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**An investigation into the effects of silver nanoparticles on antibiotic resistance of
naturally occurring bacteria in an estuarine sediment**

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27 Key words:

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31 **ABSTRACT**

32 The aim of this study was to test whether silver nanoparticles (Ag-NPs) released into
33 estuarine environments result in increased antibiotic resistance amongst the natural bacterial
34 population in estuarine sediments. A 50-day microcosm exposure experiment was carried
35 out to investigate the effects of Ag-NPs (50 nm average diameter) on the antibiotic resistance
36 of bacteria in sediments from an estuary in southwest England. Experimental microcosms
37 were constructed using 3.5 kg sediment cores with 20 l of overlaying seawater treated with
38 (final) Ag-NPs concentrations of 0, 50 or 2000 $\mu\text{g l}^{-1}$ ($n = 3$). Sediment samples were
39 screened at the end of the exposure period for the presence of bacteria resistant to eight
40 different antibiotics. Multivariate statistical analyses showed that there was no increase in
41 antibiotic resistance amongst the bacterial population in the sediment due to the dosing of
42 the microcosms with Ag-NPs. This study indicates that, under the tested conditions, Ag-NPs
43 released into the coastal marine environment do not increase antibiotic resistance among
44 naturally occurring bacteria in estuarine sediments. These results contrast previous findings
45 where antimicrobial effects of Ag-NPs on key bacterial species in laboratory experiments
46 have been demonstrated, and reasons for this are discussed. The negligible effects
47 demonstrated on bacterial populations under the selected estuarine conditions, provide
48 important information on no observed effect concentrations (NOECs) for environmental
49 regulation.

50 INTRODUCTION

51 Nanoparticles (NPs), which can broadly be defined as having one dimension between 1 and
52 100 nm (Masciangioli and Zhang, 2003), are increasingly used in consumer goods.
53 However, there are great uncertainties about the environmental fate and effects of
54 anthropogenically produced NPs which stems from a lack of understanding the behaviour of
55 these NPs (Colvin, 2003; Owen and Handy, 2007). One of the NPs that has been utilised
56 increasingly during recent years in a wide range of products are silver NPs (Ag-NPs). This
57 relates to the antimicrobial properties of Ag which, for example, has been used for a long
58 time as a biocide in the treatment of burns (Klasen, 2000) and in wound dressings. The
59 antimicrobial activity of Ag is thought to be due to the binding of the Ag⁺ cation to electron
60 donor groups in biological molecules containing sulphur, oxygen or nitrogen (e.g. enzymes)
61 which, in turn, results in the loss of their function (Uchida *et al.* 2003; Kumar and Münstedt
62 2005). Another explanation is based on the generation of free radicals from the surface of the
63 Ag-NPs which then lead to a breakdown of bacterial membrane function (Kim *et al.* 2007).
64 However, McHugh and colleagues reported as early as 1975 the emergence of a silver nitrate
65 resistant strain of *Salmonella typhimurium* in a hospital burns unit which also proved to be
66 resistant to several antibiotics (McHugh *et al.* 1975). Moreover, it is well documented that
67 metal contaminated sites have increased numbers of antibiotic resistant bacteria (Wright *et*
68 *al.* 2006, Baker-Austin *et al.* 2006). It is important to understand that, despite the absence of
69 any antibiotic at these sites, the resistance to antibiotics is often not lost by the bacterial cell
70 as the co-selection of heavy metal and antibiotic resistance is frequently due to plasmid
71 encoded resistance genes for both metal and antibiotic resistance (e.g. Davis *et al.* 2005,
72 Hernández *et al.* 1998). Therefore, it is not surprising that heavy metal contaminated soils
73 have been found to harbour more plasmids than uncontaminated soils (Rasmussen and
74 Sorensen 1998). Furthermore, the linkage of these genes on plasmids allows them to be

transferred to other cells via horizontal gene transfer, thus facilitating the spreading of both resistance patterns despite the presence of only one selective pressure.

Nevertheless, Ag in form of Ag-NPs is being increasingly integrated into products widely available on the market, such as cosmetics, paints, food containers and clothing. One of the products of most environmental concern are washing machines used to impregnate clothes with Ag-NPs to avoid the growth of odour-producing bacteria as the Ag-NPs can leach out of fabrics (Benn and Westerhoff 2008). Consequently the most probable environmental exposure route of Ag-NPs is through wastewaters and surface waters (Boxall *et al.* 2007).

Based on this knowledge and the fact that in England and Wales it has been estimated that more than one-third (ca. 37.5 %) of the total sewage treatment plant effluents are discharged into estuarine and coastal environments (Environment Agency, 1999), it was decided to investigate the potential impact of Ag-NP pollution on the natural bacterial populations on the surface of estuarine sediment where Ag-NPs are likely to accumulate over time. A 30-day microcosm study was designed in which Ag-NPs were added from a stock solution to estuarine sediment samples (in triplicate) for 20 days yielding a final cumulative treatment of either 0 $\mu\text{g l}^{-1}$ (control), 25 $\mu\text{g l}^{-1}$ or 1000 $\mu\text{g l}^{-1}$ (Bradford *et al.*, 2009). The experimental tanks were left for a further 10 days to allow for any recovery. Although the overall concentration of Ag-NPs in the water column increased steadily during the 20 days of dosing there was no influence on the abundance of heterotrophic prokaryotes in the estuary water.

