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Biological effects and bioaccumulation of gold in gilthead seabream (*Sparus aurata*) – Nano versus ionic form

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Highlights

- Gold (nano or ionic form) induced lipid peroxidation and genotoxicity on fish;
- Ionic gold induced more adverse effects than a nano form of the metal;
- Citrate coated nanoparticles were more bioactive than PVP coated nanoparticles.

Abstract

The question of whether gold (Au) is more toxic as nanoparticles or in its ionic form remains unclear and controversial. The present work aimed to clarify the effects of 96 h exposure to 4, 80 and 1600 μg.L^{-1} of 7 nm gold nanoparticles (AuNPs) – (citrate coated (cAuNPs) or polyvinylpyrrolidone coated (PVP-AuNPs)) – and ionic Au (iAu) on gilthead seabream (*Sparus aurata*). Effects at different levels of biological organization (behaviour, neurotransmission, biotransformation, oxidative stress/damage and genotoxicity) were assessed. cAuNPs induced oxidative stress and damage (lipid peroxidation increase), even at 4 μg.L^{-1}, and reduced the ability of *S. aurata* to swim against a water flow at 1600 μg.L^{-1}. Exposure to cAuNPs induced more adverse effects than exposure to PVP-AuNPs. All tested concentrations of Au (nano or ionic form) induced DNA breaks and cytogenetic damage in erythrocytes of *S. aurata*. Generally, iAu induced significantly more effects in fish than the nano form, probably associated with the significantly higher accumulation in the fish tissues. No fish mortality was observed following exposure to AuNPs, but mortality was observed in the group exposed to 1600 μg.L^{-1} of iAu.

Keywords: nanotoxicity; gold; marine fish; seawater; biomarkers

1. Introduction

Throughout its history, gold (Au) has been recognized as an inert, non-toxic and biocompatible noble metal with therapeutic properties (Daniel and Astruc 2004; Fratoddi et al. 2015). However, when Au decreases to nanometer

dimensions, the safety of the resulting nanomaterials has been questioned (Boverhof et al. 2015). Gold nanoparticles (AuNPs) have been widely used in medicine and biological research (Fratoddi et al. 2015), including targeted delivery of drugs (Ghosh et al. 2008), imaging and diagnosis (Bhattacharya and Mukherjee 2008). Its application in aquaculture as antimicrobial agent (Saleh et al. 2016) and to detect contaminants (Loganathan and John 2017) has also been investigated. Despite the widespread use of AuNPs and consequent release to the environment, there is a limited understanding of their consequences for environmental health (Barreto et al. 2019a; Barreto et al. 2019b; Iswarya et al. 2016; Na-Phatthalung et al. 2018; Teles et al. 2016). In addition, the question of whether AuNPs are more toxic than ionic Au (iAu) remains unresolved (Barbasz and Oćwieja 2016; Botha, James, and Wepener 2015; Dedeh et al. 2015; Farkas et al. 2010; Luis et al. 2016). Table 1 presents the example of several *in vitro* and *in vivo* studies where different toxicity outputs were achieved.

Table 1. Examples of studies assessing the toxicity of nano versus ionic gold. Ref. – Reference; PVP – Polyvinylpyrrolidone; BSA – Bovine serum albumin; ROS – Reactive oxygen species. 1 – Barbasz et al. (2016); 2 – Luis et al. (2016); 3 – Botha et al. (2015); 4 – Dedeh et al. (2015); 5 – Farkas et al. (2010).

Test type	Cell/ Organism	Exposure time	Endpoint/ Parameter	Coating	Size/Shape (nm)	Dose	More toxic: Ionic or nano form	Ref.
In vitro	Human promyelocytic cells of the HL-60 line Human histiocytic lymphoma cell line U-937	24, 48 and 72 h	Cytotoxicity Nitric oxide and reduced glutathione levels	Tannic acid	Spherical 21	0.75 to 25 ppm	Nano	1
In	*Mytilus*	10 min	Enzymatic activities	Citrate, PVP and	Spherical	54 $ng \cdot L^{-1}$ to	Ionic	2

vitro	*galloprovincialis* hemolymph and subcellular fraction of gills			BSA	7	2.5 $mg \cdot L^{-1}$		
In vivo	*Daphnia pulex, D. magna, Danio rerio, Poecilia reticulata, Labeobarbus aeneus, Pseudocrenilabrus philander, Tilapia sparrmanii, Oreochromis mossambicus*	48 and 96 h	Species sensitivity distributions	Citrate	Spherical 14	0.0005 to 200 $mg.L^{-1}$	Ionic	3
In vivo	*Danio rerio*	20 d	Gene expression	Citrate	Spherical 14	0.25 and 0.8 $\mu g.L^{-1}$	Nano	4
In vitro	Hepatocyte cell culture of *Oncorhynchus mykiss*	2 and 48 h	Cytotoxicity and ROS formation	Citrate	Spherical 5-10	0.063 to 19 $mg.L^{-1}$	Ionic	5

Thus, the aim of the present study was to add value to the shortage of studies available comparing both Au forms and to investigate the effects of Au on the top predator *Sparus aurata* after 96 h exposure to 7 nm AuNPs (citrate coated (cAuNPs) or polyvinylpyrrolidone coated (PVP-AuNPs)) and iAu. To the best of our knowledge, the present study is the first one testing the effects of both forms of Au to a marine fish. AuNPs of small size were chosen due to the reported highest effects attributed to small sizes (Coradeghini et al. 2013; Iswarya et al. 2016; Xia et al. 2017). Two coatings of AuNPs were tested to clarify whether they determine the effects of AuNPs in the fish. Swimming performance, the activity of enzymes involved in neurotransmission (cholinesterases – ChE), in biotransformation (glutathione *S*-transferases – GST) and antioxidant defence (glutathione reductase (GR), catalase (CAT) and glutathione peroxidase (GPx)), non-enzymatic defence (non-protein thiols – NPT), oxidative damage (in DNA and cellular membranes), DNA strand breaks and nuclear abnormalities were assessed. The concentration of Au was also

quantified in relevant tissues (gills, liver, spleen and muscle). The main specific aims were: 1) to clarify which Au form is more toxic and bioaccumulative to this marine fish (nano versus ionic); and 2) to clarify the effect of coating in the AuNPs toxicity (cAuNPs versus PVP-AuNPs).

2. Material and Methods

2.1. Gold nanoparticles (AuNPs) – Synthesis and characterization

cAuNPs with 7 nm diameter were synthesized based on the method described by Shiba et al. (2013). The citrate reduction method, one of the most widely used in AuNPs synthesis, was chosen due to the reasons described on previous publications (Barreto et al. 2019a; Barreto et al. 2019b). PVP-AuNPs were obtained by coating part of cAuNPs with polyvinylpyrrolidone (PVP) as described in detail by Barreto et al. (2015). PVP is a water-soluble, nontoxic and biodegradable homopolymer. It is an excellent coating agent, especially for noble metals NPs (Das et al. 2017; Min et al. 2009). This polymer is frequently used as AuNPs coating agent to increase its stability and to promote biological interactions (Min et al. 2009). The characterisation of AuNPs stock suspensions and AuNPs in the experimental media (artificial seawater – ASW) and in ultrapure water was performed as described in previous publications (Barreto et al. 2019a; Barreto et al. 2019b)

2.2. Bioassay

2.2.1. Fish

Juvenile gilthead seabream (*Sparus aurata*) with length 7.6 ± 0.1 cm, acquired from an aquaculture facility in Spain (Santander), were acclimated for

1 month in aquaria containing aerated and filtered artificial seawater (ASW, prepared by dissolving the salt in reverse osmosis water to obtain a salinity of 30), under controlled temperature (17ºC) and natural photoperiod. During this period, the fish were fed daily at a ratio of 1 g per 100 g of fish with commercial fish food (Sorgal, Portugal).

2.2.2. Experimental design

During the bioassay, temperature, salinity, conductivity, pH, dissolved oxygen and aeration conditions were similar to conditions of the acclimation period. The experiment followed, in general, the OECD guideline (number 203) for fish acute bioassays (OECD 1992). Fish (n=12 per condition) were randomly distributed in the experimental aquaria (3 per condition) in the ratio 1 g of fish per 1 L of ASW and exposed for 96 h to the following experimental conditions: 0, 4, 80 and 1600 $\mu g.L^{-1}$ AuNPs (citrate and PVP coating) and iAu. The lowest concentration tested (4 $\mu g.L^{-1}$) was a compromise between the predicted values of AuNPs for the aquatic environment (0.14 $\mu g.L^{-1}$) (García-Negrete et al. 2013; Tiede et al. 2009) and the potentially detectable Au concentration in fish tissues. The other concentrations tested were 20-fold increases.

Part of the experimental media (approx. 80%) was renewed daily to prevent significant AuNPs alteration and to reduce the build-up of metabolic residues, after checking fish mortality and behaviour alterations and assessing the water parameters (temperature, salinity, pH and dissolved oxygen). Water samples were collected daily (at 0 and 24 h) from each experimental aquarium for the gold quantification. Water samples collected at 0 h, correspond to the water collected at the beginning of the assay and every time renewal of the media

took place (immediately after renewal). Water samples collected at 24 h, correspond to the water collected 24 h after the beginning of the test and 24 h after the renewal of the media.

2.3. Assessment of swimming performance

After 96 h exposure, fish were individually introduced into a long flume and induced to swim against a water flow of 19 $L.min^{-1}$. The time (in seconds) that fish spent swimming against the water flow was recorded. More information about this behavioural assessment can be found in a previous study of Barreto et al. (2019b).

2.4. Collection of biological material

After a 2 h recovery period, fish were anesthetized with tricaine methanesulfonate (MS-222), blood samples were collected from the posterior cardinal vein and then the animals were euthanized by spinal section. For the comet assay, blood samples were diluted with saline phosphate buffer. Blood smears were prepared for the assessment of erythrocytic nuclear abnormalities (ENAs). Liver, gills, muscle and brain were removed from seven fish and stored at -80ºC until biochemical biomarkers analysis. Liver, gills, spleen and muscle were taken from five animals and kept at -20ºC until gold quantification.

2.4.1. Biochemical biomarkers analysis

Liver and gills were homogenized in potassium phosphate buffer (0.1 mM, pH 7.4) using an ultrasonic homogenizer. The homogenate was then divided into three aliquots for: lipid peroxidation (LPO) assay, NPT quantification and

post-mitochondrial supernatant (PMS) preparation. To prevent oxidation, the aliquot of homogenate for LPO evaluation was transferred to a microtube with 4% BHT (2,6-di-tert-butyl-4-methylphenol) in methanol. The aliquots for LPO and NPT levels determination were stored at -80ºC until analysis. PMS was accomplished by centrifugation (12 000 g for 20 min at 4ºC) and aliquots were stored at -80ºC until determination of GST, CAT, GPx and GR activities.

Muscle and brain tissues were homogenized in potassium phosphate buffer (0.1 mM, pH 7.2). Part of the homogenate was transferred to a microtube with 4% BHT and stored at -80ºC until LPO quantification. The remaining part was centrifuged (3300 g for 3 min at 4°C), and the obtained supernatant was collected and stored at -80ºC until ChE activity determination. Protein concentration of all the samples was determined according to Bradford (1976), adapted to microplate, using bovine γ-globuline as standard. ChE activity was determined according to the Ellman's method (1961) adapted to microplate (Guilhermino et al. 1996). CAT activity was assayed as described by Claiborne (1985). GR activity was estimated according the method of Carlberg and Mannervik (1975) adapted to microplate (Lima et al. 2007). GPx activity was measured according to the method described by Mohandas et al. (1984), modified by Athar and Iqbal (1998). NPT levels were determined based on the method of Sedlak and Lindsay (1968), adopted by Parvez et al. (2003). GST activity was determined by the method of Habig et al. (1974) adapted to microplate (Frasco and Guilhermino 2002). LPO levels were assessed by the formation of thiobarbituric acid reactive substances (TBARS) based on Ohkawa et al. (1979), adapted by Filho et al. (2001). More details on the biochemical biomarkers analysis can be found on the study of Barreto et al. (2019b).

