Effects and bioaccumulation of gold nanoparticles in the gilthead seabream (*Sparus aurata*) – single and combined exposures with gemfibrozil

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Abstract

Gold nanoparticles (AuNPs) are found in a wide range of applications and therefore expected to present increasing levels in the environment. There is however limited knowledge concerning the potential toxicity of AuNPs as well as

their combined effects with other pollutants. Hence, the present study aimed to

investigate the effects of AuNPs alone and combined with the pharmaceutical gemfibrozil (GEM) on different biological responses (behaviour,

neurotransmission, biotransformation and oxidative stress) in one of the most consumed fish in southern Europe, the seabream *Sparus aurata*. Fish were exposed for 96 h to waterborne 40 nm AuNPs with two coatings – citrate and polyvinylpyrrolidone (PVP), alone or combined with GEM. Antioxidant defences were induced in liver and gills upon both AuNPs exposure. Decreased swimming performance (1600 $\mu g.L^{-1}$) and oxidative damage in gills (4 and 80 $\mu g.L^{-1}$) were observed following exposure to polyvinylpyrrolidone coated gold nanoparticles (PVP-AuNPs). Generally, accumulation of gold in fish tissues and deleterious effects in *S. aurata* were higher for PVP-AuNPs than for cAuNPs exposures. Although AuNPs and GEM combined effects in gills were generally low, in liver, they were higher than the predicted. The accumulation and effects of AuNPs showed to be dependent on the size, coating, surface charge and aggregation/agglomeration state of nanoparticles. Additionally, it was tissue' specific and dependent on the presence of other contaminants. Although, gold intake by humans is expected to not exceed the estimated tolerable daily intake, it is highly recommended to keep it on track due to the increasing use of AuNPs.

Keywords: emerging contaminants; fate; toxicity; nanoparticle coating; mixtures

1. Introduction

Estuarine and coastal areas are expected to represent the ultimate recipient for many contaminants, including nanoparticles (NPs) and pharmaceuticals. NPs are currently considered emerging contaminants of concern (Sauve and Desrosiers 2014) due to: 1) its increased development, production and use; 2) their characteristics, fate, uptake and biological impact, which are dependent of the medium they are present in; and 3) the uncertainty of their potential toxicological effects (Alkilany and Murphy 2010; Canesi et al. 2012; Maynard et al. 2006). In particular, there is limited knowledge about concentrations, behaviour and bioavailability of NPs and consequently bioaccumulation and toxicological effects in marine organisms, mostly in top predators (Canesi et al. 2012).

The unique physical and chemical properties of AuNPs make them attractive to a wide range of applications. Currently AuNPs are extensively used in electronics, cosmetics, food and textile industries and biomedicine (Lapresta Fernández et al. 2012), among others. AuNPs are widely used as catalytic in several reactions and as biosensors (Chu et al. 2017; Qin et al. 2018). Biomedicine applications include diagnostic assays, cancer treatment, detection of cells and molecules and drug delivery (Cabuzu et al. 2015). Some studies have been carried out on the use of AuNPs as antimicrobials (Saleh et al. 2016) or to detect the insecticide malachite green (Loganathan and John 2017), in aquaculture. Due to this widespread use, AuNPs have the potential to become a significant persistent nanomaterial in the environment (Klaine et al. 2008; Hull

et al. 2011). Some authors have reported AuNPs as being non-toxic and biocompatible (Lapresta-Fernández et al. 2012), while other studies have highlighted their possible toxicity, with oxidative stress induction, cytotoxicity, genotoxicity and protein modifications, raising important concerns about possible impact on human health and ecosystems (Farkas et al. 2010; Paino et al. 2012; García-Cambero et al. 2013; Iswarya et al. 2016; Teles et al. 2016).

There is therefore a need for increased research on their toxicological effects, including those related with their presence alongside with other environmental contaminants.

Pharmaceuticals, another group of emerging contaminants of concern, are regularly found in aquatic habitats (Fent et al. 2006; Gonzalez-Rey et al. 2014).

Lipid regulators belong to one of the most prescribed classes of pharmaceuticals in human medicine and are commonly reported in wastewater and surface waters due to their increased use in recent years (Andreozzi et al. 2003; Sanderson et al. 2003; Lin and Reinhard 2005; Togola and Budzinski 2007; Gros et al. 2006; Schmidt et al. 2011). The presence of lipid regulators in the environment has been attracting attention within the scientific community aiming at improving the knowledge about their possible adverse effects to aquatic organisms (Fent et al. 2006). Earlier studies have indicated the possible toxicity of gemfibrozil (GEM) to aquatic organisms (Mimeault et al. 2005; Zurita et al. 2007; Quinn et al. 2008; Schmidt et al. 2011; Schmidt et al. 2014; Henriques et al. 2016; Barreto et al. 2017). Previous studies showed that short term exposure to GEM at environmentally relevant concentrations can cause behavioural alterations, genotoxicity and oxidative stress responses in *S. aurata*

(Barreto et al. 2017, 2018). There is however limited information about the mechanisms involved in GEM toxicity and, to the authors' knowledge, no study has addressed the toxicity of GEM combined with NPs.

As the precise modes of action involved in the AuNPs toxicity remain unclear, particularly in aquatic organisms, the evaluation of a range of responses, describing biochemical and biological processes, is useful to assess effects and to understand possible mechanisms of action. Behavioural, neurological and oxidative stress and damage biomarkers have been shown to be sensitive indicators of AuNPs toxicity (Klaper et al. 2009; Pan et al. 2012; Volland et al. 2015; Iswarya et al. 2016). To evaluate possible biological effects of AuNPs in aquatic systems, the swimming performance and several biochemical markers were evaluated in the fish species *Sparus aurata* following a short-term exposure (96 h) to AuNPs (citrate coated (cAuNPs) or polyvinylpyrrolidone coated (PVP-AuNPs), alone or in combination with GEM. Commercially used AuNPs may have different coatings, which may lead to different behaviour in marine media. It is therefore important to understand how such differences may lead to dissimilar effects on aquatic organisms (Barreto et al. 2015). The assessed enzymatic biomarkers were related to neurotransmission processes (cholinesterases – ChE), biotransformation (glutathione *S*-transferases – GST) and antioxidant defence (glutathione reductase – GR, catalase – CAT and glutathione peroxidase – GPx). Non-enzymatic defence processes were assessed through the quantification of non-protein thiols – NPT) and oxidative damage was assessed as lipid peroxidation (LPO). The levels of gold were also determined in different tissues (gills, muscle, liver and spleen) of *S. aurata*, as well as the bioaccumulation factors and an estimation of gold intake by humans.

2. Material and Methods

2.1. Test organisms

Juvenile gilthead seabream (*Sparus aurata*), with a length of 9±0.5 cm,

acquired from an aquaculture facility (Santander, Spain), were acclimated for 4

weeks in aquaria containing aerated and filtered (Eheim filters) artificial 128 seawater (ASW, Ocean Fish, Prodac) prepared by dissolving the salt in reverse

osmosis purified water to obtain a salinity of 35, in a controlled room

temperature (20 ºC) and natural photoperiod. During this period, animals were

fed daily with commercial fish food (Sorgal, Portugal) at a ratio of 1 g per 100 g

of fish. The ASW used to maintain fish during the acclimation period was also 133

used during toxicity tests.

2.2. Synthesis and characterisation of AuNPs

All glass material used in AuNPs synthesis was previously washed with *aqua regia* and later rinsed thoroughly with ultrapure water. AuNPs of around 40 nm were prepared by sodium citrate reduction of gold (III) chloride trihydrate (Lekeufack et al. 2010). Part of the resulting cAuNPs were coated with polyvinylpyrrolidone (PVP) as described by Barreto et al. (2015). cAuNPs and 140 PVP-AuNPs were centrifuged and the pellet resuspended in ultrapure water.

The citrate reduction method, one of the most widely used in AuNPs synthesis, was chosen due to the known non-toxicity of citrate, the use of water as solvent

and the fact that cAuNPs have been frequently used in diverse areas, namely in 144 biomedical applications (Turkevich et al. 1951; Li et al. 2011; Hanžić et al. 2015). PVP was selected as coating agent because it is a water-soluble, nontoxic and biodegradable homopolymer (Min et al. 2009). This polymer may adsorb on the surface of metal NPs and generate a covering layer by interaction of C–N and C=O groups with NPs surface (Lu et al. 2009; Behera and Ram 149 2013).

After synthesis, the AuNPs stock suspensions and AuNPs in the experimental media (ASW) and in ultrapure water were characterised at 0, 24 and 96 h. Suspensions of AuNPs combined with GEM were also characterised 153in ASW and ultrapure water. The AuNPs were characterised by UV-Vis spectra

(Cintra 303, GBC Scientific); size, assessed by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern), transmission electron microscopy (TEM; Hitachi, H9000 NAR) and scanning electron microscopy (SEM; Hitachi, SU70); and zeta 157 potential (ZP; Zetasizer Nano ZS, Malvern).

