Universidade de Aveiro Departamento de Biologia
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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Marcelino Miguel Oliveira, Investigador Auxiliar do Departamento de Biologia & CESAM da Universidade de Aveiro, Professora Doutora Susana Loureiro, Professora Auxiliar com Agregação do Departamento de Biologia & CESAM da Universidade de Aveiro, Professor Doutor Ketil Hylland, Professor do Departamento de Biociências da Universidade de Oslo.

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Dedico este trabalho às pessoas mais importantes da minha vida:
aos meus pais, aos meus irmãos e ao Bruno.

o júri

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Doutor Marcelino Miguel Guedes de Jesus Oliveira (orientador)
Investigador Auxiliar do Departamento de Biologia da Universidade de Aveiro

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palavras-chave

Nanopartículas de ouro; gemfibrozil; exposições combinadas; *Sparus aurata*; *in vivo*; *in vitro*; comportamento; stress oxidativo; dano oxidativo; genotoxicidade; proteoma.

resumo

As áreas estuarinas e costeiras são o receptor final para muitos contaminantes, incluindo contaminantes emergentes como as nanopartículas (NPs) e os fármacos. As nanopartículas de ouro (AuNPs) são usadas numa ampla gama de aplicações, podendo ser libertadas para o ambiente. Um dos requisitos fundamentais para o vasto uso das AuNPs é a sua presumível natureza não tóxica e biocompatível, embora estudos recentes tenham mostrado a sua possível toxicidade, incluindo stress oxidativo, genotoxicidade e alterações em proteínas. Estes resultados levantam preocupações acerca do impacto das AuNPs em organismos aquáticos e para a saúde humana.

Tendo em conta a informação contraditória acerca da toxicidade das AuNPs, a importância das suas características nos efeitos produzidos e o conhecimento limitado acerca dos seus efeitos em espécies de peixe marinhas/estuarinas, esta tese teve como objetivo responder à pergunta geral: Irá a exposição a AuNPs afetar respostas moleculares, bioquímicas e comportamentais da dourada (*Sparus aurata*)?

Para responder a esta questão, foram realizados ensaios *in vitro* (24 h; culturas de fígado) e *in vivo* (96 h de exposição) testando AuNPs de 7 e 40 nm, revestidas com citrato ou polivinilpirrolidona (PVP), individualmente e combinadas com o fármaco gemfibrozil (GEM). As gamas de concentrações testadas variaram entre 4 a 7200 $\mu g.L^{-1}$ e 4 a 1600 $\mu g.L^{-1}$ nos ensaios *in vitro* e *in vivo*, respetivamente. Na exposição *in vitro*, foram avaliados parâmetros de stress/dano oxidativo, biotransformação e genotoxicidade. *In vivo*, foram avaliados efeitos a diferentes níveis de organização biológica (comportamento, neurotransmissão, biotransformação, stress/dano oxidativo, genotoxicidade e alteração em proteínas).

Em meio de cultura, o tamanho das AuNPs testadas alterou-se nas primeiras 12 h de incubação com a formação de agregados/aglomerados maiores que 100 nm. Os agregados/aglomerados das nanopartículas de ouro de 7 nm revestidas com polivinilpirrolidona (PVP-AuNPs) apresentaram tamanhos menores e induziram mais efeitos do que as nanopartículas de 7 nm revestidas com citrato (cAuNPs) e as AuNPs de 40 nm. Os resultados dos ensaios com culturas de fígado mostraram que as AuNPs têm a capacidade de induzir as atividades da catalase (CAT) e glutationa redutase (GR), induzir quebras na cadeia de ADN e peroxidação lipídica (LPO).