2.4.2. Comet and erythrocytic nuclear abnormalities (ENAs) assays

The alkaline comet assay was conducted according to the method of Singh et al. (1988) with some adaptations, as previously described by Barreto et al. (2017). The sensitivity and specificity of the assay was improved by the incubation of the lysed cells (nucleoids) with a lesion-specific endonuclease, formamidopyrimidine DNA glycoslyase (Fpg). Fpg was chosen because it is a protein recommended for the detection of oxidative DNA base damage, in particular 8-OH guanine, as well as other damaged purines and abasic sites (AP sites) and ring-opened N-7 guanine adducts (Albertini et al. 2000; Epe et al. 1993; Li, Laval, and B. Ludlum 1997; Speit et al. 2004; Tchou et al. 1994; Tice et al. 2000; Tudek et al. 1998). The method for enzyme Fpg conjugated with comet assay was performed according to procedures previously reported (Collins 2014; Collins et al. 1997). Two replicate comet slides were made for each blood sample; one slide was treated with Fpg and the other without Fpg. A positive control (fish blood treated with 25 µM hydrogen peroxide (H_2O_2) for 10 min), with and without Fpg treatment, was also included in the assay. H_2O_2 is a recognized genotoxic agent, producing both strand breaks and oxidative DNA damage (Barreto et al. 2017; Termini 2000). After the lysis step, for the enzyme treatment, the correspondent slides were removed from lysis buffer and were washed 3 times in cold (4°C) enzymatic buffer solution (40 mM HEPES; 0.1 M KCl; 0.5 mM EDTA; 0.2 $mg.mL^{-1}$ bovine serum albumin, pH 8.0). Fpg (45 µL, 1:60 diluted in enzymatic buffer solution) was added to the slides, which were individually sealed with a coverslip and incubated during 30 min at 37°C. The other steps involved in the comet assay were common to slides with or without

Fpg. Cells were classified according to tail length, into five classes (Collins 2004): class 0 – undamaged, without a tail; class 1 – with a tail shorter than the diameter of the nucleus; class 2 – with a tail length 1–2 times the diameter of the nucleus; class 3 – with a tail longer than twice the diameter of the nucleus; class 4 – comets with no nucleus. A damage index (DI), in arbitrary units, was assigned to each slide (for 100 cells) and consequently for each treatment, using the formula:

$$DI = (0 \times n0) + (1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4)$$

where: *n* = number of cells in each class. DI can range from 0 to 400 (de Andrade, de Freitas, and da Silva 2004).

The DNA damage index in cells treated with Fpg with the correspondent cells without the enzymatic treatment were compared to detect possible DNA oxidative damage.

The ENAs assay was carried out in mature peripheral erythrocytes according previous procedures and nuclear lesions were scored as micronuclei, lobed, segmented, kidney-shaped and vacuolated nuclei (Barreto et al. 2017; Pacheco and Santos 1996). Results were expressed as the ENAs frequency (‰) to each replicate (for 1000 cells) and consequently for each treatment using the formula:

$$ENAs(‰) = \frac{\text{Number of cells containing ENAs}}{\text{Total number of cells counted}}$$

2.5. Gold (Au) quantification

The determination of Au in the stock suspensions, in the experimental media and fish tissues was performed according to the NIST NCL Method PCC-8 (NIST 2010). An iCAP^{TM} Q ICP-MS (inductively coupled plasma mass spectrometry) instrument (Thermo Fisher Scientific, Bremen, Germany) was used for the analysis. The ICP-MS instrumental conditions were as follow: argon flow rate (14 L.min^{-1}); auxiliary argon flow rate (0.8 L.min^{-1}); nebulizer flow rate (1.03 mL.min^{-1}); RF power (1550 W) and dwell time (100 ms). The elemental isotope ^{197}Au was monitored for analytical determination; ^{159}Tb and ^{209}Bi were used as internal standards. The instrument was tuned daily for maximum signal sensitivity and stability. More information about Au quantification can be found in the study of Barreto et al. (2019b).

Stock suspensions theoretical concentrations and number of nanoparticles (NPs) were also estimated based on their UV-Vis spectra and sizes (Barreto et al. 2015; Liu et al. 2007; Paramelle et al. 2014).

2.6. Total gold (Au) content, bioaccumulation factor and estimated intake for humans

Total Au content ($[Au]_{total}$), in µg.g^{-1}, was calculated, as described in a previous study from Barreto et al. (2019b), according to the formula:

$$[Au]_{total} = [Au]_g + [Au]_l + [Au]_s + [Au]_{ms}$$

Where $[Au]_g$ is the concentration of Au in gills, $[Au]_l$ the concentration of Au in liver, $[Au]_s$ the concentration of Au in spleen and $[Au]_{ms}$ the concentration of Au in muscle.

The bioaccumulation factor (BAF), in $L.g^{-1}$, was calculated according previous studies (Barreto et al 2019b; Yoo-Iam, Chaichana, and Satapanajaru 2014):

$$BAF = [Au]_t / [Au]_{ASW}$$

Where $[Au]_t$ is the content of Au in the specific fish tissue and $[Au]_{ASW}$ its concentration in the exposure media – ASW (collected daily at 0 h and quantified). More information about BAF calculation can be found in the study of Barreto et al. (2019b).

As *Sparus aurata* is a fish for human consumption an extrapolation of Au intake for humans was calculated, using the following formula (Barreto et al 2019b; Vieira et al. 2015; WHO 2008):

$$Au\ intake = \frac{Amount\ of\ fish\ ingested * Au\ content\ in\ the\ ingested\ fish}{Kilograms\ body\ weight}$$

A human body weight of 60 kg was assumed (IPCS 2004) and the average amount of fish ingested by each Portuguese person per year was set at 59 kg (Failler et al. 2007; Vieira et al. 2015). Au content in the ingested fish corresponds to the content of Au determined in the fish muscle ($\mu g.g^{-1}$). The calculated Au intake values were compared with the maximum amount of Au that each person may be exposed daily over their lifetimes without considerable health risk – "tolerable daily intake" (TDI). This value was previously calculated and in detail explained in the study of Barreto et al. (2019b): 322 $\mu g.kg^{-1}$.

2.7. Statistical analysis

Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) using the Sigma Plot software package (SigmaPlot 12.0, 2011). Differences between treatments and control and between all the treatments were analysed using one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey´s test whenever applicable. Significant differences were assumed for $p<0.05$.

3. Results

3.1. Gold nanoparticles (AuNPs) – Characterisation and behaviour

The synthetized cAuNPs presented a well-defined surface plasmon resonance (SPR) peak – Figure 1A. Dynamic light scattering (DLS) analysis showed an average hydrodynamic size of the particles of 7 nm and a strongly negative surface charge (-43 mV). Transmission electron microscopy (TEM) analysis confirmed that almost all cAuNPs presented spherical shape and the particles had similar sizes between them (Figure 1B). There was a slight shift in the SPR peak to a longer wavelength for PVP-AuNPs (521 nm) when compared with cAuNPs (519 nm). DLS measurements showed a size of around 8 nm and a less negative ZP than cAuNPs (-13 mV). Scanning electron microscopy (SEM) analysis allowed the visualization of a PVP layer around some AuNPs metal core (Figure 1C).

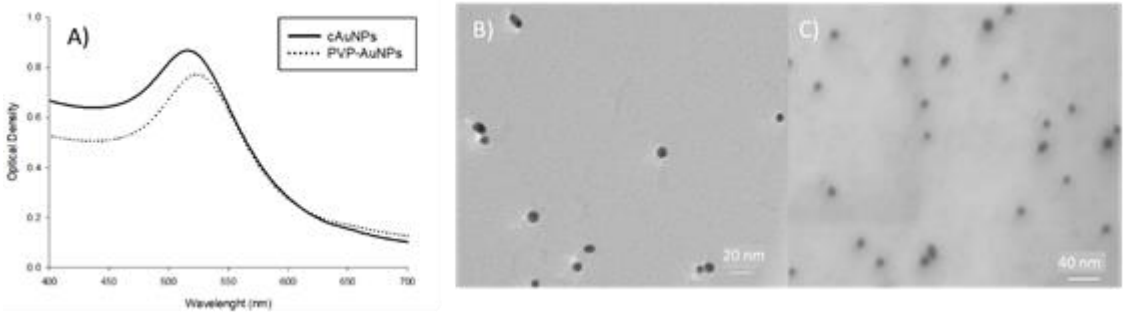

Figure 1. UV–Vis spectra **(A),** transmission electron microscopy image of citrate coated gold nanoparticles – cAuNPs **(B)** and scanning electron microscopy image of polyvinylpyrrolidone coated – PVP-AuNPs **(C)**.

In the experimental media (ASW), 80 and 1600 $\mu g.L^{-1}$ cAuNPs changed the colour from red to light blue, as a result of NPs agglomeration/aggregation, whereas PVP-AuNPs did not show colour alteration. At 4 $\mu g.L^{-1}$, it was not possible to detect any colour change. Moreover, the hydrodynamic size of cAuNPs (1600 $\mu g.L^{-1}$) in ASW at 0 h increased to around 160 nm, maintaining this size till the end of the test (96 h) – Table 2. The characteristic surface plasmon resonance (SPR) peak detected in ultrapure water was not detected in ASW (Table 2). Additionally, different peaks corresponding to different charges were found in the zeta potential (ZP) analysis of the cAuNPs in ASW. Within 24 h, in the aquaria containing 1600 $\mu g.L^{-1}$ of cAuNPs, a dark layer was visible as a consequence of the sedimentation of the NPs aggregates/agglomerates. PVP-AuNPs (1600 $\mu g.L^{-1}$) in ASW had similar characteristics (e. g. hydrodynamic size, UV-Vis spectra and ZP) as the PVP-AuNPs in ultrapure water (Table 2). At 4 and 80 $\mu g.L^{-1}$, it was not possible characterise the AuNPs because of the detection limits of the techniques used.

Table 2. Characteristics of gold nanoparticles (AuNPs), at 1600 $\mu g.L^{-1}$, in ultrapure water and artificial seawater after 96 h. cAuNPs – Citrate coated gold nanoparticles; PVP-AuNPs – Polyvinylpyrrolidone coated gold nanoparticles; PdI – Polydispersity Index; SPR – Surface Plasmon Resonance; ZP – Zeta Potential; N. D. – Not detected

	Size (nm)	PdI	SPR (nm)	ZP (mV)
Ultrapure water				
cAuNPs	6.7	0.5	519.0	-43.3
PVP-AuNPs	7.8	0.5	521.0	-12.8
Artificial seawater				
cAuNPs	159.8	0.8	N. D.	N. D.
PVP-AuNPs	8.1	0.5	521.4	-12.6

3.2. Gold (Au) quantification in experimental media

AuNPs theoretical concentration versus measured concentrations (by ICP-MS) and the number of particles present in the AuNPs stock suspensions are shown in Table S1. The nominal versus measured concentrations of Au in the experimental media are presented in Table 3.

Table 3. Nominal versus measured Au concentrations ($\mu g.L^{-1}$) in experimental media (artificial seawater) at 0 and 24 h, after exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. <d.l. – Below the detection limit.

