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

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Abstract

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

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

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Introduction

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

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

Bivalve species are used as indicators of coastal environmental quality in numerous national and international programmes monitoring environmental pollution (Buffet et al. 2013) . Thus, evidence of bioaccumulation and toxicity of AgNPs in bivalve species coupled with data on the behaviour of NPs in seawater, such as agglomeration, dissolution and deposition onto the sediment surface (Gagne et al. 2013; Gomes et al. 2014; Buffet et al. 2013) provide essential information, in order to understand the bioavailable dose and correlate this with the observed toxicity. Bivalve species are considered excellent environmental biomonitors due to their feeding mechanism, which involves filtration of large volumes of water and favours the uptake and bioaccumulation of toxic chemicals (de Lafontaine et al. 2000). Bivalves have the capacity

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

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

In this study, we assessed the effects of exposure to AgNPs on rock oyster (*Saccostrea glomerata*) through a range of biomarkers for the first time, including assessing the AgNPs behaviour in artificial seawater (ASW) and their bioaccumulation in rock oysters. The study was performed using a AgNP concentration of 12.5 μgL^{-1}, which was chosen considering the maximum concentration of total silver recorded in the literature (8.9 μgL^{-1} in Galveston bay, 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

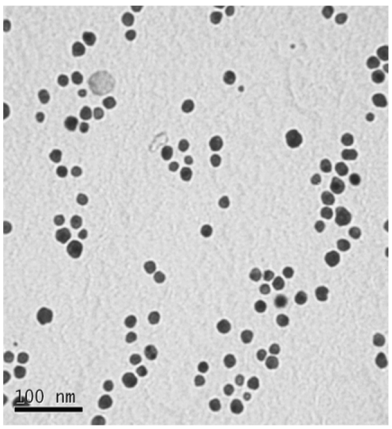
Inductively Coupled Plasma Optical Emission Spectroscopy (ICP-OES) and transmission electron microscopy (TEM).

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.

At the end of the acclimation period (7 days), oysters were fed for the last time; afterwards, water in all the tanks was partially renewed (except approximately 2 L per tanks). Two dosing concentrations of AgNPs (12.5 µgL^{-1} and 125 µgL^{-1}) were added to 4 tanks (performed in

duplicate, 4 tanks per treatment) and mixed by aeration. Following the same procedure, four additional tanks were used as the unexposed blank and $AgNO_3$ as positive ionic control (12.5 μgL^{-1}). The experimental exposure was performed in separate tanks, having four replicates per condition used. We pooled the results for all the oysters that were exposed to the same conditions and concentration of the material. Fifteen oysters were cultured per tank, using three oysters for every biomarkers' time point (0, 3, 6, and 7 days) (n=12). Finally, at day 7, the remaining three oysters were sacrificed for bioaccumulation analysis. The oysters were not fed during the experiment to maintain the water quality and minimize the risk of AgNPs being absorbed by other components such as food or faecal material; no mortality was registered during the experiment.

Characterisation and dissolution of the AgNPs in ASW during the exposure

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

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

Bioaccumulation analysis and sublethal effects

After 7 days of exposure, the gills and digestive glands of three oysters per tank and treatment (12 total) were dried at 60°C for 3 days and their dry weight was recorded. Details about the tissue extraction are shown in SI†. The samples were then acid-digested with 4mL of (1:4) H_2O_2 and HNO_3 (w/v) for 2h at 60°C using a microwave (CEM Mars-5 Microwave accelerated

reaction system) (Dogra et al. 2016) . The solutions were filtered with a syringe filter with 0.2µm Supor® membrane and the total Ag concentration was measured by ICP-OES.

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

Statistical analysis

The statistical analysis software package (SPSS 22) was used to identify significant treatment effects. Normality of the data and homogeneity of variance were analysed prior to the use of parametric tests. A two-way analysis of variance (ANOVA) was performed to evaluate the effect concentration, kind of Ag used (NP or ionic) and time of exposure upon the biomarkers. A parametric ANOVA test or T-test was used to identify significant differences between the treatments and clean day zero control, followed by a multiple comparison using Dunett′s tests and Pearson correlation analysis, applied to assess the correlations between the biomarkers, bioaccumulation and concentration of exposure. Significance level was set at $p \leq 0.05$ and $p \leq 0.01$ to identify statistically significant treatment effects.

