, recovered at the end of the exposures (after 120 h) whereas the total prokaryotic abundance was always statistically significantly higher in the control treatment (TC) than for NM-300 treatment (T1).

3.3.2. Respirometry assay

The O₂ uptake of the whole microbial community inhabiting the water column was measured during the course of the microcosm
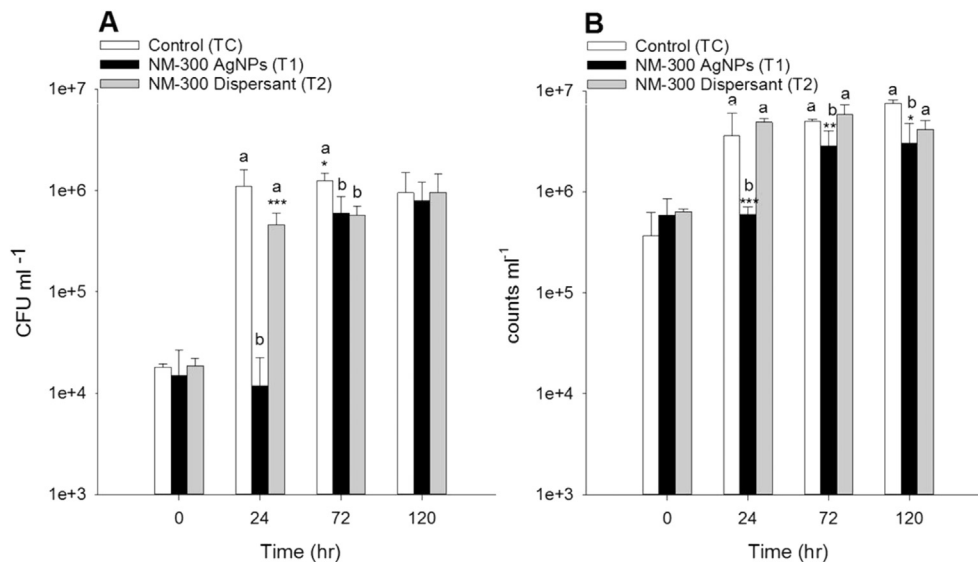
Fig. 3. Abundance of heterotrophic bacterial groups in Marine Agar (A) and total bacterial abundance in the water column (B) expressed as the mean ± SD of the log 10 (CFU or counts ml⁻¹). Columns with different letters are significantly different, (One Way ANOVA, p < 0.05, n = 4). *, **, *** symbolizes statistically significant differences (p < 0.05), (p < 0.01) p < 0.001), respectively.

exposures. The O_2 uptake rate of natural bacterial communities did not increase in presence of NM-300 (T1), whereas the O_2 uptake increased steadily during the first 48 h and progressively decreased afterwards in the Control (TC) and Dispersant treatments (T2) (Fig. 4).

4. Discussion

4.1. Nanoparticle characterization

As expected, on the basis of previous studies with metallic nanoparticles, the particle size (Z-average) reported by the DLS in Milli-Q water was greater than the size values reported with microscopy techniques (AFM and TEM). This is because the Z-average refers to the value of the hydrodynamic diameter (d_H) whereas AFM and TEM measured the nanoparticle core size. Moreover, as DLS is biased towards large particles or agglomerates, which scatter more, the analysis of smaller nanoparticles can be overlooked due to their relative decrease in intensity. The results obtained with the different characterization techniques showed the following results, where the particle size or diameter is represented by the symbol Ø: $Ø_{DLS} > Ø_{TEM} > Ø_{AFM}$. Differences between TEM and AFM analysis have been reported in previous studies and it is acknowledged that TEM measurements can be overestimated due to observational biases (Domingos et al., 2009). Our results are in agreement with previous work that compared the particle size of metallic nanoparticles (Fe_3O_4 NPs) analysed with DLS and microscopy. The DLS analysis always reported the largest values followed by TEM NPs (Lim et al., 2013), while the AFM analysis provided the smallest particle size value (Pelletier et al., 2010).

The hydrodynamic diameter of the AgNPs measured by DLS increased (50% Mesosilver, 1100% NM-300) after 24 h of exposure in bacteria-free broth medium (ZM/10) suggesting nanoparticle agglomeration. The pH in the experimental medium containing bacteria remained above 7.5 throughout the experiments, therefore observed agglomeration was due to salinity rather than pH. The ZM/10 media contained low concentrations of peptone and yeast extract that contributed to nanoparticle agglomeration (Millour

et al., 2015). The surface charge (measured by means of zeta potential) of NM-300 monitored in different media ZM/10 (75% natural seawater) and NaCl ($20 \, g \, L^{-1}$, equivalent to the concentration of chloride contained in ZM/10) decreased (absolute value) in the same way. These results indicated that chloride was a major driver responsible for the reduction of the electric double layer of the AgNPs, and, as a result, the absolute value of the surface charge also lessened. A reduction of the AgNPs zeta potential enhanced nanoparticle surface interaction and AgNPs agglomeration, and thus reduced nanoparticle stability in suspension. Other environmental factors such as organic matter, which may prevent nanoparticle oxidation (Fabrega et al., 2009) and affect their agglomeration state (António et al., 2015), and sulphide concentration, negligible under the aerobic conditions (Liu et al., 2011) of the present work, will influence the fate and persistence of AgNPs.