Nominal concentrations ($\mu g.L^{-1}$)	Time (h)	Measured Au concentrations ($\mu g.L^{-1}$) cAuNPs	PVP-AuNPs	Ionic gold
0	**0**	<d.l.	<d.l.	<d.l.
	24	<d.l.	<d.l.	<d.l.
4	**0**	2.4 ± 3.8	7.5 ± 0.7	7.1 ± 0.4
	24	1.6 ± 2.8	6.4 ± 0.6	7.1 ± 0.6
80	**0**	24.1 ± 1.1	50.0 ± 2.8	92.5 ± 0.9
	24	7.0 ± 0.6	38.4 ± 1.6	89.1 ± 1.3
1600	**0**	88.9 ± 7.0	1341.1 ± 51.7	1370.2 ± 36.0
	24	34.6 ± 5.8	1140.7 ± 19.9	1285.1 ± 81.8

At 0 h, the Au quantified in ASW, in general, was lower than the nominal concentrations, with exception to 4 $\mu g.L^{-1}$ of PVP-AuNP and iAu (4 and 80 $\mu g.L^{-1}$). The difference between the nominal and measured concentrations was more noticeable in the case of the exposures to cAuNPs (Table 3). With the increasing cAuNPs concentration, the difference between the nominal and measured concentration of Au also increased. For the nominal concentration of 4 $\mu g.L^{-1}$ cAuNPs, the measured concentration of Au was 41% lower than the expected. For PVP-AuNPs and iAu, the determined concentrations of Au were 88 and 78% higher than the expected, respectively. For the 80 $\mu g.L^{-1}$ treatment, the detected concentrations of Au in ASW were 70 and 38% lower than the nominal concentrations, after cAuNPs and PVP-AuNPs exposures, respectively. For 80 $\mu g.L^{-1}$ of iAu, the measured concentration of Au was 16% higher than the expected. At 1600 $\mu g.L^{-1}$, the concentration of Au was 84, 16 and 14% lower than the expected for cAuNPs, PVP-AuNPs and iAu, respectively. The levels of Au at 0 and 24 h decreased more for cAuNPs than PVP-AuNPs (Table 3). In the nominal concentration 4 $\mu g.L^{-1}$, an Au decrease of 33 and 15% was found for cAuNPs and PVP-AuNPs, respectively. Concerning iAu, the measured concentration at 0 h was similar to the measured at 24 h. In the nominal concentration 80 $\mu g.L^{-1}$, after 24 h of exposure, the concentrations of Au decreased by 71, 23 and 4% for cAuNPs, PVP-AuNPs and iAu, respectively. For the nominal concentration 1600 $\mu g.L^{-1}$, a decrease of Au concentration after 24 h was also observed with 61% for cAuNPs, 15% for PVP-AuNPs and 6% for iAu.

3.3. Biological responses of fish after exposure to gold (Au)

After 24 h of exposure, one fish died in the 1600 µg.L^{-1} iAu treatment. As shown in Figure 2, the ability of *Sparus aurata* to continue swimming against a water flow was significantly decreased ($p<0.05$; Dunnett's test) when fish were exposed to 1600 µg.L^{-1} of cAuNPs and iAu.

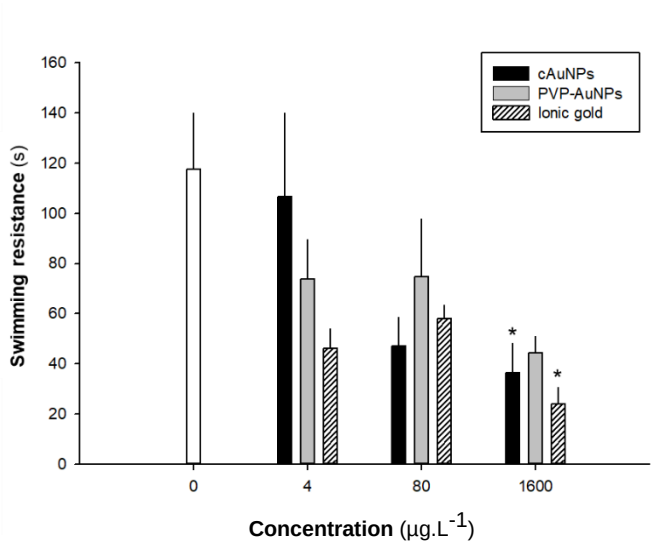

Figure 2. Resistance of *Sparus aurata* to withstand swimming against a water flow after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean time in seconds ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$).

ChE activity in brain and muscle was not significantly altered by the exposure to the both forms of Au ($p>0.05$; ANOVA; Figure 3).

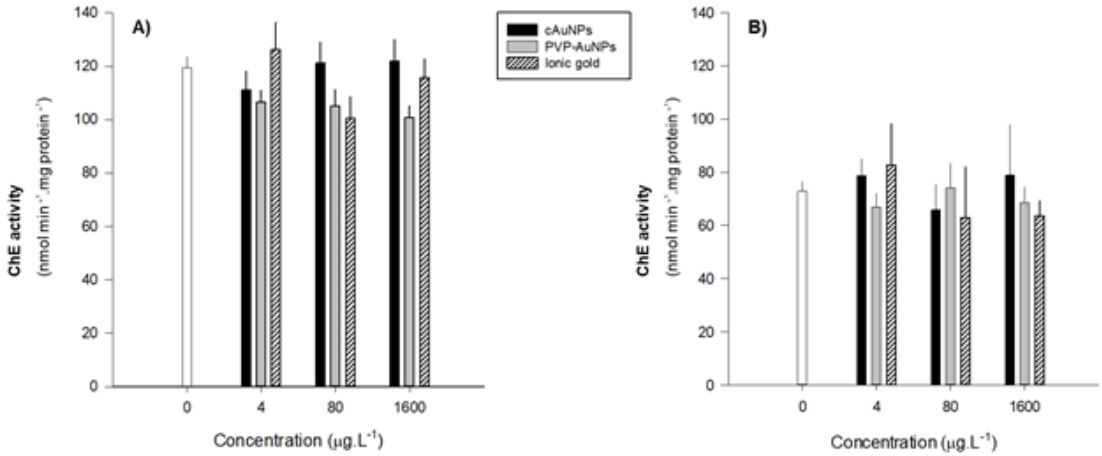

Figure 3. Brain **(A)** and muscle **(B)** cholinesterases (ChE) activity of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error.

Concerning the enzymatic antioxidant defence, iAu (4 and 1600 µg.L^{-1}) significantly increased gills CAT activity ($p<0.05$; Dunnett's test; Figure 4A), whereas, liver CAT activity was not significantly affected by the exposure to both forms of Au ($p>0.05$; ANOVA; Figure 4B). PVP-AuNPs, at 80 µg.L^{-1}, significantly decreased the gills CAT activity ($p<0.05$; Dunnett's test; Figure 4A).

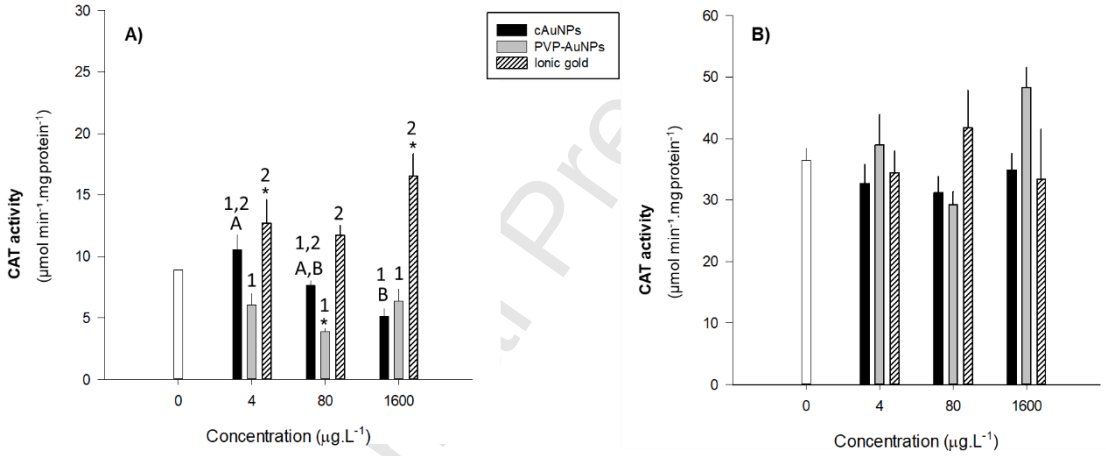

Figure 4. Gills **(A)** and liver **(B)** catalase (CAT) activity of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). Different letters correspond to significant differences between the treatments of each type of AuNPs and ionic form (Tukey´s test, $p<0.05$). Different numbers correspond to significant differences between each treatment within the same concentration (Tukey´s test, $p<0.05$).

GR activity in gills was significantly increased after exposure to 1600 µg.L^{-1} of cAuNPs ($p<0.05$; Dunnett's test; Figure 5A) whereas in the liver, GR activity was significantly increased by 80 and 1600 µg.L^{-1} of cAuNPs ($p<0.05$; Dunnett's

test; Figure 5B). PVP-AuNPs, 80 and 1600 µg.L^{-1}, significantly decreased gills GR activity ($p<0.05$; Dunnett's test; Figure 5A).

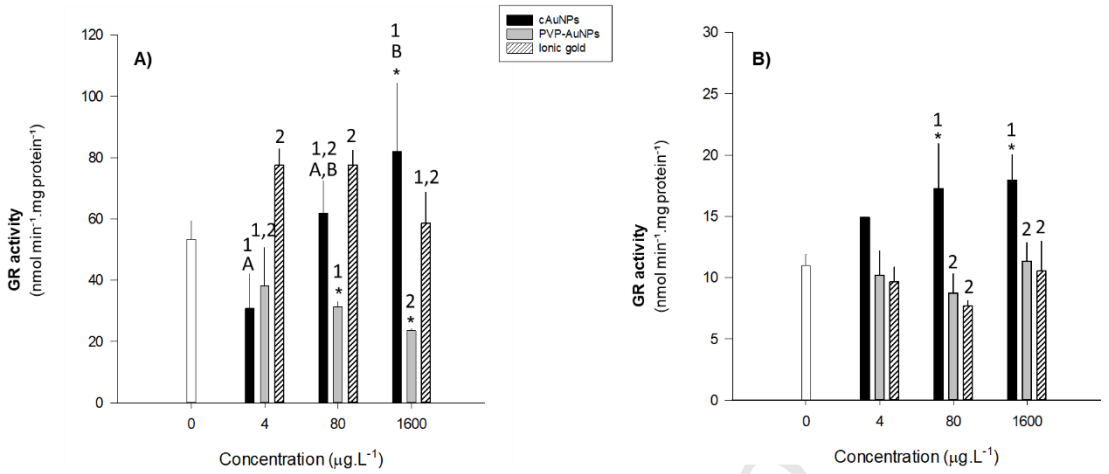

Figure 5. Gills **(A)** and liver **(B)** glutathione reductase (GR) activity of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). Different letters correspond to significant differences between the treatments of each type of AuNPs and ionic form (Tukey´s test, $p<0.05$). Different numbers correspond to significant differences between each treatment within the same concentration (Tukey´s test, $p<0.05$).

cAuNPs and iAu at 4 and 80 µg.L^{-1}, respectively, significantly increased gills GPx activity ($p<0.05$; Dunnett's test; Figure 6A). In the liver, 80 µg.L^{-1} of PVP-AuNPs decreased the GPx activity ($p<0.05$; Dunnett's test; Figure 6B).

