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Bioaccumulation and toxic effects of nanoparticulate and ionic silver in Saccostrea glomerata (rock oyster)

Carrazco-Quevedo, Ana; Römer, Isabella; Salamanca, Maria J; Poynter, Alexander; Lynch, Iseult; Valsami-Jones, Eugenia

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1 Bioaccumulation and toxic effects of nanoparticulate and ionic silver in *Saccostrea glomerata* (rock
2 oyster)

3

4 Ana Carrazco-Quevedo, ‡ [a] Isabella Römer, ‡ [a] Maria J. Salamanca,[a] Alexander Poynter,[a]
5 Iseult Lynch[a] and Eugenia Valsami-Jones[a*]

6 [a.] School of Geography, Earth and Environmental Sciences, University of Birmingham,
7 Edgbaston, Birmingham B15 2TT, UK.

8 ‡ Contributed equally

9

10 **Abstract**

11 The increasing production of Ag nanoparticle (AgNP) containing products has inevitably led
12 to a growing concern about their release into the aquatic environment, along with their
13 potential behaviour, toxicity, and bioaccumulation in marine organisms exposed to NPs
14 released from these products. Hence, this study is focused on the effects of AgNPs to
15 *Saccostrea glomerata* (rock oyster) in artificial seawater (ASW), and includes the evaluation
16 of the NP's stability, dissolution and assessment of the bioaccumulation rate. AgNPs
17 NM300K (20 ± 5 nm) in concentrations of 12.5 μgL^{-1} and 125 μgL^{-1} were used to conduct the
18 experiments, and were compared to a blank and a positive control of 12.5 μgL^{-1} $AgNO_3$.
19 Dissolution in ASW was measured by ICP-OES and stability was assessed by TEM after 1h
20 and 3, 5, and 7 days of exposure. Bioaccumulation in gills and digestive glands was
21 measured after 7 days of exposure. The higher concentration of AgNPs induced more
22 aggregation, underwent less dissolution, and showed less bioaccumulation, while the lower
23 concentration showed less aggregation, more dissolution and higher bioaccumulation. Five
24 biomarkers (EROD: ethoxyresorufin-o-deethylase, DNA strand breaks, LPO: lipid
25 peroxidation, GST: glutathione S-transferase and GR: glutathione reductase) were analysed
26 at 0, 3, 5 and 7 days. Significant differences compared to the initial day of exposure (day 0)
27 were reported in DNA strand breaks after 5 and 7 days of exposure, GST, from the third day
28 of exposure, in all the Ag samples, and in some samples for LPO and GR biomarkers, while
29 no significant induction of EROD was observed. A combined effect for each type of
30 treatment and time of exposure was also reported for DNA strand breaks and GST
31 biomarkers measured at the digestive glands. In general, the significant inductions
32 measured showed the following trend: 125 μgL^{-1} AgNPs > 12.5 μgL^{-1} AgNPs ~ 12.5 μgL^{-1}
33 $AgNO_3$ even though bioaccumulation followed the opposite trend.

34 **Keywords:** *Saccostrea glomerata*, toxicity, seawater, rock oyster, bioaccumulation,
35 biomarker responses, bivalves, nanoparticulate, silver nanoparticles.

36 *Corresponding author.
37 *E-mail address:* e.valsamijones@bham.ac.uk
38

39 Introduction

40 Nanoparticles (NPs) are defined as particles with one or more dimensions of the order of
41 100 nm or less (Rauscher et al. 2017). Particles in this size range present unique properties,
42 which differ significantly from their bulk form, and can be a function of their size, shape, and
43 structure. These properties are of special interest for industrial applications and a wide range
44 of commercial products such as paints, cosmetics, detergents, electronic devices and
45 pharmaceuticals (McCarthy et al.2013). Silver nanoparticles (AgNPs) are considered the
46 most widely used nanomaterials, with applications relevant to their broad antimicrobial
47 activity, as well as their distinct physico-chemical properties, including high electrical and
48 thermal conductivity, catalytic activity and non-linear optical behaviour (Fabrega et al. 2011).

49 As a result of the extensive number of applications for AgNPs, there are significant concerns
50 about their release from consumer products, resulting in large amounts of nanosilver in
51 rivers, lakes, estuaries and coasts through sewage and industrial discharges (Liu et al.
52 2014). In 2012, it was estimated that more than 15% of the AgNPs released into European
53 waters came from biocidal plastics, textiles and bleaching agents, leading to an estimated
54 concentration of AgNPs of 0.01 μgL^{-1} in some exposed areas (Gomes et al. 2014; Katsumiti
55 et al. 2015). The rates of dissolution and aggregation are important factors in assessing the
56 toxic effects of AgNPs in aquatic environments; factors such as the stability of ionic silver
57 (Ag^+), pH, ionic strength, presence of natural organic matter (NOM) and other ligands as well
58 as salinity may influence these effects, including the association of AgNPs with other
59 particulate materials (Römer et al. 2016; Canesi and Corsi 2016). Currently, it is not well
60 understood whether the toxicity of AgNPs is a result of the particle-specific physicochemical
61 properties, the release of Ag^+, or a combination of both (Fabrega et al. 2011; Gomes et al.
62 2012; Misra et al. 2012).

63 Bivalve species are used as indicators of coastal environmental quality in numerous national
64 and international programmes monitoring environmental pollution (Buffet et al. 2013) . Thus,
65 evidence of bioaccumulation and toxicity of AgNPs in bivalve species coupled with data on
66 the behaviour of NPs in seawater, such as agglomeration, dissolution and deposition onto
67 the sediment surface (Gagne et al. 2013; Gomes et al. 2014; Buffet et al. 2013) provide
68 essential information, in order to understand the bioavailable dose and correlate this with the
69 observed toxicity. Bivalve species are considered excellent environmental biomonitors due
70 to their feeding mechanism, which involves filtration of large volumes of water and favours

71 the uptake and bioaccumulation of toxic chemicals (de Lafontaine et al. 2000). Bivalves have
72 the capacity to concentrate/bioaccumulate small particles in their tissues, which has led to
73 concern as to whether AgNPs may bioaccumulate into the food chain and affect other
74 organisms (Rocha et al. 2015). The bioaccumulation of AgNPs in bivalves may be
75 influenced by several factors, including the concentration, exposure route, and size of the
76 NPs (Buffet et al. 2013). *Saccostrea glomerata* (rock oyster) is known as a relatively tolerant
77 species, able to survive in chronically contaminated areas, and is widely used as bioindicator
78 in the assessment of environmental pollution (Edge et al. 2012). Biological responses
79 including cellular biomarkers and reproductive endpoints have been used for biomonitoring
80 studies to identify pollutant (e.g. metals and polycyclic aromatic hydrocarbon) related effects,
81 as well as potential sensitivity to contaminants (Edge et al. 2012), and as such should be
82 applicable for NPs also.

