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

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2 **Highlights**

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4 • Gold (nano or ionic form) induced lipid peroxidation and genotoxicity on fish;

5 • Ionic gold induced more adverse effects than a nano form of the metal;

6 • Citrate coated nanoparticles were more bioactive than PVP coated

7   nanoparticles.

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9

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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

35 dimensions, the safety of the resulting nanomaterials has been questioned

36 (Boverhof et al. 2015). Gold nanoparticles (AuNPs) have been widely used in

37 medicine and biological research (Fratoddi et al. 2015), including targeted

38 delivery of drugs (Ghosh et al. 2008), imaging and diagnosis (Bhattacharya and

39 Mukherjee 2008). Its application in aquaculture as antimicrobial agent (Saleh et

40 al. 2016) and to detect contaminants (Loganathan and John 2017) has also

41 been investigated. Despite the widespread use of AuNPs and consequent

42 release to the environment, there is a limited understanding of their

43 consequences for environmental health (Barreto et al. 2019a; Barreto et al.

44 2019b; Iswarya et al. 2016; Na-Phatthalung et al. 2018; Teles et al. 2016). In

45 addition, the question of whether AuNPs are more toxic than ionic Au (iAu)

46 remains unresolved (Barbasz and Oćwieja 2016; Botha, James, and Wepener

47 2015; Dedeh et al. 2015; Farkas et al. 2010; Luis et al. 2016). Table 1 presents

48 the example of several *in vitro* and *in vivo* studies where different toxicity

49 outputs were achieved.

50

51 **Table 1**. Examples of studies assessing the toxicity of nano versus ionic

52 gold. Ref. – Reference; PVP – Polyvinylpyrrolidone; BSA – Bovine serum

53 albumin; ROS – Reactive oxygen species. 1 – Barbasz et al. (2016); 2 – Luis et

54 al. (2016); 3 – Botha et al. (2015); 4 – Dedeh et al. (2015); 5 – Farkas et al.

55 (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·L^{-1} to	Ionic	2

vitro	*galloprovincialis* hemolymph and subcellular fraction of gills			BSA	7	2.5 mg·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 µg.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 (17°C) 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

122 took place (immediately after renewal). Water samples collected at 24 h,

123 correspond to the water collected 24 h after the beginning of the test and 24 h

124 after the renewal of the media.

125

126 **2.3. Assessment of swimming performance**

127 After 96 h exposure, fish were individually introduced into a long flume and

128 induced to swim against a water flow of 19 L.min^{-1}. The time (in seconds) that

129 fish spent swimming against the water flow was recorded. More information

130 about this behavioural assessment can be found in a previous study of Barreto

131 et al. (2019b).

132

133 **2.4. Collection of biological material**

134 After a 2 h recovery period, fish were anesthetized with tricaine

135 methanesulfonate (MS-222), blood samples were collected from the posterior

136 cardinal vein and then the animals were euthanized by spinal section. For the

137 comet assay, blood samples were diluted with saline phosphate buffer. Blood

138 smears were prepared for the assessment of erythrocytic nuclear abnormalities

139 (ENAs). Liver, gills, muscle and brain were removed from seven fish and stored

140 at -80ºC until biochemical biomarkers analysis. Liver, gills, spleen and muscle

141 were taken from five animals and kept at -20ºC until gold quantification.

142

143 **2.4.1. Biochemical biomarkers analysis**

144 Liver and gills were homogenized in potassium phosphate buffer (0.1 mM,

145 pH 7.4) using an ultrasonic homogenizer. The homogenate was then divided

146 into three aliquots for: lipid peroxidation (LPO) assay, NPT quantification and

7

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).

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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

197 Fpg. Cells were classified according to tail length, into five classes (Collins

198 2004): class 0 – undamaged, without a tail; class 1 – with a tail shorter than the

199 diameter of the nucleus; class 2 – with a tail length 1–2 times the diameter of

200 the nucleus; class 3 – with a tail longer than twice the diameter of the nucleus;

201 class 4 – comets with no nucleus. A damage index (DI), in arbitrary units, was

202 assigned to each slide (for 100 cells) and consequently for each treatment,

203 using the formula:

204

205
$$DI = (0 \times n0) + (1 \times n1) + (2 \times n2) + (3 \times n3) + (4 \times n4)$$

206

207 where: n = number of cells in each class. DI can range from 0 to 400 (de

208 Andrade, de Freitas, and da Silva 2004).

209 The DNA damage index in cells treated with Fpg with the correspondent cells

210 without the enzymatic treatment were compared to detect possible DNA

211 oxidative damage.

212 The ENAs assay was carried out in mature peripheral erythrocytes according

213 previous procedures and nuclear lesions were scored as micronuclei, lobed,

214 segmented, kidney-shaped and vacuolated nuclei (Barreto et al. 2017; Pacheco

215 and Santos 1996). Results were expressed as the ENAs frequency (‰) to each

216 replicate (for 1000 cells) and consequently for each treatment using the formula:

217

218
$$ENAs(‰) = \frac{\text{Number of cells containing ENAs}}{\text{Total number of cells counted}}$$

219

220 **2.5. Gold (Au) quantification**

221   The determination of Au in the stock suspensions, in the experimental media

222   and fish tissues was performed according to the NIST NCL Method PCC-8

223   (NIST 2010). An iCAP^{TM} Q ICP-MS (inductively coupled plasma mass

224   spectrometry) instrument (Thermo Fisher Scientific, Bremen, Germany) was

225   used for the analysis. The ICP-MS instrumental conditions were as follow:

226   argon flow rate (14 L.min^{-1}); auxiliary argon flow rate (0.8 L.min^{-1}); nebulizer flow

227   rate (1.03 mL.min^{-1}); RF power (1550 W) and dwell time (100 ms). The

228   elemental isotope ^{197}Au was monitored for analytical determination; ^{159}Tb and

229   ^{209}Bi were used as internal standards. The instrument was tuned daily for

230   maximum signal sensitivity and stability. More information about Au

231   quantification can be found in the study of Barreto et al. (2019b).