Em água salgada, as cAuNPs de 7 nm, quase imediatamente agregaram/aglomeraram e aumentaram o seu tamanho (aggregados/aglomerados de 160 nm), induzindo mais efeitos em *S. aurata* do que as de PVP-AuNPs (7 e 40 nm), apesar da estabilidade das PVP-AuNPs neste meio. As cAuNPs de 7 nm causaram também mais efeitos do que as cAuNPs de 40 nm que formaram agregados/aglomerados de 340 nm em água salgada. *In vivo*, a acumulação de ouro nas brânquias, fígado e baço da dourada foi maior do que no músculo. A acumulação de ouro nos tecidos foi dependente das características das AuNPs, principalmente com o revestimento, verificando-se uma maior acumulação de ouro após exposição a PVP-AuNPs (comparando com cAuNPs). De um modo geral, a indução das defesas enzimáticas (CAT, GR, glutationa peroxidase (GPx) e glutationa *S*-transferases (GST)) e não enzimáticas (tióis não proteicos (NPT)) foi detetada depois da exposição *in vivo* a AuNPs, nas brânquias e fígado. A diminuição da capacidade natatória da dourada face a um fluxo de água constante foi observada após 96 h de exposição às AuNPs, o que pode ser considerado um efeito ecológico relevante após exposição a NPs. Dano oxidativo nas brânquias e fígado (níveis de LPO aumentados), aumento das quebras na cadeia de ADN e da frequência de anomalias nucleares nos eritrócitos foram detetados depois da exposição *in vivo* às AuNPs.

resumo

As AuNPs induziram também alterações na abundância de proteínas presentes no fígado da dourada, com cAuNPs de 7 nm induzindo mais efeitos que as PVP-AuNPs de 7 nm e as AuNPs de 40 nm. A análise das respostas por tecido mostrou que as brânquias da dourada foram mais sensíveis do que o fígado, nas exposições às AuNPs (individuais e em combinação com o fármaco).

Nos ensaios *in vitro* e *in vivo*, a avaliação dos efeitos combinados das AuNPs (7 ou 40 nm) e GEM mostrou que as percentagens esperadas de efeito (a soma da percentagem das exposições individuais) foram, para a maioria dos parâmetros avaliados, diferentes das percentagens de efeito observadas, representando possíveis padrões antagonistas – no caso das anomalias nucleares e dano no ADN dos eritrócitos, ou sinergistas – nas atividades da CAT e GR no fígado depois da exposição às AuNPs de 40 nm com o GEM e níveis de NPT nas brânquias depois da exposição às AuNPs de 7 nm com o GEM.

De uma forma geral, os efeitos da exposição a AuNPs dependeu da concentração, tamanho e revestimento das NPs e da presença de outros contaminantes. Os resultados dos ensaios *in vitro* e *in vivo* mostraram que as AuNPs de tamanho menor (7 nm) induziram mais alterações e, em termos de revestimento, foram encontradas respostas específicas em cada ensaio, com o revestimento PVP e citrato a ser mais biologicamente ativo no ensaio *in vitro* e *in vivo*, respetivamente. Os resultados mostraram que as AuNPs não são inertes, mesmo a concentrações baixas como 4 $\mu g.L^{-1}$, levantando preocupações acerca da segurança do seu uso em aquacultura, aplicações biomédicas ou outras áreas.

Os resultados mostraram que a abordagem multiparamétrica usada nesta tese, integrando a avaliação de efeitos *in vivo* de biomarcadores comportamentais e de stress/dano oxidativo, genotoxicidade e alterações proteícas, juntamente com a caracterização e bioacumulação de NPs, foi essencial para aumentar o conhecimento acerca da toxicidade das NPs para espécies de peixe marinhas. Adicionalmente, as culturas de fígado foram sensíveis a concentrações baixas dos contaminantes testados e permitiu diferenciar as respostas a AuNPs com diferentes características, realçando o seu uso como alternativa aos testes *in vivo*.

keywords

Gold nanoparticles; gemfibrozil; combined exposures; *Sparus aurata*; *in vivo*; *in vitro*; behaviour; oxidative stress; oxidative damage; genotoxicity; proteome.

abstract

Estuarine and coastal areas are the ultimate recipient for most contaminants, including emerging contaminants of concern such as nanoparticles (NPs) and pharmaceuticals. Gold nanoparticles (AuNPs) are used for a wide range of applications and have the potential to be extensively released into the environment. One of the fundamental requirements for the wide use of AuNPs is their presumed non-toxic and biocompatible nature, but recent studies have highlighted their possible toxicity, including oxidative stress, genotoxicity and protein modifications. These findings raise concerns about the potential impact of AuNPs on aquatic organisms and ultimately on human health.