83 Numerous studies in aquatic organisms have demonstrated the toxic effects of AgNPs,
84 including alterations to the hepatopancreas (digestive gland) and gills in bivalves (McCarthy
85 et al. 2013; Rocha et al. 2015; Buffet et al. 2013), cytotoxic, genotoxic, and embryo
86 developmental effects in fish (Fabrega et al. 2011), and DNA damage, alterations in genes,
87 and antioxidant capacity in other aquatic vertebrates (Canesi et al. 2012; Renault 2015;
88 Canesi and Corsi 2016). Biomarkers allow the assessment of the initial responses to
89 environmental perturbations and contamination of different xenobiotics in organisms,
90 including changes in the antioxidant defences such as oxidative stress (de Lafontaine et al.
91 2000). Therefore, the use of biomarkers to detect adverse responses in bivalves under
92 laboratory conditions can help detect the effects of a contaminant within organisms at the
93 gene or cellular level before deeper alterations in the ecosystem occur (van de Oost et al.
94 2003). The metabolism of toxic metallic compounds in organisms results in cellular toxicity
95 due to the formation of reactive oxygen species (ROS), which are neutralised by antioxidant
96 defences, antioxidant substances (glutathione, vitamin E and carotenoids) and enzymes
97 (catalase -CAT, glutathione reductase - GR, and superoxide dismutase - SOD). When the
98 rate of generation of ROS exceeds the antioxidant defence system, the oxidative stress
99 occurs (Finkel and Holbrook 2000) causing deleterious effects, such as protein and DNA
100 oxidation as well as peroxidation of lipids in the cell membrane (Bonnail et al. 2018).

101 In this study, we assessed the effects of exposure to AgNPs on rock oyster (*Saccostrea*
102 *glomerata*) through a range of biomarkers for the first time, including assessing the AgNPs
103 behaviour in artificial seawater (ASW) and their bioaccumulation in rock oysters. The study
104 was performed using a AgNP concentration of 12.5 μgL^{-1}, which was chosen considering the
105 maximum concentration of total silver recorded in the literature (8.9 μgL^{-1} in Galveston bay,
106 Texas) (Buffet et al. 2013). A high concentration of 125 μgL^{-1} was also chosen to mimic

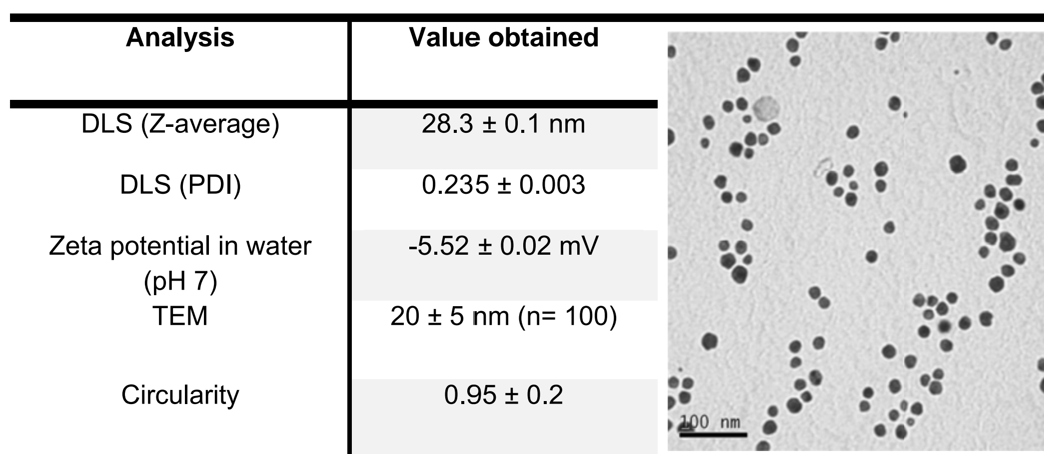
future scenarios, and additionally a blank and a positive (ionic) control of 12.5 µgL^{-1} $AgNO_3$ were used. AgNP dissolution and stability in ASW was measured at 1h and at 3, 5 and 7 days by Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) and transmission electron microscopy (TEM).

Materials and methods

Characterisation of the pristine AgNPs and experimental design

The AgNP used was the OECD representative material Ag NM300K (JRC 2011), obtained from the European Commission Joint Research Centre (JRC) as part of the NanoMILE project (www. http://nanomile.eu-vri.eu/). The Ag NM300K consisted of a spherical colloidal dispersion with a nominal silver content of 122.2 mgmL^{-1}. The NPs were stabilised with 4% (w/w) of the surfactant polyoxyethylene glycerol trioleate and polyoxyethylene sorbitan monolaurate (Tween 20). TEM indicated a size of 20 ± 5 nm, an image is shown in Table 1.

Table 1. Characterisation of the pristine Ag NPs (NM 300K) performed in the laboratory in ultrahigh purity water.



Analysis	Value obtained
DLS (Z-average)	28.3 ± 0.1 nm
DLS (PDI)	0.235 ± 0.003
Zeta potential in water (pH 7)	-5.52 ± 0.02 mV
TEM	20 ± 5 nm (n= 100)
Circularity	0.95 ± 0.2

Abbreviations: Average of Zeta potential (Z-average). Polydispersity Index (PDI). 100 nanoparticles were used to calculate a size distribution (n=100). TEM image used for the analysis is also included.