232   Stock suspensions theoretical concentrations and number of nanoparticles

233   (NPs) were also estimated based on their UV-Vis spectra and sizes (Barreto et

234   al. 2015; Liu et al. 2007; Paramelle et al. 2014).

235

236   **2.6. Total gold (Au) content, bioaccumulation factor and estimated intake**

237   **for humans**

238   Total Au content ([Au]$_{total}$), in µg.g^{-1}, was calculated, as described in a

239   previous study from Barreto et al. (2019b), according to the formula:

240

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

241

242

243   Where [Au]$_g$ is the concentration of Au in gills, [Au]$_l$ the concentration of Au in

244   liver, [Au]$_s$ the concentration of Au in spleen and [Au]$_{ms}$ the concentration of Au

245   in muscle.

11

246   The bioaccumulation factor (BAF), in $L.g^{-1}$, was calculated according

247 previous studies (Barreto et al 2019b; Yoo-Iam, Chaichana, and Satapanajaru

248 2014):

249

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

250

251   Where $[Au]_t$ is the content of Au in the specific fish tissue and $[Au]_{ASW}$ its

252 concentration in the exposure media – ASW (collected daily at 0 h and

253 quantified). More information about BAF calculation can be found in the study of

254 Barreto et al. (2019b).

255   As *Sparus aurata* is a fish for human consumption an extrapolation of Au

256 intake for humans was calculated, using the following formula (Barreto et al

257 2019b; Vieira et al. 2015; WHO 2008):

258

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

259

260

261   A human body weight of 60 kg was assumed (IPCS 2004) and the average

262 amount of fish ingested by each Portuguese person per year was set at 59 kg

263 (Failler et al. 2007; Vieira et al. 2015). Au content in the ingested fish

264 corresponds to the content of Au determined in the fish muscle ($\mu g.g^{-1}$). The

265 calculated Au intake values were compared with the maximum amount of Au

266 that each person may be exposed daily over their lifetimes without considerable

267 health risk – "tolerable daily intake" (TDI). This value was previously calculated

268 and in detail explained in the study of Barreto et al. (2019b): 322 $\mu g.kg^{-1}$.

269

270 **2.7. Statistical analysis**

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271 Data were tested for normality (Shapiro-Wilk test) and homogeneity of

272 variance (Levene's test) using the Sigma Plot software package (SigmaPlot

273 12.0, 2011). Differences between treatments and control and between all the

274 treatments were analysed using one-way analysis of variance (ANOVA),

275 followed by Dunnett's or Tukey´s test whenever applicable. Significant

276 differences were assumed for $p < 0.05$.

277

278 **3. Results**

279 **3.1. Gold nanoparticles (AuNPs) – Characterisation and behaviour**

280 The synthetized cAuNPs presented a well-defined surface plasmon

281 resonance (SPR) peak – Figure 1A. Dynamic light scattering (DLS) analysis

282 showed an average hydrodynamic size of the particles of 7 nm and a strongly

283 negative surface charge (-43 mV). Transmission electron microscopy (TEM)

284 analysis confirmed that almost all cAuNPs presented spherical shape and the

285 particles had similar sizes between them (Figure 1B). There was a slight shift in

286 the SPR peak to a longer wavelength for PVP-AuNPs (521 nm) when compared

287 with cAuNPs (519 nm). DLS measurements showed a size of around 8 nm and

288 a less negative ZP than cAuNPs (-13 mV). Scanning electron microscopy

289 (SEM) analysis allowed the visualization of a PVP layer around some AuNPs

290 metal core (Figure 1C).

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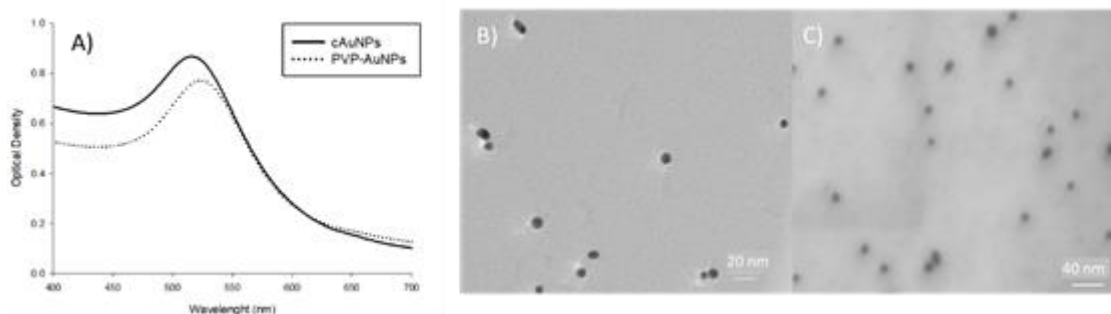
Figure 1. UV–Vis spectra **(A),** transmission electron microscopy image of citrate coated gold nanoparticles – cAuNPs **(B)** and scanning electron microscopy image of polyvinylpyrrolidone coated – PVP-AuNPs **(C)**.