2.3. Fish bioassay

The bioassay followed the OECD guideline (number 203) for fish acute bioassays (OECD 1992). Fish specimens (n=10 per condition) were randomly distributed in the experimental aquaria and exposed for 96 h to the following 9 experimental conditions: 4, 80 and 1600 $\mu g.L^{-1}$ AuNPs (citrate and PVP coating); 150 $\mu g.L^{-1}$ GEM; mixture of 150 $\mu g.L^{-1}$ GEM with 80 $\mu g.L^{-1}$ AuNPs (citrate and PVP coating). Test suspensions of AuNPs were prepared in ASW, by dilution of cAuNPs and PVP-AuNPs stock suspensions containing 97 $mg.L^{-1}$

and 58 mg.L^{-1} of gold, respectively. The ASW was also as negative control. The

AuNPs lowest concentration tested (4 µg.L^{-1}) was near to the predicted values

for water (0.14 µg.L^{-1}) and soil (5.99 µg.kg^{-1}) (García-Negrete et al. 2013; Tiede

et al. 2009). The other concentrations tested were 20-fold increases. A stock

solution of GEM (50 g.L^{-1}) was prepared in dimethyl sulfoxide (DMSO). The test

solutions with GEM (150 µg.L^{-1}) were prepared by appropriate dilutions of the

stock solution in ASW. A solvent control with DMSO (at 0.003%, the

concentration of DMSO used in the GEM treatments) was also included. The

concentration of GEM tested (150 µg.L^{-1}) is about 100 times higher than

relevant environmentally concentrations of GEM and has been shown to induce

significant effects, in terms of genotoxicity and oxidative stress in *S. aurata*

(Barreto et al. 2017; 2018).

Every 24 h, after checking fish mortality and assessing the water parameters

(temperature, salinity, conductivity, pH and dissolved oxygen), approximately

80% of the experimental media was renewed in order to prevent significant NPs

alteration and/or GEM degradation and to reduce the build-up of metabolic

residues. Water samples – experimental media from each aquarium (15 mL of

aquaria with single exposures and 30 mL of aquaria with combined exposures)

184 – were collected daily (at 0 and 24 h) for the quantification of gold and GEM.

During the bioassay, photoperiod, temperature and aeration conditions were

similar to those used in the acclimation period. No food was provided to the

fish during the exposure period.

2.4. Assessment of swimming performance

After 96 h exposure, fish were individually transferred to a 1.2 m long track race flume with 6.7 cm diameter and induced to swim against a water flow (20 $L.min^{-1}$), generally following the procedure described by Oliveira et al. (2012). The time spent by the fish swimming against the water flow was recorded and presented in seconds. After this behavioural test, fish were transferred back to their original test aquaria where they were left for an additional 2 h period.

2.5. Collection of biological material for biomarkers determination and gold quantification

After this 2-h recovery period, animals were anesthetised with tricaine methanesulfonate (MS-222) and euthanised by spinal section. Liver, gills, muscle and brain were taken from seven fish, snap frozen in liquid nitrogen to prevent enzyme or tissue degradation and stored at -80 ºC until further processing. Liver, gills, muscle and spleen were collected from three animals and stored at -20 ºC until further quantification of gold.

2.6. Quantification of gold and GEM

The determination of gold in ASW and fish samples was performed according to the NIST NCL Method PCC-8 (NIST 2010). A MLS-1200 Mega microwave digestion unit (Milestone, Sorisole, Italy) was used for closed-vessel acid digestion of the fish samples and an iCAP™ Q ICP-MS (inductively coupled plasma mass spectrometry; Thermo Fisher Scientific, Bremen, Germany) was used for gold determination in both fish digests and water samples. The

elemental isotope ^{197}Au was monitored for analytical determination; ^{159}Tb and 212 ^{209}Bi were used as internal standards.

The analysis of GEM in water samples was performed by solid phase extraction (SPE), using Strata X cartridges (200 mg, 3 mL) (Phenomenex, USA), and following the procedure described in Barreto et al. (2017). GEM was quantified by ultra-high performance liquid chromatography tandem-mass spectrometry (UHPLC-MS/MS) using internal standard calibration. A Nexera UHPLC system with a triple-quadrupole mass spectrometer detector LCMS219 8030 (Shimadzu Corporation, Kyoto, Japan) was used. Detailed information about the chromatographic and mass spectrometry experimental conditions as well as the validation parameters can be found elsewhere (Barreto et al. 2017).

2.7. Total gold content and bioaccumulation factor

Total gold content ($[Au]_{total}$), expressed as $\mu g.g^{-1}$, was calculated as the sum 224 of the gold content in each fish tissue, according the formula:

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

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

gold in liver, $[Au]_s$ the concentration of gold in spleen and

$[Au]_m$ the 228 concentration of gold in muscle.

The bioaccumulation factor (BAF), in $L.g^{-1}$, was determined according to Yoo230 iam et al. (2014), by dividing the gold content ($\mu g.g^{-1}$) in each tissue of the fish

(gills, liver, spleen or muscle) by the initial concentration of gold in the exposure 232 media ($\mu g.L^{-1}$):

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

Where $[Au]_t$ is the content of gold in the specific fish tissue and $[Au]_{ASW}$ its concentration in the exposure media – ASW (collected daily at 0 h and quantified).

2.8. Biomarkers determination

Liver and gills were homogenized in potassium phosphate buffer (0.1 mM, 239 pH 7.4) using an ultrasonic homogenizer (Branson Ultrasonics Sonifier S-250A). The homogenate was then divided into three aliquots: for the quantification of LPO, NPT and for the preparation of post-mitochondrial supernatant (PMS). The aliquot of homogenate for LPO evaluation was transferred to a microtube with 4% BHT (2,6-Di-tert-butyl-4-methylphenol) in methanol, to prevent oxidation. The aliquots for LPO and NPT assays were stored at -80 ºC until analysis. PMS was accomplished by centrifugation at 12 000 g for 20 min at 4 246 ºC. PMS aliquots were stored at -80 ºC until GST, CAT, GPx and GR activities determination.

Muscle and brain were used for ChE activity determination. Tissues were homogenized in potassium phosphate buffer (0.1 mM, pH 7.2), centrifuged at 3 300 g for 3 min at 4 ºC and the obtained supernatant was collected and stored at -80 ºC.

Protein content was determined for all samples according to Bradford (1976), by measuring the absorbance at 600 nm, using a microplate-adapted 254 procedure, with bovine □-globulin as the standard.

ChE activity was determined according to the Ellman's method (1961) adapted to microplate reader (Guilhermino et al. 1996). The rate of thiocholine

production was assessed at 412 nm as nmol of thiocholine formed per min per mg of protein ($\varepsilon=1.36\times10^4$ $M^{-1}.cm^{-1}$), using acetylthiocholine as substrate.

CAT activity was assayed as described by Claiborne (1985). Changes in the absorbance at 240 nm caused by the dismutation of hydrogen peroxide (H_2O_2) were recorded and CAT activity was calculated as µmol H_2O_2 consumed per min per mg of protein ($\varepsilon=40$ $M^{-1}.cm^{-1}$).

GR activity was estimated according to the method of Carlberg and Mannervik (1975) adapted to microplate reader (Lima et al. 2007), measuring the reduced nicotinamide-adenine dinucleotide phosphate (NADPH) decrease at 340 nm and was expressed as nmol of oxidized nicotinamide-adenine dinucleotide phosphate ($NADP^+$) formed per min per mg of protein ($\varepsilon=6.22\times10^3$ $M^{-1}.cm^{-1}$).

GPx activity was measured according to the method described by Mohandas et al. (1984) as modified by Athar and Iqbal (1998). Oxidation of NADPH was recorded spectrophotometrically at 340 nm and the enzyme activity was calculated as nmol NADPH oxidized per min per mg of protein ($\varepsilon=6.22 \times 10^3$ $M^{-1}.cm^{-1}$).

To determine NPT levels, protein content in the homogenate was precipitated with trichloroacetic acid (10% m/v) for 1 h and then centrifuged at 12 000 g for 5 min at 4 °C. NPT were spectrophotometrically determined in the resulting supernatant at 412 nm by the method of Sedlak and Lindsay (1968) as adopted by Parvez et al. (2003) and results were expressed as nmol per mg of protein.

GST activity was determined spectrophotometrically by the method of Habig

et al. (1974) adapted to microplate reader (Frasco and Guilhermino 2002), following the conjugation of the substrate, 1-chloro-2, 4-dinitrobenzene (CDNB), with reduced glutathione. Absorbance was recorded at 340 nm and the GST activity was calculated as nmol of CDNB conjugate formed per min per mg of protein ($\varepsilon=9.6\times10^{-3}$ $M^{-1}.cm^{-1}$).

LPO levels were assessed by the production of thiobarbituric acid reactive substances (TBARS) based on Ohkawa et al. (1979) method, adapted by Filho et al. (2001). Absorbance was measured at 535 nm and LPO was expressed as nmol of TBARS formed per mg of protein ($\varepsilon=1.56\times10^{5}$ $M^{-1}.cm^{-1}$).

2.9. Estimated gold intake by humans

Since *S. aurata* is one of the most consumed fish in south Europe, an estimation of gold intake by humans, expressed as µg per kg of body weight per year, was calculated, using the conventional formula (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). Gold content in the ingested fish corresponds to the content of gold determined in the fish muscle ($\mu g.g^{-1}$).

To stablish an estimated maximum amount of gold that each individual may be exposed daily over their lifetimes without considerable health risk – “tolerable 301 daily intake” (TDI) (IPCS 2004), the following formula was used (FDA 2015):

$$TDI = \frac{NOAEL}{100}$$

Where TDI is expressed in µg per kg body weight per day and estimated based on the "No Observed Adverse Effect Level" (NOAEL) for humans which is derivate from the most sensitive species of experimental animals and for the most sensitive adverse effect relevant to human (FDA 2015). Then, NOAEL is divided by a safety factor, usually 100, which results in a large margin of safety.