Adult specimens of *Saccostrea glomerata* (rock oysters) were purchased in a local oyster farm in West Mersea, in the United Kingdom. Twelve oysters were acclimated in 40L experimental tanks supplied with 20L of artificial seawater (ASW) for 7 days. The ASW was prepared by adding enough salt (Instant Ocean® Sea Salt) to obtain a salinity of 35‰; ASW salt contents are shown in Table S1† (supporting information, SI) (Atkinson and Bingman 2008). Salinity was adjusted with a hydrometer (Aquarium Systems Hydrometer®). The water was kept under aeration during the culturing; the temperature (20 °C), salinity (32-35‰), and pH (7.8) of the water were measured daily.

130 At the end of the acclimation period (7 days), oysters were fed for the last time; afterwards, 131 water in all the tanks was partially renewed (except approximately 2 L per tanks). Two 132 dosing concentrations of AgNPs (12.5 μgL^{-1} and 125 μgL^{-1}) were added to 4 tanks 133 (performed in duplicate, 4 tanks per treatment) and mixed by aeration. Following the same 134 procedure, four additional tanks were used as the unexposed blank and $AgNO_3$ as positive 135 ionic control (12.5 μgL^{-1}). The experimental exposure was performed in separate tanks, 136 having four replicates per condition used. We pooled the results for all the oysters that were 137 exposed to the same conditions and concentration of the material. Fifteen oysters were 138 cultured per tank, using three oysters for every biomarkers' time point (0, 3, 6, and 7 days) 139 (n=12). Finally, at day 7, the remaining three oysters were sacrificed for bioaccumulation 140 analysis. The oysters were not fed during the experiment to maintain the water quality and 141 minimize the risk of AgNPs being absorbed by other components such as food or faecal 142 material; no mortality was registered during the experiment.

143 **Characterisation and dissolution of the AgNPs in ASW during the exposure**

144 Water samples (100μL) were taken from the tank at 1h, 3, 5 and 7 days post mixing, and 145 TEM samples were prepared by partially drying a drop of the removed tank water on a 146 copper mesh 400 holey carbon grid (Agar scientific) at room temperature (Römer et al. 147 2013). Grids were carefully washed several times with ultra-high purity (UHP) water and re- 148 dried. Images were obtained using a JEOL 1200EX (accelerating voltage 80 kV), and 149 recorded using Gatan Digital Micrograph software, and images were analysed by Image J, 150 100 particles were analysed per image (n=100). Recorded images are shown in Fig S1, S2 151 and S3†.

152 AgNP dissolution in the ASW containing the rock oysters was assessed after 1 hour and 3, 5 153 and 7 days, in parallel with the total Ag concentration. Samples of ASW (5mL) were taken 154 consecutively from each of the tanks and added into Amicon 15 centrifugal filter units 155 (Millipore®; with regenerated cellulose acetate membranes with 3kDa cut off value and PP 156 filter housing). The units were centrifuged for 30 min at 4444 g-force (Eppendorf 5804-R) 157 (Dogra et al. 2016). The supernatants were acidified to a final concentration of 2% (w/v) 158 HNO_3 and then the Ag content was determined using ICP-OES. To measure the total Ag 159 concentration in ASW, a water sample (5mL) was taken at the same time as the dissolution 160 samples, acid digested with 10% (w/v) HNO_3 overnight, diluted to a final concentration of 2% 161 (w/v) HNO_3, and measured by ICP-OES. Full details of the process, the calibration and the 162 standard solutions used are provided in the SI†.

163 **Bioaccumulation analysis and sublethal effects**

164 After 7 days of exposure, the gills and digestive glands of three oysters per tank and
165 treatment (12 total) were dried at 60°C for 3 days and their dry weight was recorded. Details
166 about the tissue extraction are shown in SI†. The samples were then acid-digested with 4mL
167 of (1:4) H_2O_2 and HNO_3 (w/v) for 2h at 60°C using a microwave (CEM Mars-5 Microwave
168 accelerated reaction system) (Dogra et al. 2016) . The solutions were filtered with a syringe
169 filter with 0.2μm Supor® membrane and the total Ag concentration was measured by ICP-
170 OES.

171 The cytochrome P450 enzyme ethoxyresorufin O-deethylase (EROD) , DNA strand breaks,
172 lipid peroxidation (LPO), glutathione S-transferases (GST), glutathione reductase (GR) and
173 total proteins were measured in the gills and the digestive gland of three oysters per tank
174 after 3, 5 and 7 days of exposure, as well as for twelve day zero unexposed oysters,
175 following their homogenization and centrifugation (15000xg for 30 minutes at 4ºC) (Gagné
176 and Blaise 1993). Full details of the protocols used for all assays are provided in the SI†.

177 **Statistical analysis**

178 The statistical analysis software package (SPSS 22) was used to identify significant
179 treatment effects. Normality of the data and homogeneity of variance were analysed prior to
180 the use of parametric tests. A two-way analysis of variance (ANOVA) was performed to
181 evaluate the effect concentration, kind of Ag used (NP or ionic) and time of exposure upon
182 the biomarkers. A parametric ANOVA test or T-test was used to identify significant
183 differences between the treatments and clean day zero control, followed by a multiple
184 comparison using Dunett's tests and Pearson correlation analysis, applied to assess the
185 correlations between the biomarkers, bioaccumulation and concentration of exposure.
186 Significance level was set at $p \leq 0.05$ and $p \leq 0.01$ to identify statistically significant treatment
187 effects.

188 **Results**

189 **Stability and dissolution of AgNPs in ASW**

190 The stability of the particle dispersions was measured for both concentrations of AgNPs.
191 Large agglomerates were observed after 1 hour when measured by TEM (Fig S1†). The
192 images obtained for the AgNPs at 12.5 μgL^{-1} (12.5 AgNPs) showed agglomerates with a size
193 distribution between 0.2 to 0.5μm, which started to decrease in size by day 3 (Fig. S1†, A, B,
194 C, D†). In the case of AgNPs 125 μgL^{-1} (125 AgNPs) large agglomerates that ranged
195 between 0.1 to 0.5 μm in size were observed, as well as the presence of some individual
196 NPs of 19 ± 5 nm (n=100) after 1h (Fig. S1†. E, F, G, H† and Table S2†). After 3 days, both
197 smaller or slightly smaller free NPs (11 ± 6 nm, n=100) and slightly larger free particles (46 ±

198 12 nm) were observed. More images for the free AgNPs after 1h and 3 days can be found in
199 Fig. S2† and Fig. S3† in SI, a table with a summary of the results can also be found in Table
200 S2†. The larger free particles also have a smaller circularity compared to the pristine AgNPs,
201 (0.7 ± 0.2 compared to the initial value of 0.9 ± 0.1), which were significantly different ($p < 0.05$), and show very uneven surfaces. The smaller free particles had a circularity of 0.9 ±
203 0.2, which was much closer to the values obtained for the pristine NPs.

