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

3

4

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24 Effects of silver nanoparticles on bacterial antibiotic resistance

25

26

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30

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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<sup>+</sup> 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

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

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

83 Based on this knowledge and the fact that in England and Wales it has been estimated that  
84 more than one-third (ca. 37.5 %) of the total sewage treatment plant effluents are discharged  
85 into estuarine and coastal environments (Environment Agency, 1999), it was decided to  
86 investigate the potential impact of Ag-NP pollution on the natural bacterial populations on  
87 the surface of estuarine sediment where Ag-NPs are likely to accumulate over time. A 30-  
88 day microcosm study was designed in which Ag-NPs were added from a stock solution to  
89 estuarine sediment samples (in triplicate) for 20 days yielding a final cumulative treatment of  
90 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  
91 tanks were left for a further 10 days to allow for any recovery. Although the overall  
92 concentration of Ag-NPs in the water column increased steadily during the 20 days of dosing  
93 there was no influence on the abundance of heterotrophic prokaryotes in the estuary water.  
94 Similarly, despite the predicted accumulation of the Ag-NPs in the surface layer of the  
95 sediment (~top 3 mm), there were only negligible differences in bacterial diversity between  
96 treatments, suggesting that under the selected experimental regime Ag-NPs present little or  
97 no impact on estuarine sediment bacterial diversity (Bradford *et al.*, 2009). Although the  
98 chemical fate of the Ag-NPs after their release into the microcosms was not examined, it is  
99 believed that physicochemical parameters in the brackish environment (such as salinity and

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

108

## 109 **MATERIALS AND METHODS**

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

115

### 116 **Experimental Design**

117 Glass microcosms were prepared in triplicate for each exposure concentration, and were  
118 maintained covered and continuously aerated in a constant-temperature culture room at  
119 15°C. The following three exposure treatments were carried out: 0  $\mu\text{g l}^{-1}$  of Ag-NPs in the  
120 overlaying estuary water (control tanks T01, T02, T03); 50  $\mu\text{g l}^{-1}$  Ag-NPs (T04, T05, T06);  
121 and 2000  $\mu\text{g l}^{-1}$  Ag-NPs (T07, T08, T09) to represent a low-level contamination (50  $\mu\text{g l}^{-1}$ ),  
122 while the 2000  $\mu\text{g l}^{-1}$  concentration was selected as it is much higher than predicted future  
123 environmental concentrations resulting from anthropogenic pollution (Boxall *et al.*, 2007).

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

137 The sediment samples were exposed to a cumulative dose of Ag-NPs that totalled 0  
138 (control), 50 and 2000  $\mu\text{g l}^{-1}$  of overlaying intertidal seawater. The dosing was carried out in  
139 two steps. 1/40<sup>th</sup> of the final concentration of Ag-NPs was added daily to the overlaying  
140 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  
141 were added to the 50 and 2000  $\mu\text{g l}^{-1}$  tanks, respectively. The microcosms were then left  
142 untreated for 10 days, followed by renewed daily dosing (1/40<sup>th</sup> of the final concentration)  
143 for another 20 days (i.e. a total exposure time of 50 days).

144

#### 145 **Characterisation of Ag-NPs**

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

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

151

## 152 **Analysis of water samples and metals in sediments**

153 Water samples (10 ml) were collected for metal analysis before and after each daily addition  
154 of Ag-NPs, and samples were also collected for general nutrient analyses of the seawater (50  
155 ml samples from each tank). Nutrient analyses were performed according to Woodward  
156 (2001) using a Technicon segmented flow colorimetric autoanalyser. The following water  
157 quality parameters were measured at 8 time points (days 0, 1, 4, 10, 15, 20, 25, 30) during  
158 the first 30 days of the incubation: salinity (mean  $\pm$  S.E.M:  $29.47 \pm 0.15 \text{ ‰}$ ), dissolved  
159 oxygen ( $100.7 \pm 0.97 \text{ ‰}$ ), pH ( $7.82 \pm 0.12$ ), ammonium ( $23.9 \pm 1.9 \text{ } \mu\text{M}$ ), nitrate ( $58 \pm 2.1$   
160  $\mu\text{M}$ ), 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  
161 ( $13.17 \pm 0.06 \text{ } ^\circ\text{C}$ ).

162 Metal analysis was performed on water and sediment samples by inductively coupled  
163 plasma-optical emission mass spectrometry (ICP-OES, Varian 725-ES) using matrix  
164 matched standards and 1 % yttrium ( $\text{Y}^{39}$ ) oxide as an internal standard in samples (see  
165 Bradford et al., 2009, for further details). A certified estuarine reference material (LGC  
166 6137, Laboratory of the Government Chemist) was treated likewise as an internal control.  
167 Measured trace element concentrations in the sediments at the start (i.e. day 0) of the  
168 exposure experiment were very similar: (mg metal  $\text{kg}^{-1}$  dry weight, mean  $\pm$  S. E. M.,  $n =$   
169 144); Ca,  $8477 \pm 459$ ; Cu,  $160 \pm 7$ ; Fe,  $36994 \pm 1682$ ; K,  $2801 \pm 152$ ; Mg,  $9402 \pm 490$ ; Na,  
170  $30642 \pm 2332$ ; Zn  $235 \pm 11$ .