In the experimental media (ASW), 80 and 1600 µg.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 µg.L^{-1}, it was not possible to detect any colour change. Moreover, the hydrodynamic size of cAuNPs (1600 µg.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 µg.L^{-1} of cAuNPs, a dark layer was visible as a consequence of the sedimentation of the NPs aggregates/agglomerates. PVP-AuNPs (1600 µg.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 µg.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 µg.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

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	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

318

3.2. Gold (Au) quantification in experimental media

320 AuNPs theoretical concentration versus measured concentrations (by ICP-

321 MS) and the number of particles present in the AuNPs stock suspensions are

322 shown in Table S1. The nominal versus measured concentrations of Au in the

323 experimental media are presented in Table 3.

324

325 **Table 3.** Nominal versus measured Au concentrations ($\mu g.L^{-1}$) in
326 experimental media (artificial seawater) at 0 and 24 h, after exposure to gold
327 nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-
328 AuNPs) and ionic gold. Results are expressed as mean ± standard error. <d.l. –
329 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

15

At 0 h, the Au quantified in ASW, in general, was lower than the nominal concentrations, with exception to 4 µg.L^{-1} of PVP-AuNP and iAu (4 and 80 µg.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 µg.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 µg.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 µg.L^{-1} of iAu, the measured concentration of Au was 16% higher than the expected. At 1600 µg.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 µg.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 µg.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 µg.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)

355   After 24 h of exposure, one fish died in the 1600 µg.L^{-1} iAu treatment. As

356   shown in Figure 2, the ability of *Sparus aurata* to continue swimming against a

357   water flow was significantly decreased ($p < 0.05$; Dunnett's test) when fish were

358   exposed to 1600 µg.L^{-1} of cAuNPs and iAu.
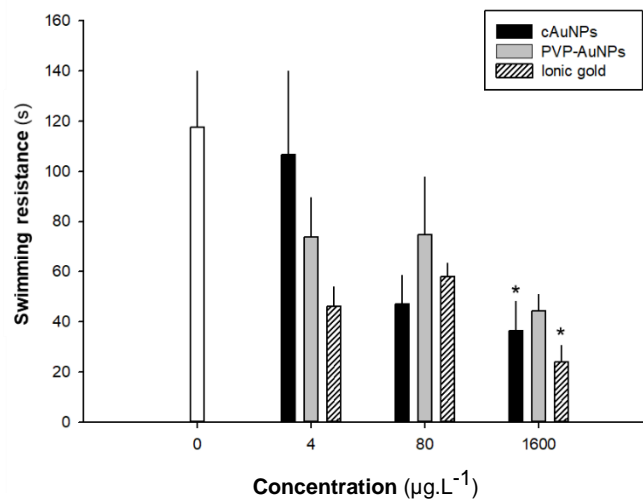
359

360   **Figure 2.** Resistance of *Sparus aurata* to withstand swimming against a

361   water flow after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs

362   and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are

363   expressed as mean time in seconds ± standard error. *Significant differences to

364   control (Dunnett´s test, $p < 0.05$).

365

366   ChE activity in brain and muscle was not significantly altered by the exposure

367   to the both forms of Au ($p > 0.05$; ANOVA; Figure 3).

368

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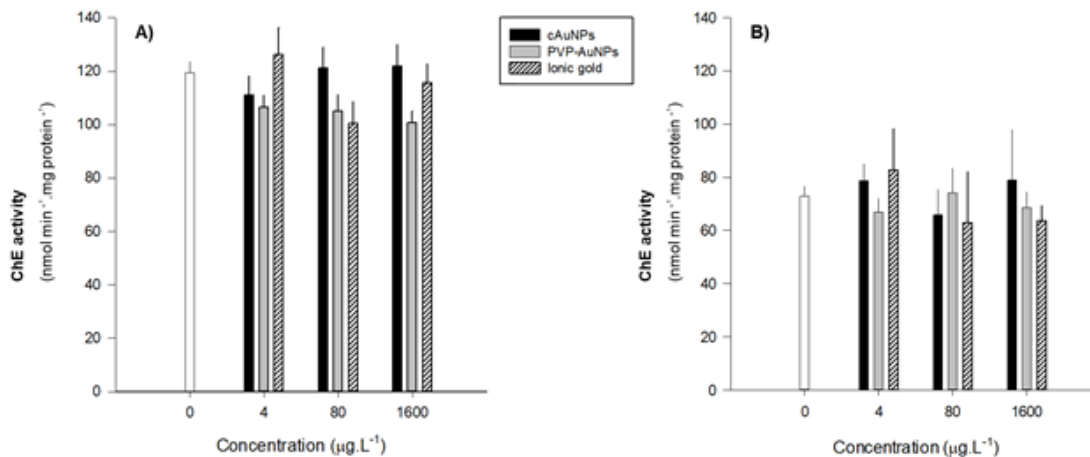
Figure 3. Brain **(A)** and muscle **(B)** cholinesterases (ChE) activity of *Sparus*

