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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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25

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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

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 ($1/40^{\text{th}}$ 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. $1/40^{\text{th}}$ 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 ($1/40^{\text{th}}$ 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 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 ± 0.12), ammonium ($23.9 \pm 1.9 \text{ } \mu\text{M}$), nitrate (58 ± 2.1
160 μM), 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 (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 kg^{-1} dry weight, mean \pm S. E. M., $n =$
169 144); Ca, 8477 ± 459 ; Cu, 160 ± 7 ; Fe, 36994 ± 1682 ; K, 2801 ± 152 ; Mg, 9402 ± 490 ; Na,
170 30642 ± 2332 ; Zn 235 ± 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 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 μl 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°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).

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 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.

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 CdCl₂ and NiCl₂. 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 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

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.

344

345

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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 mg l^{-1} : microcosms 7, 8, 9).

Microcosm →	1	2	3	4	5	6	7	8	9
Antibiotic¹⁾									
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

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