Similarly, despite the predicted accumulation of the Ag-NPs in the surface layer of the sediment (~top 3 mm), there were only negligible differences in bacterial diversity between treatments, suggesting that under the selected experimental regime Ag-NPs present little or no impact on estuarine sediment bacterial diversity (Bradford *et al.*, 2009). Although the chemical fate of the Ag-NPs after their release into the microcosms was not examined, it is believed that physicochemical parameters in the brackish environment (such as salinity and

organic matter content) might render Ag-NPs less toxic and unreactive within a short time frame of their release (see physico-chemistry in Handy *et al.*, 2008). Nevertheless, it can be hypothesised that pollution of an estuarine environment with Ag-NPs can lead to an increase in the presence of silver-resistant bacteria in the sediment surface and, through co-selection, lead to an enrichment of antibiotic resistant bacteria in the natural population. Therefore, we investigated in the present study the potential impact of Ag-NPs on the occurrence of antibiotic resistance in natural bacterial assemblages using subsamples from the estuarine sediment surfaces from the experiment, but with a prolonged exposure time.

MATERIALS AND METHODS

Details on the collection of environmental samples, the experimental design of the microcosm exposure and sampling as well as the experimental and statistical analyses of the samples from the microcosms are described by Bradford *et al.* (2009). A summary of relevant information, as well as modifications to the methods used previously, are outlined below.

Experimental Design

Glass microcosms were prepared in triplicate for each exposure concentration, and were maintained covered and continuously aerated in a constant-temperature culture room at 15°C. The following three exposure treatments were carried out: 0 $\mu\text{g l}^{-1}$ of Ag-NPs in the overlaying estuary water (control tanks T01, T02, T03); 50 $\mu\text{g l}^{-1}$ Ag-NPs (T04, T05, T06); and 2000 $\mu\text{g l}^{-1}$ Ag-NPs (T07, T08, T09) to represent a low-level contamination (50 $\mu\text{g l}^{-1}$), while the 2000 $\mu\text{g l}^{-1}$ concentration was selected as it is much higher than predicted future environmental concentrations resulting from anthropogenic pollution (Boxall *et al.*, 2007).

The microcosm tanks consisted of sediment (ca. L 28 x W 17 x D 7 cm) with 20 l of overlaying estuarine seawater. Each microcosm and all of the equipment used for sampling or aeration was thoroughly acid washed (0.5 % nitric acid, over night) to remove trace metals from the surface of the glassware. In order to minimise the loss of any added Ag-NP material by adsorption onto the tanks, the microcosms, except those used as controls, were saturated with Ag-NPs, by pre-soaking them with an Ag-NP solution ($1/40^{\text{th}}$ of the final concentration of Ag-NPs used – see below) for 1 h. The estuarine sediment samples were collected from an intertidal area at the mouth of the Tamar Estuary (St John's lake mud flats, OSGB grid ref. SX412539). Care was taken to ensure that there was minimal disturbance of the surface layer and stratification of the sediment, and that any large invertebrates were avoided during collection. Upon returning to the laboratory the sediment samples were placed into the individual microcosm tanks and samples were allocated randomly to treatments to minimise any sampling bias.

The sediment samples were exposed to a cumulative dose of Ag-NPs that totalled 0 (control), 50 and 2000 $\mu\text{g l}^{-1}$ of overlaying intertidal seawater. The dosing was carried out in two steps. $1/40^{\text{th}}$ of the final concentration of Ag-NPs was added daily to the overlaying seawater over a period of 20 days; i.e. 0.25 ml and 10 ml of a 100 $\mu\text{g ml}^{-1}$ stock solution were added to the 50 and 2000 $\mu\text{g l}^{-1}$ tanks, respectively. The microcosms were then left untreated for 10 days, followed by renewed daily dosing ($1/40^{\text{th}}$ of the final concentration) for another 20 days (i.e. a total exposure time of 50 days).

Characterisation of Ag-NPs

The Ag-NP test material (Sigma Aldrich Silver nano; < 100 nm) was selected as the Ag-NPs were representative in size to those used by Yoon *et al.* (2007) and Pal *et al.* (2007). TEM

revealed that the sonicated Ag-NPs stock solution (100 mg l^{-1}) used for the dosing was well dispersed and that the individual particles (if assumed to be spheroidal) had an average diameter of 58.6 nm (standard deviation = 18.6 nm; $n = 64$).

