

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

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

DOI:

[10.1016/j.ecoenv.2019.04.032](https://doi.org/10.1016/j.ecoenv.2019.04.032)

License:

Creative Commons: Attribution-NonCommercial-NoDerivs (CC BY-NC-ND)

Document Version

Peer reviewed version

Citation for published version (Harvard):

Carrasco-Quevedo, A, Römer, I, Salamanca, MJ, Poynter, A, Lynch, I & Valsami-Jones, E 2019, 'Bioaccumulation and toxic effects of nanoparticulate and ionic silver in *Saccostrea glomerata* (rock oyster)', *Ecotoxicology and Environmental Safety*, vol. 179, pp. 127-134. <https://doi.org/10.1016/j.ecoenv.2019.04.032>

[Link to publication on Research at Birmingham portal](#)

Publisher Rights Statement:

Checked for eligibility: 24/05/2019

General rights

Unless a licence is specified above, all rights (including copyright and moral rights) in this document are retained by the authors and/or the copyright holders. The express permission of the copyright holder must be obtained for any use of this material other than for purposes permitted by law.

- Users may freely distribute the URL that is used to identify this publication.
- Users may download and/or print one copy of the publication from the University of Birmingham research portal for the purpose of private study or non-commercial research.
- User may use extracts from the document in line with the concept of 'fair dealing' under the Copyright, Designs and Patents Act 1988 (?)
- Users may not further distribute the material nor use it for the purposes of commercial gain.

Where a licence is displayed above, please note the terms and conditions of the licence govern your use of this document.

When citing, please reference the published version.

Take down policy

While the University of Birmingham exercises care and attention in making items available there are rare occasions when an item has been uploaded in error or has been deemed to be commercially or otherwise sensitive.

If you believe that this is the case for this document, please contact UBIRA@lists.bham.ac.uk providing details and we will remove access to the work immediately and investigate.

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 AgNO₃.
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 AgNO₃ 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 $0.01 \mu\text{gL}^{-1}$ 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

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

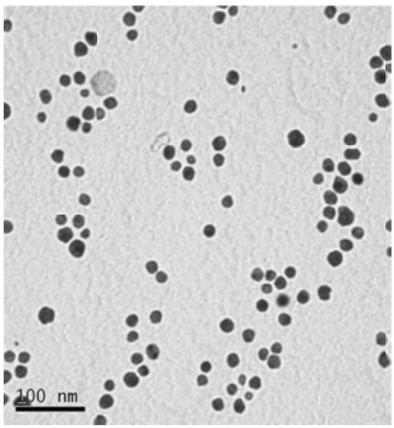
111 **Materials and methods**

112 **Characterisation of the pristine AgNPs and experimental design**

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

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

Analysis	Value obtained
DLS (Z-average)	$28.3 \pm 0.1 \text{ nm}$
DLS (PDI)	0.235 ± 0.003
Zeta potential in water (pH 7)	$-5.52 \pm 0.02 \text{ mV}$
TEM	$20 \pm 5 \text{ nm}$ (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.

121

122 Adult specimens of *Saccostrea glomerata* (rock oysters) were purchased in a local oyster
 123 farm in West Mersea, in the United Kingdom. Twelve oysters were acclimated in 40L
 124 experimental tanks supplied with 20L of artificial seawater (ASW) for 7 days. The ASW was
 125 prepared by adding enough salt (Instant Ocean® Sea Salt) to obtain a salinity of 35‰; ASW
 126 salt contents are shown in Table S1† (supporting information, SI) (Atkinson and Bingman
 127 2008). Salinity was adjusted with a hydrometer (Aquarium Systems Hydrometer®). The
 128 water was kept under aeration during the culturing; the temperature ($20 \text{ }^\circ\text{C}$), salinity (32-
 129 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 ($12.5 \mu\text{gL}^{-1}$ and $125 \mu\text{gL}^{-1}$) 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 ($12.5 \mu\text{gL}^{-1}$). 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 ($100\mu\text{L}$) 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 S1†. The samples were then acid-digested with 4mL
167 of (1:4) H₂O₂ and HNO₃ (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 4°C) (Gagné
176 and Blaise 1993). Full details of the protocols used for all assays are provided in the S1†.