2.10. Statistical analysis

Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene’s test) using the Sigma Plot 12.0 software package. Differences between controls (negative and solvent) were tested using a Student t-test. Differences between treatments and controls were tested using one-way analysis of variance (ANOVA), followed by Dunnett’s test whenever applicable. Differences between single and combined exposures were tested using one-way analysis of variance (ANOVA), followed by Tukey´s test 316 whenever applicable. Significant differences were assumed for $p<0.05$.

Observed percentages of effect in the combined exposures, corresponding to measured effects, were compared with the correspondent predicted percentages of effect which were derived by the sum of single exposure effects.

These comparisons were performed to understand if the combined effect of AuNPs and GEM was similar, lower or greater than the sum of both single exposure effects.

3. Results and Discussion

3.1. Characterisation and behaviour of AuNPs

The cAuNPs displayed a well-defined absorption band with the surface plasmon resonance (SPR) peak at 534 nm. DLS analysis showed an average hydrodynamic size of the particles of 35 nm and a negative surface charge (-44 mV). TEM analysis confirmed that almost all of the colloidal cAuNPs had the same size and were approximately spherical (Figure 1A).

There was a slight shift in the SPR peak to a longer wavelength (535 nm) for PVP-AuNPs when compared with the original cAuNPs as previously observed (Barreto et al. 2015; Nghiem et al. 2010). DLS measurements showed an increased size of PVP-AuNPs to 50 nm when compared with cAuNPs (35 nm), also in agreement with previous studies (Barreto et al. 2015; Mahl et al. 2010).

SEM analysis allowed the visualization of a PVP layer around the metal core of AuNPs (Figure 1B). PVP is an uncharged homopolymer and the presence of the PVP layer led to a less negative ZP value (-17 mV).

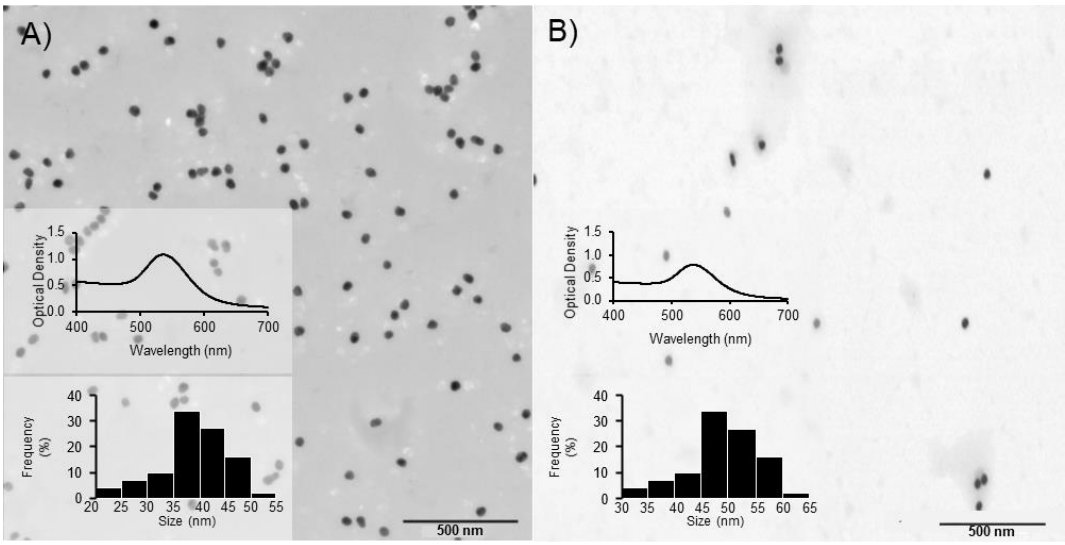

Fig. 1. UV-Vis spectrum, size distribution histogram and electron microscopy image of citrate coated gold nanoparticles – cAuNPs (A) and polyvinylpyrrolidone (B). (1.5 column)

Charged species present in media will interact with NPs and may change their physiochemical properties (e.g. size and surface charge) (Alkilany and Murphy 2010). In high ionic strength media, such as estuarine and marine environments, NPs tend to aggregate or agglomerate (Lee et al. 2012; Yoo-Iam et al. 2014) as a consequence of a modulated balance between repulsive and attractive forces (Krysanov et al. 2010; Moore et al. 2015).

At the lowest concentrations (4 and 80 $\mu g.L^{-1}$) the media did not display the typical red colour of AuNPs suspensions. It was therefore not possible to observe the typical changes in colour due to agglomeration/aggregation (Barreto et al. 2015). UV-Vis spectrophotometry and DLS also did not allow the study of the behaviour of the NPs at these concentrations because of the weakness of its signal. The methodological challenge of assessing the behaviour of NPs at low concentrations using UV-Vis spectra and DLS has

been reported earlier (García-Negrete et al. 2013). An evaluation of NPs behaviour using microscopy would also be challenging due to sample preparation requirements associated with the low number of particles as well as the presence of salt crystals in ASW. Previous studies have similarly reported the difficulty of finding NPs on a dried copper grid, at low concentrations (Botha et al. 2015). Nonetheless, the results in García-Negrette et al. (2013) indicated that 20 nm cAuNPs can be considered resistant to salt-induced aggregation in a of low µg.L^{-1} range, with the concentration of 60 µg L^{-1} showing no significant differences in morphology or size regarding AuNPs primary particles. The same study reported that, at 600 µg L^{-1}, a fine sediment was found after two days in ASW (García-Negrete et al. 2013). In the present study, the cAuNPs highest tested concentration (1600 µg.L^{-1}) displayed an immediate change in colour from red to light blue, typical of NPs agglomeration/aggregation. The SPR peak that was initially detected at longer wavelengths disappeared after few minutes. The hydrodynamic size of AuNPs increased to about 340 nm and different peaks corresponding to different charges were found in the ZP analysis. Within 24 h a dark layer was visible in the aquaria containing the highest concentration of cAuNPs, probably due to sedimentation of aggregates/agglomerates. At the end of the assay (i.e., after 96 h), the size of aggregates/agglomerates was still around 340 nm without a detectable SPR peak.

PVP-AuNPs (at 1600 µg.L^{-1}) did not display change in colour, in agreement with the previous study of Barreto et al. (2015) which demonstrated that 40 nm PVP-AuNPs were stable in ASW during 30 d. The conjugation with PVP

promoted stability of AuNPs in ASW, as assessed through UV-Vis spectra, size and ZP, parameters that were similar to those of PVP-AuNPs in ultrapure water after 96 h. Thus, the present study confirms that PVP-AuNPs at 1600 µg.L^{-1} may remain stable in suspension in a nano size range in ASW, whereas cAuNPs immediately alter their characteristics and aggregate/agglomerate, increasing their size.

A study of the interaction of GEM and AuNPs was not possible at the tested concentrations of 80 and 150 µg.L^{-1} (AuNPs and GEM, respectively), because of the detection limits. A UV-Vis spectrophotometric analysis of a mixture of these two compounds in ultrapure water, at the same ratio but a ten-fold higher concentration (800 and 1500 µg.L^{-1}, respectively), revealed that the characteristic SPR peak of AuNPs was maintained and the peak corresponding to GEM was detected at the expected wavelength (around 276 nm). In addition, the size, as determined by DLS, and ZP of AuNPs were maintained when they were mixed with GEM. In ASW, cAuNPs with GEM also aggregated/agglomerated, presenting similar behaviour and characteristics as when they were single in ASW. PVP-AuNPs combined with GEM remained stable in ASW, such as when they were single in the medium. The absence of changes in UV-Vis spectra, size and ZP of AuNPs when they were mixed with GEM suggests that GEM and AuNPs did not have a physical association.

3.2. Quantification of gold and GEM in ASW

The measured concentrations of gold and GEM in the experimental media (ASW) are present in the Table 1. At 0 h, the gold quantified in the media was lower than the nominal concentrations, except for PVP-AuNPs at 4 µg.L^{-1}. The

difference between the nominal and measured concentrations was more evident for cAuNPs. For the nominal concentration of 4 μg.L^{-1} cAuNPs, the measured concentration of gold was 32% lower than the predicted. For the 80 μg.L^{-1}, the detected gold concentration in ASW was 62 and 15% lower than the nominal concentration for cAuNPs and PVP-AuNPs, respectively. At the highest tested concentration, the concentration of gold was 92 and 9% lower than the predicted for cAuNPs and PVP-AuNPs, respectively. The concentration of GEM at 0 h was around 60% higher than the nominal concentration (150 μg.L-1), for both single and combined exposures. In the combined exposures with GEM, at 0 h, the concentration of gold in ASW was 56 and 20% lower than the predicted for cAuNPs and PVP-AuNPs, respectively.

After 24 h of exposure, comparing with the gold quantified at 0 h, gold concentration decreased more after the exposure to cAuNPs than to PVP AuNPs (Table 1). In the nominal concentration 4 μg.L^{-1}, this decrease was 51 and 19% for cAuNPs and PVP-AuNPs, respectively. In the nominal concentration 80 μg.L^{-1}, after 24 h of exposure, the concentrations of gold decreased by 83 and 16% for cAuNPs and PVP-AuNPs, respectively. For the nominal concentration 1600 μg.L^{-1}, a decrease of gold concentration after 24 h was also observed with 47% for cAuNPs and 35% for PVP-AuNPs. After 24 h, the measured GEM concentration was similar to the measured concentration at 0 h, for both single and combined exposures. In the combined exposures with

GEM, comparing with 0 h, the concentration of gold decreased 55 and 27% in ASW after 24 h for cAuNPs and PVP-AuNPs, respectively.

Table 1. Nominal and measured concentrations ($\mu g.L^{-1}$) of gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and gemfibrozil (GEM) in experimental media (artificial seawater) at 0 and after 24 h.