Results

Stability and dissolution of AgNPs in ASW

The stability of the particle dispersions was measured for both concentrations of AgNPs. Large agglomerates were observed after 1 hour when measured by TEM (Fig S1†). The images obtained for the AgNPs at 12.5 μgL^{-1} (12.5 AgNPs) showed agglomerates with a size distribution between 0.2 to 0.5µm, which started to decrease in size by day 3 (Fig. S1†, A, B, C, D†). In the case of AgNPs 125 μgL^{-1} (125 AgNPs) large agglomerates that ranged between 0.1 to 0.5 µm in size were observed, as well as the presence of some individual NPs of 19 ± 5 nm (n=100) after 1h (Fig. S1†. E, F, G, H† and Table S2†). After 3 days, both smaller or slightly smaller free NPs (11 ± 6 nm, n=100) and slightly larger free particles (46 ± 12 nm) were observed. More images for the free AgNPs after 1h and 3 days can be found in Fig. S2† and Fig. S3† in SI, a table with a summary of the results can also be found in Table S2†. The larger free particles also have a smaller circularity compared to the pristine AgNPs, (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 ± 0.2, which was much closer to the values obtained for the pristine NPs.

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

Bioaccumulation of Ag in tissues

Bioaccumulation is an important process to enable understanding of the potential effects resulting from the exposure of an organism to pollutants such as AgNPs. The bioaccumulation of a xenobiotic is considered a precursor of toxicity; and AgNPs are not the exception to this

generality (Fabrega et al. 2011). Here, the bioaccumulation of total Ag in gills and the digestive gland after 7 days of exposure was analysed and compared to the $AgNO_3$ treatment (Fig. 1B) As expected, the bioaccumulation of the $AgNO_3$ treatment in both tissues was higher compared to the AgNPs, despite the NPs undergoing partial dissolution. The 12.5 AgNPs concentration presented a lower accumulation in the gills, 0.840 ± 0.008 μgKg^{-1}, compared with the 125 AgNPs that presented almost double the bioaccumulation (1.42 ± 0.03 μgKg^{-1}) despite the x10 higher concentration potentially available (Fig. 1A).

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

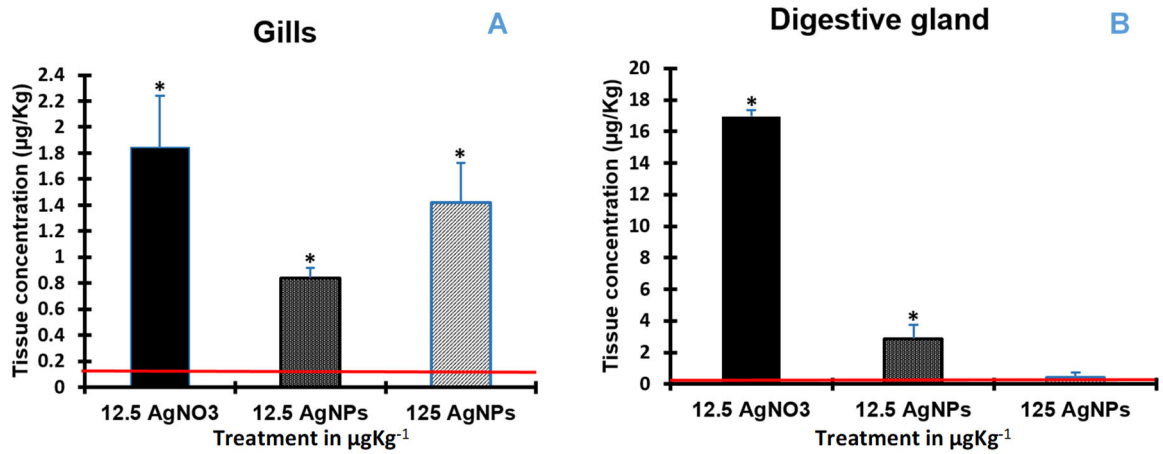


Fig 1. The bioaccumulation of total Ag for all treatments (μgKg^{-1} dry weight) after 7 days of exposure, (A) in gills and (B) in digestive gland. The graphs represent the mean of 12 oysters per treatment (3 oysters per tank used). Control (ASW only) is not included as Ag values were under limit detection of the ICP-OES. Standard deviation was calculated from the 12 samples measured. The red line represents the limit of detection of the ICP-OES (0.014 μgKg^{-1} for gills and 0.005 μgKg^{-1} for digestive glands) while significant differences compared to the detection limit ($p<0.01$) are indicated with asterisks.

Biomarkers

The results obtained can be found in Table S3† and Fig. 2 and 3. It can be observed that the AgNPs and $AgNO_3$ treatments used in this study had a significant influence upon EROD measured in gills only for the exposure time, however no interaction between concentration

and exposure time was observed (Table S3†). No correlations were found between EROD and the other biomarkers measured or Ag bioaccumulated in the tissues.