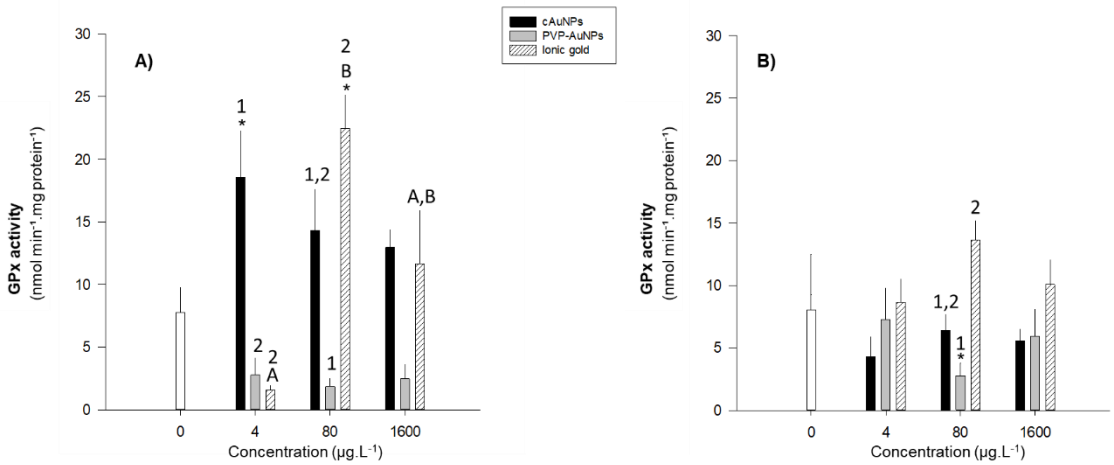

Figure 6. Gills **(A)** and liver **(B)** glutathione peroxidase (GPx) activity of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05). Different letters correspond to significant differences between the treatments of each type of AuNPs and ionic form (Tukey´s test, p<0.05). Different numbers correspond to significant differences between each treatment within the same concentration (Tukey´s test, p<0.05).

Concerning the non-enzymatic antioxidant defence, all tested concentrations of cAuNPs and 1600 µg.L^{-1} of iAu significantly increased gills NPT levels (p<0.05; Dunnett's test; Figure 7A). In liver, only 1600 µg.L^{-1} of cAuNPs significantly increased the levels of NPT (p<0.05; Dunnett's test; Figure 7B).

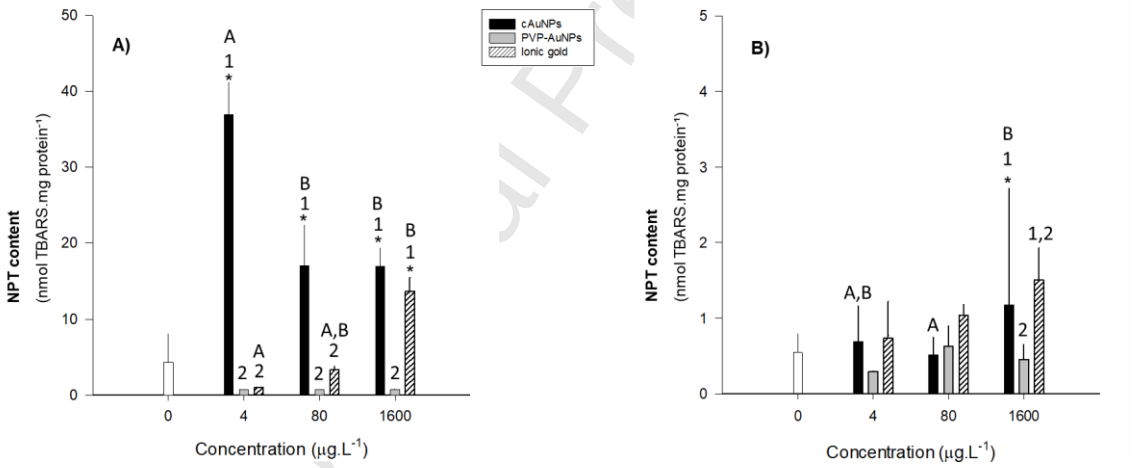

Figure 7. Gills **(A)** and liver **(B)** non-protein thiols (NPT) levels of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05). Different letters correspond to significant differences between the treatments of each type of AuNPs and ionic form (Tukey´s test, p<0.05). Different numbers correspond to significant differences between each treatment within the same concentration (Tukey´s test, p<0.05).

cAuNPs (4 µg.L^{-1}) and iAu (1600 µg.L^{-1}) significantly increased gills GST activity ($p<0.05$; Dunnett's test; Figure 8A). In liver, 1600 µg.L^{-1} iAu significantly increased the activity of this enzyme ($p>0.05$; ANOVA; Figure 8B).

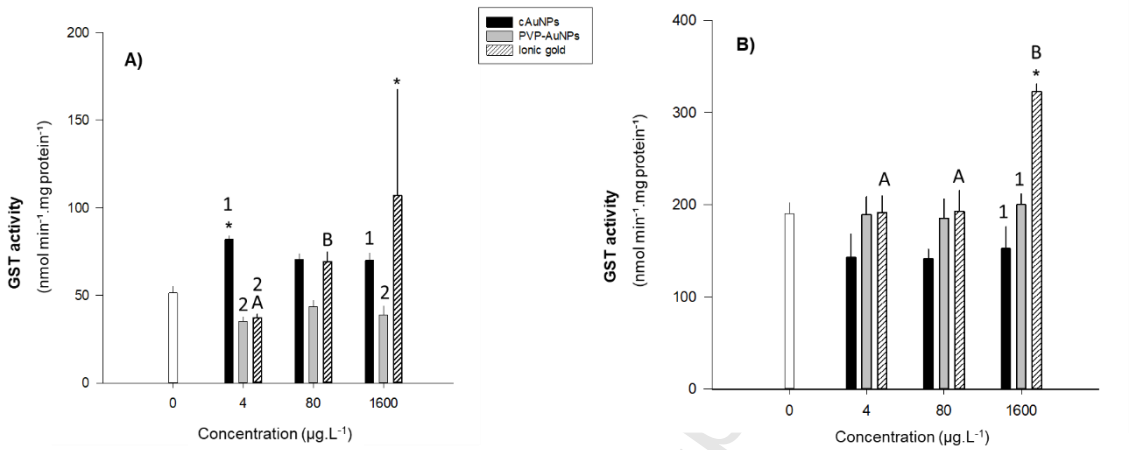

Figure 8. Gills **(A)** and liver **(B)** glutathione *S*-transferases (GST) activity of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). Different letters correspond to significant differences between the treatments of each type of AuNPs and ionic form (Tukey´s test, $p<0.05$). Different numbers correspond to significant differences between each treatment within the same concentration (Tukey´s test, $p<0.05$).

As shown in Figure 9A, oxidative damage (assessed as TBARS levels) was found in gills after the exposure to all tested concentrations of cAuNPs ($p<0.05$; Dunnett's test). In liver, LPO levels significantly increased after the exposure to 1600 µg.L^{-1} of cAuNPs ($p<0.05$; Dunnett's test; Figure 9B).

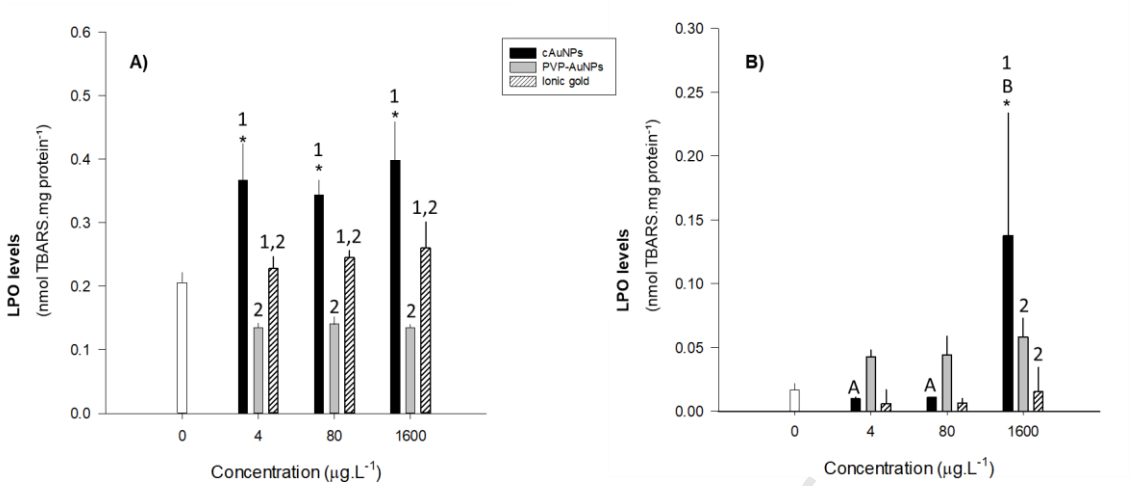

Figure 9. Gills **(A)** and liver **(B)** lipid peroxidation (LPO) levels of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). Different letters correspond to significant differences between the treatments of each type of AuNPs and ionic form (Tukey´s test, $p<0.05$). Different numbers correspond to significant differences between each treatment within the same concentration (Tukey´s test, $p<0.05$).

Increase of LPO levels was also detected in brain following exposure to 4 and 80 µg.L^{-1} of iAu ($p<0.05$; Dunnett's test; Figure 10A) and in muscle after the exposure to 80 µg.L^{-1} of iAu ($p<0.05$; Dunnett's test; Figure 10B).

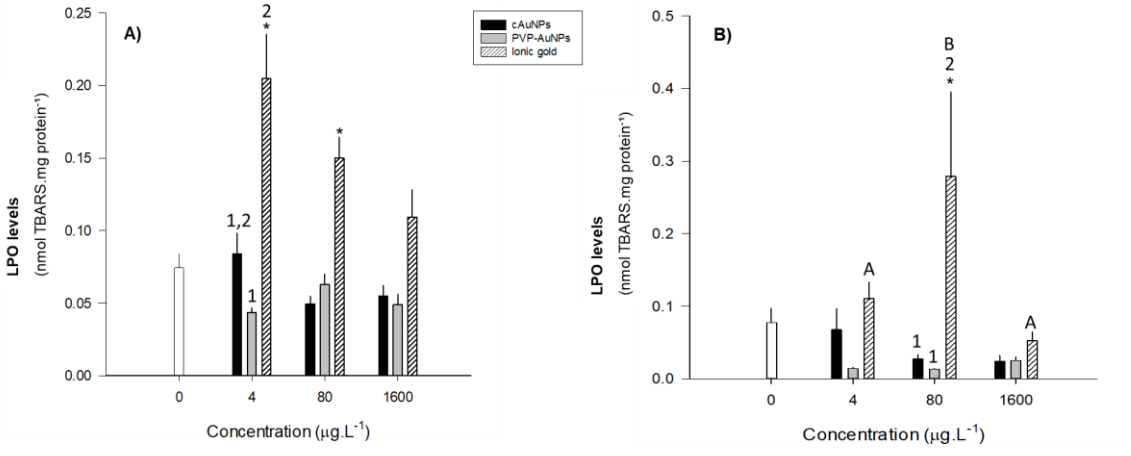

Figure 10. Brain **(A)** and muscle **(B)** lipid peroxidation (LPO) levels of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). Different letters correspond to significant differences between the treatments of each type of AuNPs and ionic form (Tukey´s test, $p<0.05$). Different numbers correspond to significant differences between each treatment within the same concentration (Tukey´s test, $p<0.05$).