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≤0.05 and p≤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 ($p <$
 202 0.05), and show very uneven surfaces. The smaller free particles had a circularity of $0.9 \pm$
 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, $26.6 \pm$
 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 $45 \pm 2\%$. However, by 5 and 7 days, a rapid decrease in free
 208 Ag^+ was observed, from $15 \pm 2\%$ 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 $31 \pm 6\%$; this was double the percentage of dissolution registered
 211 at 1h ($14 \pm 2\%$). At day 5, a decrease was registered, going from $31 \pm 6\%$ on day 3 to $24 \pm$
 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 $28 \pm 4\%$ 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 $85.4 \pm 0.7\%$ 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

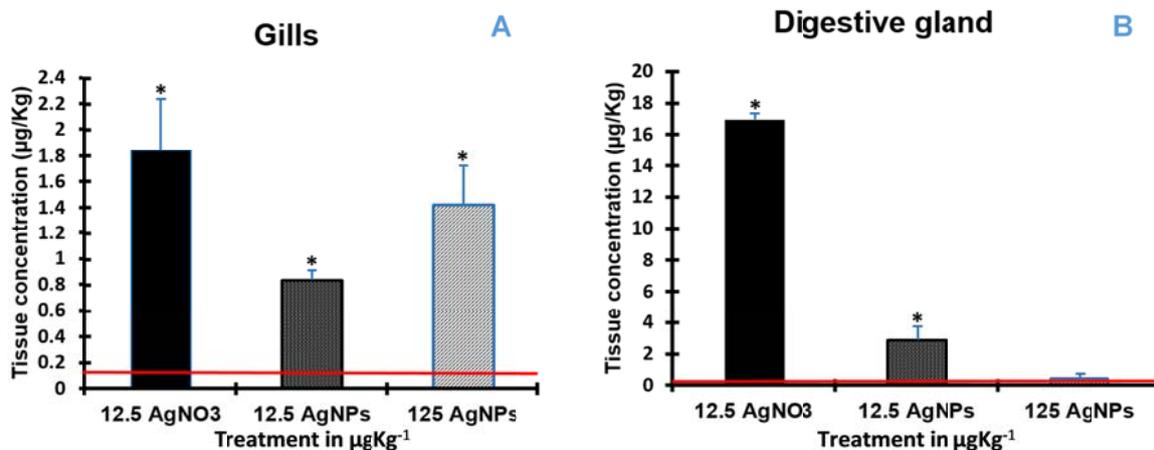
*<0.5 represents the ICP-OES detection limit for Ag. Obtained concentrations are presented in μgL^{-1} , unless otherwise stated.

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 ($1.42 \pm 0.03 \mu\text{gKg}^{-1}$) 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 $2.87 \pm 0.09 \mu\text{gKg}^{-1}$, compared to $0.41 \pm 0.03 \mu\text{gKg}^{-1}$ 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 ($16.95 \pm 0.04 \mu\text{gKg}^{-1}$)
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