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

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

184

#### 185 **Screening for antibiotic resistant bacteria**

186 A 5-ml syringe (without the plunger and with the tip removed) was used to remove a core  
187 sample of the sediment on day 50 of the incubation from each of the nine microcosms. A  
188 100-mg aliquot of the surface layer of each of these samples from the sediment cores was  
189 collected and transferred into 10 ml of filtered ( $0.2 \mu\text{m}$ ) seawater and mixed by vortexing. 50  
190  $\mu\text{l}$  of this suspension and of two 10-fold dilutions of it were transferred onto a 10-times  
191 diluted version of marine broth (per litre of estuarine (29 ‰ salinity) seawater: 0.5 g  
192 peptone, 0.1 g yeast extract, 0.01 g  $\text{FePO}_4\text{-H}_2\text{O}$  was added and was then solidified using  
193 1.5% (w/v) agar). Each of the medium plates contained one of 8 different antibiotics (as well  
194 as cycloheximide ( $20 \mu\text{g ml}^{-1}$ ; Melford Laboratories Ltd., Chelworth, UK) to inhibit fungal  
195 growth). The concentration of the antibiotics was based on those suggested by the DIN –  
196 Deutsches Institut für Normung e.V. (1998). Antibiotics (Sigma-Aldrich, Poole, UK) used  
197 were erythromycin ( $20 \mu\text{g ml}^{-1}$ ), oxytetracycline ( $20 \mu\text{g ml}^{-1}$ ), sulfadiazine ( $32 \mu\text{g ml}^{-1}$ ),

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

202

### 203 **Statistical analyses**

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

213

## 214 **RESULTS**

215

### 216 **Environmental parameters of the microcosms**

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

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

224

### 225 **Antibiotic resistance in the estuarine sediment samples**

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

244

### 245 **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  $\beta$ -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.

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

285

286 It is unlikely that the exposure time (50 days) to the Ag-NPs was too short to generate  
287 antibiotic resistant bacteria, as bacteria undergo multiple replications each day and antibiotic  
288 resistance can be located on plasmids. This is further confirmed by the fact that  
289 Stepanauskas *et al.* (2006) found an increase in antibiotic resistant bacteria after a 7-day  
290 incubation to 1 mM concentrations of CdCl<sub>2</sub> and NiCl<sub>2</sub>. Additionally, the time period of the  
291 exposure is also thought to be sufficient for bacteria to acquire the relevant resistance genes  
292 through horizontal gene transfer as the genes responsible for resistance to heavy metals, such  
293 as the *silE* gene which has been shown to confer resistance to Ag (e.g. Davis *et al.* 2005), are  
294 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  $\text{Ag}^+$ ,  $\text{AgCl}^0$ ,  $\text{AgCl}_2^-$ ,  
306  $\text{AgCl}_3^{2-}$  depend strongly on the salinity of the water, with higher salinity favouring the latter  
307 two complexes. The relative abundance of the  $\text{Ag}^+$  (1.7 %) and  $\text{AgCl}^0$  (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% ( $\text{Ag}^+$ : 0.004%;  
310  $\text{AgCl}^0$  1.4 %) with the Ag complexes comprising the remaining 98% ( $\text{AgCl}_2^-$ : 49 %;  $\text{AgCl}_3^{2-}$   
311 : 49 %). If  $\text{AgCl}_2^-$  and  $\text{AgCl}_3^{2-}$  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  $\text{Ag}^+$  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

320 complexation with organics and, in particular, the formation of silver sulfide clusters (Roza  
321 *et al.*, 2000) can reduce toxicity to *Daphnia magna* (Bowles *et al.*, 2002; Biachini *et al.*,  
322 2002). Such complexation could also lead to reduced antimicrobial activity (due to loss of  
323 bioavailability), and potentially to a reduction in antibiotic resistant bacteria.

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

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

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475 *Escherichia coli* and *Bacillus subtilis* to silver and copper nanoparticles. *Science of the*  
476 *Total Environment* 373, 572-575.

477 Table 1. Bacterial counts in form of colony forming units (cfus) on medium containing  
 478 cycloheximide and eight different antibiotics. The experiments were carried out in triplicate  
 479 (control/0  $\mu\text{g l}^{-1}$  overlying estuary water: microcosms 1, 2, 3; 25  $\mu\text{g l}^{-1}$ : microcosms 4, 5, 6;  
 480 1  $\text{mg l}^{-1}$ : microcosms 7, 8, 9).

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

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

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

485 Table 2. Results from permutation-based Analysis of Variance tests for differences among  
 486 groups of samples, using Type III sums of squares based on 999 permutations of residuals  
 487 under a reduced model. The factors are antibiotic and Ag-NPs. (SS = square sum, MS =  
 488 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 ×Ag-NPs	14	13591	971	1.2	0.264
Residual	48	39565	824		
Total	71	147700			

489

490

491 Table 3. Results of pairwise tests for differences in counts between antibiotic treatments  
 492 from permutation-based Analysis of Variance tests using Type III sums of squares based on  
 493 999 permutations of residuals under a reduced model. Values of t and its significance is  
 494 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
<b>Erythromycin</b>	6.91***							
<b>Oxytetracycline</b>	6.61***	4.90**						
<b>Sulfadiazine</b>	5.44***	6.49***	4.90**					
<b>Trimethoprim</b>	4.34***	4.91***	4.22**	1.68n				
<b>Lincomycin</b>	4.74***	5.76**	4.82***	1.45n	0.46n			
<b>Ceftazidime</b>	6.38***	5.64**	2.00n	3.74**	3.69**	4.10**		
<b>Amoxicillin</b>	6.57***	3.10*	0.11n	4.56**	4.13**	4.66**	1.52n	
<b>Vancomycin</b>	3.26**	2.33*	2.03*	0.95n	0.13n	0.38n	1.81n	2.02*

495