All the treatments induced genotoxic effects ($p<0.05$; Dunnett's test), assessed by DNA strand breakage – Table 4. A dose response pattern was found, with damage index increasing with the increase of Au concentration (both forms). A DNA damage index around 319 was detected in animals exposed to 1600 $\mu g.L^{-1}$ of iAu, the highest value detected considering all the treatments (Table 4). In terms of damage classes, as shown in Table 4, the most abundant classes in the negative control group were class 0 and 1. Class 2 was the most detected in the exposures to 4 $\mu g.L^{-1}$ and classes 2 and 3 in the exposures to 80 $\mu g.L^{-1}$ ($p<0.05$; Dunnett's test). At 1600 $\mu g.L^{-1}$, Au exposures induced a DNA damage classified, mostly, in classes 3 and 4 ($p<0.05$; Dunnett's test). No significant oxidative DNA damage was found ($p>0.05$; ANOVA). Comparing the DNA damage index in cells treated with Fpg with the correspondent cells without the enzymatic treatment, no significant differences were found ($p>0.05$; ANOVA) – Table 4. However, comparing the DNA damage index in cells treated with H_2O_2 with and without treatment with Fpg, in the cells with Fpg the DNA damage index was significantly higher than those without Fpg ($p<0.05$; Tukey´s test).

Table 4. DNA damage classes, measured by the comet assay, of peripheral blood cells from *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. *Significant differences to control (Dunnett's test, $p<0.05$); data are presented as mean ± standard error. Different letters correspond to significant differences between the treatments of each type of AuNPs and ionic form (Tukey´s test, $p<0.05$). A. U. – Arbitrary units; Fpg – Formamidopyrimidine DNA glycoslyase.

Treatment group	DNA damage classes (%)					DNA damage index (A. U.)	DNA damage index (A. U.) with Fpg
	0	1	2	3	4		
Control	35.9±2.6	58.2±2.9	5.7±0.9	0.2±0.1	-	70.1±2.5	69.9±4.2
4 µg.L^{-1} cAuNPs	0.8±0.4*	37.2±3.5*	47.6±4.8*	14.0±2.7*	0.4±0.2	176.0±4.0*^{A}	192.0±2.6*^{A,1}
80 µg.L^{-1} cAuNPs	0.2±0.2*	8.8±4.2*	42.0±2.6*	40.0±2.7*	9.0±0.5	248.8±6.9*^{B}	261.8±2.9*^{B}
1600 µg.L^{-1} cAuNPs	0.6±0.4*	11.4±4.4*	31.6±3.2*	32.4±2.5*	30.0±5.0*	291.8±15.3*^{B}	301.4±13.8*^{B}
4 µg.L^{-1} PVP-AuNPs	1.0±0.4*	28.8±3.9*	52.0±3.6*	16.2±2.0*	2.0±1.8	189.4±6.3*^{A}	196.0±6.3*^{A,1,2}
80 µg.L^{-1} PVP-AuNPs	0.2±0.2*	9.8±3.9*	31.0±2.5*	49.6±5.0*	7.8±2.1	251.8±7.7*^{B}	257.4±7.2*^{B}
1600 µg.L^{-1} PVP-AuNPs	0.6±0.4*	11.2±4.5*	25.6±1.7*	37.8±2.8*	30.8±4.9*	299.0±14.2*^{C}	305.2±12.6*^{C}
4 µg.L^{-1} ionic gold	0.8±0.4*	16.2±5.1*	50.2±2.9*	27.6±4.8*	5.2±2.5	220.2±13.7*^{A}	240.2±7.9*^{A,2}
80 µg.L^{-1} ionic gold	0.2±0.2*	11.4±4.4*	31.6±3.2*	32.4±2.5*	30.0±5.0*	291.8±15.3*^{B}	290.6±13.0*^{B}
1600 µg.L^{-1} ionic gold	-*	1.0±0.8*	18.4±3.1*	41.6±5.1*	39.0±2.1*	318.6±3.1*^{B}	322.4±4.0*^{B}

All the treatments, with the exception to 4 µg.L^{-1} of cAuNPs and iAu, led to significantly higher ENAs frequency ($p<0.05$; Dunnett's test), as shown in Figure 11. The frequency of ENAs increased with the increase of Au concentration (both forms).

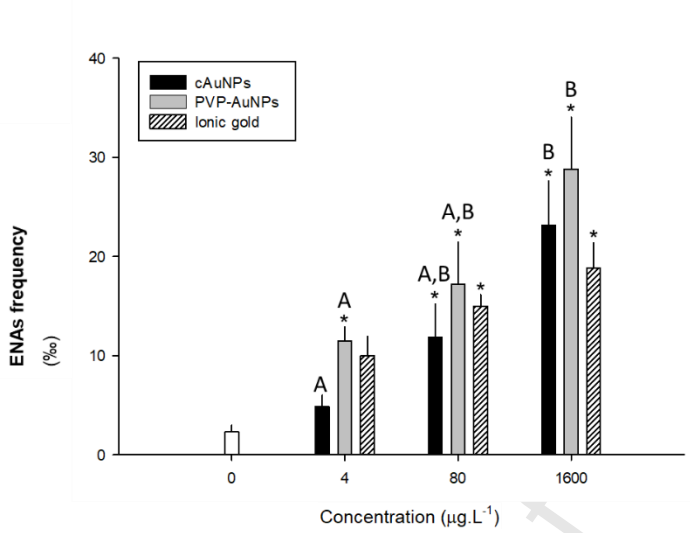

Figure 11. Erythrocytic nuclear abnormalities (ENAs) frequency in *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). Different letters correspond to significant differences between the treatments of each type of AuNPs and ionic form (Tukey´s test, $p<0.05$).

As shown in Table 5, the lobed nuclei abnormality was the most commonly detected in all the treatments, followed by kidney-shaped nuclei ($p<0.05$; Dunnett's test). The segmented, micronuclei and vacuolated nuclei abnormalities were the less detected – Table 5.

Table 5. Erythrocytic nuclear abnormalities (ENAs) detected in *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. *Significant differences to control (Dunnett's test, $p<0.05$); data are presented as mean ± standard error. K – kidney-shaped nuclei; S – segmented nuclei; L – lobed nuclei; V – vacuolated nuclei; MN – micronuclei.

Treatment group	ENAs frequency (‰)				
	K	S	L	V	MN
Control	0.8±0.2	0.1±0.1	1.5±0.5	0.1±0.1	0.0±0.0
4 µg.L^{-1} cAuNPs	2.0±0.8	0.3±0.2	2.3±0.4	0.3±0.2	0.0±0.0
80 µg.L^{-1} cAuNPs	4.0±1.1*	0.2±0.2	7.7±2.5*	0.0±0.0	-0.0±0.0
1600 µg.L^{-1} cAuNPs	7.6±1.3*	0.1±0.1	15.4±3.7*	0.0±0.0	0.0±0.0
4 µg.L^{-1} PVP-AuNPs	4.3±0.7*	0.0±0.0-	7.2±1.1*	0.0±0.0	0.0±0.0
80 µg.L^{-1} PVP-AuNPs	4.6±0.8*	0.4±0.2	12.2±3.8*	0.0±0.0	0.0±0.0
1600 µg.L^{-1} PVP-AuNPs	10.3±1.6*	0.0±0.0	18.3±4.1*	0.0±0.0	0.2±0.2
4 µg.L^{-1} ionic gold	3.3±0.5*	0.4±0.2*	6.3±1.4*	0.0±0.0	0.0±0.0
80 µg.L^{-1} ionic gold	4.7±8*	0.3±0.2	9.7±0.9*	0.3±0.2	0.0±0.0
1600 µg.L^{-1} ionic gold	4.5±1.0*	0.3±0.3	14.2±1.2*	0.5±0.3	0.0±0.0

3.4. Total gold (Au) content, bioaccumulation factor and estimated intake for humans

Au did not accumulate significantly in the assessed tissues of *S. aurata* ($p>0.05$; ANOVA; Table 6) after the exposure to AuNPs. However, Au significantly accumulated in gills, liver and spleen of *S. aurata* after the exposure to 1600 µg.L^{-1} iAu ($p<0.05$; Dunnett's test; Table 6). The highest calculated BAF value (2 L.g^{-1}) was found for the nominal concentration exposure of 4 µg.L^{-1} cAuNPs, in the spleen (Table 6).

Table 6. Gold content in tissues (gills, liver, spleen and muscle) of *Sparus aurata* exposed to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold for 96 h and respective estimated bioaccumulation factor (BAF). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). <d.l. – Below the detection limit.

Nominal concentrations ($\mu g.L^{-1}$)	Tissues	Gold content ($\mu g.g^{-1}$)			BAF ($L.g^{-1}$)		
		cAuNPs	PVP-AuNPs	Ionic gold	cAuNPs	PVP-AuNPs	Ionic gold
0	Gills	<d.l.	<d.l.	<d.l.	-	-	-
	Liver	<d.l.	<d.l.	<d.l.	-	-	-
	Spleen	<d.l.	<d.l.	<d.l.	-	-	-
	Muscle	<d.l.	<d.l.	<d.l.	-	-	-
4	Gills	<d.l.	0.2 ± 0.0	0.1 ± 0.0	-	0.0	0.0
	Liver	0.1 ± 0.0	<d.l.	0.1 ± 0.0	0.0	-	0.0
	Spleen	4.8 ± 0.4	<d.l.	0.4 ± 0.0	2.0	-	0.1
	Muscle	<d.l.	<d.l.	<d.l.	-	-	-
80	Gills	1.3 ± 0.1	0.2 ± 0.1	1.2 ± 0.4	0.1	0.0	0.0
	Liver	0.1 ± 0.0	<d.l.	0.8 ± 0.4	0.0	-	0.0
	Spleen	0.5 ± 0.1	0.4 ± 0.0	1.6 ± 1.0	0.0	0.0	0.0
	Muscle	0.1 ± 0.0	0.1 ± 0.0	0.2 ± 0.0	0.0	0.0	0.0
1600	Gills	3.3 ± 0.3	0.8 ± 0.2	8.2 ± 4.5 *	0.0	0.0	0.0
	Liver	0.5 ± 0.0	2.4 ± 2.1	8.4 ± 2.5 *	0.0	0.0	0.0
	Spleen	3.3 ± 1.9	1.1 ± 1.0	6.4 ± 4.0 *	0.0	0.0	0.0
	Muscle	0.1 ± 0.0	<d.l.	1.1 ± 0.6	0.0	-	0.0

The highest $[Au]_{total}$ value (around 24 $\mu g.g^{-1}$) was detected after exposure to 1600 $\mu g.L^{-1}$ iAu ($p<0.05$; Dunnett's test).

The highest estimated value for Au intake by a Portuguese citizen would be for the condition 1600 $\mu g.L^{-1}$ of iAu (Table 7).

Table 7. Estimated gold intake (µg per kg body weight per year), by each Portuguese person, after the ingestion of *Sparus aurata*, taking into account the total content of gold detected in muscle of fish after 96 h exposure to gold

nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold.