Analysis of water samples and metals in sediments

Water samples (10 ml) were collected for metal analysis before and after each daily addition of Ag-NPs, and samples were also collected for general nutrient analyses of the seawater (50 ml samples from each tank). Nutrient analyses were performed according to Woodward (2001) using a Technicon segmented flow colorimetric autoanalyser. The following water quality parameters were measured at 8 time points (days 0, 1, 4, 10, 15, 20, 25, 30) during the first 30 days of the incubation: salinity (mean $\pm$ S.E.M: $29.47 \pm 0.15 \text{ ‰}$), dissolved oxygen ($100.7 \pm 0.97 \text{ ‰}$), pH (7.82 ± 0.12), ammonium ($23.9 \pm 1.9 \text{ } \mu\text{M}$), nitrate ($58 \pm 2.1 \text{ } \mu\text{M}$), nitrite ($9.7 \pm 1.1 \text{ } \mu\text{M}$), phosphate ($4.7 \pm 0.1 \text{ } \mu\text{M}$), silicate ($28.5 \pm 1.3 \text{ } \mu\text{M}$), temperature ($13.17 \pm 0.06 \text{ } ^\circ\text{C}$).

Metal analysis was performed on water and sediment samples by inductively coupled plasma-optical emission mass spectrometry (ICP-OES, Varian 725-ES) using matrix matched standards and 1 % yttrium (Y^{39}) oxide as an internal standard in samples (see Bradford et al., 2009, for further details). A certified estuarine reference material (LGC 6137, Laboratory of the Government Chemist) was treated likewise as an internal control. Measured trace element concentrations in the sediments at the start (i.e. day 0) of the exposure experiment were very similar: (mg metal kg^{-1} dry weight, mean $\pm$ S. E. M., $n = 144$); Ca, 8477 ± 459 ; Cu, 160 ± 7 ; Fe, 36994 ± 1682 ; K, 2801 ± 152 ; Mg, 9402 ± 490 ; Na, 30642 ± 2332 ; Zn 235 ± 11 .

ICP-OES of seawater samples from the microcosms treated with $2000 \text{ } \mu\text{g l}^{-1}$ Ag-NPs revealed that Ag accumulated in the overlaying seawater during the first period of daily

dosing (i.e. first 20 days) to approximately $50 \mu\text{g l}^{-1}$ and decreased then to less than $10 \mu\text{g l}^{-1}$ at day 30, after which another 20 days of dosing was carried out (no analyses were carried out for this time period). Parallel analyses of extracts of samples from the surface of the sediment collected during the first 30 days of the exposure time revealed that the Ag accumulated in the surface layer of the sediment. Whilst Ag could be detected and quantified in all of the seawater and sediment samples from the $2000 \mu\text{g l}^{-1}$ dosed microcosms (following the initial dosing), in the $50 \mu\text{g l}^{-1}$ treated microcosms, Ag was below the detection limit (approx. 1 ng l^{-1}) of the method used in any of the overlaying seawater samples and was detected only in the sediment sample collected on day 25, providing further evidence that the Ag-NPs accumulate at the surface of the sediment. Ag was not detectable in either the overlaying seawater or the sediment from any of the control microcosms.

184

185 **Screening for antibiotic resistant bacteria**

A 5-ml syringe (without the plunger and with the tip removed) was used to remove a core sample of the sediment on day 50 of the incubation from each of the nine microcosms. A 100-mg aliquot of the surface layer of each of these samples from the sediment cores was collected and transferred into 10 ml of filtered ($0.2 \mu\text{m}$) seawater and mixed by vortexing. 50 μl of this suspension and of two 10-fold dilutions of it were transferred onto a 10-times diluted version of marine broth (per litre of estuarine (29 ‰ salinity) seawater: 0.5 g peptone, 0.1 g yeast extract, 0.01 g $\text{FePO}_4\cdot\text{H}_2\text{O}$ was added and was then solidified using 1.5% (w/v) agar). Each of the medium plates contained one of 8 different antibiotics (as well as cycloheximide ($20 \mu\text{g ml}^{-1}$; Melford Laboratories Ltd., Chelworth, UK) to inhibit fungal growth). The concentration of the antibiotics was based on those suggested by the DIN – Deutsches Institut für Normung e.V. (1998). Antibiotics (Sigma-Aldrich, Poole, UK) used were erythromycin ($20 \mu\text{g ml}^{-1}$), oxytetracycline ($20 \mu\text{g ml}^{-1}$), sulfadiazine ($32 \mu\text{g ml}^{-1}$),

trimethoprim ($32 \mu\text{g ml}^{-1}$), lincomycin ($32 \mu\text{g ml}^{-1}$), ceftazidime ($32 \mu\text{g ml}^{-1}$), amoxicillin ($20 \mu\text{g ml}^{-1}$) and vancomycin ($32 \mu\text{g ml}^{-1}$). Additionally, to evaluate the efficacy of the antibiotic treatments, aliquots were also transferred onto marine broth plates containing only the fungicide cycloheximide ($20 \mu\text{g ml}^{-1}$). Plates were incubated at 15°C for seven days.

Statistical analyses

The interactive effects of different antibiotic treatments and the presence or absence of Ag were analysed using counts (colony forming units – cfus) from antibiotic-containing plates at the end of the seven-day incubation. A Euclidean distance matrix of differences between every pair of samples was calculated and used to conduct tests of differences between groups of samples, using permutation-based 2-way crossed Analysis of Variance (Anderson 2001; McArdle and Anderson 2001). This procedure is formally equivalent to a standard ANOVA but the flexibility and robustness of the permutation approach ameliorates the necessity for variables to fulfil standard assumptions, such as normality. The analyses were implemented in PRIMER 6 & PERMANOVA+ 1 software (www.primers-e.com).