Results are expressed as mean ± standard error. N.D. – Not determined. (Double column)

Nominal concentrations ($\mu g.L^{-1}$)		**Measured concentrations ($\mu g.L^{-1}$)**		
		cAuNPs	**PVP-AuNPs**	**GEM**
4 AuNPs	**0h**	2.7 ± 0.3	4.2 ± 0.2	N.D.
	24h	1.3 ± 0.3	3.4 ± 0.1	N.D.
80 AuNPs	**0h**	30.5 ± 4.7	67.8 ± 6.1	N.D.
	24h	5.1 ± 0.2	56.9 ± 3.0	N.D.
1600 AuNPs	**0h**	115.2 ± 4.2	1458.7 ± 41.8	N.D.
	24h	61.1 ± 10.1	943.0 ± 11.7	N.D
150 GEM	**0h**	N.D.	N.D.	240.0 ± 9.3
	24h	N.D	N.D	236.0 ± 2.3
80 AuNPs + 150 GEM	**0h**	35.1 ± 4.1	63.9 ± 18.0	235.0 ± 7.9
	24h	15.9 ± 3.5	46.7 ± 2.7	229.0 ± 1.1

Du et al. (2012) reported an 80% decrease of the number of 40 nm cAuNPs in suspension in phosphate buffered saline (PBS) after 30 min. In the same study, the number of AuNPs coated with PVP (10 to 50 $mg.L^{-1}$) in suspension in

PBS media showed a lower decrease than for cAuNPs (Du et al. 2012). In the present study, the higher decrease of the gold in suspension in the ASW media

after 24 h and more pronounced difference between the nominal and the measured concentrations, observed in the exposures to cAuNPs, may be explained by the aggregation of these particles and subsequent sedimentation. Since the PVP-AuNPs did not aggregate, the concentration of gold in suspension in the medium after 24 h was closer to the initial concentration than for cAuNPs.

3.3. Total gold content and bioaccumulation factor

The highest concentrations of gold in fish tissues were detected when *S. aurata* was exposed to PVP-AuNPs (Table 2). At the lowest tested concentration (4 µg.L^{-1}), PVP-AuNPs significantly accumulated ($p<0.05$; Dunnett's test) in the liver. However, at 80 and 1600 µg.L^{-1} PVP-AuNPs significantly accumulated in the gills ($p<0.05$; Dunnett's test). cAuNPs also significantly accumulated in the gills following exposure to 1600 µg.L^{-1} ($p<0.05$; Dunnett's test). In the single exposures to AuNPs, liver was the organ that accumulated most gold. Concerning the combined exposures to AuNPs and GEM, PVP-AuNPs significantly accumulated in gills and muscle whereas cAuNPs significantly accumulated in the liver and spleen ($p<0.05$; Dunnett's test). Muscle was the tissue that accumulated most gold (particularly for PVP462 AuNPs) (Table 2). The calculated BAF showed that bioaccumulation generally

was higher for PVP-AuNPs than for cAuNPs and the highest value was 464 observed for the nominal concentration 4 µg.L^{-1} in the liver (3.44) (Table 2).

Table 2. Gold concentration in tissues of *Sparus aurata* (gills, liver, spleen and muscle) exposed to gold nanoparticles (citrate coated – cAuNPs and

polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM) for 96 h and respective estimated bioaccumulation factor (BAF). Results are expressed as means ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$).

(Double column)

Nominal Concentrations ($\mu g.L^{-1}$)	Tissues	Gold Content ($\mu g.g^{-1}$) PVP-AuNPs	Gold Content ($\mu g.g^{-1}$) cAuNPs	BAF ($L.g^{-1}$) PVP-AuNPs	BAF ($L.g^{-1}$) cAuNPs
0 AuNPs	**Gills**	0.0 ± 0.0	0.0 ± 0.0	0.00	0.00
	Liver	0.0 ± 0.0	0.0 ± 0.0	0.00	0.00
	Spleen	0.0 ± 0.0	0.0 ± 0.0	0.00	0.00
	Muscle	0.0 ± 0.0	0.0 ± 0.0	0.00	0.00
4 AuNPs	**Gills**	0.2 ± 0.0	0.3 ± 0.1	0.05	0.06
	Liver	0.2 ± 0.0	14.6 ± 0.6 *	0.05	3.44
	Spleen	1.7 ± 0.5	0.6 ± 0.0	0.60	0.15
	Muscle	0.1 ± 0.0	0.4 ± 0.1	0.02	0.10
80 AuNPs	**Gills**	0.2 ± 0.0	3.6 ± 0.4 *	0.01	0.05
	Liver	0.2 ± 0.0	0.6 ± 0.4 0.0	0.01	0.00
	Spleen	± 0.0	0.2 ± 0.1	0.00	0.00
	Muscle	0.3 ± 0.1	0.5 ± 0.0	0.01	0.00
1600 AuNPs	**Gills**	2.9 ± 0.3 *	32.8 ± 3.7 *	0.02	0.02
	Liver	0.7 ± 0.3	0.2 ± 0.1 0.4	0.00	0.00
	Spleen	± 0.1	0.3 ± 0.1	0.00	0.00
	Muscle	0.3 ± 0.1	1.4 ± 0.4	0.00	0.00
80 AuNPs + 150 GEM	**Gills**	0.2 ± 0.1	7.9 ± 1.3 *	0.01	0.12
	Liver	5.9 ± 1.7 *	0.8 ± 0.1	0.17	0.01
	Spleen	3.0 ± 1.5 *	1.7 ± 0.4	0.08	0.03
	Muscle	1.0 ± 0.3	16.5 ± 15.4 *	0.02	1.82

The $[Au]_{total}$ values were also higher for PVP-AuNPs and the highest value was observed for the combined exposure to PVP-AuNPs and GEM (Figure 2).

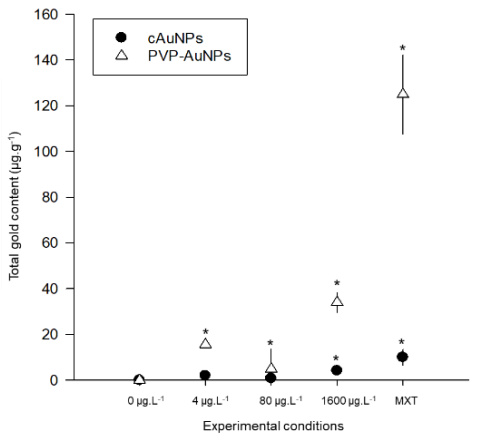

Fig. 2. Total gold content on *Sparus aurata* after 96 h of individual or combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM. (Single column)

It is known that NPs may accumulate in aquatic organisms (Krysanov et al. 2010); however, the information about accumulation of nanomaterials in the tissues is scarce and currently contradictory (Krysanov et al. 2010). In fish, NPs

may be taken up mostly through gills or the gastrointestinal tract and may accumulate in different tissues such as liver, spleen, brain and muscle (Lee et al. 2012; Yoo-Iam et al. 2014). Their accumulation is dependent on the NPs characteristics but also on their behaviour upon contact with the fish intestinal fluids, where nutrient absorption occurs, or other surfaces. After 96 h exposure to 5 nm AuNPs (0.2 $mg.L^{-1}$), the mean concentrations of gold detected in the whole body of the marine fish *Pomatoschistus microps* ranged from 0.129 to 0.546 $\mu g.g^{-1}$ (Ferreira et al. 2016). Bioaccumulation of AuNPs has been observed in the digestive gland (61 $\mu g.g^{-1}$), gills (0.5 $\mu g.g^{-1}$) and mantle (0.02 $\mu g.g^{-1}$) of the marine mussel *Mytilus edulis* following 24 h exposure to 13 nm

AuNPs (750 μg.L^{-1}) (Tedesco et al. 2008). In zebrafish (*Danio rerio*) exposed to a diet containing 4.5 μg.g^{-1} AuNPs (12 nm) for 36 d, gold was detected in brain

and liver at concentrations of 4.6 and 3.0 μg.g^{-1}, respectively (Geffroy et al. 2012). In *D. rerio* exposed for 20 d to sediment spiked with 14 nm AuNPs at a concentration of 16 and 55 μg.g^{-1}, gold was detected in the gills (between 0.01 and 0.03 μg.g^{-1}), digestive tract (between 0.22 and 1.40 μg.g^{-1}) but not in brain and muscle (Dedeh et al. 2015). Variable results have been found concerning the accumulation of other types of NPs in fish tissues. Iron oxide NPs with different sizes were found to accumulate at higher concentrations in spleen than in muscle of tilapia (*Oreochromis niloticus*) following 30 and 60 d of exposure (Ates et al. 2016). Scown et al. (2010) reported that silver NPs with different sizes accumulated more in the liver than in the gills of rainbow trout (*Oncorhynchus mykiss*) after 10 d of exposure. The study of Ates et al. (2013) using an *in vitro* model to determine the possible uptake of titanium dioxide NPs (exposure for 96 h) showed that NPs accumulated more in the gills and intestine and there was no significant accumulation in muscle and brain of the goldfish (*Carassius auratus*).