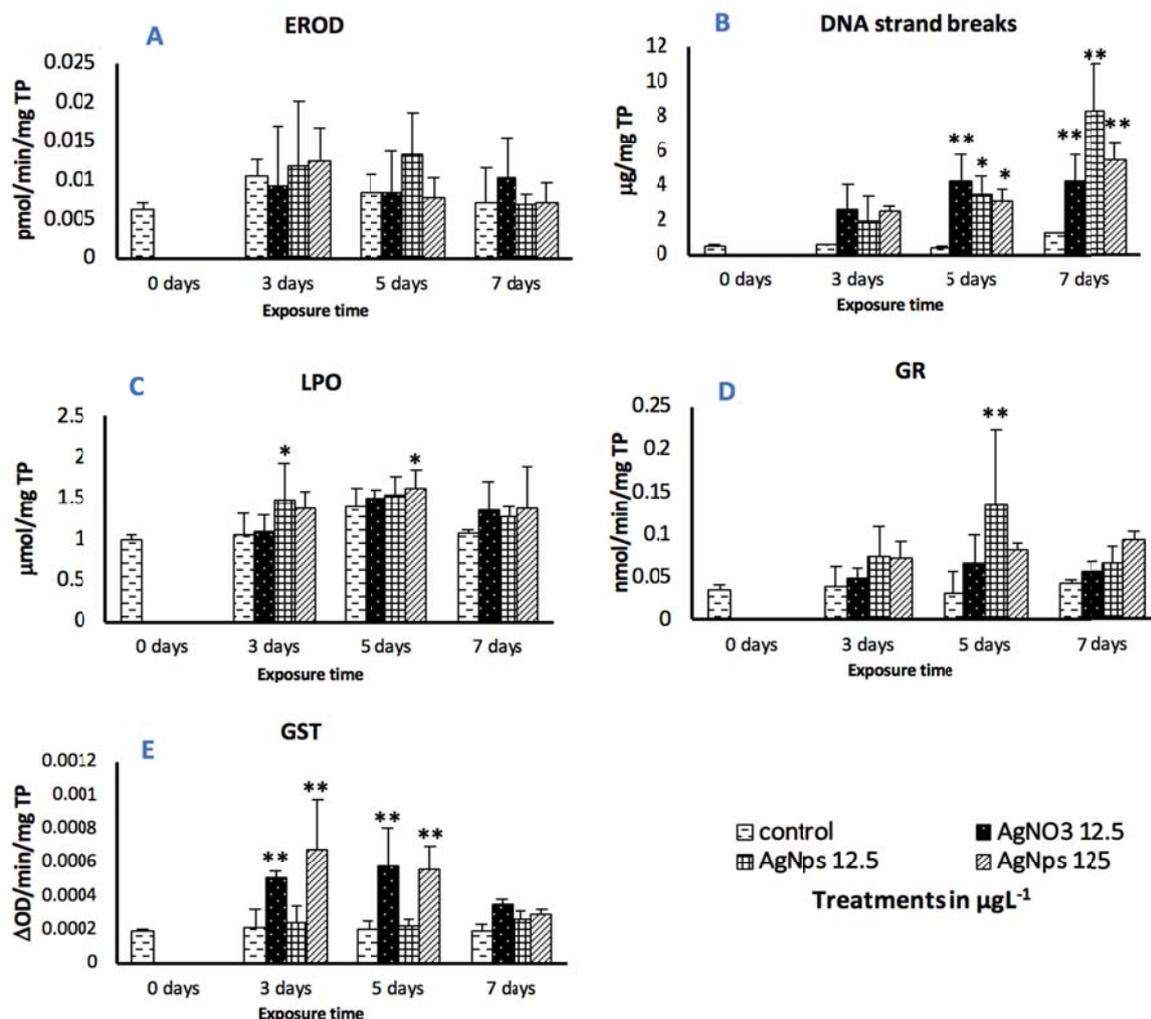
245 (0.014 μgKg^{-1} for gills and 0.005 μgKg^{-1} for digestive glands) while significant differences
246 compared to the detection limit ($p<0.01$) are indicated with asterisks.
247

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 AgNO₃ 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 AgNO₃ 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