204 The dissolution data indicate that both concentrations of AgNPs underwent partial
205 dissolution, with an average dissolution of around 20-30% (Table 2). For 12.5 AgNPs, 26.6 ±
206 0.2% of Ag measured was present as dissolved Ag after 1 hour; at 3 days, the percentage of
207 dissolved Ag showed a peak of 45 ± 2%. However, by 5 and 7 days, a rapid decrease in free
208 Ag^+ was observed, from 15 ± 2 % to a concentration below the ICP-OES detection limit (<0.5
209 μgL^{-1}) (PerkinElmer 2008). The highest percentage of dissolved Ag in the case of 125
210 AgNPs was at day 3 with 31 ± 6%; this was double the percentage of dissolution registered
211 at 1h (14 ± 2 %). At day 5, a decrease was registered, going from 31 ± 6% on day 3 to 24 ±
212 5% on day 5 (Table 2). In addition, the dissolution of 125 AgNPs at day 7 presented a
213 different pattern in comparison to 12.5 AgNPs that showed a dissolution of 28 ± 4% in
214 contrast with the concentration below the ICP-OES detection limit observed for 12.5 AgNPs.
215 In the case of the $AgNO_3$ treatment, after 3 days there was already an 85.4 ± 0.7 % loss of
216 silver, presumably due to bivalve's filtration, bioaccumulation in tissues and/or abiotic
217 precipitation or adhesion to the tank's surface. In both AgNP cases, the concentration of
218 silver detected in the tank water by day 7 was <10% of the initial concentration of silver
219 applied.

Table 2. AgNPs and $AgNO_3$ concentration and dissolution in artificial seawater. Total silver concentration was measured by ICP-OES, as well as the dissolved silver after centrifugal filtration. All samples were taken from the tanks containing the oysters, which may have absorbed part of the Ag from the solution. All values show the mean and the standard deviation of 4 measurements.

Treatment (μgL^{-1})	**Analysis (μgL^{-1})**	**Time points measured**			
		1 Hour	**3 Days**	**5 Days**	**7 Days**
12.5 AgNPs	Total Ag	12.5 ± 0.01	2.03 ± 0.09	2.2 ± 0.1	1.1 ± 0.1
	Ag dissolution	3.32 ± 0.02	1.05 ± 0.1	0.35 ± 0.06	<0.5
	Percentage (%)	26.6 ± 0.2	45 ± 2	15 ± 2	<0.5
125 AgNPs	Total Ag	125.2 ± 0.1	14 ± 2	11 ± 2	8.1 ± 0.8
	Ag dissolution	17 ± 2	4 ± 1	2.4 ± 0.5	2.2 ± 0.4
	Percentage (%)	13 ± 2	31 ± 6	24 ± 5	28 ± 4
12.5 $AgNO_3$	Total Ag	12.5 ± 0.01	1.83 ± 0.09	1.5 ± 0.1	<0.5
	Sample loss (%)	0	85.4 ± 0.7	88.8 ± 0.4	<0.5

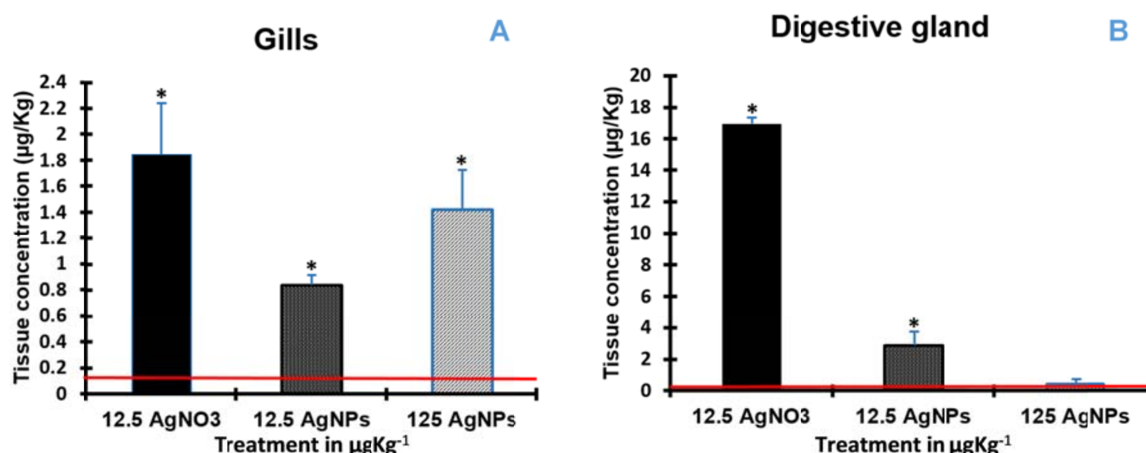
<0.5 represents the ICP-OES detection limit for Ag. Obtained concentrations are presented in μgL^{-1}, unless otherwise stated.

220

221 **Bioaccumulation of Ag in tissues**

222 Bioaccumulation is an important process to enable understanding of the potential effects
223 resulting from the exposure of an organism to pollutants such as AgNPs. The
224 bioaccumulation of a xenobiotic is considered a precursor of toxicity; and AgNPs are not the
225 exception to this generality (Fabrega et al. 2011). Here, the bioaccumulation of total Ag in
226 gills and the digestive gland after 7 days of exposure was analysed and compared to the
227 $AgNO_3$ treatment (Fig. 1B) As expected, the bioaccumulation of the $AgNO_3$ treatment in both
228 tissues was higher compared to the AgNPs, despite the NPs undergoing partial dissolution.
229 The 12.5 AgNPs concentration presented a lower accumulation in the gills, 0.840 ± 0.008
230 μgKg^{-1}, compared with the 125 AgNPs that presented almost double the bioaccumulation
231 (1.42 ± 0.03 μgKg^{-1}) despite the x10 higher concentration potentially available (Fig. 1A).