Nominal concentrations ($\mu g.L^{-1}$)	Estimated gold intake ($\mu g.kg$ body weight per year)		
	cAuNPs	PVP-AuNPs	Ionic gold
4	-	-	-
80	0.05	0.05	0.15
1600	0.14	0.03	1.10

4. Discussion

The coating of 7 nm AuNPs with PVP resulted in a slight shift in the SPR peak to a longer wavelength when compared with the original cAuNPs as previously observed for the same AuNPs (Barreto et al. 2015). DLS measurements showed an increased size of PVP-AuNPs and a less negative ZP value when compared with cAuNPs, also in agreement with a previous study (Barreto et al. 2015). The detected size difference may be explained by the fact that PVP presenting a larger size than citrate (Iswarya et al. 2016; Tejamaya et al. 2012). In terms of ZP, the observed difference between cAuNPs and PVP-AuNPs may be explained by the fact that PVP is an uncharged molecule thus making the PVP-AUNPs less negative than cAuNPs (Mahl et al. 2010). In the experimental media (ASW), 80 and 1600 $\mu g.L^{-1}$ cAuNPs changed the colour, as a result of NPs agglomeration/aggregation. PVP-AuNPs, at 80 and 1600 $\mu g.L^{-1}$, did not show colour alteration in ASW. These results are in agreement with the previous study of Barreto et al. (2015) which demonstrated that 7 nm PVP-

AuNPs were stable in ASW for more than 30 days. Thus, the present study confirmed that PVP-AuNPs may remain stable in suspension in a nano size range in ASW, whereas cAuNPs immediately alter their characteristics and aggregate/agglomerate, increasing their size to more than 100 nm. These characteristics and behaviour of different AuNPs may influence their accumulation and effects to the organisms. NPs size may affect its bioavailability to the organisms. When aggregates become too large for direct transport across the cell membrane, uptake may be prevented (Vale et al. 2016).

Although the stability of the tested AuNPs was different in ASW, no significant differences were found in terms of Au accumulation in the tissues of *S. aurata* after the exposure to cAuNPs and PVP-AuNPs. Another interesting result was the highest BAF in the spleen calculated for the lowest nominal concentration exposure of cAuNPs (4 $\mu g.L^{-1}$). This may be due to the lower aggregation/agglomeration processes, with higher ability of Au entering in the tissues (Barreto et al. 2019a). As already reported, aggregation/agglomeration is expected to increase with the increase in the number of particles per volume (Barreto et al. 2015). Therefore, since cAuNPs, at the highest tested concentrations (80 and 1600 $\mu g.L^{-1}$), are more likely to aggregate/agglomerate, they are less available for the uptake by fish. The detected accumulation of Au in the spleen of *S. aurata* (although not significantly) show the potential role of the spleen in Au elimination. Despite the lack of Au accumulation after the exposure to AuNPs, particles were bioactive to *S. aurata*. The present data reveal that 7 nm cAuNPs induced more pronounced effects, in terms of oxidative stress and damage responses, than PVP-AuNPs. This result was

unexpected considering the stability of the particles, despite the fact that PVP coating is considered safer and more biocompatible than citrate coating (Min et al. 2009). The 7 nm PVP-AuNPs remained stable in ASW, dispersed in the water column and, therefore, more available for the uptake by fish. An opposite pattern was previously observed for 40 nm AuNPs with PVP-AuNPs inducing more adverse effects than cAuNPs to *S. aurata* (Barreto et al. 2019b). However, a previous study, where different organisms were tested (bacteria, algae, SiHa cell line and mice), also showed that cAuNPs had more adverse effects than PVP-AuNPs (Iswarya et al. 2016). Wang et al. (2011) reported that the toxicity of AuNPs is related to the co-existence of citrate and Au^{3+} ions. When NPs are coated with PVP, there is an absence of reactive citrate ions on the surface of AuNPs. In the present study, the tested concentrations of cAuNPs decreased the swimming resistance, induced enzymatic and non-enzymatic responses involved in the oxidative defence/damage and genotoxicity in fish. Therefore, it seems that the formed agglomerates/aggregates in the ASW (less than 200 nm) may be incorporated through the cellular membranes.

Comparing the ionic with the nano form, Au significantly accumulated in almost all assessed tissues of *S. aurata* after the exposure to 1600 $\mu g.L^{-1}$ iAu, whereas after the exposure to AuNPs, Au did not significantly accumulate. Despite the significantly higher effects of cAuNPs in some endpoints (such as gills GPx and liver GR activities, gills NPT and gills/liver LPO levels), even at the lowest tested concentration (i.e., 4 $\mu g\ L^{-1}$), iAu induced, in general, more effects on the fish (gills CAT, GR and liver GST activities and muscle/brain LPO levels). Additionally, no fish mortality was detected after the exposures with AuNPs, whereas one fish died after the exposure to 1600 $\mu g.L^{-1}$ of iAu. The

results demonstrated a tissue specificity, cAuNPs induced LPO in gills and liver, while iAu induced LPO in brain and muscle. There are few available studies about the mechanisms involved in the toxicity of iAu. Nonetheless, the iAu ability to undergo redox reactions with peptides and proteins, particularly involving sulphur amino acids, to deprotonate and bind to peptide amide bonds and cross-link histidine imidazole rings, has been already reported (Best and Sadler 1996; Luis et al. 2016). Some authors, using *in vitro* tests, reported that iAu induced effects to mussel (*Mytilus galloprovincialis*) and rainbow trout (*Oncorhynchus mykiss*) whereas the nano form did not have any effect (Farkas et al. 2010; Luis et al. 2016). Botha el al. (2015), using different aquatic species (daphnia and fish), also showed that iAu was more toxic than nano form. However, Barbasz et al. (2016), using two types of human cell lines, showed a higher cytotoxicity of AuNPs than iAu. Dedeh et al. (2014) described that, in spite of iAu having accumulated more in the tissues of zebrafish (*Danio rerio*) than the AuNPs, the latter had more effects on the fish, in terms of gene expression and neurotransmission.

In terms of genotoxicity, all the treatments induced DNA strand breaks, assessed by comet assay, in *S. aurata* peripheral blood cells. Concerning cytogenetic damage, ENAs frequency increased with the increase of Au concentration (nano or ionic form). A previous study also showed the potential genotoxicity of 40 nm AuNPs (both PVP-AuNPs and cAuNPs) to *S. aurata* (Barreto et al. 2019a). Comet assay is a rapid method to detect low levels of DNA damage. However, this technique gives limited information about the kind of DNA damage, if it is a direct consequence of the damaging agent or indirect effects, such as oxidative damage, apurinic/pyrimidinic sites or DNA repair

(Smith, O'Donovan, and Martin 2006). As previously described, the genotoxic effects of AuNPs may be caused directly following the entry of NPs into the nuclei, binding to DNA; or indirectly, through oxidative stress, which may consequently induce DNA oxidative damage (Auffan et al. 2009; Barreto et al. 2019a; Cardoso et al. 2014). The production of reactive oxygen species (ROS) following AuNPs exposure has been demonstrated in studies involving aquatic organisms (Farkas et al. 2010; Tedesco et al. 2008, 2010; Pan et al. 2012). Modification of the comet assay with the incorporation of lesion specific endonucleases, such as Fpg, increases its sensitivity and specificity through the recognition of damaged bases and introduction of additional breaks (Azqueta et al. 2013; Smith, O'Donovan, and Martin 2006; Speit et al. 2004). The present study showed that Au (nano and ionic form) induced DNA breaks, but oxidative DNA damage was not observed. This result was previously described in studies with different types of NPs (Ag, CeO_2, Co_3O_4 and SiO_2) and metal ions (Al^{3+}, Ni^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+}) (Grin et al. 2009; Kain, Karlsson, and Möller 2012) and may be due to: 1) the low potential of the tested conditions to induce oxidative damage on the erythrocyte DNA of *S. aurata*, which is not supported by the LPO data; 2) the oxidative DNA lesions caused by the exposure to Au may have been already been repaired by cellular DNA repair systems (Catalán et al. 2014); 3) NPs and ionic forms may interact with Fpg, not allowing the binding of the enzyme with DNA (Asmuss et al. 2000; Kain, Karlsson, and Möller 2012).

Overall, after Au exposures, enzymatic and non-enzymatic responses involved in the defence of *S. aurata* against oxidative damage were more activated in the gills than in the liver. Additionally, oxidative damage (LPO

increase) was more clearly expressed in gills than in liver. Gills are the first organ to be exposed and provide a large surface area for contaminants such as AuNPs, being considered a good candidate to an early assessment of the effects of waterborne contaminants (Oliveira, Pacheco, and Santos 2008).

Since *S. aurata* is one of the most consumed fish in south Europe, an estimation of Au intake by humans via food chain is an important assessment (Barreto et al. 2019b). The highest estimated value for Au intake by each Portuguese person (1.10 µg.kg body weight per year) would be relevant following an exposure of the fish to 1600 $\mu g.L^{-1}$ iAu. Based on the tested conditions and present results, the estimated maximum Au intake by humans per day was around 0.003 $\mu g.kg^{-1}$ body weight. So, this value did not exceed the estimated TDI value for Au (322 $\mu g.kg^{-1}$). Future studies should carry out the assessment of Au intake by humans via food chain since AuNPs use is increasing worldwide and thus it is expected to find increased concentration of them in the environment.

5. Conclusions

The present results showed that short-term exposure to gold (nano or ionic form), at low levels such as 4 $\mu g.L^{-1}$, was able to induce oxidative stress and damage, as well as genotoxicity to the marine/estuarine fish *Sparus aurata.* Citrate coated gold nanoparticles (cAuNPs), even aggregating/agglomerating in seawater, induced significantly more effects to fish (oxidative stress and damage) than the polyvinylpyrrolidone coated gold nanoparticles (PVP-AuNPs), which maintained its nano size in seawater. The exposures to ionic gold resulted in higher accumulation in the fish tissues and induced more effects to

fish than nano form. After gold exposures, responses involved in fish defence against oxidative damage were more activated in the gills than in the liver. Furthermore, oxidative damage (lipid peroxidation increase) was more detected in gills than in the liver. The results showed that gold (nano and ionic form) is not inert and a distinct response was found in the assessed tissues. Further chronic tests must be performed to complement the present findings.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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6. References

Albertini, R. J., D. Anderson, G. R. Douglas, L. Hagmar, K. Hemminki, F. Merlo, A. T. Natarajan, H. Norppa, D. E. G. Shuker, R. Tice, Michael D. Waters, and Antero Aitio. 2000. 'IPCS guidelines for the monitoring of genotoxic effects of carcinogens in humans', *Mutation Research/Reviews in Mutation Research*, 463: 111-72.

Asmuss, M., L. H. F. Mullenders, A. Eker, and A. Hartwig. 2000. 'Differential effects of toxic metal compounds on the activities of Fpg and XPA, two zinc finger proteins involved in DNA repair', *Carcinogenesis*, 21: 2097-104.

Athar, M., and M. Iqbal. 1998. 'Ferric nitrilotriacetate promotes N-diethylnitrosamine-induced renal tumorigenesis in the rat: implications for the involvement of oxidative stress', *Carcinogenesis*, 19: 1133-39.

Auffan, M., J. Rose, T. Orsiere, M. De Meo, A. Thill, O. Zeyons, O. Proux, A. Masion, P. Chaurand, O. Spalla, A. Botta, M. R. Wiesner, and J.-Y. Bottero. 2009. 'CeO_2 nanoparticles induce DNA damage towards human dermal fibroblasts *in vitro*', *Nanotoxicology*, 3: 161-71.

Azqueta, A., L. Arbillaga, A. L. de Cerain, and An. Collins. 2013. 'Enhancing the sensitivity of the comet assay as a genotoxicity test, by combining it with bacterial repair enzyme FPG', *Mutagenesis*, 28: 271-77.

Barbasz, A., and M. Oćwieja. 2016. 'Gold nanoparticles and ions – friends or foes? As they are seen by human cells U-937 and HL-60', *Journal of Experimental Nanoscience*, 11: 564-80.

Barreto, A., L. G. Luis, A. M. V. M. Soares, P. Paíga, L. H. M. L. M. Santos, C. Delerue-Matos, K. Hylland, S. Loureiro, and M. Oliveira. 2017. 'Genotoxicity

of gemfibrozil in the gilthead seabream (*Sparus aurata*)', *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 821: 36-42.