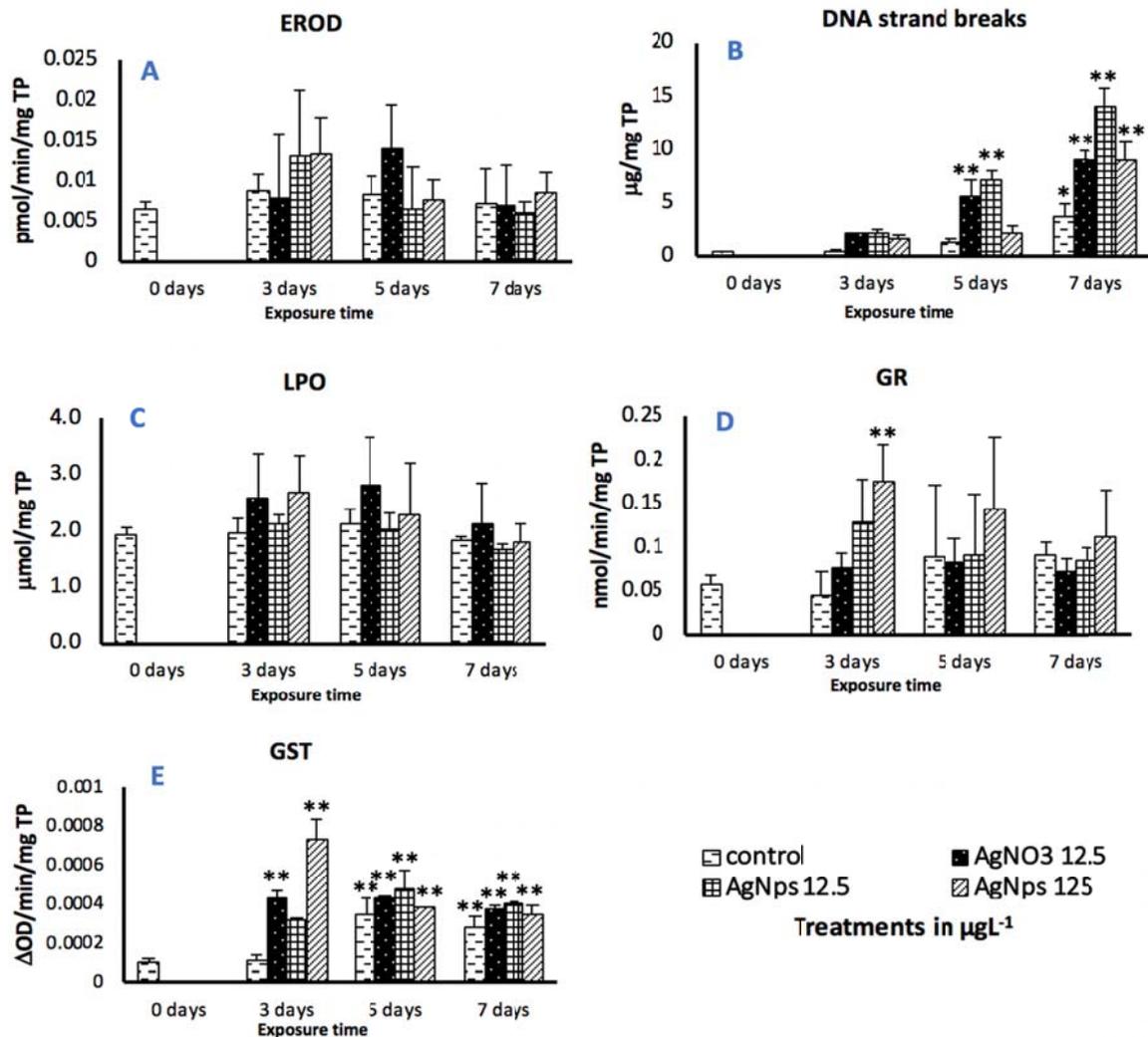
273 **Fig 2.** Mean and standard deviation (n=12) values for Ethoxyresosufine-o-deethylase activity
 274 (EROD), DNA damage (strand breaks), lipid peroxidation (LPO), glutathione-S-transferase
 275 (GST) and glutathione reductase (GR) analysed in gills for all the materials used (in μgL^{-1})
 276 and 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 ($5 \mu\text{gL}^{-1}$)
311 than for higher ones ($100 \mu\text{gL}^{-1}$) (Zook et al. 2011). Heithmar (2011) showed that when 50
312 nm citrate-capped AgNPs were diluted from $16 \mu\text{gL}^{-1}$ to 160ngL^{-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 $125 \mu\text{gL}^{-1}$ 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 ($12.5 \mu\text{gL}^{-1}$). 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 AgNO₃ 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 AgNO₃ 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 AgNO₃ 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 AgNO₃,
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.

438 **Acknowledgements**

439 AC-Q and AP acknowledge the University of Birmingham for financial support. AC-Q would
440 also like to thank The National Council for Science and Technology (CONACyT) in Mexico
441 for funding. IR was supported by the European Commission's 7th Framework Program

442 project “NanoMILE” (contract no. NMP4-LA-2013-310451). MJS was supported by the
443 Andalucía Talent Hub Program launched by the Andalusian Knowledge Agency (Spain).