232 The digestive gland showed an opposite effect compared to the results obtained for the gills.
233 Here, the digestive gland presented a higher accumulation in the case of the 12.5 AgNPs,
234 2.87 ± 0.09 μgKg^{-1}, compared to 0.41 ± 0.03 μgKg^{-1} for 125 AgNPs, which was the only
235 measured sample that did not present significant differences ($p<0.01$) compared to the
236 control (which was assumed to be the detection limit of the ICP-OES, Fig. 1). In the case of
237 the $AgNO_3$ treatment, we observed the highest bioaccumulation rate (16.95 ± 0.04 μgKg^{-1})
238 which was almost 6 times higher than the concentrations obtained for 12.5 AgNPs (Fig 1B).



239

240 **Fig 1.** The bioaccumulation of total Ag for all treatments (μgKg^{-1} dry weight) after 7 days of
241 exposure, (A) in gills and (B) in digestive gland. The graphs represent the mean of 12
242 oysters per treatment (3 oysters per tank used). Control (ASW only) is not included as Ag
243 values were under limit detection of the ICP-OES. Standard deviation was calculated from
244 the 12 samples measured. The red line represents the limit of detection of the ICP-OES

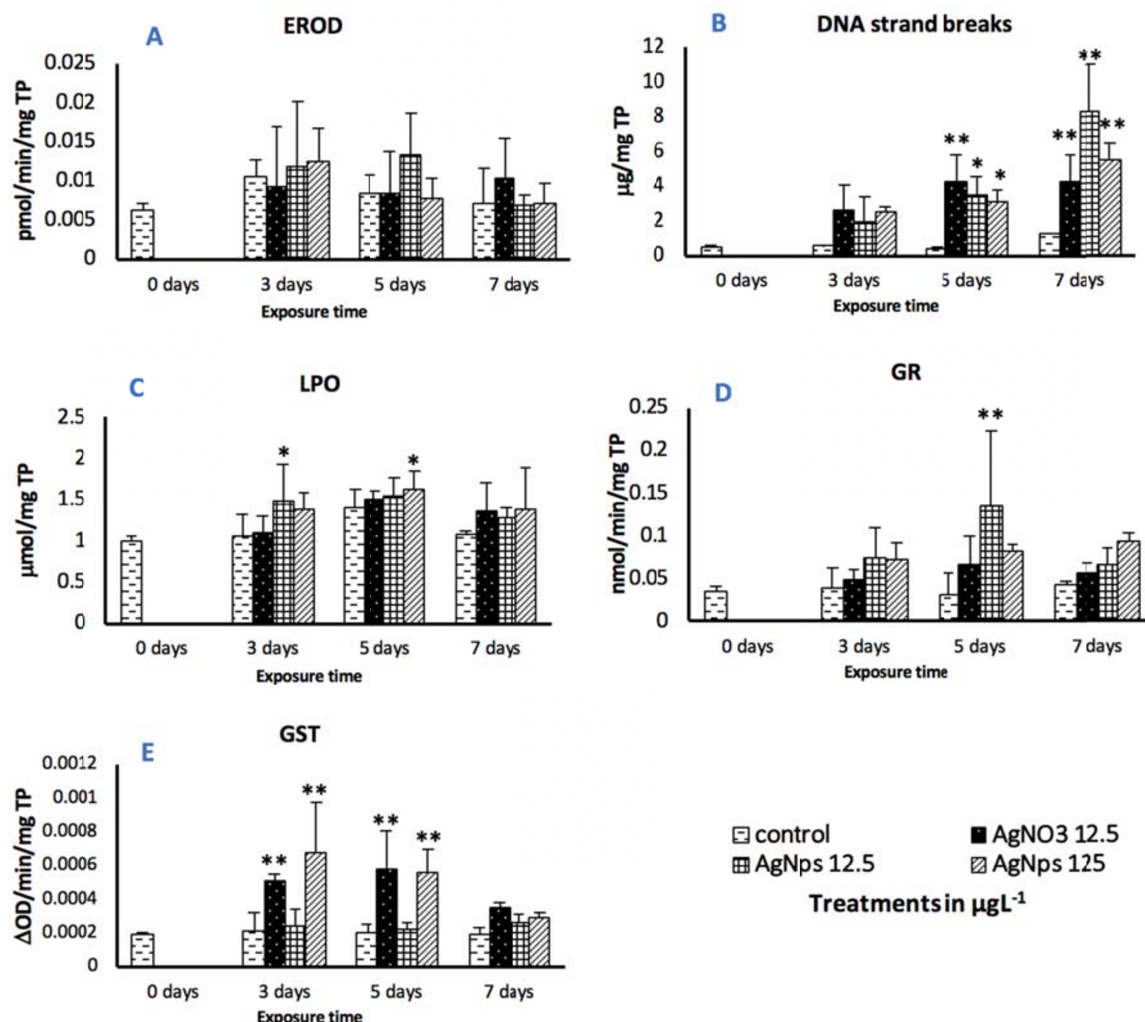
245 (0.014 μgKg^{-1} for gills and 0.005 μgKg^{-1} for digestive glands) while significant differences
246 compared to the detection limit ($p<0.01$) are indicated with asterisks.
247

248 **Biomarkers**

249 The results obtained can be found in Table S3† and Fig. 2 and 3. It can be observed that the
250 AgNPs and $AgNO_3$ treatments used in this study had a significant influence upon EROD
251 measured in gills only for the exposure time, however no interaction between concentration
252 and exposure time was observed (Table S3†). No correlations were found between EROD
253 and the other biomarkers measured or Ag bioaccumulated in the tissues.