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

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

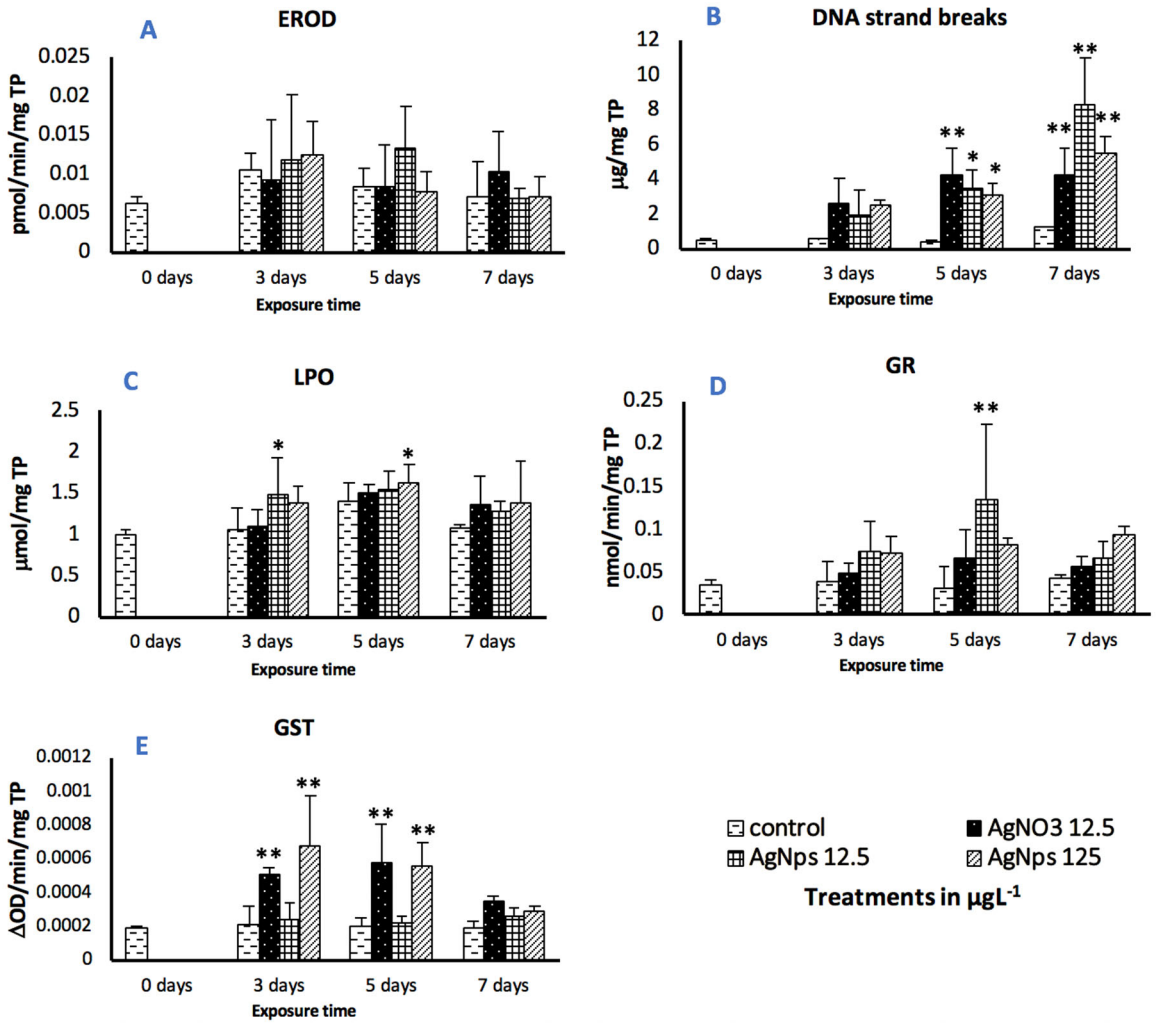


Fig 2. Mean and standard deviation (n=12) values for Ethoxyresosufine-o-deethylase activity (EROD), DNA damage (strand breaks), lipid peroxidation (LPO), glutathione-S-transferase (GST) and glutathione reductase (GR) analysed in gills for all the materials used (in μgL^{-1}) and blank compared to the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