444

445 **References**

- 446 Atkinson, M.J, and C. Bingman. 2008. 'Elemental composition of commercial seasalts', *Journal of*
447 *Aquariculture and Aquatic Sciences*, 8: 39-43.
- 448 Bonnal, Estefanía, Ricardo Cunha Lima, Esther Bautista-Chamizo, María José Salamanca, and Pablo
449 Cruz-Hernández. 2018. 'Biomarker responses of the freshwater clam *Corbicula fluminea* in
450 acid mine drainage polluted systems', *Environmental Pollution*, 242: 1659-68.
- 451 Buffet, P. E., O. F. Tankoua, J. F. Pan, D. Berhanu, C. Herrenknecht, L. Poirier, C. Amiard-Triquet, J. C.
452 Amiard, J. B. Berard, C. Risso, M. Guibbolini, M. Romeo, P. Reip, E. Valsami-Jones, and C.
453 Mouneyrac. 2011. 'Behavioural and biochemical responses of two marine invertebrates
454 *Scrobicularia plana* and *Hediste diversicolor* to copper oxide nanoparticles', *Chemosphere*,
455 84: 166-74.
- 456 Buffet, Pierre-Emmanuel, Jin-Fen Pan, Laurence Poirier, Claude Amiard-Triquet, Jean-Claude Amiard,
457 Pierre Gaudin, Christine Risso-de Faverney, Marielle Guibbolini, Douglas Gilliland, Eugenia
458 Valsami-Jones, and Catherine Mouneyrac. 2013. 'Biochemical and behavioural responses of
459 the endobenthic bivalve *Scrobicularia plana* to silver nanoparticles in seawater and
460 microalgal food', *Ecotoxicology and Environmental Safety*, 89: 117-24.
- 461 Buffet, Pierre-Emmanuel, Aurore Zalouk-Vergnoux, Amélie Châtel, Brigitte Berthet, Isabelle Métais,
462 Hanane Perrein-Ettajani, Laurence Poirier, Andrea Luna-Acosta, Hélène Thomas-Guyon,
463 Christine Risso-de Faverney, Marielle Guibbolini, Douglas Gilliland, Eugenia Valsami-Jones,
464 and Catherine Mouneyrac. 2014. 'A marine mesocosm study on the environmental fate of
465 silver nanoparticles and toxicity effects on two endobenthic species: The ragworm *Hediste*
466 *diversicolor* and the bivalve mollusc *Scrobicularia plana*', *Science of The Total Environment*,
467 470–471: 1151-59.
- 468 Canesi, Laura, Caterina Ciacci, Rita Fabbri, Antonio Marcomini, Giulio Pojana, and Gabriella Gallo.
469 2012. 'Bivalve molluscs as a unique target group for nanoparticle toxicity', *Marine*
470 *Environmental Research*, 76: 16-21.
- 471 Canesi, Laura, and Ilaria Corsi. 2016. 'Effects of nanomaterials on marine invertebrates', *Science of*
472 *the Total Environment*, 565: 933-40.
- 473 de Lafontaine, Yves, François Gagné, Christian Blaise, Georges Costan, Pierre Gagnon, and H. M.
474 Chan. 2000. 'Biomarkers in zebra mussels (*Dreissena polymorpha*) for the assessment and
475 monitoring of water quality of the St Lawrence River (Canada)', *Aquatic Toxicology*, 50: 51-
476 71.
- 477 Dogra, Yuktee, Kenton P. Arkill, Christine Elgy, Bjorn Stolpe, Jamie Lead, Eugenia Valsami-Jones,
478 Charles R. Tyler, and Tamara S. Galloway. 2016. 'Cerium oxide nanoparticles induce oxidative
479 stress in the sediment-dwelling amphipod *Corophium volutator*', *Nanotoxicology*, 10: 480-
480 87.
- 481 Edge, Katelyn J., Emma L. Johnston, Anthony C. Roach, and Amy H. Ringwood. 2012. 'Indicators of
482 environmental stress: cellular biomarkers and reproductive responses in the Sydney rock
483 oyster (*Saccostrea glomerata*)', *Ecotoxicology*, 21: 1415-25.
- 484 Fabrega, Julia, Samuel N. Luoma, Charles R. Tyler, Tamara S. Galloway, and Jamie R. Lead. 2011.
485 'Silver nanoparticles: Behaviour and effects in the aquatic environment', *Environment*
486 *International*, 37: 517-31.
- 487 Finkel, Toren, and Nikki J. Holbrook. 2000. 'Oxidants, oxidative stress and the biology of ageing',
488 *Nature*, 408: 239.

489 Gagne, F., J. Auclair, P. Turcotte, and C. Gagnon. 2013. 'Sublethal Effects of Silver Nanoparticles and
490 Dissolved Silver in Freshwater Mussels', *Journal of Toxicology and Environmental Health-Part*
491 *a-Current Issues*, 76: 479-90.

492 Gagné, François, and Christian Blaise. 1993. 'Hepatic metallothionein level and mixed function
493 oxidase activity in fingerling rainbow trout (*Oncorhynchus mykiss*) after acute exposure to
494 pulp and paper mill effluents', *Water Research*, 27: 1669-82.

495 Gomes, Tânia, Catarina G. Pereira, Cátia Cardoso, José P. Pinheiro, Ibon Cancio, and Maria João
496 Bebianno. 2012. 'Accumulation and toxicity of copper oxide nanoparticles in the digestive
497 gland of *Mytilus galloprovincialis*', *Aquatic Toxicology*, 118–119: 72-79.