4.2. Effects of NM-300 on marine bacteria

No information regarding the ecotoxicity of the NM-300/NM-300K AgNPs in the marine environment has been found in the literature with the exception of our previous study (Echavarri-Bravo et al., 2015). Most of the available work has been developed in freshwater or terrestrial environments (Voelker et al., 2015).

The toxicity tests with pure bacterial cultures produced useful information relating to the sensitivity of different bacterial species to NM-300. The knowledge gained in the individual bacterial strain work supported the analysis of the results obtained from the microcosm experiments, as they provided an insight into the susceptibility of natural bacterial populations to NM-300 AgNPs.

In the present study it was observed that the antibacterial properties of AgNPs are species-specific, as described by us and others in earlier studies, and can be associated with differences in the bacterial envelope between Gram positive and Gram negative species (Morones et al., 2005; Suresh et al., 2010; Tamboli and Lee, 2013). Gram positive bacteria can be more susceptible to silver ions as they are less efficient in terms of efflux pumps (O'Shea and Moser, 2008). P. aliena exhibited the greatest resistance to AgNPs in the present study. In addition to being Gram negative, members of the Pseudoalteromonas genus are known to produce exopolysaccharides (EPS) (Gutierrez et al., 2008) that bind to metal ions reducing metal bioavailability and enhancing the bacterial resistance to metals and also to AgNPs (Joshi et al., 2012). In the present study P. aliena could be considered as an r-strategist or opportunistic strain compared to the other strains, as it has a shorter generation time and can grow at higher concentrations of AgNPs.

The IC_{50} of NM-300 calculated in the present study with marine bacterial species is lower (A. agilis $IC_{50} < 0.15 \, mg \, L^{-1}$; C. fucicola $IC_{50} < 0.6 \, mg \, L^{-1}$) than the IC_{50} and/or the half maximum effective concentration (EC_{50}, required to reduced bacterial response) of the same nanoparticle type (NM-300 and NM-300K series) observed by other authors (summarised in Table 3).

In the present work the total silver adhered to the walls of 50 ml the flasks was measured at the end of the exposures in parallel experiments (further information provided in section 4 of the SI, Fig. S11), and accounted for up to 50% of the initial silver concentration. This loss of silver from solution due to adsorption to the flask wall is in agreement with previous work (Sekine et al., 2015).

4.2.1. Toxicity tests with single bacterial strains vs. microcosms experiments

A recovery in the cell viability and bacterial abundance was observed in the toxicity tests developed with single strain cultures (e.g. C. fucicola.) and in the microcosm experiment with natural bacterial populations after 72 h exposure. This effect, of AgNPs inducing bacterial growth inhibition during the first hours or days
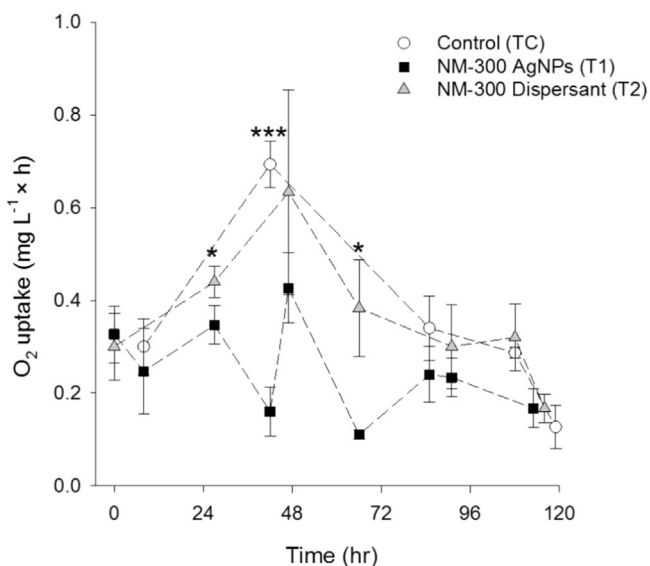
Fig. 4. O_2 uptake rate of the microbial communities during the course of the experiment expressed as the Mean value of the oxygen uptake ±S.D., n = 3. The differences between treatments where analysed with the Student's t-test for independent samples. *, **, *** symbolizes statistically significant differences at a p-value < 0.05, 0.01, 0.001 respectively.