Considering the conflicting information about the toxicity of AuNPs, the relevance of their characteristics on the induced effects and the limited knowledge pertaining to any effects on marine/estuarine fish species, this thesis aimed to answer this general question: Will exposure to AuNPs affect molecular, biochemical and behavioural responses of *Sparus aurata*?

To answer this general question, *in vitro* (24 h liver organ culture) and *in vivo* (96 h of exposure) assays were performed testing 7 and 40 nm AuNPs with either citrate or polyvinylpyrrolidone (PVP) coating, alone and combined with the pharmaceutical gemfibrozil (GEM). Tested concentrations ranged from 4 to 7200 $\mu g.L^{-1}$ and 4 to 1600 $\mu g.L^{-1}$ in the *in vitro* and *in vivo* assays, respectively. *In vitro*, oxidative stress/damage and biotransformation responses as well as genotoxicity were evaluated. *In vivo*, effects at different levels of biological organization (behaviour, neurotransmission, biotransformation, oxidative stress/damage, genotoxicity and proteins alterations) were evaluated.

In cell culture media, the size of all tested AuNPs was altered within 12 h of incubation with the formation of aggregates/agglomerates larger than 100 nm. Aggregates/agglomerates of 7 nm polyvinylpyrrolidone coated gold nanoparticles (PVP-AuNPs) had smaller sizes and induced more effects than 7 nm citrate coated gold nanoparticles (cAuNPs) and 40 nm AuNPs. The results from *S. aurata* liver organ culture assays showed that AuNPs induced catalase (CAT) and glutathione reductase (GR) activities, DNA strand breaks and lipid peroxidation (LPO).

In seawater, 7 nm cAuNPs, almost immediately aggregated/agglomerated and increased their sizes (160 nm), inducing more effects on *S. aurata* than PVP-AuNPs (7 and 40 nm), despite PVP-AuNPs observed stability. Also, 7 nm cAuNPs induced more effects than 40 nm cAuNPs which formed agglomerates/aggregates of 340 nm in seawater. *In vivo*, gold accumulation in *S. aurata* gills, liver and spleen was higher than in muscle. The observed gold accumulation was dependent on the characteristics of AuNPs, mostly on the coating, with higher accumulation after exposure to PVP-AuNPs compared to cAuNPs. Overall, induction of enzymatic (e.g. CAT, GR, glutathione peroxidase (GPx) and glutathione *S*-transferases (GST)) and non-enzymatic (non-protein thiols – NPT) defences was found after *in vivo* exposure to AuNPs, both in gills and liver. Decreased ability of *S. aurata* to continue swimming against a water flow, which can be considered an ecologically relevant effect of NPs exposure, was observed after 96 h AuNPs exposure. Gills and liver oxidative damage (increased LPO levels) and increased erythrocytes DNA strand breaks and frequency of nuclear abnormalities were detected after AuNPs *in vivo* exposure.

abstract

AuNPs also induced alterations in the abundance of *S. aurata* liver proteins, with 7 nm cAuNPs inducing more effects than 7 nm PVP-AuNPs and 40 nm AuNPs. The analysis of the tissues responses showed that the gills of *S. aurata* were more sensitive than the liver, both in the single and combined exposures to AuNPs.

In the *in vitro* and *in vivo* experiments, the assessment of the combined effects of AuNPs (7 or 40 nm) and GEM showed that the predicted percentages of effect (the sum of the percentage of the single exposures) were, for most of the tested endpoints, different than the observed percentages of effect, representing possible antagonistic (e.g. erythrocytic nuclear abnormalities and DNA damage) or synergistic (e.g. hepatic CAT and GR activities for 40 nm AuNPs with GEM and gills NPT content for 7 nm AuNPs with GEM) patterns.

Overall, the effects of exposure to AuNPs depended on the concentration, size and coating of NPs and the presence of other contaminants. Data from the *in vitro* and the *in vivo* assays showed that the smaller AuNPs (7 nm) induced more alterations and, in terms of coating, assay specific responses were found, with PVP and citrate coating AuNPs being more biologically active in the *in vitro* and *in vivo* assay, respectively. The results showed that AuNPs are not inert, even at low concentrations as 4 $\mu g.L^{-1}$, raising concern about its safety for use in aquaculture, biomedical applications or other areas.