254 AgNPs, as well as $AgNO_3$ treatment, induced DNA damage in the organisms exposed from
255 the 5th day of exposure, showing significant differences compared to the control day zero
256 organisms (Fig. 2B and 3B). A significant difference was only found between the untreated
257 control in the digestive glands at 7 days of exposure. In both organs (gills and digestive
258 gland), an effect due to concentration, along exposure time and as combination of both
259 ($p<0.05$) was suggested by the two-way ANOVA (Table S3†). A positive correlation was
260 found between DNA strand breaks measured in the digestive gland and in the gills
261 (R^2=0.863).

262 According to our results, no significant dependence between LPO treatment and
263 concentration was found for either tissue analysed. In the gills, the two-way ANOVA showed
264 a relation between LPO induction and exposure time ($p<0.05$), while in the digestive gland
265 samples it seems to have an accumulative effect between concentration and exposure time
266 ($p<0.05$). The results obtained for the gills and the digestive gland were compared to the day
267 zero control (Fig. 2C and 3C); a statistically significant difference was only observed for the
268 gills on the 3rd day of exposure, and for 12.5 AgNPs and 125 AgNPs on the 5th day of
269 exposure. LPO measured in the digestive gland was positively correlated with other
270 biomarkers measured: DNA strand breaks measured in the digestive gland (with R^2= 0.539)
271 and GST in the gills (R^2=0.729).



272

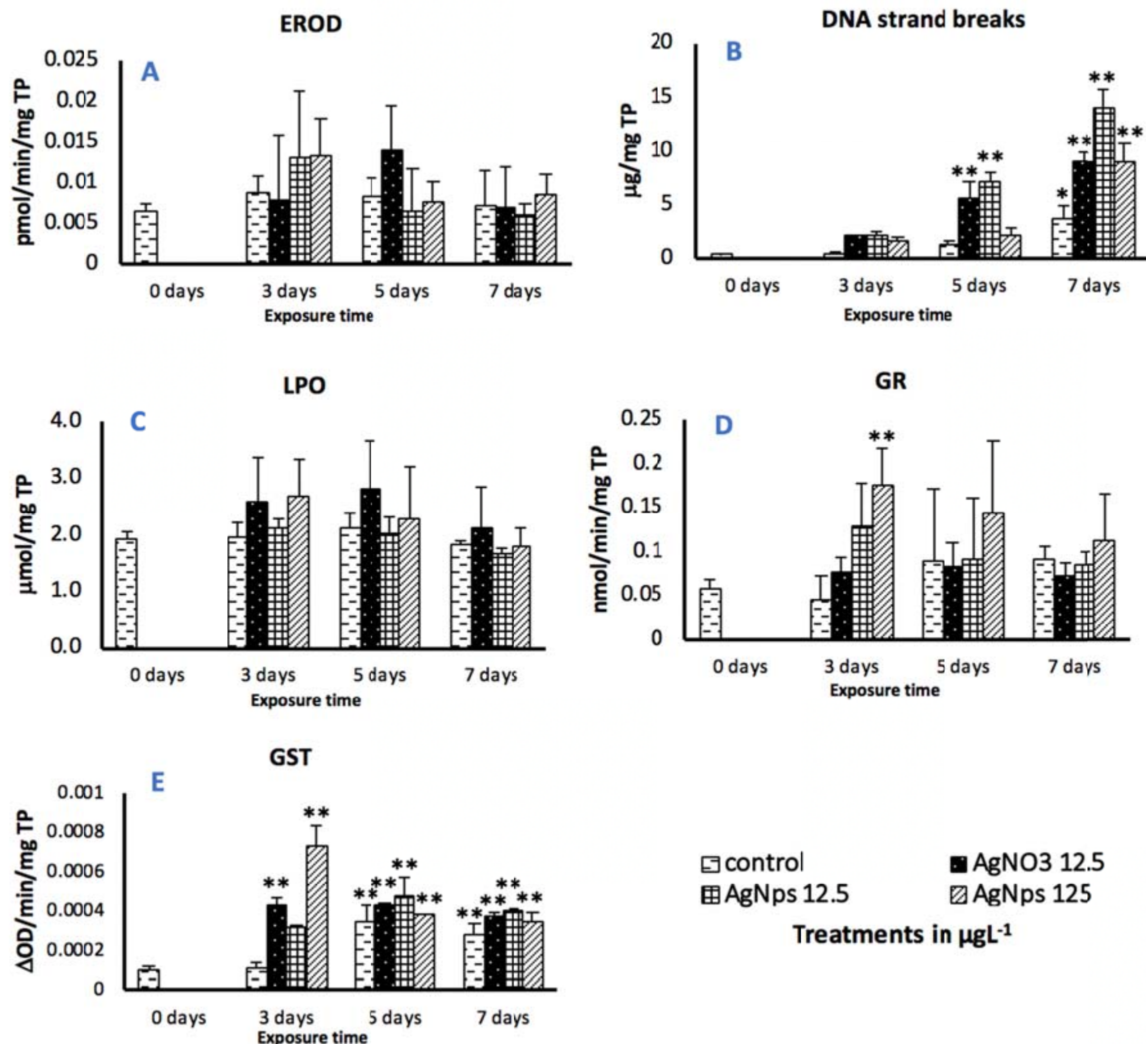
273 **Fig 2.** Mean and standard deviation (n=12) values for Ethoxyresosufine-o-deethylase activity
274 (EROD), DNA damage (strand breaks), lipid peroxidation (LPO), glutathione-S-transferase
275 (GST) and glutathione reductase (GR) analysed in gills for all the materials used (in μgL^{-1})
276 and blank compared to the day zero activity levels. Significant differences are indicated with
277 an asterisk (p>0.05 *, p>0.01**).

278

279 GST measured in the gills and the digestive gland showed a dependence on exposure time
280 and a combination of the effect of exposure time and concentration (Table S3†, $p<0.05$). For
281 the digestive gland, a relationship between the concentration or type of material used and
282 the induction of this biomarker was also observed (Table S3†, $p<0.05$). 12.5 AgNPs and
283 $AgNO_3$ treatments significantly induced the activity of phase II detoxification in the gills and
284 the digestive gland in comparison to the day zero control. This is in agreement with the
285 major inductions that were observed on day 3 and 5 for 125 AgNPs and $AgNO_3$ for gills, and
286 for digestive glands at 3, 5 and 7 day of exposure. For the untreated control and 12.5
287 AgNPs, we found significant inductions in the samples for day 5 and 7 measured in the

288 digestive gland (Fig. 3E). A positive correlation was found between GST measured in the
289 gills and the LPO from the digestive gland (R^2=0.729).