498 Gomes, Tânia, Catarina G. Pereira, Cátia Cardoso, Vânia Serrão Sousa, Margarida Ribau Teixeira, José
499 P. Pinheiro, and Maria João Bebianno. 2014. 'Effects of silver nanoparticles exposure in the
500 mussel *Mytilus galloprovincialis*', *Marine Environmental Research*, 101: 208-14.

501 Griffitt, Robert J., Jing Luo, Jie Gao, Jean-Claude Bonzongo, and David S. Barber. 2008. 'Effects of
502 particle composition and species on toxicity of metallic nanomaterials in aquatic organisms',
503 *Environmental toxicology and chemistry*, 2008 v.27 no.9: pp. 1972-78.

504 Heithmar. 2011. "Screening Methods for Metal-Containing Nanoparticles in U.S Water." In.:
505 Environmental Protection Agency, Washington, DC, EPA/600/R-11/096, 2011.

506 Jimeno-Romero, A., E. Bilbao, U. Izagirre, M. P. Cajaraville, I. Marigomez, and M. Soto. 2017.
507 'Digestive cell lysosomes as main targets for Ag accumulation and toxicity in marine mussels,
508 *Mytilus galloprovincialis*, exposed to maltose-stabilised Ag nanoparticles of different sizes',
509 *Nanotoxicology*, 11: 168-83.

510 Johnson, B. D., S. L. Gilbert, B. Khan, D. L. Carroll, and A. H. Ringwood. 2015. 'Cellular responses of
511 eastern oysters, *Crassostrea virginica*, to titanium dioxide nanoparticles', *Mar Environ Res*,
512 111: 135-43.

513 JRC. 2011. 'NM-Series of Representative Manufactured Nanomaterials NM-300 Silver
514 Characterisation, Stability, Homogeneity ', Accessed 02/09/2016.
515 <http://publications.jrc.ec.europa.eu/repository/handle/JRC60709>.

516 Katsumiti, Alberto, Douglas Gilliland, Inmaculada Arostegui, and Miren P. Cajaraville. 2015.
517 'Mechanisms of Toxicity of Ag Nanoparticles in Comparison to Bulk and Ionic Ag on Mussel
518 Hemocytes and Gill Cells', *PLoS ONE*, 10: e0129039.

519 Kvítek, Libor, Aleš Panáček, Jana Soukupová, Milan Kolář, Renata Večeřová, Robert Pucek, Mirka
520 Holecová, and Radek Zbořil. 2008. 'Effect of Surfactants and Polymers on Stability and
521 Antibacterial Activity of Silver Nanoparticles (NPs)', *The Journal of Physical Chemistry C*, 112:
522 5825-34.

523 Li, Xuan, and John J. Lenhart. 2012. 'Aggregation and Dissolution of Silver Nanoparticles in Natural
524 Surface Water', *Environmental Science & Technology*, 46: 5378-86.

525 Liu, Y., M. Tourbin, S. Lachaize, and P. Guiraud. 2014. 'Nanoparticles in wastewaters: Hazards, fate
526 and remediation', *Powder Technology*, 255: 149-56.

527 McCarthy, Melissa P., David L. Carroll, and Amy H. Ringwood. 2013. 'Tissue specific responses of
528 oysters, *Crassostrea virginica*, to silver nanoparticles', *Aquatic Toxicology*, 138–139: 123-28.

529 McFarland, VA, LS Inouye, CH Lutz, AS Jarvis, JU Clarke, and DD McCant. 1999. 'Biomarkers of
530 oxidative stress and genotoxicity in livers of field-collected brown bullhead, *Ameiurus
531 nebulosus*', *Archives of Environmental Contamination and Toxicology*, 37: 236-41.

532 Misra, Superb K., Agnieszka Dybowska, Deborah Berhanu, Samuel N. Luoma, and Eugenia Valsami-
533 Jones. 2012. 'The complexity of nanoparticle dissolution and its importance in
534 nanotoxicological studies', *Science of The Total Environment*, 438: 225-32.