The findings showed that the multiparametric approach used in this thesis, integrating the evaluation of the *in vivo* effects of behavioural and oxidative stress/damage biomarkers, genotoxicity and proteins alterations, together with NPs characterisation and bioaccumulation, was essential to increase the knowledge about the toxicity of NPs to marine fish species. Addittionally, the liver organ culture of *S. aurata* was sensitive to low concentrations of the tested contaminants and could be used to differentiate responses to AuNPs with different characteristics, supporting its use as an alternative to *in vivo* testing.

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1. GENERAL INTRODUCTION

1.1. Marine environment

The oceans are vital for humans, both economically (e.g. shipping industry; areas and resources for aquaculture production; food for human consumption) and socially (e.g. recreation/tourism activities) (Janssen et al. 2011). However, it is crucial that the biodiversity, resources and environmental quality of marine ecosystems are conserved, protected and sustainably managed, as the anthropogenic pressures continue to increase (Janssen et al. 2011). One of the main anthropogenic associated pressures affecting the marine environment is pollution: the release and subsequent effects of chemicals and particles from industrial, agricultural and domestic waste into lakes, rivers, transitional waters and the ocean (Jiang, Lee, and Fang 2014). Coastal environments are considered the ultimate sink for contaminants released into the environment. Some of the contaminants may induce harmful effects to aquatic species and, ultimately, human health through food-chain transfer. Adverse effects may lead to alterations on the structure and functioning of the ecosystems, reducing biodiversity and productivity with consequent reduction and depletion of marine food resources (Janssen et al. 2011).

Aquatic pollution is currently a global concern and most developing nations are still producing huge pollution loads with increasing trends expected (Shahidul Islam and Tanaka 2004).

1.2. Emerging Contaminants of Concern

The contaminants commonly found in marine ecosystems include persistent organic pollutants, metals and pesticides. Numerous contaminants have not yet been regulated, which have been referred as emerging contaminants of concern (ECs) by the United States Environmental Protection Agency (EPA) or Emerging Substances by EU NORMAN network (Brumovský et al. 2017). Over the last decades, the term ECs has been increasingly used, incorporating not only new synthetic or natural compounds or microorganisms, but also other contaminants

recently detected in the environment due to the development of new detection methods (Richardson and Ternes 2014). Thus, ECs entering the aquatic environment due to their continuous and widespread use and limited elimination or degradation include a wide range of man-made compounds, as well as metabolites and transformation products. ECs have been categorized into more than 20 classes related to their characteristics (e.g. purpose and nature) such as personal care products, corrosion inhibitors, industrial chemicals, illicit drugs, food additives disinfection by-products and biocides (Alygizakis et al. 2016; Arpin-Pont et al. 2016; Brumovský et al. 2016; Brumovský et al. 2017; Gogoi et al. 2018). Some common categories of ECs are presented in Table 1.

Table 1. Classes and examples of emerging contaminants of concern (ECs).

Classes	Examples	Definition
Pesticides	Dimethoate, deltamethrin	Agents that deter, incapacitate, kill, or otherwise discourage pests.
Pharmaceuticals	Diclofenac, gemfibrozil, fluoxetine	Substances used to prevent, diagnose and treat diseases.
Plastics (micro and nano)	Polyethylene, polystyrene particles	Polymeric particles with sizes smaller than 5 mm.
Nanoparticles	Gold, silver, graphene	Particles typically smaller than 100 nm.

More than 700 ECs, their metabolites and transformation products, have been listed as present in the European aquatic environment. In the European Union, a watch list of ECs has been compiled from national monitoring programs, as a requirement of the Marine Framework Directive. This list presents ECs requiring further attention due to their high frequency of occurrence, the expected risk for human health and/or aquatic life, and/or for a lack of analytical techniques (Geissen et al. 2015).