RESULTS

Environmental parameters of the microcosms

Analyses of biotic and abiotic parameters of the overlaying water samples were undertaken at 8 time points (days 0, 1, 4, 10, 15, 20, 25, 30) during the first 30 days of the incubation. There were no significant differences in water quality between the tanks (ANOVA, $P > 0.05$) and the results from the measurement of dissolved oxygen, pH temperature and salinity were in the normal range for this estuarine site. Similarly, there were no significant differences

between microcosms in terms of bacterial numbers in the overlaying water ($P = 0.725$),
though their numbers fluctuated over the time course of the incubation.

Antibiotic resistance in the estuarine sediment samples

The 10-fold dilutions of the sediment sample suspensions were found to provide the most reliable numbers for subsequent statistical analyses. Undiluted suspensions produced >700 colonies per plate, which were graded the same (i.e. 700 cfus) thereby underestimating within-treatment variability. The 100-fold diluted samples often produced too few cfus to be counted. The cfu counts from the undiluted and the 10-fold dilution of the sediment samples are listed in Table 1. Data from the test on erythromycin-resistant bacteria were excluded from the analysis as no bacteria grew on the plate. Permutation-based ANOVA (Table 2) showed that there is no interaction between the effects of antibiotics and Ag. The Ag treatments, therefore, had no significant effect on bacterial counts. Different antibiotic treatments, however, did have significantly different effects on the counts. These were examined in more detail (Table 3). As expected, all counts from samples treated with all antibiotics differed significantly from those in samples treated solely with the antifungal agent cycloheximide. No colonies grew in the erythromycin treatment. Samples treated with other antibiotics effectively fell into two groups. Counts were significantly lower in samples treated with oxytetracycline or amoxycillin than they were in samples treated with sulfadiazine, trimethoprim, lincomycin or vancomycin, although the effects of the latter were variable. Samples treated with ceftazidime tended to have intermediate counts that overlapped the two groups (Table 1).

DISCUSSION

246

247 **Antibiotic resistance in estuarine microcosms and the impact of Ag-NPs**

248 The presented results reiterate the now commonly accepted concept that environmental
 249 populations of bacteria include a wide range of antibiotic resistant strains. Although it is well
 250 known that some of this antibiotic resistance is intrinsic and natural to bacteria (D'Costa *et*
 251 *al.* 2006), it has also been demonstrated that it is caused, to a large extent, through the
 252 release of antibiotics from agricultural or human prescriptions into the environment (e.g.
 253 Aarestrup *et al.* 2000; Goni-Urriza *et al.*, 2000).

254 Another factor that has been found to result in increased antibiotic resistance is pollution
 255 with heavy metals in soils (Berg *et al.* 2005) or in sediments, biofilms and the water column
 256 of polluted rivers (Stepanauskas *et al.* 2005; Wright *et al.* 2006). This correlation has also
 257 been demonstrated in heavy metal (cadmium, nickel) spiked freshwater microcosm
 258 experiments which showed an increase of antibiotic resistant bacteria with increasing heavy
 259 metal concentrations (Stepanauskas *et al.* 2006). Given the increasingly wide-spread use of
 260 Ag-NPs in consumer and medical products it is of great importance to evaluate the potential
 261 link between environmental pollution by Ag-NPs and antibiotic resistance.

262 The present experiment, however, did not find such a correlation with any of the antibiotics
 263 tested, which were selected based on their high prescription rate among human patients
 264 (amoxicillin), their importance as an “antibiotic of last resort” (vancomycin), their high
 265 usage in farming (oxytetracycline), and on the fact that bacteria resistant to β -lactam based
 266 antibiotics are increasingly found in clinical and environmental environments (ceftazidime).
 267 Other antibiotics were recommended for investigation by Dr Alistair Boxall (University of
 268 York, personal communication; see also Boxall *et al.*, 2006) due to their persistence in the
 269 environment (e.g. lincomycin) or their previous detection in other estuaries (e.g.

erythromycin). The fact that the bacterial counts for amoxicillin were at the lower end of all of the antibiotics is likely to be due to its fast degradation both inside the human body and upon release into the environment (Boxall, personal communication). Similarly, in field experiments the concentration of free oxytetracycline was also found to decrease within the first few days after spiking into agricultural soils (Boxall *et al.*, 2006) or into manure samples (Loke *et al.* 2003). In contrast to these, trimethoprim and lincomycin are well known to be less degradable in the environment (Ingerslev and Halling-Sørensen 2000, Boxall *et al.*, 2006). This correlates with the high abundance of the antibiotic resistant bacteria found in this study. However, the history of any antibiotic contamination at the sampling site prior to this study is unknown. The relatively high number of bacterial counts of vancomycin-resistant bacteria is of particular concern, as this antibiotic is one of very few that are still active against some strains of methicillin-resistant *Staphylococcus aureus* (e.g. Maclayton and Hall II 2007). Investigations into bacterial diversity in the sediment surface throughout the experimental exposure period did not reveal any significant changes (Bradford *et al.*, 2009).