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).
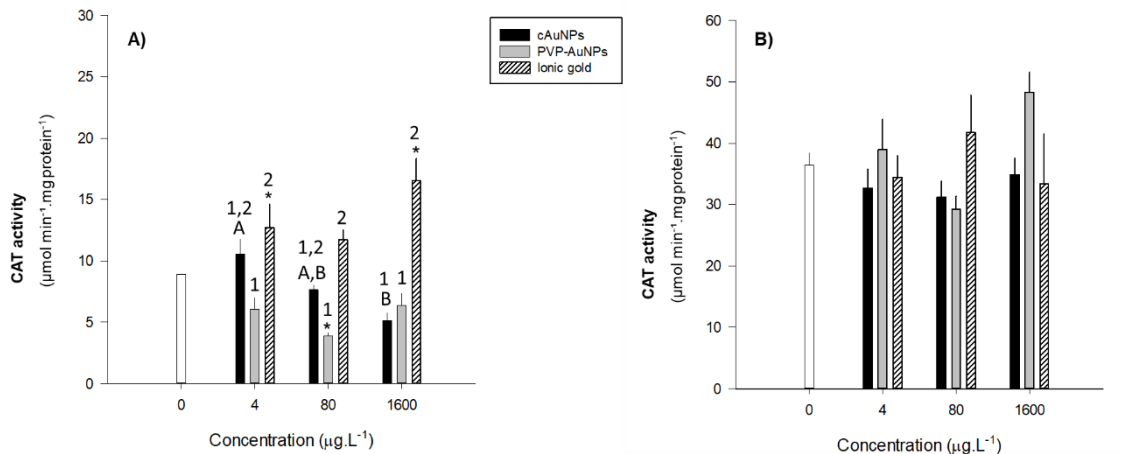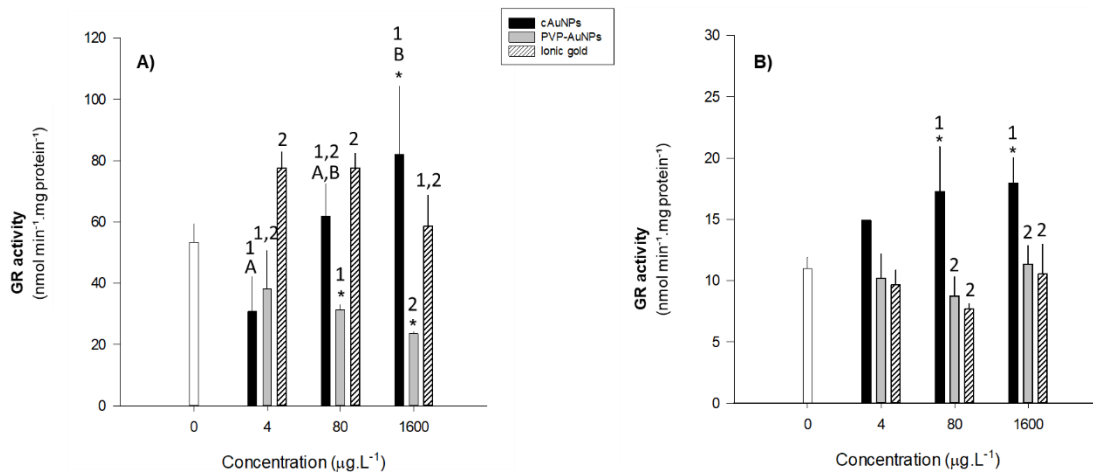
Figure 4. Gills **(A)** and liver **(B)** catalase (CAT) activity of *Sparus aurata* after

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

392   test; Figure 5B). PVP-AuNPs, 80 and 1600 µg.L^{-1}, significantly decreased gills

393   GR activity (p<0.05; Dunnett's test; Figure 5A).

394

395   **Figure 5.** Gills **(A)** and liver **(B)** glutathione reductase (GR) activity of *Sparus*

396   *aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and

397   polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are

398   expressed as mean ± standard error. *Significant differences to control

399   (Dunnett´s test, p<0.05). Different letters correspond to significant differences

400   between the treatments of each type of AuNPs and ionic form (Tukey´s test,

401   p<0.05). Different numbers correspond to significant differences between each

402   treatment within the same concentration (Tukey´s test, p<0.05).

403

404   cAuNPs and iAu at 4 and 80 µg.L^{-1}, respectively, significantly increased gills