Barreto, A., L. G. Luis, A. V. Girão, T. Trindade, A. M. V. M. Soares, and M. Oliveira. 2015. 'Behavior of colloidal gold nanoparticles in different ionic strength media', *Journal of Nanoparticle Research*, 17: 1-13.

Barreto, A., L. G. Luis, E. Pinto, A. Almeida, P. Paíga , L. H. M. L. M. Santos, C. Delerue-Matos, T. Trindade, A. M. V. M. Soares, K. Hylland, S. Loureiro, M. Oliveira. 2019a. 'Genotoxicity of gold nanoparticles in the gilthead seabream (*Sparus aurata*) after single exposure and combined with the pharmaceutical gemfibrozil', *Chemosphere*, 220:11-19.

Barreto, A., L. G. Luis, E. Pinto, A. Almeida, P. Paíga , L. H. M. L. M. Santos, C. Delerue-Matos, T. Trindade, A. M. V. M. Soares, K. Hylland, S. Loureiro, M. Oliveira. 2019b. 'Effects and bioaccumulation of gold nanoparticles in the gilthead seabream (*Sparus aurata*) – Single and combined exposures with gemfibrozil', *Chemosphere*, 215:248-60.

Best, S. L., and P. J. Sadler. 1996. 'Gold drugs: Mechanism of action and toxicity', *Gold Bulletin*, 29: 87-93.

Bhattacharya, R., and P. Mukherjee. 2008. 'Biological properties of “naked” metal nanoparticles', *Advanced Drug Delivery Reviews*, 60: 1289-306.

Botha, T. L., T. E. James, and V. Wepener. 2015. 'Comparative Aquatic Toxicity of Gold Nanoparticles and Ionic Gold Using a Species Sensitivity Distribution Approach', *Journal of Nanomaterials*, 2015: 986902.

Boverhof, D. R., C. M. Bramante, J. H. Butala, S. F. Clancy, M. Lafranconi, J. West, and S. C. Gordon. 2015. 'Comparative assessment of nanomaterial

definitions and safety evaluation considerations', *Regulatory Toxicology and Pharmacology*, 73: 137-50.

Bradford, M. M. 1976. 'A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding', *Analytical Biochemistry*, 72: 248-54.

Bulloch, D. N., R. Lavado, K. L. Forsgren, S. Beni, D. Schlenk, and C. K. Larive. 2012. 'Analytical and Biological Characterization of Halogenated Gemfibrozil Produced through Chlorination of Wastewater', *Environmental Science & Technology*, 46: 5583-89.

Cardoso, E., G. T. Rezin, E. T. Zanoni, F. de S. Notoya, D. D. Leffa, A. P. Damiani, F. Daumann, J. C. O. Rodriguez, R. Benavides, L. da Silva, V. M. Andrade, and P. M. M. da Silva. 2014. 'Acute and chronic administration of gold nanoparticles cause DNA damage in the cerebral cortex of adult rats', *Mutation research*, 766-767: 25-30.

Carlberg, I., and B Mannervik. 1975. 'Purification and characterization of the flavoenzyme glutathione reductase from rat liver', *Journal of Biological Chemistry*, 250: 5475-80.

Catalán, J., S. Suhonen, A. Huk, and M. Dusinska. 2014. 'Analysis of nanoparticle-induced DNA damage by the comet assay.' in L. María Sierra and Isabel Gaivão (eds.), *Genotoxicity and DNA Repair: A Practical Approach* (Springer New York: New York, NY).

Claiborne, A. 1985. 'Catalase activity', *CRC handbook of methods for oxygen radical research*, 1: 283-84.

Collins, A. R. 2014. 'Measuring oxidative damage to DNA and its repair with the comet assay', *Biochimica et Biophysica Acta (BBA) - General Subjects*, 1840: 794-800.

Collins, A. R., V. L. Dobson, M. Dušinská, G. Kennedy, and R. Štětina. 1997. 'The comet assay: what can it really tell us?', *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis*, 375: 183-93.

Collins, A. R. 2004. 'The comet assay for DNA damage and repair', *Molecular Biotechnology*, 26: 249-61.

Coradeghini, R., S. Gioria, C. P. García, P. Nativo, F. Franchini, D. Gilliland, J. Ponti, and F. Rossi. 2013. 'Size-dependent toxicity and cell interaction mechanisms of gold nanoparticles on mouse fibroblasts', *Toxicology Letters*, 217: 205-16.

Daniel, M.-C., and D. Astruc. 2004. 'Gold nanoparticles: assembly, supramolecular chemistry, quantum-size-related properties, and applications toward biology, catalysis, and nanotechnology', *Chemical Reviews*, 104: 293-346.

Das, T., V. Kolli, S. Karmakar, and N. Sarkar. 2017. 'Functionalisation of polyvinylpyrrolidone on gold nanoparticles enhances its anti-amyloidogenic propensity towards hen egg white lysozyme', *Biomedicines*, 5: 19.

de Andrade, V. M., T. R. O. de Freitas, and J. da Silva. 2004. 'Comet assay using mullet (*Mugil* sp.) and sea catfish (*Netuma* sp.) erythrocytes for the detection of genotoxic pollutants in aquatic environment', *Mutation Research/Genetic Toxicology and Environmental Mutagenesis*, 560: 57-67.

Dedeh, A., A. Ciutat, M. Treguer-Delapierre, and J.-P. Bourdineaud. 2015. 'Impact of gold nanoparticles on zebrafish exposed to a spiked sediment', *Nanotoxicology*, 9: 71-80.

Ellman, G. L., K. D. Courtney, V. Andres jr, and R. M. Featherstone. 1961. 'A new and rapid colorimetric determination of acetylcholinesterase activity', *Biochemical Pharmacology*, 7: 88-95.

Epe, B., M. Pflaum, M. Häring, J. Hegler, and H. Rüdiger. 1993. 'Use of repair endonucleases to characterize DNA damage induced by reactive oxygen species in cellular and cell-free systems', *Toxicology Letters*, 67: 57-72.

Failler, P., G. V. de Walle, N. Lecrivain, A. Himbes, and R. Lewins. 2007. 'Future prospects for fish and fishery products. 4. Fish consumption in the European Union in 2015 and 2030. Part 1', *European overview FAO Fisheries Circular (FAO)*.

Farkas, J., P. Christian, J. A. G. Urrea, N. Roos, M. Hassellöv, K. E. Tollefsen, and K. V. Thomas. 2010. 'Effects of silver and gold nanoparticles on rainbow trout (*Oncorhynchus mykiss*) hepatocytes', *Aquatic Toxicology*, 96: 44-52.

FDA. 2015. 'Q3D Elemental Impurities Guidance for Industry'.

Filho, D., T. Tribess, C. Gáspari, F. D. Claudio, M. A. Torres, and A. R. M. Magalhães. 2001. 'Seasonal changes in antioxidant defenses of the digestive gland of the brown mussel (*Perna perna*)', *Aquaculture*, 203: 149-58.

Frasco, M. F., and L. Guilhermino. 2002. 'Effects of dimethoate and beta-naphthoflavone on selected biomarkers of *Poecilia reticulata*', *Fish Physiology and Biochemistry*, 26: 149-56.

Fratoddi, I., I. Venditti, C. Cametti, and M. V. Russo. 2015. 'How toxic are gold nanoparticles? The state-of-the-art', *Nano Research*, 8: 1771-99.

García-Negrete, C. A., J. Blasco, M. Volland, T. C. Rojas, M. Hampel, A. Lapresta-Fernández, M. C. Jiménez de Haro, M. Soto, and A. Fernández. 2013. 'Behaviour of Au-citrate nanoparticles in seawater and accumulation in bivalves at environmentally relevant concentrations', *Environmental Pollution*, 174: 134-41.

Ghosh, P., G. Han, M. De, C. K. Kim, and V. M. Rotello. 2008. 'Gold nanoparticles in delivery applications', *Advanced Drug Delivery Reviews*, 60: 1307-15.

Grin, I., P. G Konorovsky, G. Nevinsky, and D. O Zharkov. 2009. 'Heavy metal ions affect the activity of DNA glycosylases of the Fpg family', *Biochemistry*, 74: 1253-9.

Guilhermino, L., M. C. Lopes, A. P. Carvalho, and A. M. V. M. Soares. 1996. 'Acetylcholinesterase activity in juveniles of *Daphnia magna* straus', *Bulletin of Environmental Contamination and Toxicology*, 57: 979-85.

Habig, W. H., Michael J. Pabst, and W. B. Jakoby. 1974. 'Glutathione S-Transferases. The first enzymatic step in mercapturic acid formation', *Journal of Biological Chemistry*, 249: 7130-39.

Hanžić, N., T. Jurkin, A. Maksimović, and M. Gotić. 2015. 'The synthesis of gold nanoparticles by a citrate-radiolytical method', *Radiation Physics and Chemistry*, 106: 77-82.

Ido, A., Y. Hiromori, L. Meng, H. Usuda, H. Nagase, M. Yang, J. Hu, and T. Nakanishi. 2017. 'Occurrence of fibrates and their metabolites in source and drinking water in Shanghai and Zhejiang, China', *Scientific Reports*, 7: 45931.

IPCS. 2004. 'Harmonization project document no. 1 - IPCS risk assessment terminology', *WHO, Geneva*.

Iswarya, V., J. Manivannan, A. De, S. Paul, R. Roy, J. B. Johnson, R. Kundu, N. Chandrasekaran, A. Mukherjee, and A. Mukherjee. 2016. 'Surface capping and size-dependent toxicity of gold nanoparticles on different trophic levels', *Environmental Science and Pollution Research*, 23: 4844-58.

Jackevicius, C. A., J. V. Tu, J. S. Ross, D. T. Ko, D. Carreon, and H. M. Krumholz. 2011. 'Use of Fibrates in the United States and Canada', *Jama*, 305: 1217-24.

Kain, J., H. L. Karlsson, and L. Möller. 2012. 'DNA damage induced by micro- and nanoparticles-interaction with FPG influences the detection of DNA oxidation in the comet assay', *Mutagenesis*, 27: 491-500.

Kim, K., H. K. Kleinman, H.-J. Lee, and K. Pahan. 2017. 'Safety and potential efficacy of gemfibrozil as a supportive treatment for children with late infantile neuronal ceroid lipofuscinosis and other lipid storage disorders', *Orphanet Journal of Rare Diseases*, 12: 113.

Li, C., D. Li, G. Wan, J. Xu, and W. Hou. 2011. 'Facile synthesis of concentrated gold nanoparticles with low size-distribution in water: temperature and pH controls', *Nanoscale Research Letters*, 6: 1-10.

Li, Q., J. Laval, and D. B. Ludlum. 1997. 'Fpg protein releases a ring-opened N-7 guanine adduct from DNA that has been modified by sulfur mustard', *Carcinogenesis*, 18:1035-38.

Lima, I., S. M. Moreira, J. R.-V. Osten, A. M. V. M. Soares, and L. Guilhermino. 2007. 'Biochemical responses of the marine mussel *Mytilus*

galloprovincialis to petrochemical environmental contamination along the North-western coast of Portugal', *Chemosphere*, 66: 1230-42.

Liu, X., M. Atwater, J. Wang, and Q. Huo. 2007. 'Extinction coefficient of gold nanoparticles with different sizes and different capping ligands', *Colloids and Surfaces B: Biointerfaces*, 58: 3-7.

Loganathan, C., and S. A. John. 2017. 'Naked eye and spectrophotometric detection of chromogenic insecticide in aquaculture using amine functionalized gold nanoparticles in the presence of major interferents', *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, 173: 837-42.