It is unlikely that the exposure time (50 days) to the Ag-NPs was too short to generate antibiotic resistant bacteria, as bacteria undergo multiple replications each day and antibiotic resistance can be located on plasmids. This is further confirmed by the fact that Stepanauskas *et al.* (2006) found an increase in antibiotic resistant bacteria after a 7-day incubation to 1 mM concentrations of CdCl₂ and NiCl₂. Additionally, the time period of the exposure is also thought to be sufficient for bacteria to acquire the relevant resistance genes through horizontal gene transfer as the genes responsible for resistance to heavy metals, such as the *silE* gene which has been shown to confer resistance to Ag (e.g. Davis *et al.* 2005), are encoded on plasmids or other highly transferable gene cassettes and integrons which

295 represent mobile genetic elements that facilitate horizontal gene transfer (Fluit and Schmitz
296 2004).

297 There are, however, a number of potential explanations for the results presented which, taken
298 together, suggest that the impact of Ag-NPs on natural bacterial populations is unpredictable
299 and probably needs to be investigated for each environment individually. We believe that the
300 main reason for this unpredictability lies in the specific abiotic parameters of a particular
301 environment. Estuaries, in particular, differ from other environments in that the mixing of
302 river water and seawater generates complex gradients which control the formation of
303 colloids and their removal through flocculation (e.g. Sholkovitz 1978, Stolpe and Hassellöv
304 2007).

305 Ward and Kramer (2002) showed that the ratio of the Ag species Ag^+ , AgCl^0 , AgCl_2^- ,
306 AgCl_3^{2-} depend strongly on the salinity of the water, with higher salinity favouring the latter
307 two complexes. The relative abundance of the Ag^+ (1.7 %) and AgCl^0 (42 %) species is
308 considerably higher at a salinity of 1‰ (Ward and Kramer, 2002) as compared to a salinity
309 of 25‰ where their concentration will be reduced to approximately 1.4% (Ag^+ : 0.004%;
310 AgCl^0 1.4 %) with the Ag complexes comprising the remaining 98% (AgCl_2^- : 49 %; AgCl_3^{2-}
311 : 49 %). If AgCl_2^- and AgCl_3^{2-} prove indeed to lack antimicrobial activity then Ag-NPs
312 would not exert selective pressure on natural bacterial population in marine environments
313 and, therefore, would not result in co-selection of antibiotic resistance. Moreover, the
314 charged nature of these colloids may also favour attachment to DOM and POM and thus
315 further reduce their bioavailability. In contrast, the free Ag^+ cation and AgCl appear to be
316 highly bioavailable (Engel *et al.* 1981, reviewed by Luoma *et al.* 1995) and possess
317 antimicrobial activity (Uchida *et al.* 2003; Kumar and Münstedt 2005). Thus toxicity in
318 estuarine environments could be more than 2 orders of magnitude lower than in non-saline
319 water. However, this does not necessarily mean that in freshwater environments

complexation with organics and, in particular, the formation of silver sulfide clusters (Rozan *et al.*, 2000) can reduce toxicity to *Daphnia magna* (Bowles *et al.*, 2002; Biachini *et al.*, 2002). Such complexation could also lead to reduced antimicrobial activity (due to loss of bioavailability), and potentially to a reduction in antibiotic resistant bacteria.

Lok *et al.* (2007) have observed the loss of antibacterial activity of Ag-NPs against *E. coli* in high salinity media. They attributed this to aggregation of the NPs which could be overcome by premixing the Ag-NPs with bovine serum albumin (BSA). In the present study it was preferred to maintain unmodified environmental conditions as the dispersant is unlikely to occur in relevant concentrations in the environment; therefore, we used sonication of the stock solution prior to dosing of the microcosms.

In conclusion, the microcosm experiments undertaken in this study demonstrate that Ag-NPs do not result in an increase in antibiotic resistance of naturally occurring bacteria in estuarine sediments. This is in contrast to previous findings from heavy metal polluted natural environments or from heavy metal amended microcosm experiments, which showed that heavy metal contamination results in increased occurrence of antibiotic resistant bacteria. The study, therefore, indicates that a crucial step in the effects of Ag-NPs, and possibly other NPs, is connected to the bioavailability of the particles. A clearer understanding of this aspect, particularly of the chemical behaviour of the NPs once released into the environment and the abiotic factors that may deactivate the antimicrobial effect of the particles, is required to better understand their behaviour, to validate their NOECs and to be able to predict the potential consequences of environmental pollution from the emerging applications of Ag-NPs and nanoparticles in general. Until then it will not be possible to extrapolate the results from laboratory experiments carried out on bacterial isolates to the natural environment.