290 For GR activity, a significant relation was found between induction and exposure time for all
291 the samples (Table S3†, p<0.05). For the gills, it seems that concentration and material used
292 are also important for induction (Table S3†, p<0.05), not only time, but we did not find
293 correlation between both factors.



294

295 **Fig 3.** Mean and standard deviation (n=12) values for Ethoxyresosufine-o-deethylase activity
296 (EROD), DNA damage (strand breaks), lipid peroxidation (LPO), glutathione-S-transferase
297 (GST) and glutathione reductase (GR) analysed in the digestive glands for all the materials
298 used (in μgL^{-1}) and blank compared to the day zero activity levels. Significant differences are
299 indicated with an asterisk (p>0.05 *, p>0.01**).

300 AgNPs significantly induced GR activity at day 3 (for 125 AgNPs) in the digestive gland
301 compared to day zero levels (Fig 3D, p<0.01). After 5 days of exposure to 12.5 AgNPs,
302 significant differences in GR expression in the gills were detected, compared to the day zero

303 control individuals (Fig 2D, p<0.01). GR activity measured in the digestive gland positively
304 correlated with the bioaccumulation in the digestive gland (R^2=0.501).

305 **Discussion**

306 During the AgNPs and $AgNO_3$ exposure to oysters and stability analysis, we found that 12.5
307 μgL^{-1} AgNPs tend to dissolve more and at a faster rate (Table 2) than more concentrated
308 particles, which suggests that particle concentrations in media also influence their
309 aggregation/dissolution (Heithmar 2011). Zook et al. (2011) found that dissolution rates in
310 environmentally relevant media were much higher for lower AgNPs concentrations (5 μgL^{-1})
311 than for higher ones (100 μgL^{-1}) (Zook et al. 2011). Heithmar (2011) showed that when 50
312 nm citrate-capped AgNPs were diluted from 16 μgL^{-1} to 160 ngL^{-1}, agglomeration was
313 suppressed and the degree of apparent dissolution increased 5-fold (Heithmar 2011). After 3
314 days, in the case of the 125 μgL^{-1} AgNPs, we still found non-aggregated AgNPs. We
315 observed smaller or slightly smaller particles compared to the pristine NPs which could be
316 formed by dissolution or photofragmentation of the pristine NPs, and slightly larger particles,
317 possibly formed by secondary precipitation and/or reduction, which could be the first step
318 before forming larger aggregates (Li and Lenhart 2012; Tejamaya et al. 2012). Experimental
319 data by Johnson et al. (2015) showed that agglomeration and particle size were directly
320 related to particle concentration, and that particle concentration affected particle size more
321 than ionic strength (Johnson et al. 2015).

322 Silver nanoparticles in contact with biological systems may suffer from two main
323 transformations: dissolution and release of Ag ions and/or NPs aggregation, which is
324 strongly related with the fact that when the dissolution rate decreases, the NPs' aggregation
325 state increases (Kvítek et al. 2008; Römer et al. 2011) . Media composition has a strong
326 influence on NP agglomeration, and therefore is considered an important factor that may
327 induce different effects from NPs in organisms (Römer et al. 2011). It is well known that
328 seawater has a high ionic strength and will induce aggregation very quickly (Buffet et al.
329 2011). In addition, it has been observed that the antibacterial activity of AgNPs decreases
330 when aggregates are formed (Kvítek et al. 2008; Li and Lenhart 2012). The presence of
331 organisms in the media can affect particle dissolution and agglomeration in the exposure
332 medium (Griffitt et al. 2008), highlighting the importance of studying dissolution rates of
333 AgNPs in the presence of organisms (Buffet et al. 2011).

334 We observed that gills were more likely to bioaccumulate at high AgNP concentration (125
335 μgL^{-1}), compared to the digestive gland that bioaccumulated at a higher rate at the lower
336 concentration (12.5 μgL^{-1}). We believe that large aggregates in the ASW may be trapped in
337 the gills impeding their entrance into the digestive gland. Different studies have shown a

338 major likelihood of bivalves to bioaccumulate phytoplankton and small particles, in the range
339 of 1-5 μm via their gills, therefore small agglomerates of NPs have the potential to be
340 internalised easily by this organ (Ward and Kach 2009; Johnson et al. 2015). Thus, the
341 preferential accumulation of NPs is via capture and ingestion, followed by a substantial
342 accumulation in the gills and digestive gland respectively (Canesi et al. 2012).
343 Bioaccumulation of Ag when exposed to NPs in bivalves may be influenced by different
344 factors including the NPs' size, concentration, exposure route, media, structure and NP
345 dispersion, dissolution and aggregation (Fabrega et al. 2011).

346 On the other hand, we also observed that the $AgNO_3$ treatment presented a higher
347 propensity to bioaccumulate in the organisms, as described in previous studies (Jimeno-
348 Romero et al. 2017; Canesi et al. 2012; Gomes et al. 2012). This is consistent with the data
349 displayed for 12.5 AgNPs, which presented a higher dissolution rate, and thus a higher silver
350 concentration in the digestive gland. We associate this to the fact that dissolved silver does
351 not present any difficulty to be internalised by the gills due to its chemical form that makes it
352 more amenable to uptake via cell membrane transportation (Fabrega et al. 2011).

353 Regarding the biomarker activities, the only significant induction in the blank treatment (in
354 only ASW) was detected in the case of GST activity in the digestive glands after 5 and 7
355 days of exposure and DNA strand breaks after 7 days of experiment. This can be related to
356 the stress caused by the laboratory conditions (e.g. starvation), but considering the other
357 biomarker activities, it can be assumed that the induction in the case of the exposed
358 samples is caused by the presence of silver and not due to the laboratory conditions.