Luis, L. G., A. Barreto, T. Trindade, A. M. V. M. Soares, and M. Oliveira. 2016. 'Effects of emerging contaminants on neurotransmission and biotransformation in marine organisms – An *in vitro* approach', *Marine Pollution Bulletin*, 106: 236-44.

Mahl, D., C. Greulich, W. Meyer-Zaika, M. Koller, and M. Epple. 2010. 'Gold nanoparticles: dispersibility in biological media and cell-biological effect', *Journal of Materials Chemistry*, 20: 6176-81.

Min, Z., W. Baoxiang, R. Zbigniew, X. Zhaohui, F. J. Otto, Y. Xiaofeng, and R. Steinar. 2009. 'Minute synthesis of extremely stable gold nanoparticles', *Nanotechnology*, 20: 505606.

Mohandas, J., J. J. Marshall, G. G. Duggin, J. S. Horvath, and D. J. Tiller. 1984. 'Differential distribution of glutathione and glutathione-related enzymes in rabbit kidney', *Biochemical Pharmacology*, 33: 1801-07.

Na-Phatthalung, P., M. Teles, L. Tort, and M. Oliveira. 2018. 'Gold nanoparticles exposure modulates antioxidant and innate immune gene expression in the gills of *Sparus aurata*', *Genomics*, 110: 430-34.

NIST. 2010. 'NCL Method PCC-8, Determination of gold in rat tissue with inductively coupled plasma mass spectrometry'.

Nunes, B., F. Carvalho, and L. Guilhermino. 2004. 'Acute and chronic effects of clofibrate and clofibric acid on the enzymes acetylcholinesterase, lactate dehydrogenase and catalase of the mosquitofish, *Gambusia holbrooki*', *Chemosphere*, 57: 1581-89.

OECD. 1992. *Test No. 203: Fish, Acute Toxicity Test* (OECD Publishing).

Ohkawa, H., N. Ohishi, and K. Yagi. 1979. 'Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction', *Analytical Biochemistry*, 95: 351-58.

Oliveira, M., C. Gravato, and L. Guilhermino. 2012. 'Acute toxic effects of pyrene on *Pomatoschistus microps* (Teleostei, Gobiidae): Mortality, biomarkers and swimming performance', *Ecological Indicators*, 19: 206-14.

Oliveira, M., M. Pacheco, and M. A. Santos. 2008. 'Organ specific antioxidant responses in golden grey mullet (*Liza aurata*) following a short-term exposure to phenanthrene', *Science of The Total Environment*, 396: 70-78.

Ozansoy, G., B. Akin, F. Aktan, and Ç. Karasu. 2001. 'Short-term gemfibrozil treatment reverses lipid profile and peroxidation but does not alter blood glucose and tissue antioxidant enzymes in chronically diabetic rats', *Molecular and Cellular Biochemistry*, 216: 59-63.

Pacheco, M., and M. A. Santos. 1996. 'Induction of micronuclei and nuclear abnormalities in the erythrocytes of *Anguilla anguilla* L. exposed either to cyclophosphamide or to bleached kraft pulp mill efluent', *Fresenius Environmental Bulletin*, 5: 746-51.

Pan, J.-F., P.-E. Buffet, L. Poirier, C. Amiard-Triquet, D. Gilliland, Y. Joubert, P. Pilet, M. Guibbolini, C. R. de Faverney, M. Roméo, E. Valsami-Jones, and C. Mouneyrac. 2012. 'Size dependent bioaccumulation and ecotoxicity of gold nanoparticles in an endobenthic invertebrate: The Tellinid clam *Scrobicularia plana*', *Environmental Pollution*, 168: 37-43.

Paramelle, D., A. Sadovoy, S. Gorelik, P. Free, J. Hobley, and D. G. Fernig. 2014. 'A rapid method to estimate the concentration of citrate capped silver nanoparticles from UV-visible light spectra', *Analyst*, 139: 4855-61.

Parvez, S., I. Sayeed, S. Pandey, A. Ahmad, B. Bin-Hafeez, R. Haque, I.Ahmad, and S. Raisuddin. 2003. 'Modulatory effect of copper on nonenzymatic antioxidants in freshwater fish *Channa punctatus* (Bloch.)', *Biological Trace Element Research*, 93: 237-48.

Prindiville, J. S., J. A. Mennigen, J. M. Zamora, T. W. Moon, and J.-M. Weber. 2011. 'The fibrate drug gemfibrozil disrupts lipoprotein metabolism in rainbow trout', *Toxicology and Applied Pharmacology*, 251: 201-08.

Qu, B., Q.-T. Li, K. P. Wong, T. M. C. Tan, and B. Halliwell. 2001. 'Mechanism of clofibrate hepatotoxicity: mitochondrial damage and oxidative stress in hepatocytes', *Free Radical Biology and Medicine*, 31: 659-69.

Quinn, B., W. Schmidt, K. O'Rourke, and R. Hernan. 2011. 'Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (*Dreissena polymorpha*) and their comparison with standardised toxicity tests', *Chemosphere*, 84: 657-63.

Roy, A., and K. Pahan. 2009. 'Gemfibrozil, stretching arms beyond lipid lowering', *Immunopharmacology and immunotoxicology*, 31: 339-51.

Saleh, M., G. Kumar, A.-A. Abdel-Baki, S. Al-Quraishy, and M. El-Matbouli. 2016. '*In vitro* antimicrosporidial activity of gold nanoparticles against *Heterosporis saurida*', *BMC Veterinary Research*, 12: 44.

Sedlak, J., and R. H. Lindsay. 1968. 'Estimation of total, protein-bound, and nonprotein sulfhydryl groups in tissue with Ellman's reagent', *Analytical Biochemistry*, 25: 192-205.

Shiba, F. 2013. 'Size control of monodisperse Au nanoparticles synthesized via a citrate reduction process associated with a pH-shifting procedure', *CrystEngComm*, 15: 8412-15.

SigmaPlot 12.0. 2011. 'Statistical Package for the Social Sciences - SigmaPlot for Windows', 12.0 ed, Chicago, IL, USA.

Singh, N. P., M. T. McCoy, R. R. Tice, and E. L. Schneider. 1988. 'A simple technique for quantitation of low levels of DNA damage in individual cells', *Experimental Cell Research*, 175: 184-91.

Skolness, S. Y., E. J. Durhan, K. M. Jensen, M. D. Kahl, E. A. Makynen, D.L. Villeneuve, and G. T. Ankley. 2012. 'Effects of gemfibrozil on lipid metabolism, steroidogenesis, and reproduction in the fathead minnow (*Pimephales promelas*)', *Environmental Toxicology and Chemistry*, 31: 2615-24.

Smith, C. C., M. R. O'Donovan, and E. A. Martin. 2006. 'hOGG1 recognizes oxidative damage using the comet assay with greater specificity than FPG or ENDOIII', *Mutagenesis*, 21: 185-90.

Speit, G., P. Schütz, I. Bonzheim, K. Trenz, and H. Hoffmann. 2004. 'Sensitivity of the FPG protein towards alkylation damage in the comet assay', *Toxicology Letters*, 146: 151-58.

Sutken, E., M. Inal, and F. Ozdemir. 2006. 'Effects of vitamin E and gemfibrozil on lipid profiles, lipid peroxidation and antioxidant status in the elderly and young hyperlipidemic subjects', *Saudi Medical Journal*, 27: 453-59.

Tchou, J., V. Bodepudi, S. Shibutani, I. Antoshechkin, J. Miller, A. P. Grollman, and F. Johnson. 1994. 'Substrate specificity of Fpg protein. Recognition and cleavage of oxidatively damaged DNA', *Journal of Biological Chemistry*, 269: 15318-24.

Tedesco, S., H. Doyle, G. Redmond, and D. Sheehan. 2008. 'Gold nanoparticles and oxidative stress in *Mytilus edulis*', *Marine Environmental Research*, 66: 131-33.

Tedesco, S., H. Doyle, J. Blasco, G. Redmond, and D. Sheehan. 2010. 'Oxidative stress and toxicity of gold nanoparticles in *Mytilus edulis*', *Aquatic Toxicology*, 100: 178-86.

Tejamaya, M., I. Römer, R. C. Merrifield, and J. R. Lead. 2012. 'Stability of citrate, PVP, and PEG coated silver nanoparticles in ecotoxicology media', *Environmental Science & Technology*, 46: 7011-17.

Teles, M., C. Fierro-Castro, P. Na-Phatthalung, A. Tvarijonaviciute, T. Trindade, A. M. V. M. Soares, L. Tort, and M. Oliveira. 2016. 'Assessment of gold nanoparticle effects in a marine teleost (*Sparus aurata*) using molecular and biochemical biomarkers', *Aquatic Toxicology*, 177: 125-35.

Termini, J. 2000. 'Hydroperoxide-induced DNA damage and mutations', *Mutation Research - Fundamental and Molecular Mechanisms of Mutagenesis*, 450: 107-24.

Tice, R. R., E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J. C. Ryu, and Y. F. Sasaki. 2000. 'Single

cell gel/comet assay: Guidelines for *in vitro* and *in vivo* genetic toxicology testing', *Environmental and Molecular Mutagenesis*, 35: 206-21.

Tiede, K., M. Hassellöv, E. Breitbarth, Q. Chaudhry, and A. B. A. Boxall. 2009. 'Considerations for environmental fate and ecotoxicity testing to support environmental risk assessments for engineered nanoparticles', *Journal of Chromatography A*, 1216: 503-09.

Tudek, B., A. A. VanZeeland, J. T. Kusmierek, and J. Laval. 1998. 'Activity of *Escherichia coli* DNA-glycosylases on DNA damaged by methylating and ethylating agents and influence of 3-substituted adenine derivatives', *Mutation Research/DNA Repair*, 407: 169-76.

Turkevich, J., P. C. Stevenson, and J. Hillier. 1951. 'A study of the nucleation and growth processes in the synthesis of colloidal gold', *Discussions of the Faraday Society*, 11: 55-75.

Vale, G., K. Mehennaoui, S. Cambier, G. Libralato, S. Jomini, and R. F. Domingos. 2016. 'Manufactured nanoparticles in the aquatic environment-biochemical responses on freshwater organisms: A critical overview', *Aquatic Toxicology*, 170: 162-74.

Vieira, H. C., F. Morgado, A. M. V. M. Soares, and S. N. Abreu. 2015. 'Fish consumption recommendations to conform to current advice in regard to mercury intake', *Environmental Science and Pollution Research*, 22: 9595-602.

Wang, S., R. Lawson, P.C. Ray, H. Yu. 2011. 'Toxic effects of gold nanoparticles on *Salmonella typhimurium* bacteria', *Toxicology and Industrial Health*, 27: 547-54.

WHO. 2008. 'Guidance for identifying populations at risk from mercury exposure', *Switzerland, Geneva*.

Xia, Q., H. Li, Y. Liu, S. Zhang, Q. Feng, and K. Xiao. 2017. 'The effect of particle size on the genotoxicity of gold nanoparticles', *Journal of Biomedical Materials Research Part A*, 105: 710-19.

Yoo-Iam, M., R. Chaichana, and T. Satapanajaru. 2014. 'Toxicity, bioaccumulation and biomagnification of silver nanoparticles in green algae (*Chlorella* sp.), water flea (*Moina macrocopa*), blood worm (*Chironomus* spp.) and silver barb (*Barbonymus gonionotus*)', *Chemical Speciation and Bioavailability*, 26: 257-65.

Zeng, S., M. Cai, H. Liang, and J. Hao. 2012. 'Size-dependent colorimetric visual detection of melamine in milk at 10 ppb level by citrate-stabilized Au nanoparticles', *Analytical Methods*, 4: 2499-505.

Declaration of competing interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Graphical abstract