516 The greater accumulation of gold in tissues when fish were exposed to PVP517 AuNPs is probably related to a higher bioavailability of PVP-AuNPs, compared to cAuNPs. PVP-AuNPs remained stable in ASW, maintaining their nano size, being dispersible in the water column and, therefore, more available for the uptake by fish, as indicated by the gold levels in the tissues of *S. aurata*. On the contrary, cAuNPs immediately aggregated/agglomerated in ASW, the aggregates/agglomerates (with sizes higher than 300 nm) were deposited on the tanks' bottom, leading to a lower concentration of AuNPs in the water

column and, consequently, a lower uptake by fish. It has already been described that the NPs size have a crucial role in its bioavailability and consequent effects to the organisms (Vale et al. 2016). When aggregates/agglomerates become too large for direct transport across the cell membrane, uptake may be reduced and less effects to the organisms are expected (Vale et al. 2016).

In combined exposures, the accumulation of gold in the tissues was different compared to the single exposures to AuNPs. This is a relevant finding because it may indicate changes in the internalization processes of AuNPs when GEM is present, as the characterization for both AuNPs indicated no interaction in ASW with GEM.

3.4. Effects of AuNPs on *S. aurata*

The dissimilar behaviour of cAuNPs and PVP-AuNPs found in the present study may lead to different effects in *S. aurata*. As shown in Figure 3, the ability of *S. aurata* to continue swimming against a water flow was significantly decreased ($p<0.05$; Dunnett's test), about 80%, when fish were exposed to 1600 $\mu g.L^{-1}$ of PVP-AuNPs. cAuNPs did not show any effects on their swimming performance.

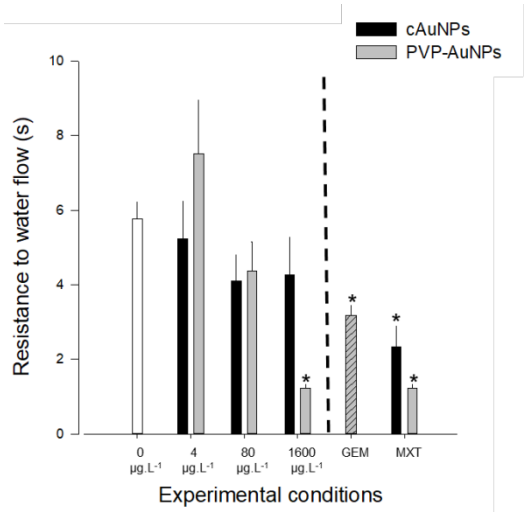

Fig. 3. Resistance of *Sparus aurata* to withstand swimming against a water flow after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM. (Single column)

Previous studies have shown that nanosized materials may affect the behaviour of fish: erratic swimming and slow opercular movements of cichlid fish (*Etroplus maculatus*) after 96 h exposure to 100 µg.L^{-1} fullerene NPs (Sumi and Chitra 2015); reduction of the ability of the *D. rerio* embryos to maintain their orientation within a water current after 4 h exposure to copper and silver NPs (50, 150 and 225 µg.L^{-1}) (McNeil et al. 2014); significantly greater disruption of the olfactory-mediated behavioural response of *O. mykiss* after 12 h exposure to 50 µg.L^{-1} copper NPs (Sovová et al. 2014). In terms of AuNPs, no study has so far reported alterations on the swimming behaviour of fish 563 although a decreased feeding performance was reported for marine fish *P. microps* (Ferreira et al. 2016). Among other factors, the changes detected in the

swimming performance of *S. aurata* could be a result of a direct effect of NPs on the brain (Kashiwada 2006; Mattsson et al. 2015). Fish exposed to NPs can take up the particles through the gills, and the particles can be transported to the different organs, including the brain (Kashiwada 2006). At the brain, a lipid rich organ, NPs may affect the organization and function of tissue membranes because of its strong affinity to lipids (Mattsson et al. 2015). The interaction between NPs and biological membranes depend on their physicochemical properties, such as, size and surface charge (Broda et al. 2016).

A decrease in the activity of ChE, some of which are critical enzymes for neurological function (Hernández-Moreno et al. 2011; Oliveira et al. 2013), could be another explanation to the decrease of *S. aurata* resistance against the water flow. However, the activity of ChE (both in brain and muscle) was not significantly altered by the exposure to AuNPs ($p>0.05$; ANOVA; Figure 4), suggesting the involvement of other factors.

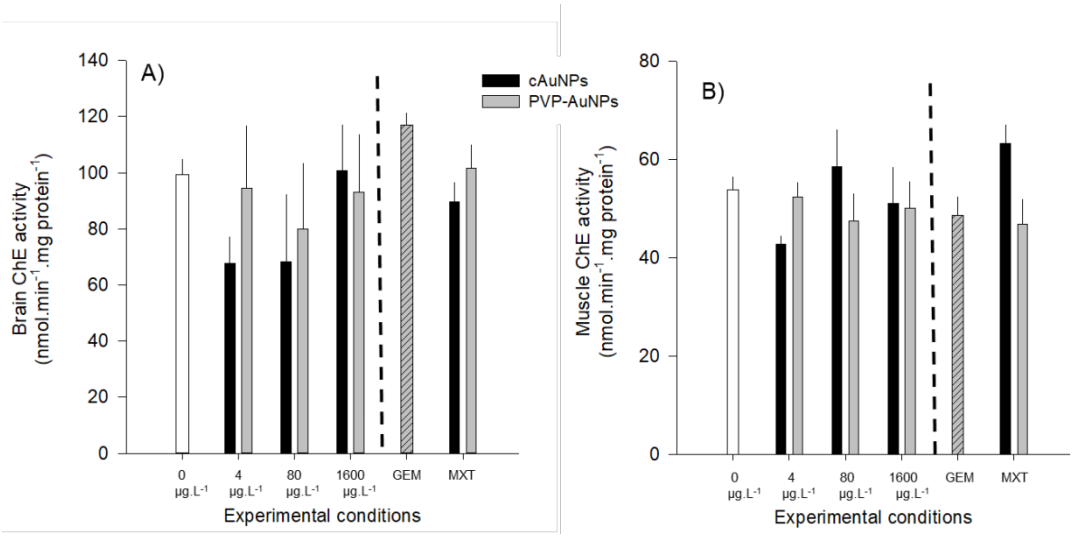

Fig. 4. Brain (A) and muscle (B) cholinesterases (ChE) activity of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM).

Results are expressed as mean ± standard error. MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM. (1.5 column)

Despite the scarcity of studies on the effects of NPs in the ChE activity (Wang et al. 2009; Pan et al. 2012; Šinko et al. 2014; Luis et al. 2016), the lack of association between altered fish behaviour and ChE activity after exposure to NPs was also reported in Boyle et al. (2013) for *O. mykiss* exposed to titanium NPs and in Ferreira et al. (2016) with *P. microps* after the exposure to AuNPs. However, some authors consider that ChE may be used as a potential biomarker for NPs exposure (Wang et al. 2009). In the clams *Scrobicularia plana,* ChE activity was significantly increased after 16 d exposure to 100 $\mu g.L^{-1}$ of 5, 15 and 40 nm cAuNPs (Pan et al. 2012). Although, in the present study, with 40 nm cAuNPs at a similar concentration (80 $\mu g.L^{-1}$), different results were obtained possibly due to the shorter exposure period (96 h versus 16 d) and different organisms tested (invertebrate versus vertebrate). An *in vitro* approach with mussels (*Mytilus galloprovincialis*) showed that cAuNPs and PVP-AuNPs (in concentrations ranging from 54 $ng \cdot L^{-1}$ to 2.5 $mg \cdot L^{-1}$) did not alter the activity of ChE (Luis et al. 2016). There is still no clear explanation on how NPs interact with ChE. In general, NPs have binding affinity to ChE due to its lipophilicity and the hydrophobicity of the environment in ChE molecules (Šinko et al. 2014). However, different types of NPs have shown different affinities to the ChE (Wang et al. 2009). A study with silver NPs also reported that the effect of these NPs on the ChE activity was dependent on the surface coating of the NPs (Šinko et al. 2014).

Concerning the enzymatic defence responses, AuNPs did not induced

significant alteration in the gills CAT activity of *S. aurata* ($p>0.05$; ANOVA; Figure 5A). However, in the liver, CAT activity was significantly increased ($p<0.05$; Dunnett's test) after fish exposure to 1600 µg.L^{-1} AuNPs (both citrate and PVP coating) – Figure 5B. In the case of PVP-AuNPs, a dose-response relationship was apparent.

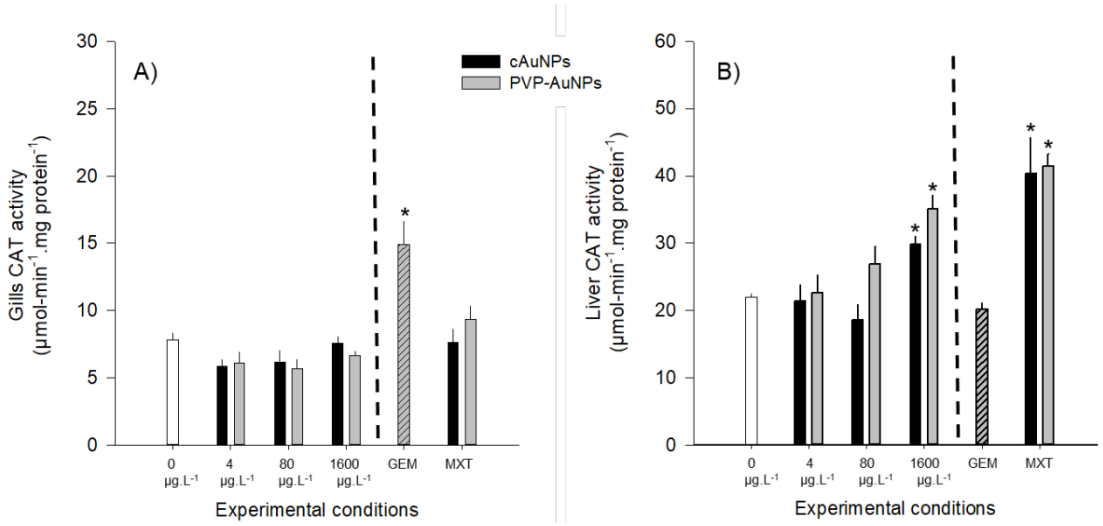

Fig. 5. Gills (A) and liver (B) catalase (CAT) activity of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are 627 expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, 628 $p<0.05$). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM. (1.5 column)

The activity of GR (both in gills and liver) was not affected by the exposure to AuNPs ($p>0.05$; ANOVA; Figure 6).