345

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351 **REFERENCES**

- 352 Aarestrup, F. M., Kruse, H., Tast, E., Hammerum, A. M., 2000. Jensen, L. B., Associations
 353 between the use of antimicrobial agents for growth promotion and the occurrence of
 354 resistance among *Enterococcus faecium* from broilers and pigs in Denmark, Finland, and
 355 Norway. Microbial Drug Resistance 6, 63-70.
- 356 Anderson, M.J., 2001. A new method for non-parametric multivariate analysis of variance.
 357 Austral Ecology 26, 32–46.
- 358 Baker-Austin, C., Wright, M.S., Stepanauskas, R., McArthur, J.V., 2006. Co-selection of
 359 antibiotic and metal resistance. Trends in Microbiology 14, 176-182.
- 360 Benn, T.M., Westerhoff, P., 2008. Nanoparticle silver released into water from commercially
 361 available sock fabrics. Environmental Science and Technology 42, 4133-4139.
- 362 Berg, J., Tom-Petersen, A., Nybroe, O., 2005. Copper amendment of agricultural soil selects
 363 for bacterial antibiotic resistance in the field. Letters in Applied Microbiology 40, 146-
 364 151.
- 365 Bianchini, A., Bowles, K.C., Brauner, C.J., Gorsuch, J.W., Kramer, J.R., Wood, C.M., 2000.
 366 Evaluation of the effect of reactive sulfide on the acute toxicity of silver (I) to *Daphnia*
 367 *magna*. Part 2: toxicity results. Environmental Toxicology and Chemistry 21, 1294-1300.
- 368 Bowles, K.C., Bianchini, A., Brauner, C.J., Kramer, J.R., Wood, C.M., 2000. Evaluation of
 369 the effect of reactive sulfide on the acute toxicity of silver (I) to *Daphnia magna*. Part 1:

- 370 description of the chemical system. *Environmental Toxicology and Chemistry* 21, 1286-
371 1293.
- 372 Boxall, A.B.A., Tiede, K., Chaudhry, Q., 2007. Engineered nanomaterials in soils and water:
373 how do they behave and could they pose a risk to human health? *Nanomedicine* 2, 919-
374 927.
- 375 Boxall, A.B.A., Fogg, L.A., Baird, D.J., Lewis, C., Telfer, T.C., Kolpin, D., Gravell, A.,
376 Pemberton, E., Boucard, T., 2006. Targeted monitoring study for veterinary medicines in
377 the environment, Science Report: SC030183/SR, Bristol: Environment Agency, 120 pp.
- 378 Boxall, A. B. A., Chaudhry, Q., Sinclair, C., Jones, A., Aitken, R., Jefferson, B., Watts, W.,
379 2007. Current and Future Predicted Environmental Exposure to Engineered
380 Nanoparticles; Central Science Laboratory: York; [http://randd.defra.gov.uk/Document](http://randd.defra.gov.uk/Document.aspx?DocumentID)2291).
381 [asp? DocumentID\)2291](http://randd.defra.gov.uk/Document.aspx?DocumentID)2291).
- 382 Bradford, A., Handy, R., Readman, J., Atfield, A., Mühling, M., 2009. Impact of silver
383 nanoparticle contamination on the genetic diversity of natural bacterial assemblages in
384 brackish sediments. *Environmental Science & Technology*
385 (<http://pubs.acs.org/doi/abs/10.1021/es9001949>).
- 386 Colvin, V.L., 2003. The potential environmental impact of engineered nanomaterials. *Nature*
387 *Biotechnology* 21, 1166-1170.
- 388 Davis, I.J., Richards, H., Mullany, P., 2005. Isolation of silver- and antibiotic-resistant
389 *Enterobacter cloacae* from teeth. *Oral Microbiology and Immunology* 20, 191-194.
- 390 D'Costa, V.M., McGrann, K.M., Hughes, D.W., Wright, G.D., 2006. Sampling the antibiotic
391 resistome. *Science* 311, 374-377.
- 392 DIN – Deutsches Institut für Normung e.V. (1998) Methoden zur Empfindlichkeitsprüfung
393 von mikrobiellen Krankheitserregern gegen Chemotherapeutika, DIN 58940. Vol. 3,
394 Beuth Verlag, Berlin.