405   GPx activity (p<0.05; Dunnett's test; Figure 6A). In the liver, 80 µg.L^{-1} of PVP-

406   AuNPs decreased the GPx activity (p<0.05; Dunnett's test; Figure 6B).

407
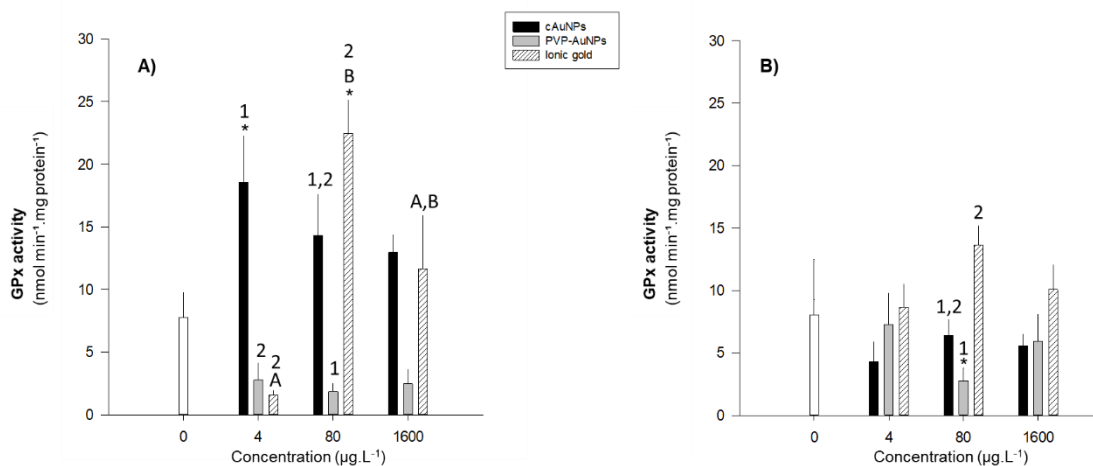
Figure 6. Gills **(A)** and liver **(B)** glutathione peroxidase (GPx) activity of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to 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 $\mu g.L^{-1}$ of iAu significantly increased gills NPT levels ($p < 0.05$; Dunnett's test; Figure 7A). In liver, only 1600 $\mu g.L^{-1}$ of cAuNPs significantly increased the levels of NPT ($p < 0.05$; Dunnett's test; Figure 7B).
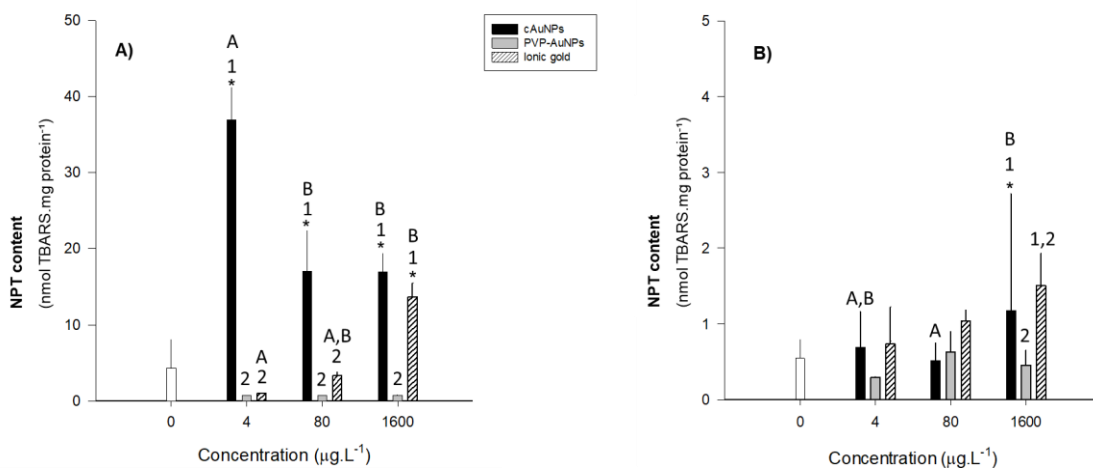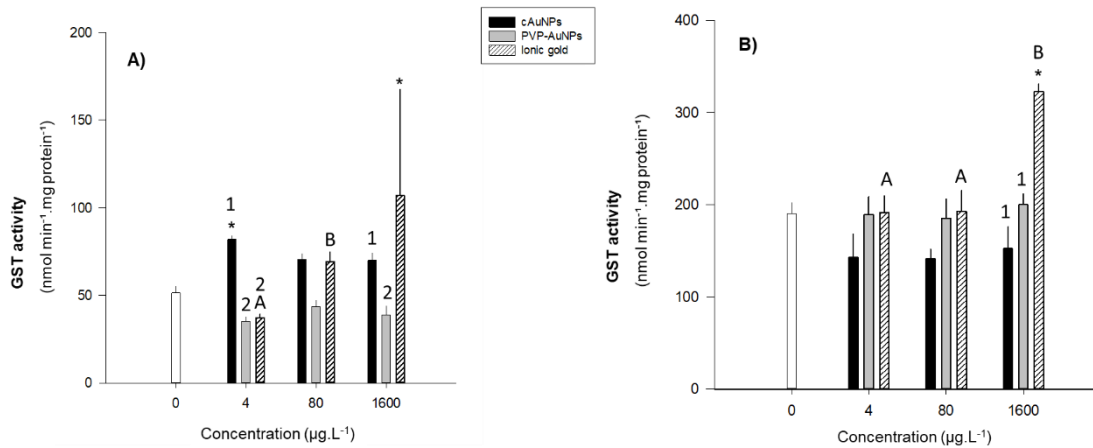

Figure 7. Gills **(A)** and liver **(B)** non-protein thiols (NPT) levels of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (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, $p < 0.05$).

431 cAuNPs (4 µg.L^{-1}) and iAu (1600 µg.L^{-1}) significantly increased gills GST

432 activity (p<0.05; Dunnett's test; Figure 8A). In liver, 1600 µg.L^{-1} iAu significantly

433 increased the activity of this enzyme (p>0.05; ANOVA; Figure 8B).