535 Mouneyrac, Catherine, Pierre-Emmanuel Buffet, Laurence Poirier, Aurore Zalouk-Vergnoux, Marielle
536 Guibbolini, Christine Risso-de Faverney, Douglas Gilliland, Déborah Berhanu, Agnieszka
537 Dybowska, Amélie Châtel, Hanane Perrein-Ettajni, Jin-Fen Pan, Hélène Thomas-Guyon, Paul
538 Reip, and Eugénia Valsami-Jones. 2014. 'Fate and effects of metal-based nanoparticles in
539 two marine invertebrates, the bivalve mollusc *Scrobicularia plana* and the annelid

540 polychaete *Hediste diversicolor*', *Environmental science and pollution research international*,
541 21: 7899-912.

542 PerkinElmer. 2008. 'Atomic Spectroscopy - A Guide to Selecting the Appropriate Technique and
543 System', Accessed 07/09/2016. [http://www.perkinelmer.co.uk/lab-](http://www.perkinelmer.co.uk/lab-solutions/resources/docs/BRO_WorldLeaderAAICPMSICPMS.pdf)
544 [solutions/resources/docs/BRO_WorldLeaderAAICPMSICPMS.pdf](http://www.perkinelmer.co.uk/lab-solutions/resources/docs/BRO_WorldLeaderAAICPMSICPMS.pdf).

545 Rauscher, Hubert, Kirsten Rasmussen, and Birgit Sokull-Klüttgen. 2017. 'Regulatory Aspects of
546 Nanomaterials in the EU', 89: 224-31.

547 Regoli, F., M. E. Giuliani, M. Benedetti, and A. Arukwe. 2011. 'Molecular and biochemical biomarkers
548 in environmental monitoring: a comparison of biotransformation and antioxidant defense
549 systems in multiple tissues', *Aquat Toxicol*, 105: 56-66.

550 Renault, T. 2015. 'Immunotoxicological effects of environmental contaminants on marine bivalves',
551 *Fish & Shellfish Immunology*, 46: 88-93.

552 Rocha, Thiago Lopes, Tânia Gomes, Vânia Serrão Sousa, Nélia C. Mestre, and Maria João Bebianno.
553 2015. 'Ecotoxicological impact of engineered nanomaterials in bivalve molluscs: An
554 overview', *Marine Environmental Research*, 111: 74-88.

555 Römer, Isabella, Alex J. Gavin, Thomas A. White, Ruth C. Merrifield, James K. Chipman, Mark R.
556 Viant, and Jamie R. Lead. 2013. 'The critical importance of defined media conditions in
557 *Daphnia magna* nanotoxicity studies', *Toxicology Letters*, 223: 103-08.

558 Römer, Isabella, Zhi Wei Wang, Ruth C. Merrifield, Richard E. Palmer, and Jamie Lead. 2016. 'High
559 Resolution STEM-EELS Study of Silver Nanoparticles Exposed to Light and Humic Substances',
560 *Environmental Science & Technology*, 50: 2183-90.

561 Römer, Isabella, Thomas A. White, Mohammed Baalousha, Kevin Chipman, Mark R. Viant, and Jamie
562 R. Lead. 2011. 'Aggregation and dispersion of silver nanoparticles in exposure media for
563 aquatic toxicity tests', *Journal of chromatography. A*, 1218: 4226-33.

564 Tejamaya, M., I. Romer, R. C. Merrifield, and J. R. Lead. 2012. 'Stability of citrate, PVP, and PEG
565 coated silver nanoparticles in ecotoxicology media', *Environ Sci Technol*, 46: 7011-7.

566 Unfried, Klaus, Catrin Albrecht, Lars-Oliver Klotz, Anna Von Mikecz, Susanne Grether-Beck, and Roel
567 P. F. Schins. 2007. 'Cellular responses to nanoparticles: Target structures and mechanisms',
568 *Nanotoxicology*, 1: 52-71.

569 van der Oost, Ron, Jonny Beyer, and Nico P. E. Vermeulen. 2003. 'Fish bioaccumulation and
570 biomarkers in environmental risk assessment: a review', *Environmental Toxicology and*
571 *Pharmacology*, 13: 57-149.

572 Ward, J. E., and D. J. Kach. 2009. 'Marine aggregates facilitate ingestion of nanoparticles by
573 suspension-feeding bivalves', *Marine Environmental Research*, 68: 137-42.

574 Zook, J. M., S. E. Long, D. Cleveland, C. L. Geronimo, and R. I. MacCuspie. 2011. 'Measuring silver
575 nanoparticle dissolution in complex biological and environmental matrices using UV-visible
576 absorbance', *Anal Bioanal Chem*, 401: 1993-2002.

577