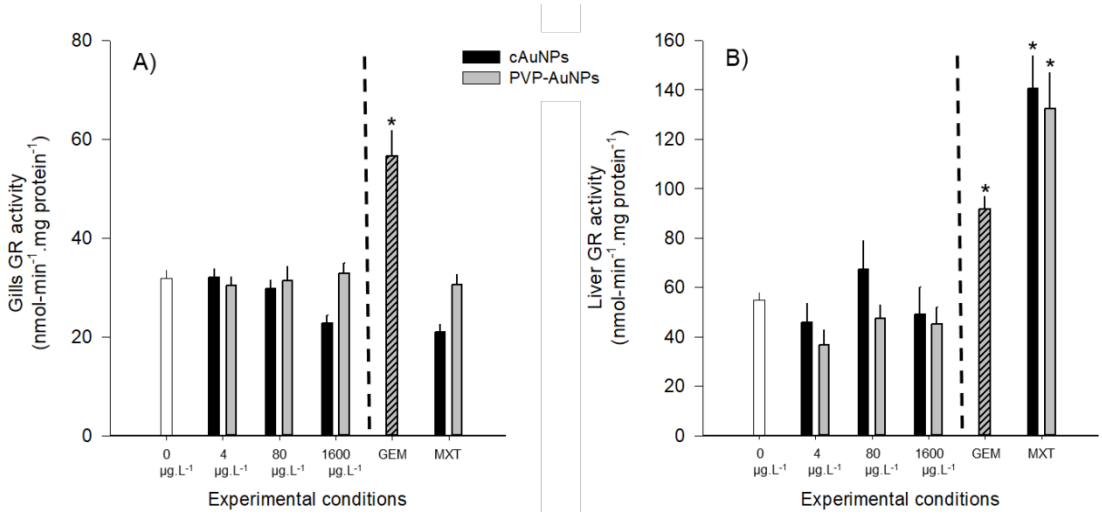

Fig. 6. Gills (A) and liver (B) glutathione reductase (GR) activity of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and 640 polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. MXT – AuNPs (cAuNPs or PVP-

AuNPs) with GEM. (1.5 column)

Regarding GPx, in gills, only 80 µg.L^{-1} PVP-AuNPs significantly increased

this enzyme activity ($p<0.05$; Dunnett's test; Figure 7A). In the liver, PVP-AuNPs

exposure (4 and 1600 µg.L^{-1}) significantly increased the GPx activity ($p<0.05$;

Dunnett's test; Figure 7B).

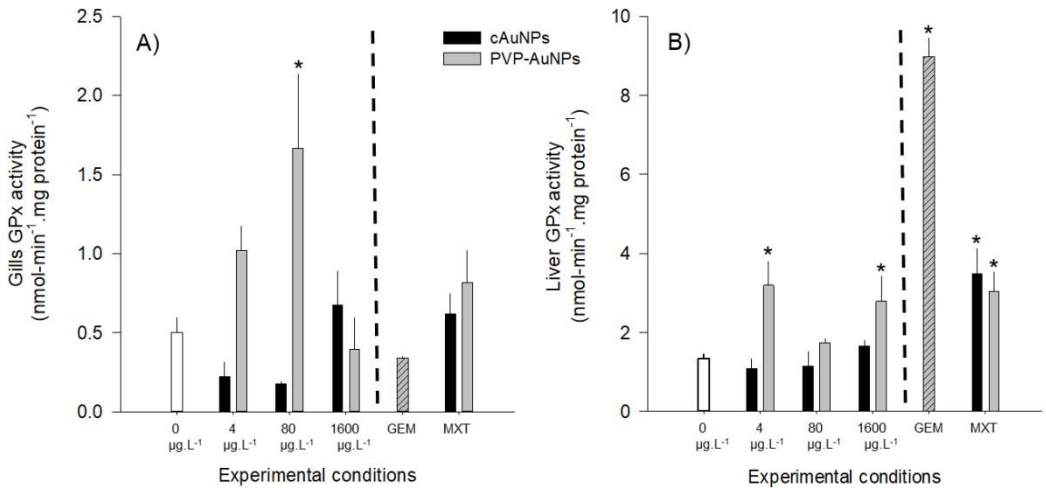

Fig. 7. Gills (A) and liver (B) glutathione peroxidase (GPx) activity of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM).

Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM. (1.5 column)

Concerning non-enzymatic defence response, cAuNPs (80 and 1600 µg.L^{-1}) significantly increased the NPT levels ($p<0.05$; Dunnett's test; Figure 8A), both in liver and gills, while PVP-AuNPs had no significant effect ($p>0.05$; ANOVA; Figure 8B).

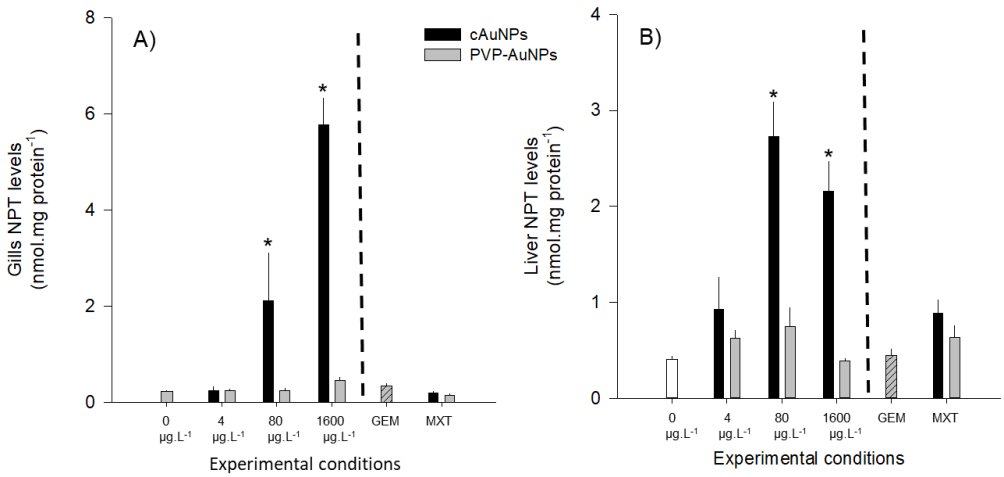

Fig. 8. Gills (A) and liver (B) non-protein thiols (NPT) levels of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone

coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are

expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM. (1.5 column)

In the activity of GST, group of enzymes involved in the xenobiotics biotransformation, AuNPs exposures did not have significant effects on gills (p>0.05; ANOVA; Figure 9A). In liver, 1600 µg.L^{-1} of PVP-AuNPs significantly increased the GST activity (p<0.05; Dunnett's test; Figure 9B). A dose-response

relationship could be found. On the contrary, the activity of GST remained unchanged after the exposure to cAuNPs (p>0.05; ANOVA; Figure 9B).

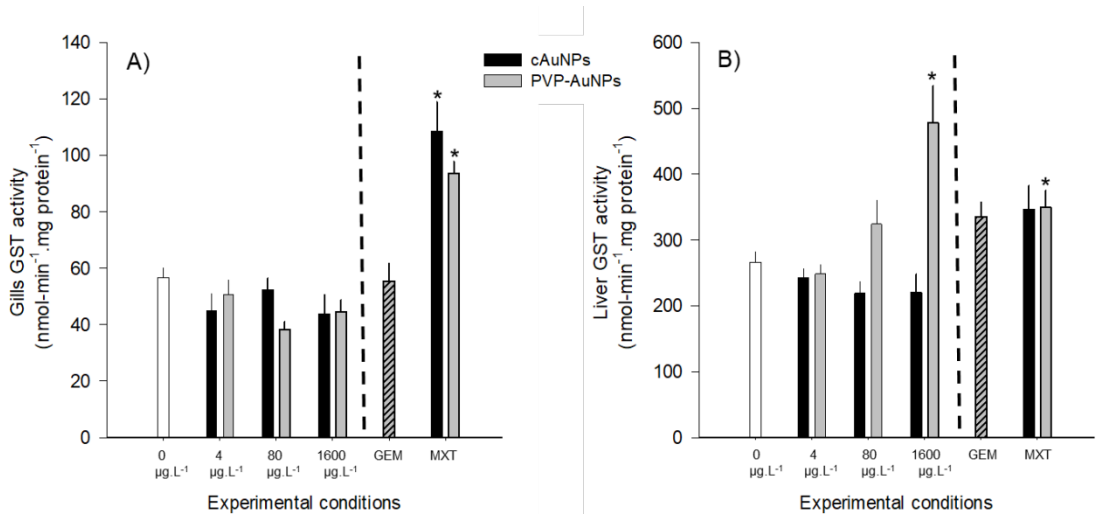

Fig. 9. Gills (A) and liver (B) glutathione *S*-transferases (GST) activity of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM).

Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM. (1.5 column)

As shown in Figure 10A, oxidative damage (assessed as TBARS levels) was found in gills. PVP-AuNPs (4 and 80 µg.L^{-1}) induced increased LPO levels (p<0.05; Dunnett's test), with PVP-AuNPs concentration increase, it was

observed a tendency to the LPO levels decreased. For cAuNPs, the LPO levels remained unchanged (p>0.05; ANOVA; Figure 10A) despite the increase in NPs

concentration. In liver, oxidative damage was not identified (Figure 10B). The obtained results, in liver, suggest that, after 96 h, the defence system (enzymatic and non-enzymatic) was efficient protecting this organ from oxidative damage.

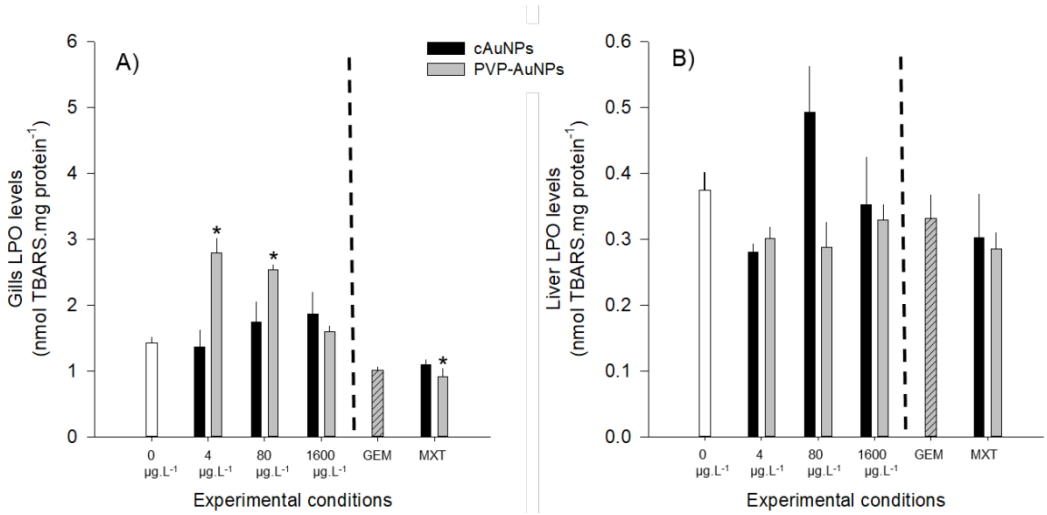

Fig. 10. Gills (A) and liver (B) lipid peroxidation (LPO) levels of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone

coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM. (1.5 column)

Comparing the present results with previous studies on AuNPs exposure in aquatic organisms, dissimilar results were found (Tedesco et al. 2008; Tedesco

et al. 2010b; Pan et al. 2012; Volland et al. 2015), which may be explained by different factors, being species specific, dependent on time of exposure and

NPs characteristics. Pan et al. (2012) reported a significant increase in CAT and

GST activity in clams *S. plana* after a 16 d exposure to 100 µg.L^{-1} of 40 nm

cAuNPs. In the present study, the exposure to 80 µg.L^{-1} did not induce

significant alterations in those enzymes' activity in gills and liver *S. aurata*. A

study with marine fish *P. microps* showed no significant differences in GST

activity, determined in all the body of fish, after 96 h exposure to 5 nm AuNPs

(0.2 mg.L^{-1}) (Ferreira et al. 2016). In mussels *M. edulis*

µg.L^{-1} of 13 nm cAuNPs, the CAT activity in their digestive gland was stimulated

(Tedesco et al. 2008). Volland et al. (2015) reported that 20 nm cAuNPs

exposure (0.75 µg.L^{-1} for 24 h) induced increased GR and GPx activity in the

digestive gland of the marine bivalve (*Ruditapes philippinarum*). However, in the

gills, cAuNPs did not show any effect on these enzymes activity (Volland et al.

2015). In the present study, PVP-AuNPs increased GPx activity in gills and liver

of *S. aurata*. Some authors also reported that AuNPs may cause damage in

aquatic organisms in the form of LPO (Tedesco et al. 2010b). However, no

oxidative damage has been reported in other studies (Ferreira et al. 2016; Pan

et al. 2012; Tedesco et al. 2008). A lack of significant changes in LPO levels

was found after 96 h exposure to 5 nm AuNPs (0.2 mg.L^{-1}) in *P. microps*

(Ferreira et al. 2016). Pan et al. (2012) reported that the defence system of *S. plana* was effective and thus the AuNPs did not induce oxidative damage in

clams. Similarly, Tedesco et al. (2008) reported in *M. edulis* exposed for 24 h to

750 µg.L^{-1} cAuNPs (13 nm) a moderate level of oxidative stress, without

increased LPO levels. However, mussels exposed to 5 nm cAuNPs displayed LPO in digestive gland, gills and mantle (Tedesco et al. 2010b).

Some authors suggest that NPs do not possess a unique toxicity mechanism. The current hypothesized nanotoxicity mechanisms include suppression of energy metabolism, oxidative damage of crucial proteins and enzymes, and increased membrane permeability, causing cell disruption (Tang et al. 2007; Khalili Fard et al. 2015). However, reactive oxygen species (ROS) generation, whose overproduction can lead to oxidative stress on the organism tissues, is the most widely accepted nanotoxicity mechanism. AuNPs have been shown to induce ROS production to different aquatic organisms (Pan et al. 2007; Tedesco et al. 2008; Farkas et al. 2010; Tedesco et al. 2010b). This toxicity seems to be dependent mainly on NPs size, aggregation/agglomeration state, coating and surface charge (Fu et al. 2014). Although PVP is considered safer and more biocompatible than citrate (Min et al. 2009; Iswarya et al. 2016), in the present study, PVP-AuNPs showed to have more effects in *S. aurata* than cAuNPs. The swimming performance of fish, LPO levels (in gills) and some enzymatic antioxidant/biotransformation responses (such as GPx and GST activities) were only affected at exposure to PVP-AuNPs. Other studies also reported the coating-dependent toxicity of NPs, with Teles et al. (2016) showing a significant impact of PVP-AuNPs in the hepatic expression of antioxidant, immune and apoptosis related genes of *S. aurata*, and no relevant effects for cAuNPs. Iswarya et al. (2016) showed that, in a swiss albino mice, PVP-AuNPs were also more toxic than cAuNPs. However, in the bacteria *Bacillus aquimaris*,

the alga *Chlorella* sp. and the cervical cancer cell line SiHa cells, cAuNPs induced more effects (Iswarya et al. 2016). In addition, some authors reported that smaller NPs with positive charge presented higher affinity for membranes and caused more biological effects (Broda et al. 2016). In the present study, the synthetized PVP-AuNPs remained in nano-size in ASW and had a ZP close to zero, while cAuNPs aggregated at the beginning of the assay, becoming bigger than 300 nm. Moreover, cAuNPs were more negative (ZP) compared to PVP AuNPs. These dissimilar characteristics and behaviour may explain the higher effects of PVP-AuNPs to *S. aurata*.

Another important issue regards the potential changes of AuNPs properties inside the organism due to a different physico-chemical environment (e.g., the presence of electrolytes, proteins and different pH). It seems that PVP may prevent the aggregation/agglomeration of AuNPs and help maintain their original characteristics *in vivo* (Schaeublin et al. 2011). For the PVP-AuNPs exposures, the gold content determined in the tissues of *S. aurata* was higher than for the exposures to cAuNPs, further supporting the previous assumptions.

These results show the importance of studying the toxicity of AuNPs with different characteristics, e.g. different sizes and coatings.

NPT levels was the only endpoint where cAuNPs caused higher effect than PVP-AuNPs. NPT is a term used to encompass all low molecular weight thiol compounds, such as reduced glutathione (GSH), which is the predominant NPT (Tedesco et al. 2010a). Despite NPT have been poorly studied (Tedesco et al.

2010a), they are known to play a pivotal role in the defence against oxidative

stress (Mulier et al. 1998). AuNPs may react directly with NPT such as GSH or may indirectly cause an imbalance in the GSH/GSSG (oxidized glutathione) ratio during oxidative stress (Renault et al. 2008; Tedesco et al. 2010a). Thiol groups are known to have high binding affinity to noble metal, in particular to gold (Sperling and Parak 2010). The presence of cAuNPs may stimulate the production of NPT and this may explain the increase of NPT levels in gills and liver of *S. aurata* after the exposure to cAuNPs. On the other hand, PVP-AuNPs may not interact with NPT as cAuNPs and, consequently, the levels of NPT remained unchanged.

Concerning combined exposures, 80 $\mu g.L^{-1}$ AuNPs and 150 $\mu g.L^{-1}$ GEM induced a significantly decreased in fish performance ($p<0.05$; Dunnett´s test; Figure 3). As in the single exposures, combined exposures did not induce significant changes in the brain and muscle ChE activity of *S. aurata* ($p>0.05$; ANOVA; Figure 4). The gills CAT activity, in the combined exposures, was similar to control ($p>0.05$; ANOVA; Figure 5A). In liver, the mixture of AuNPs (both coatings) with GEM significantly increased the CAT activity ($p<0.05$; Dunnett's test; Figure 5B). Regarding gills GR activity, in the combined exposures, the activity of this enzyme was similar to control ($p>0.05$; ANOVA; Figure 6A). On the contrary, the combined exposures to AuNPs (both coatings) with GEM significantly increased the activity of GR in liver ($p<0.05$; Dunnett's test; Figure 6B). The combination of AuNPs with GEM did not induce alterations

on the gills GPx activity ($p>0.05$; ANOVA; Figure 7A). In the liver, AuNPs (both coatings) and GEM mixture significantly increased the GPx activity ($p<0.05$; Dunnett's test; Figure 7B). The combination of AuNPs with GEM did not induce

alterations on the gills and liver NPT levels ($p>0.05$; ANOVA; Figure 8). The combined exposures significantly increased the gills GST activity ($p<0.05$; 818 Dunnett's test; Figure 9A). Concerning liver, only the mixture of PVP-AuNPs with GEM induced significant changes, increasing the GST activity ($p<0.05$; Dunnett's test; Figure 9B). When PVP-AuNPs were combined with GEM, the gills LPO levels significantly decreased ($p<0.05$; Dunnett's test; Figure 10A). As in the single exposures, combined exposures did not induce significant alterations in liver LPO levels ($p>0.05$; ANOVA; Figure 10B).