434

435

436 **Figure 8.** Gills **(A)** and liver **(B)** glutathione *S*-transferases (GST) activity of

437 *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated –

438 cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results

439 are expressed as mean ± standard error. *Significant differences to control

440 (Dunnett´s test, p<0.05). Different letters correspond to significant differences

441 between the treatments of each type of AuNPs and ionic form (Tukey´s test,

442 p<0.05). Different numbers correspond to significant differences between each

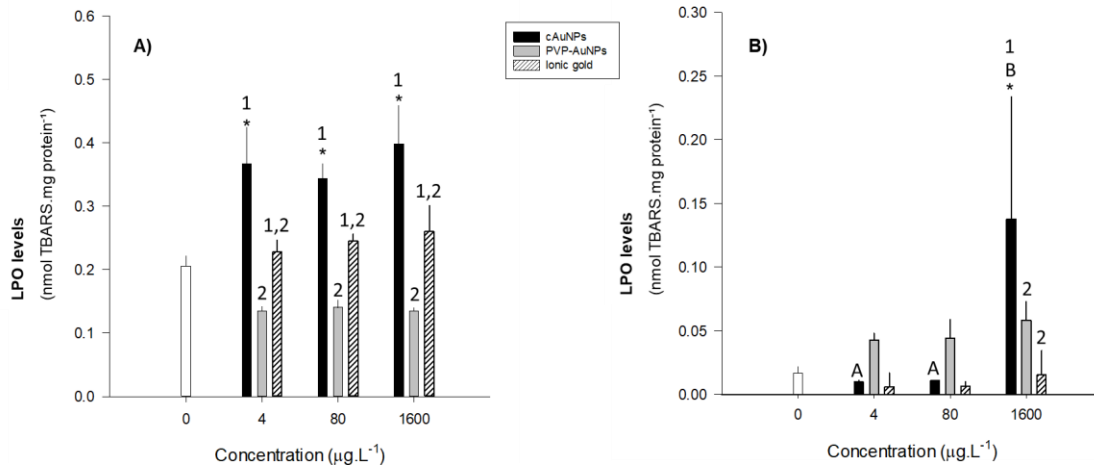443 treatment within the same concentration (Tukey´s test, p<0.05).

444

445 As shown in Figure 9A, oxidative damage (assessed as TBARS levels) was

446 found in gills after the exposure to all tested concentrations of cAuNPs (p<0.05;

447 Dunnett's test). In liver, LPO levels significantly increased after the exposure to

448 1600 µg.L^{-1} of cAuNPs (p<0.05; Dunnett's test; Figure 9B).

449

450 **Figure 9.** Gills **(A)** and liver **(B)** lipid peroxidation (LPO) levels of *Sparus*
451 *aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and
452 polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are
453 expressed as mean ± standard error. *Significant differences to control
454 (Dunnett´s test, $p < 0.05$). Different letters correspond to significant differences
455 between the treatments of each type of AuNPs and ionic form (Tukey´s test,
456 $p < 0.05$). Different numbers correspond to significant differences between each
457 treatment within the same concentration (Tukey´s test, $p < 0.05$).

458

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

462

463
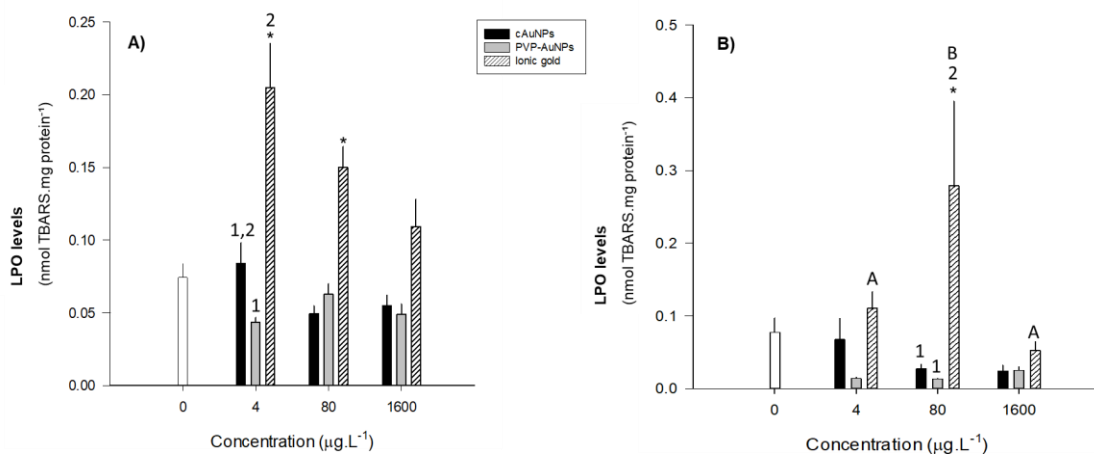
Figure 10. Brain **(A)** and muscle **(B)** lipid peroxidation (LPO) levels of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (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).
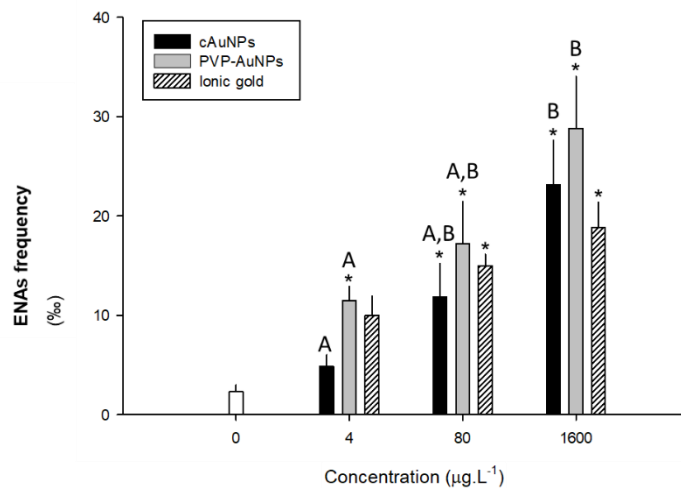
Figure 11. Erythrocytic nuclear abnormalities (ENAs) frequency in *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s 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

528

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).