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

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

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

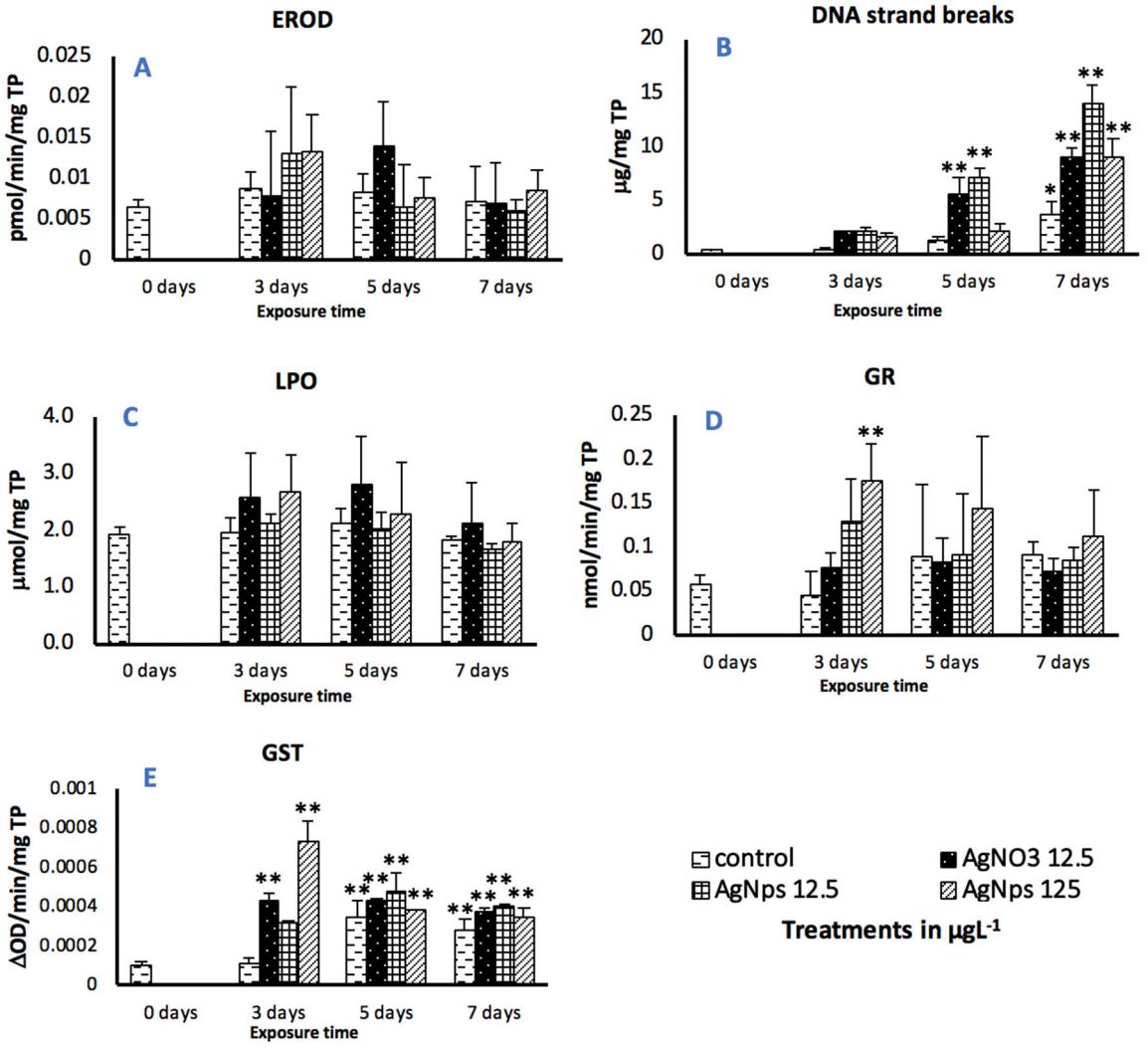


Fig 3. Mean and standard deviation (n=12) values for Ethoxyresosufine-o-deethylase activity (EROD), DNA damage (strand breaks), lipid peroxidation (LPO), glutathione-S-transferase (GST) and glutathione reductase (GR) analysed in the digestive glands for all the materials used (in μgL^{-1}) and blank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

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

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

Discussion

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

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

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

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

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

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

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

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

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

Exposure to 125 AgNPs produced the highest number of significant inductions, except for GST, followed by exposure to 12.5 AgNPs. Therefore, although there was more Ag availability in the first 5 days of exposure according to the analyses, which is reflected in greater

bioaccumulation in gills and digestive glands, the responses to AgNPs cannot be underestimated since they produce a toxic effect similar or even greater (for the same concentration) than the $AgNO_3$ treatment. This means that aggregates and free AgNPs clearly influenced the toxicity and induction of the biomarkers measured in this study.

Conclusions

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

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

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

Conflicts of interest

There are no conflicts to declare.

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