The percentage of effect on *S. aurata,* in the different assessed endpoints, after the exposure to single and combined exposures of AuNPs and GEM are shown in the Table 3. In some endpoints, the predicted percentage of effect (the sum of the percentage of the single exposures) are similar than the observed percentage of effect as in the case of swimming resistance and ChE activity (Table 3). However, in most cases, they are considerably different. For instance, in gills CAT and GR activities, the observed percentage of effect was lower than the predicted, where apparently AuNPs eliminated the adverse effects induced during GEM single exposure. In the liver CAT and GR activities, the observed percentage of effect was higher than the predicted (Table 3).

Table 3. The percentage of effect on *Sparus aurata*, in the different assessed endpoints, after a 96-h exposure to single and combined exposures of gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs)

and gemfibrozil (GEM), compared with control. Observed (O) % in the combined exposures refers to measured effects and the Predicted (P) % were derived by the sum of single exposure effects. *Significant differences to control (Dunnett´s test, $p<0.05$). #Significant differences between the combined exposure and the correspondent single exposure of nanoparticles (Tukey´s test, $p<0.05$). xSignificant differences between the combined exposure and the single exposure of GEM. (Double column)

Assessed Endpoints	% of Effect Related to Control					
	Tissues	**cAuNPs**	**PVP-AuNPs**	**GEM**	**cAuNPs + GEM**	**PVP-AuNPs + GEM**
Swimming Resistance		30	25	47 *	P: 77 O: 60 *	P: 72 O: 80 *#x
Cholinesterases Activity	**Brain**	31	19	- 18	P: 13 O: 10	P: 1 O: - 2
	Muscle	- 9	12	10	P: 1 O: - 17	P: 22 O: 13

Catalase Activity	**Gills**	22	29	-95 *	P: - 73 O: 3	P: - 66 O: - 41
	Liver	15	- 27	10	P: 25 O: - 83 *#X	P: - 17 O: - 88 *#X
Glutathione Reductase Activity	**Gills**		2	- 86 *	P: - 79 O: 34	P: - 84 O: 4
	Liver	7 - 22	17	- 67 *	P: - 89 O: - 156 *#X	P: - 50 O: - 131 *#X
Glutathione Peroxidase Activity	**Gills**	65	- 233 *		P: 97 O: - 23	P: - 201 O: - 63 #
	Liver	14	- 30	32 - 574 *	P: - 560 O: - 162 *#X	P: - 604 O: - 127 *X

Non-Protein Thiols Levels	**Gills**	-845 *	-8	- 60	P: -904 O: 10	P: -68 O: 30
	Liver	-575 *	- 84	- 12	P: - 587 O: - 119	P: - 96 O: - 56

Glutathione *S*-Transferases Activity	**Gills**	8	32	- 1	P: 7 O: - 91 *#x	P: 32 O: - 65
	Liver	18	- 22	- 26	P: - 8 O: - 30	P: - 47 O: - 31 *
Lipid Peroxidation Levels	**Gills**	- 22	- 77 *	29	P: 7 O: 23	P: - 47 O: 29 *#
	Liver	- 31	23	12	P: - 20 O: 19	P: 35 O: 24

NPs are often used to deliver drugs at high concentrations to target sites (Singh and Lillard 2009). So, it is possible that they can also carry pollutants increasing their damage to cells (Inoue and Takano 2010). The observed effects of the AuNPs and GEM combined exposures were different from the predicted. In gills, in general, the combined exposures to AuNPs and GEM were "neutral", since the fish responses were similar to the control. However, in liver, the combined exposures showed to have more effects in fish than the predicted.

These findings are highly relevant because, in the environment, there is a variety of contaminants and there is a lack of studies about the combined 855 effects of NPs and other emerging contaminants. As described above, it seems that, in ASW, GEM and AuNPs did not have a physical association. However, inside the organism they may interact and cause different effects than the predicted. In an *in vitro* study with marine mussel *M. galloprovincialis*, Luis et al.

(2016) also reported different results after the combined exposures to AuNPs and pharmaceuticals than the predicted. The GST activity increased with the exposure to carbamazepine. However, after the simultaneous exposures to AuNPs (citrate and PVP coated) and carbamazepine, the enzyme activity decreased to levels similar to the control. cAuNPs also had the same effect when combined with another pharmaceutical drug, fluoxetine (Luis et al. 2016).

Overall, after exposures to AuNPs, enzymatic and non-enzymatic responses involved in the defence of *S. aurata* against oxidative damage were more active in the liver than in gills. The oxidative damage found in gills may be explained by a generally higher accumulation of AuNPs in gills than in liver and less responsive defence mechanisms in gills than in liver. For instance, after the exposure to 1600 $\mu g.L^{-1}$ PVP-AuNPs, the activity of GST significantly increased in liver ($p<0.05$; Dunnett's test), while, in gills, it remained similar to the control. On the other hand, gills are the first organ to be exposed and are considered a good candidate to an early assessment of the effects of waterborne contaminants (Oliveira et al. 2008) while liver is the main detoxification organ. Both are known target organs of NPs toxicity (Handy et al. 2008; Lee et al. 2012; Abdel-Khalek et al. 2015; Ostaszewska et al. 2016).

3.5. Estimated gold intake by humans

The detected accumulation of AuNPs in muscle of *S. aurata*, important component of human diet, is a matter of concern. People consume the flesh of fish rather than the internal organs and thus, it is possible that NPs can be transferred to the consumer (Yoo-Iam et al. 2014; Ates et al. 2015). The highest

estimated values for gold intake were obtained for the conditions 1600 µg.L^{-1} of 883 PVP-AuNPs and 80 µg.L^{-1} of PVP-AuNPs combined with GEM (Table 4).

Table 4. 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 a 96-h single or combined exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and gemfibrozil (GEM). (Single column)

Nominal Concentrations (µg.L^{-1})	**Estimated gold intake (µg.kg body weight per year)**	
	cAuNPs	**PVP-AuNPs**
4 AuNPs	0.06	0.40
80 AuNPs	0.29	0.53
1600 AuNPs	0.28	1.41
80 AuNPs + 150 GEM	1.02	114.59

To the authors' knowledge no study is available addressing gold intake by fish consumers. However, this information is relevant and further studies are needed to understand the transfer of gold from fish to humans and to stablish the TDI of gold for humans, as already calculated for other contaminants (IPCS 2004). None of the organizations Food and Agriculture Organization of the United Nations (FAO) or World Health Organization (WHO) has established a TDI for gold due to the limited data on absorption, distribution, metabolism and excretion (ADME) as well as on the toxicological effects of gold in humans (Panel on Food Additives and Nutrient Sources Added to Food 2016).

Based in the NOAEL of gold (32.2 $mg.kg^{-1}$) in rats obtained in the study of Ahmed et al. 2012, according the formula previous presented, it was possible obtain a TDI of gold as 322 $\mu g.kg^{-1}$ body weight. In the present study, according to the calculated gold intake by humans (maximum value: 114.6 $\mu g.kg^{-1}$ body weight per year) (Table 4), this value did not exceed the estimated TDI. Based on the tested conditions and experimental results obtained, the estimated maximum gold intake by humans per day was around 0.31 $\mu g.kg^{-1}$ body weight. The results of the present study showed potential toxic effects of AuNPs both at higher and environmentally relevant concentrations. The present findings support the idea that the bioaccumulation and toxicity of AuNPs is dependent on the size, coating, surface charge and aggregation/agglomeration state of NPs, and on the presence of other chemicals. Further studies are encouraged with AuNPs presenting different characteristics, e.g. size and coatings (alone or

combined exposures) to increase the knowledge about their biological effects to

fish using different exposure conditions (such as longer exposure periods) and, being a species for human consumption, the NPs transfer to the consumer.

4. Conclusions

The present study provides relevant information about the accumulation and possible toxic effects of gold nanoparticles (AuNPs) to an economically important marine fish species, the top predator seabream *Sparus aurata*. Induction of antioxidant enzymatic and non-enzymatic responses were observed following exposure to AuNPs, both alone or in combined exposure with a common pharmaceutical drug (gemfibrozil). PVP (polyvinylpyrrolidone)

coating increased the stability of AuNPs in artificial seawater and consequently increased its bioavailability and accumulation into the fish tissues. Decreased

swimming performance of fish and increased lipid peroxidation in gills were observed following exposure to PVP-AuNPs. The present findings showed that the assessment of behavioural and oxidative stress/damage biomarkers, together with NPs characterization and bioaccumulation, represents a sensitive tool to increase the knowledge about the toxicity of NPs to marine fish species. Although the calculated gold intake by humans did not exceed the estimated tolerable daily intake, this is an important assessment and highly recommended in studies with fish for human consumption.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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