537 **Table 6.** Gold content in tissues (gills, liver, spleen and muscle) of *Sparus*
538 *aurata* exposed to gold nanoparticles (citrate coated – cAuNPs and
539 polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold for 96 h and
540 respective estimated bioaccumulation factor (BAF). Results are expressed as
541 mean ± standard error. *Significant differences to control (Dunnett´s test,
542 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

543

544   The highest [Au]total value (around 24 $\mu g.g^{-1}$) was detected after exposure to

545   1600 $\mu g.L^{-1}$ iAu (p<0.05; Dunnett's test).

546

547   The highest estimated value for Au intake by a Portuguese citizen would be

548   for the condition 1600 $\mu g.L^{-1}$ of iAu (Table 7).

549

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

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

555

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

556

557 **4. Discussion**

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

572 AuNPs were stable in ASW for more than 30 days. Thus, the present study

573 confirmed that PVP-AuNPs may remain stable in suspension in a nano size

574 range in ASW, whereas cAuNPs immediately alter their characteristics and

575 aggregate/agglomerate, increasing their size to more than 100 nm. These

576 characteristics and behaviour of different AuNPs may influence their

577 accumulation and effects to the organisms. NPs size may affect its

578 bioavailability to the organisms. When aggregates become too large for direct

579 transport across the cell membrane, uptake may be prevented (Vale et al.

580 2016).

581   Although the stability of the tested AuNPs was different in ASW, no

582 significant differences were found in terms of Au accumulation in the tissues of

583 *S. aurata* after the exposure to cAuNPs and PVP-AuNPs. Another interesting

584 result was the highest BAF in the spleen calculated for the lowest nominal

585 concentration exposure of cAuNPs (4 $\mu g.L^{-1}$). This may be due to the lower

586 aggregation/agglomeration processes, with higher ability of Au entering in the

587 tissues (Barreto et al. 2019a). As already reported, aggregation/agglomeration

588 is expected to increase with the increase in the number of particles per volume

589 (Barreto et al. 2015). Therefore, since cAuNPs, at the highest tested

590 concentrations (80 and 1600 $\mu g.L^{-1}$), are more likely to aggregate/agglomerate,

591 they are less available for the uptake by fish. The detected accumulation of Au

592 in the spleen of *S. aurata* (although not significantly) show the potential role of

593 the spleen in Au elimination. Despite the lack of Au accumulation after the

594 exposure to AuNPs, particles were bioactive to *S. aurata*. The present data

595 reveal that 7 nm cAuNPs induced more pronounced effects, in terms of

596 oxidative stress and damage responses, than PVP-AuNPs. This result was

597   unexpected considering the stability of the particles, despite the fact that PVP

598   coating is considered safer and more biocompatible than citrate coating (Min et

599   al. 2009). The 7 nm PVP-AuNPs remained stable in ASW, dispersed in the

600   water column and, therefore, more available for the uptake by fish. An opposite

601   pattern was previously observed for 40 nm AuNPs with PVP-AuNPs inducing

602   more adverse effects than cAuNPs to *S. aurata* (Barreto et al. 2019b). However,

603   a previous study, where different organisms were tested (bacteria, algae, SiHa

604   cell line and mice), also showed that cAuNPs had more adverse effects than

605   PVP-AuNPs (Iswarya et al. 2016). Wang et al. (2011) reported that the toxicity

606   of AuNPs is related to the co-existence of citrate and Au^{3+} ions. When NPs are

607   coated with PVP, there is an absence of reactive citrate ions on the surface of

608   AuNPs. In the present study, the tested concentrations of cAuNPs decreased

609   the swimming resistance, induced enzymatic and non-enzymatic responses

610   involved in the oxidative defence/damage and genotoxicity in fish. Therefore, it

611   seems that the formed agglomerates/aggregates in the ASW (less than 200 nm)

612   may be incorporated through the cellular membranes.

613   Comparing the ionic with the nano form, Au significantly accumulated in

614   almost all assessed tissues of *S. aurata* after the exposure to 1600 $\mu g.L^{-1}$ iAu,

615   whereas after the exposure to AuNPs, Au did not significantly accumulate.

616   Despite the significantly higher effects of cAuNPs in some endpoints (such as

617   gills GPx and liver GR activities, gills NPT and gills/liver LPO levels), even at

618   the lowest tested concentration (i.e., 4 $\mu g L^{-1}$), iAu induced, in general, more

619   effects on the fish (gills CAT, GR and liver GST activities and muscle/brain LPO

620   levels). Additionally, no fish mortality was detected after the exposures with

621   AuNPs, whereas one fish died after the exposure to 1600 $\mu g.L^{-1}$ of iAu. The

622   results demonstrated a tissue specificity, cAuNPs induced LPO in gills and liver,

623   while iAu induced LPO in brain and muscle. There are few available studies

624   about the mechanisms involved in the toxicity of iAu. Nonetheless, the iAu

625   ability to undergo redox reactions with peptides and proteins, particularly

626   involving sulphur amino acids, to deprotonate and bind to peptide amide bonds

627   and cross-link histidine imidazole rings, has been already reported (Best and

628   Sadler 1996; Luis et al. 2016). Some authors, using *in vitro* tests, reported that

629   iAu induced effects to mussel (*Mytilus galloprovincialis*) and rainbow trout

630   (*Oncorhynchus mykiss*) whereas the nano form did not have any effect (Farkas

631   et al. 2010; Luis et al. 2016). Botha el al. (2015), using different aquatic species

632   (daphnia and fish), also showed that iAu was more toxic than nano form.