- Engel, D.W., Sunda, W.G., Fowler, B.A., 1981. Factors affecting trace metal uptake and toxicity to estuarine organisms. I Environmental parameters, in: Vernberg, J.F., Callabrese, A., Thurberg F.P. and Vernberg, W.B. (Eds.), Biological Monitoring of Marine Pollutants. Academic Press, New York, pp. 127-144.
- Fluit, A.C. and Schmitz, F.-J. (2004) Resistance integrons and super-integrons. European Journal of Clinical Microbiology and Infectious Diseases 10, 272-288.
- Goni-Urriza, M., Capdepuy, M., Arpin, C., Raymond, N., Caumette, P., Quentin, C., 2000. Impact of an urban effluent on antibiotic resistance of riverine *Enterobacteraceae* and *Aeromonas* spp. Applied and Environmental Microbiology 66, 125-132.
- Handy, R.D., Kammer, F.v.d., Lead, J.R., Hassellöv, M., Owen, R., Crane, M. 2008. The ecotoxicology and chemistry of manufactured nanoparticles. Ecotoxicology 17, 287-314.
- Hernández, A., Mellado, R.P., Martínez, J.L., 1998. Metal accumulation and vanadium-induced multidrug resistance by environmental isolates of *Escherichia hermannii* and *Enterobacter cloacae*. Applied and Environmental Microbiology 64, 4317-4320.
- Ingerslev, F., Halling-Sørensen, B., 2000. Biodegradability properties of sulfonamides in activated sludge. Environmental Toxicology and Chemistry 19, 2467-2473
- Kalman, J., Turner, A., 2007. An evaluation of metal bioaccessibility in estuarine sediments using the commercially available protein, bovine serum albumin. Marine Chemistry 107, 486-497.
- Kim, J.S., Kuk, E., Yu, K.N., Kim, J.-H., Park, S.J., Lee, H.J., Kim, S.H., Park, Y.K., Park, Y.H., Hwang, C.Y., Kim, Y.K., Lee, Y.S., Jeong, D.H., Cho, M.H., 2007. Antimicrobial effects of silver nanoparticles. Nanomedicine 3, 95-101.
- Klasen, H.J., 2000. A historical review of the use of silver in the treatment of burns. II. Renewed interest for silver. Burns 26, 131-138.

- 419 Kumar, R., Münstedt, H., 2005. Silver ion release from antimicrobial polyamide/silver
420 composites. *Biomaterials* 26, 2081-2088.
- 421 Lok, C.N., Ho, C.-M., Chen, R., He, Q.-Y., Yu, W.-Y., Sun, H., Tam, P.K.-H., Chiu, J.F.,
422 Che, C.-M., 2007. Silver nanoparticles: partial oxidation and antibacterial activities.
423 *Journal of Biological Inorganic Chemistry* 12, 527-534.
- 424 Loke, M.-L., Jespersen, S., Vreeken, R., Halling-Sørensen, B., Tjørnelund, J., 2003.
425 Determination of oxytetracycline and its degradation products by high-performance liquid
426 chromatography–tandem mass spectrometry in manure-containing anaerobic test systems.
427 *Journal of Chromatography B* 783, 11-23.
- 428 Luoma, N.S., Ho, Y.B., Bryan, G.W., 1995. Fate, bioavailability and toxicity of silver in
429 estuarine environments. *Marine Pollution Bulletin* 31, 44-54.
- 430 Macclayton, D.O., Hall II, R.G., 2007. Pharmacologic treatment options for nosocomial
431 pneumonia involving methicillin-resistant *Staphylococcus aureus*. *The Annals of*
432 *Pharmacotherapy* 41, 235-244.
- 433 Masciangioli, T., Zhang, W.X., 2003. Environmental technologies at the nanoscale.
434 *Environmental Science and Technology* 37, 102A-108A.
- 435 McArdle, B.H., Anderson, M.J., 2001. Fitting multivariate models to community data: a
436 comment on distance-based redundancy analysis. *Ecology* 82, 290-297.
- 437 McHugh, G.L., Moellering, R.C., Hopkins, C.C., Swartz, M.N., 1975. *Salmonella*
438 *typhimurium* resistant to silver nitrate, chloramphenicol, and ampicillin. *Lancet* 1, 235-
439 240.
- 440 Owen, R., Handy, R., 2007. Formulating the problems for environmental risk assessment of
441 nanomaterials. *Environmental Science and Technology* 15, 5582-5588.

- 442 Pal, S., Tak, Y.K., Song, J.M., 2007. Does the antibacterial activity of silver nanoparticles
443 depend on the shape of the nanoparticle? A study of the gram-negative bacterium
444 *Escherichia coli*. *Applied Environmental Microbiology* 73, 1712-1720.
- 445 Rasmussen, L.D., Sorensen, S.J., 1998. The effect of longterm exposure to mercury on the
446 bacterial community in marine sediment. *Current Microbiology* 36, 291-297.
- 447 Rozan, T.F., Lassman, M.E., Ridge, D.P., Luther, G.W. III, (2000) Evidence for
448 multinuclear Fe, Cu and Zn molecular sulfide clusters in oxic river waters. *Nature* 406,
449 879-882.
- 450 Sholkovitz, E.R., 1978. The flocculation of dissolved Fe, Mn, Al, Cu, Ni, Co and Cd during
451 estuarine mixing. *Earth and Planetary Science Letters* 41, 77-86.
- 452 SonDI, I., Salopek-SonDI, B., 2004. Silver nanoparticles as antimicrobial agent: a case study
453 on *E. coli* as a model for Gram-negative bacteria. *Journal of Colloid and Interface Science*
454 275, 177-182.
- 455 Stolpe, B., Hassellöv, M., 2007. Changes in size distribution of fresh water nanoscale
456 colloidal matter and associated elements on mixing with seawater. *Geochimica*
457 *Cosmochimica Acta* 71, 3292-3301.
- 458 Stepanauskas, R., Glenn, T.C., Jagoe, C.H., Tuckfield, R.C., Lindell, A.H., McArthur, J.V.,
459 2005. Elevated bacterioplankton tolerance to metals and antibiotics in metal-contaminated
460 industrial environments. *Environmental Science and Technology* 39, 3671-3678.
- 461 Stepanauskas, R., Glenn, T.C., Jagoe, C.H., Tuckfield, R.C., Lindell, A.H., King, C.J.,
462 McArthur, J.V., 2006. Coselection for microbial resistance to metals and antibiotics in
463 freshwater microcosms. *Environmental Microbiology* 8, 1510-1514.
- 464 Uchida, M., Yamamoto, T., Taniguchi, A., 2003. Reaction of silver ions and some amino
465 acids. *Journal of Antibacterial and Antifungal Agents* 31, 695-704.