Table 3
Summary of bacterial inhibitory concentrations (IC/EC) of the NM-300/300K

Microorganism	Inhibitory concentration NM-300/NM-300K	Environmental compartment/media type	Endpoint/assay	Author
A.agilis	$IC_{50} = 0.145$ mg L^{-1} (21 h)	broth ZM/10	Growth monitored by OD600nm	Present study
C.fuciola	$IC_{50} = 0.544$ mg L^{-1} (13 h)	broth ZM/10	Growth monitored by OD600nm	Present study
Natural bacterial communities	1 mg L^{-1}, growth supressed by 2 orders of magnitude (24 h)	natural estuarine water	Growth/CFU counts	Present study
Natural bacterial communities	6 mg Ag kg^{-1} dry weight sediments	Estuarine sediments	Growth/Biolog Ecoplate	(Echavarri-Bravo et al., 2015)
Pseudomonas putida	$IC_{50} = 5$ mg L^{-1} (1 h exposure)	artificial waste water	Growth/Bioluminescence assay	(Mallevre et al., 2014)
Pseudomonas putida	$IC_{50} = 50$ mg L^{-1} (1 h exposure)	real crude wastewater	Growth/Bioluminescence assay	(Mallevre et al., 2016)
Sewage sludge microorganisms	$EC_{10} = 27.9$ mg L^{-1} (after 3 h)	sewage sludge	Respiration assay	(Schlich et al., 2013)
Arthrobacter globiformis	$EC_{50} = 33.38$ mg L^{-1} $EC_{50} = 34.16$ mg L^{-1}	broth medium broth medium + soil	dehydrogenase activity assay dehydrogenase activity assay	(Engelke et al., 2014) (Engelke et al., 2014)
Mycobacterium genus Map K10/GFP	$EC_{50} = 5.71$ mg L^{-1} (7 day)	broth medium	Growth monitored by fluorescence	(Donnellan et al., 2016)
S.Senftenberg	20 mg L^{-1} (recovery in <4 h)	broth medium	Growth/CFU counts	(Losasso et al., 2014).
S.Enteriditis	20 mg L^{-1} (recovery in 2 h)			
S.Hadar	20 mg L^{-1} (recovery 1hr)			

of exposure followed by a later recovery in bacterial abundance, has been reported in earlier studies in fresh water at AgNPs concentrations ranging between 0.05 and 0.5 mg L^{-1} (carboxy-functionalized AgNP) in the water column (Das et al., 2012), in seawater at 0.05 mg L^{-1} of polymer-coated AgNPs (Doiron et al., 2012) and in estuarine sediments (Antizar-Ladislao et al., 2015; Echavarri-Bravo et al., 2015). In the present study samples were collected from a location that has been subject to high anthropogenic pressure for more than a century, receiving water discharges from a WWTP servicing a highly populated area as well as historical industrial activities associated with the mining and petrochemical industries (Baxter et al., 2011). For this reason, owing to the continuous exposure to metals from anthropogenic but also geological origin, some of the species present in the natural populations such as C. fuciola and P. aliena may exhibit higher tolerance to metals (Knapp et al., 2011) including AgNPs.

The results obtained with C. fuciola and the recovery of the bacterial abundance in the microcosms shows the ability of some species to recover from acute exposure after the antibacterial agent has been removed (the total concentration of silver was below 0.03 mg L^{-1} in the water column of the microcosms after 48 h (Echavarri-Bravo et al., 2015)). Thus, the removal of the antibacterial agent can reactivate the bacterial division as observed with C. fuciola when exposed to the NM-300. Even though the recovery of C. fuciola exposed to 1.5 mg L^{-1} of the NM-300 AgNPs was not apparent on the basis of OD_{600} measurements, bacterial cells were still viable (CFU developed on agar plates free of AgNPs) after 24 h of exposure. The current study also shows that NM-300, at an initial concentration of 1 mg L^{-1}, negatively affect bacterial viability and abundance in the water column of the microcosms and their physiological status (O_2 uptake decreased). This concentration, 1 mg L^{-1}, is lower than the inhibitory concentrations of the NM-300/NM-300K AgNPs observed in previous work carried out with bacteria (Table 3). Nevertheless, IC_{50}/EC_{50} found in independent studies with different bacterial species should be compared cautiously due, for example, to differences in the methodology applied, and the bacterial species used as model organisms, particularly as some species may contain silver resistance genes (Losasso et al., 2014). The media composition used to conduct the toxicity tests will also influence the IC_{50}; media rich in cysteine will