633   However, Barbasz et al. (2016), using two types of human cell lines, showed a

634   higher cytotoxicity of AuNPs than iAu. Dedeh et al. (2014) described that, in

635   spite of iAu having accumulated more in the tissues of zebrafish (*Danio rerio*)

636   than the AuNPs, the latter had more effects on the fish, in terms of gene

637   expression and neurotransmission.

638   In terms of genotoxicity, all the treatments induced DNA strand breaks,

639   assessed by comet assay, in *S. aurata* peripheral blood cells. Concerning

640   cytogenetic damage, ENAs frequency increased with the increase of Au

641   concentration (nano or ionic form). A previous study also showed the potential

642   genotoxicity of 40 nm AuNPs (both PVP-AuNPs and cAuNPs) to *S. aurata*

643   (Barreto et al. 2019a). Comet assay is a rapid method to detect low levels of

644   DNA damage. However, this technique gives limited information about the kind

645   of DNA damage, if it is a direct consequence of the damaging agent or indirect

646   effects, such as oxidative damage, apurinic/pyrimidinic sites or DNA repair

647 (Smith, O'Donovan, and Martin 2006). As previously described, the genotoxic

648 effects of AuNPs may be caused directly following the entry of NPs into the

649 nuclei, binding to DNA; or indirectly, through oxidative stress, which may

650 consequently induce DNA oxidative damage (Auffan et al. 2009; Barreto et al.

651 2019a; Cardoso et al. 2014). The production of reactive oxygen species (ROS)

652 following AuNPs exposure has been demonstrated in studies involving aquatic

653 organisms (Farkas et al. 2010; Tedesco et al. 2008, 2010; Pan et al. 2012).

654 Modification of the comet assay with the incorporation of lesion specific

655 endonucleases, such as Fpg, increases its sensitivity and specificity through the

656 recognition of damaged bases and introduction of additional breaks (Azqueta et

657 al. 2013; Smith, O'Donovan, and Martin 2006; Speit et al. 2004). The present

658 study showed that Au (nano and ionic form) induced DNA breaks, but oxidative

659 DNA damage was not observed. This result was previously described in studies

660 with different types of NPs (Ag, CeO_2, Co_3O_4 and SiO_2) and metal ions (Al^{3+} ,

661 Ni^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+}) (Grin et al. 2009; Kain, Karlsson, and Möller

662 2012) and may be due to: 1) the low potential of the tested conditions to induce

663 oxidative damage on the erythrocyte DNA of *S. aurata*, which is not supported

664 by the LPO data; 2) the oxidative DNA lesions caused by the exposure to Au

665 may have been already been repaired by cellular DNA repair systems (Catalán

666 et al. 2014); 3) NPs and ionic forms may interact with Fpg, not allowing the

667 binding of the enzyme with DNA (Asmuss et al. 2000; Kain, Karlsson, and

668 Möller 2012).

669 Overall, after Au exposures, enzymatic and non-enzymatic responses

670 involved in the defence of *S. aurata* against oxidative damage were more

671 activated in the gills than in the liver. Additionally, oxidative damage (LPO

672 increase) was more clearly expressed in gills than in liver. Gills are the first

673 organ to be exposed and provide a large surface area for contaminants such as

674 AuNPs, being considered a good candidate to an early assessment of the

675 effects of waterborne contaminants (Oliveira, Pacheco, and Santos 2008).

676 Since *S. aurata* is one of the most consumed fish in south Europe, an

677 estimation of Au intake by humans via food chain is an important assessment

678 (Barreto et al. 2019b). The highest estimated value for Au intake by each

679 Portuguese person (1.10 µg.kg body weight per year) would be relevant

680 following an exposure of the fish to 1600 µg.L^{-1} iAu. Based on the tested

681 conditions and present results, the estimated maximum Au intake by humans

682 per day was around 0.003 µg.kg^{-1} body weight. So, this value did not exceed

683 the estimated TDI value for Au (322 µg.kg^{-1}). Future studies should carry out the

684 assessment of Au intake by humans via food chain since AuNPs use is

685 increasing worldwide and thus it is expected to find increased concentration of

686 them in the environment.

687

688 **5. Conclusions**

689 The present results showed that short-term exposure to gold (nano or ionic

690 form), at low levels such as 4 µg.L^{-1}, was able to induce oxidative stress and

691 damage, as well as genotoxicity to the marine/estuarine fish *Sparus aurata.*

692 Citrate coated gold nanoparticles (cAuNPs), even aggregating/agglomerating in

693 seawater, induced significantly more effects to fish (oxidative stress and

694 damage) than the polyvinylpyrrolidone coated gold nanoparticles (PVP-AuNPs),

695 which maintained its nano size in seawater. The exposures to ionic gold

696 resulted in higher accumulation in the fish tissues and induced more effects to

697 fish than nano form. After gold exposures, responses involved in fish defence

698 against oxidative damage were more activated in the gills than in the liver.

699 Furthermore, oxidative damage (lipid peroxidation increase) was more detected

700 in gills than in the liver. The results showed that gold (nano and ionic form) is

701 not inert and a distinct response was found in the assessed tissues. Further

702 chronic tests must be performed to complement the present findings.

703

704 **Conflict of interest statement**

705

706 The authors declare that there are no conflicts of interest.

707

708 **Acknowledgments**

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6. References

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