359 Differences between AgNPs and $AgNO_3$ treatment were not as high as expected, but there
360 was more DNA damage after 7 days of exposure in both studied organs (gills and digestive
361 glands) in the case of the oysters exposed to the highest AgNPs concentration than those
362 exposed to $AgNO_3$. Additionally, we found significant induction compared to day zero in the
363 case of the LPO measured in the gills and the GR activity in the gills and digestive glands of
364 AgNPs exposed organisms, while there was no significant induction in the same biomarkers
365 for $AgNO_3$. This is in accordance with the results obtained by McCarthy et al. (2013)
366 (McCarthy et al.2013). The only exception to this trend is the GST induction in the gills,
367 which was observed after 3 days of exposure to $AgNO_3$ and not to AgNPs at the same
368 concentration. The LPO and EROD measured in the digestive gland did not show significant
369 induction compared to day zero, the rest of the biomarkers measured showed a higher
370 induction throughout the exposure time. Taking into account time and dose, the biomarkers
371 that reported the highest induction (DNA strand breaks and GST in digestive glands) were
372 the same as the two-way ANOVA revealed to have the most important synergistic effect. Our

373 results agree with other cases of induction reported in bivalves after exposure to different
374 metal NPs (Katsumiti et al. 2015; Buffet et al. 2014). In general, several studies have shown
375 that NPs contribute to oxidative stress and to DNA damage (Dogra et al. 2016; McFarland et
376 al. 1999; Unfried et al. 2007). For example, Katsumiti et al. (2015) also found differences in
377 the toxicity of ionic Ag, bulk Ag and AgNPs in terms of oxidative stress, activation of
378 antioxidant mechanism and genotoxicity to mussels, suggesting that further studies are
379 necessary to assess the contribution of released Ag ions and AgNPs to observed toxic
380 effects (Katsumiti et al. 2015). It also has been observed that healthy individuals clearly
381 responded to a fall in the biomarker levels when the defence mechanisms were
382 overwhelmed (Katsumiti et al. 2015). This can be related to the results of the oysters
383 exposed in this bioassay considering the GR and GST trend followed along the exposure
384 time.

385 The indicator of oxidative stress, GST, and the effect of DNA strand breaks seem to be the
386 most sensitive biomarkers from those studied in our experiment. In general, the use of
387 biomarkers as 'early warning' tools has demonstrated that metals, including metallic NPs,
388 can be toxic to aquatic life. For example, in clams, several defence biochemical biomarkers
389 were activated in the presence of AgNPs, and GST has been classified as an antioxidant
390 defence system in various aquatic species such as bivalves (Mouneyrac et al. 2014; Regoli
391 et al. 2011). This is in accordance with our results, which showed induction of activities in
392 biotransformation enzymes, GST activity, and oxidative stress as LPO and DNA damage, at
393 different days of exposure to AgNPs and $AgNO_3$ in gills and digestive gland, indicating
394 cellular damages caused by these compounds. We observed that EROD did not present
395 significant inductions. Although this biomarker has been reported in cases of mixture of
396 contaminant, including metals, it seems that induction occurs mainly in cases of organic
397 xenobiotics, pesticides or pharmaceutical compounds (van de Oost et al. 2003). The other
398 measured biomarkers were co-related to each other, especially DNA strand breaks, GST,
399 GR and LPO, indicating that more than one detoxification mechanism can occur in parallel
400 until the systems collapses and begins to decay, which usually happens when
401 histopathological damages appear (Regoli et al. 2011). Both are associated with/or use
402 Glutathione (GSH). GR is involved in transforming oxidized GSH (e.g. GSSG) back to
403 reduced GSH. Therefore, increases in GR could reflect increases in oxidized
404 glutathione. GST conjugates GSH with toxic metabolites, so impacts on GSH related to
405 oxidative stress could impact GST and GT dynamics. Starvation also results in depleted
406 GSH levels, so increased GR could be a mechanism to generate more reduced GSH.

407 Exposure to 125 AgNPs produced the highest number of significant inductions, except for
408 GST, followed by exposure to 12.5 AgNPs. Therefore, although there was more Ag

409 availability in the first 5 days of exposure according to the analyses, which is reflected in
410 greater bioaccumulation in gills and digestive glands, the responses to AgNPs cannot be
411 underestimated since they produce a toxic effect similar or even greater (for the same
412 concentration) than the $AgNO_3$ treatment. This means that aggregates and free AgNPs
413 clearly influenced the toxicity and induction of the biomarkers measured in this study.

414 Conclusions

415 In our study, we showed that the NP's concentration in the system plays an important role on
416 the toxicity mode of action as well as in their bioaccumulation. Filter-feeding bivalves can
417 efficiently internalise, capture and ingest NPs that are incorporated into the aquatic system;
418 demonstrated by the fact that the bivalves' gills may function as a net to trap bigger NPs or
419 aggregates, but allowing the internalization of Ag ions. The bioaccumulation of $AgNO_3$ was
420 higher in the analysed tissues compared to AgNPs; the lower concentration (12.5 AgNPs)
421 had a higher dissolution rate in the presence of oysters, compared to the higher
422 concentration (125 AgNPs), which showed a higher bioaccumulation in the gills, but not the
423 digestive gland. We found that the concentration of Ag ions in the 12.5 AgNPs decreased
424 after 7 days, in contrast to the results obtained for the 125 AgNPs, which presented more
425 aggregates and consequently influenced the bioaccumulation rate.

426 Significant differences compared to the blank were reported in the DNA strand breaks, GST
427 LPO and GR biomarkers, while no significant induction in EROD was observed. We found
428 that the effects produced by AgNPs and aggregates cannot be underestimated since we
429 observed a toxic effect similar or even greater (for the same concentration) than $AgNO_3$,
430 although a higher number of significant inductions was measured for 125 AgNPs.

431 These results indicate the importance of assessing the AgNPs interactions in ASW, where
432 factors such as, pH, NP concentration, size, and salinity, may induce dissolution and
433 aggregation of AgNPs, influencing the organism's uptake and bioaccumulation. Clearly,
434 further studies are necessary to evaluate the potential role of different types of nanoparticles
435 in relevant environmental exposures.

436 Conflicts of interest

437 There are no conflicts to declare.

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