- 466 Ward, T.J., Kramer, J.R., 2002. Silver speciation during chronic toxicity tests with the
467 mysid, *Americamysis bahia*. *Comparative Biochemistry and Physiology C* 133, 75-86.
- 468 Woodward, E.M.S., Rees, A.P., 2001. Nutrient distributions in an anticyclonic eddy in the
469 northeast Atlantic Ocean, with reference to nanomolar ammonium concentrations. *Deep-*
470 *Sea Research Part II* 48, 775-793.
- 471 Wright, M.S., Peltier, G.L., Stepanauskas, R., McArthur, J.V., 2006. Bacterial tolerances to
472 metals and antibiotics in metal-contaminated and reference streams. *FEMS Microbiology*
473 *Ecology* 58, 293-302.
- 474 Yoon, K.Y., Byeon, J.H., Park, J.H., Hwang, J., 2007. Susceptibility constants of
475 *Escherichia coli* and *Bacillus subtilis* to silver and copper nanoparticles. *Science of the*
476 *Total Environment* 373, 572-575.

Table 1. Bacterial counts in form of colony forming units (cfus) on medium containing cycloheximide and eight different antibiotics. The experiments were carried out in triplicate (control/0 $\mu\text{g l}^{-1}$ overlying estuary water: microcosms 1, 2, 3; 25 $\mu\text{g l}^{-1}$: microcosms 4, 5, 6; 1 mg l^{-1} : microcosms 7, 8, 9).

Microcosm →	1	2	3	4	5	6	7	8	9
Antibiotic ¹⁾									
Cycloheximide	108	65	100	130	142	100	105	97	250
Erythromycin ²⁾	0	0	0	0	0	0	0	0	0
Oxytetracycline ²⁾	6	9	3	5	5	5	4	0	9
Amoxycillin ²⁾	1	15	2	9	5	13	0	0	3
Ceftazidime ³⁾	6	15	11	8	6	5	8	4	17
Sulfadiazine ³⁾	24	44	17	34	17	17	23	11	27
Trimethoprim ³⁾	38	24	46	94	48	17	22	28	27
Lincomycin ²⁾	28	51	23	76	27	37	25	14	23
Vancomycin ³⁾	34	42	10	49	185	17	17	5	8

¹⁾ cycloheximide (20 $\mu\text{g mL}^{-1}$) was added to any of the eight antibiotic-containing media to prevent fungal growth; additionally, to evaluate whether the samples contained comparable numbers of bacteria, aliquots of the samples were also transferred on solidified medium containing only cycloheximide (20 $\mu\text{g mL}^{-1}$)

²⁾ used in a concentration of 20 $\mu\text{g mL}^{-1}$; ³⁾ used in a concentration of 32 $\mu\text{g mL}^{-1}$

Table 2. Results from permutation-based Analysis of Variance tests for differences among groups of samples, using Type III sums of squares based on 999 permutations of residuals under a reduced model. The factors are antibiotic and Ag-NPs. (SS = square sum, MS = medium square, F = ratio of SS/MS)

Source	df	SS	MS	F	p
Antibiotic	7	91580	13083	15.9	0.001
Ag-NPs	2	2962	1481	1.8	0.164
Antibiotic $\times$ Ag-NPs	14	13591	971	1.2	0.264
Residual	48	39565	824		
Total	71	147700			

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Table 3. Results of pairwise tests for differences in counts between antibiotic treatments from permutation-based Analysis of Variance tests using Type III sums of squares based on 999 permutations of residuals under a reduced model. Values of t and its significance is indicated by: n: $p > 0.05$; *: $0.05 \geq p > 0.01$; **: $0.01 \geq p > 0.001$, ***: $p \leq 0.001$.

	Cyclo-heximide	Erythro-mycin	Oxytetra-cycline	Sulfa-diazine	Trimetho-prim	Linco-mycin	Cefta-zidime	Amoxy-cillin
Erythromycin	6.91***							
Oxytetracycline	6.61***	4.90**						
Sulfadiazine	5.44***	6.49***	4.90**					
Trimethoprim	4.34***	4.91***	4.22**	1.68n				
Lincomycin	4.74***	5.76**	4.82***	1.45n	0.46n			
Ceftazidime	6.38***	5.64**	2.00n	3.74**	3.69**	4.10**		
Amoxycillin	6.57***	3.10*	0.11n	4.56**	4.13**	4.66**	1.52n	
Vancomycin	3.26**	2.33*	2.03*	0.95n	0.13n	0.38n	1.81n	2.02*

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