complex ionic silver resulting from AgNPs dissolution, reducing their toxicity (Li et al., 2015). There will also be differences between real media (crude sewage sludge) and artificial waste water media, as observed by Mallevre et al. (2014, 2016). Moreover, bacterial species from different environmental compartments exhibit different growth requirements (e.g. fresh water bacteria will not tolerate the high concentration of NaCl present in seawater) and thus the composition of the exposure media will vary according to the chemistry of the environmental compartment. The results obtained in the present study suggest that marine bacteria are susceptible to AgNPs. At high concentrations of chloride, as it is in seawater (19.37 g L^{-1} of Cl^- is in average seawater, total salinity 35 g L^{-1}) and in the present work (17.16 g L^{-1} of Cl^-) in the water column of the microcosm, the dissolution of AgNPs and the formation of soluble silver-chloro complexes ($AgCl$, $AgCl_2^-$, $AgCl_3^{2-}$) is enhanced, as calculated with Visual MINTEQ (3.1) and supported by other authors (Levard et al., 2013). These soluble compounds are bioavailable, can cross the bacterial membranes and decrease the bacterial resistance to silver (Gupta et al., 1998). Therefore, wild marine bacteria are good candidates as model organisms to assess the hazard associated with products containing nanosilver, unlike commercial biosensor assay kits, such as Microtox®, where EC_{50} values for AgNPs could not be established at concentrations in excess of 450 mg L^{-1}, (Beddow et al., 2014).

4.2.2. Hormesis response of marine bacteria

The respirometry assays were performed with pure bacterial cultures of the Gram-positive bacteria A. agilis and S. koyangensis and the Gram negative bacterium P. aliena. The Gram positive species exhibited a different response to NM-300 exposure compared to the Gram negative strain P. aliena (see SI Fig. S10). The consumption of O_2 by A. agilis increased at the lowest concentrations of NM-300 (0.05–0.1 mg L^{-1}) and decreased at higher concentrations. It was observed that S. koyangensis behaved similarly to A. agilis in the presence of AgNPs, showing an increased O_2 uptake at low concentrations (0.1 and 0.25 mg L^{-1}), whilst it decreased by 50% at concentrations ≥ 0.5 mg L^{-1}. In contrast AgNPs did not stimulate the consumption of O_2 of the Gram negative bacterium P. aliena at low concentrations, and no differences in the O_2 consumption were observed between 0.25 and 1 mg L^{-1}. The two Gram

positive species showed a hormesis response, increasing their O_2 uptake under a low dose of NM-300 during the first 1–2 h. A hormesis response to AgNPs has been observed in previous studies with AgNPs (Xiu et al., 2012) similar to the effects of other antibiotics, such as amoxicillin (Händel et al., 2013), and may account for the apparent increased respiration in the present study.

4.3. Antibacterial activity of the Mesosilver product

In the present work the physicochemical properties of AgNPs present in a commercial product, the Mesosilver Hot tub™, (which we call 'Mesosilver') were characterised. Mesosilver inhibited bacterial growth at a concentration as low as 0.072 mg L^{-1} and it always inhibited bacterial growth at concentrations lower than the values observed for NM-300. The higher antibacterial activity of Mesosilver could be due to the initial higher concentration of ionic silver (2.2 times higher). In addition, the analysis of the nanoparticle size by AFM showed that Mesosilver was lower than NM-300 in bacterial broth media. Small size favours the interaction with the bacterial membranes and previous studies indicated that small AgNPs are more toxic (Lu et al., 2013; Morones et al., 2005). Besides, smaller nanoparticles exhibit a higher surface area that may also enhance dissolution (Dobias and Bernier-Latmani, 2013; Mitrano et al., 2014; Xiu et al., 2012). The present study shows that the use of this type of product will introduce AgNPs and ionic silver into the urban sewage system and its potential release into estuaries and coastal areas if silver is not efficiently removed in WWTPs. Our results indicate that Mesosilver is highly toxic to natural marine bacteria and highlights the need for a comprehensive risk assessment and regulation of products of domestic use that incorporate AgNPs. Our work on single bacterial cultures shows that further work is required to assess the environmental hazard of this product in the marine environment as well as additional chemical analysis to investigate if other toxic compounds, required for AgNPs synthesis, are also present and not reported by the supplier.

5. Conclusion

The present study shows that the marine bacteria we have tested are more susceptible to the antibacterial activity of AgNPs than bacterial communities from other environmental compartments, because of the different physicochemical properties of AgNPs in seawater. In addition, AgNPs present in a household product were characterised showing that it exhibits higher antibacterial activity towards non-target marine bacteria than the NM-300 AgNPs (OECD programme). The authors propose the use of natural marine bacteria as model organisms (natural microbial communities and/or single species isolates such as *A. agilis*) when conducting toxicity studies to assess the environmental hazard associated to products containing nanosilver.

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.marenvres.2017.08.006.

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