Due to their hydrophilic nature, numerous ECs are very mobile in the aqueous phase and consequently may be transported by receiving rivers to marine

environments (Brumovský et al. 2017). ECs predominantly reach marine waters via riverine inputs and sewage effluents, in addition to some substances used in aquaculture (Bueno et al. 2012; Jiang, Lee, and Fang 2014; Zheng et al. 2012). ECs are incorporated into consumer products, essential to daily life, health care, food production and sanitation. Therefore, the discharges from wastewater treatment plants (WWTPs) represent a dominant input pathway to the marine environment in developed countries (Brumovský et al. 2017; Verlicchi, Al Aukidy, and Zambello 2012). In addition, passenger ships are allowed to discharge wastewater and sewage sludge in the open sea and are therefore possible sources (Brumovský et al. 2017).

The occurrence and fate of various ECs (e.g. pharmaceuticals) in freshwater environments is already well documented (Li 2014; Murray, Thomas, and Bodour 2010; Hughes, Kay, and Brown 2013) but their presence in coastal and marine ecosystems is much less studied and understood (Arpin-Pont et al. 2016; Brumovský et al. 2017; Gaw, Thomas, and Hutchinson 2014).

Some ECs have been intentionally designed to affect living systems even at relatively low doses (e.g., pesticides, pharmaceuticals and illicit drugs). Exposure to low levels of those substances or their metabolites may impact coastal/marine ecosystem functions and structures, particularly as a result of chronic exposure (Brumovský et al. 2017; Fent, Weston, and Caminada 2006). The ability of silver nanoparticles (NPs), which are among the most studied NPs, to affect different trophic levels (e.g. in terms of growth, behaviour and mortality) in marine ecosystems has been reported previously (Gambardella et al. 2015). Effects caused by pharmaceuticals may be diverse, ranging from oxidative stress reported in clams exposed to carbamazepine (Almeida et al. 2015) to behavioural alterations of crabs after exposure to fluoxetine (Peters et al. 2017).

1.2.1. Nanoparticles

The prefix “nano” derived from the Greek “nanos” signifying “dwarf”. NPs are defined as particles having at least one dimension between one to one hundred nm (ASTM 2012) and have special physical and chemical properties compared to their bulk materials (Farkas et al. 2010; Niemeyer 2001). Nanomaterial means a

natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 to 100 nm. In specific cases and where warranted by concerns for the environment, health, safety or competitiveness the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%. For this definition, “particle”, “agglomerate” and “aggregate” are defined as follows: particle means a minute piece of matter with defined physical boundaries; agglomerate means a collection of weakly bound particles or aggregates where the resulting external surface area is similar to the sum of the surface areas of the individual components; aggregate means a particle comprising of strongly bound or fused particles (European Commission, 2011). NPs are not restricted to recent man-made materials, as they are abundant in nature. Natural occurring NPs include organic (proteins, polysaccharides, viruses, among others) and inorganic particles (e.g. iron oxyhydroxides, aluminosilicates, metals), which are produced by simple erosion, volcanic eruptions, wildfires, microbial processes and by plants and animals (Buzea, Pacheco, and Robbie 2007; Heiligtag and Niederberger 2013; Hough, Noble, and Reich 2011). NPs may present dissimilar characteristics such as size, shape, composition and surface functionalities and each type of NPs can potentially be synthesised using different methods (Wang and Wang 2014). Therefore, NPs may be classified into different categories based on e.g. their physical/chemical characteristics, morphology, composition, uniformity/agglomeration or application (Table 2).

NPs are currently considered ECs (Sauve and Desrosiers 2014) due to: their increased development, production and use; their size dependent characteristics, fate, uptake and biological impact, which are dependent of the medium where they are present; the uncertainty of their potential toxicological effects.

NPs have been increasingly important in the development of novel devices which may be used in numerous physical, biological, biomedical and pharmaceutical applications (Khan, Saeed, and Khan 2017; Loureiro et al. 2016; Nikalje 2015). Nanotechnology is present in commercially available products for home, cars, computers and other electronics devices and in cosmetic (Khan,

Saeed, and Khan 2017), and has been touted as the next revolution in many industries including food processing and packaging (Khan, Saeed, and Khan 2017). The environmental applications of NPs include environmentally and/or sustainable products for pollution prevention, remediation of contaminated environments and as sensors (Khan, Saeed, and Khan 2017; Tratnyek and Johnson 2006).

Table 2. Classifications of nanoparticles (NPs), based on Buzea, Pacheco, and Robbie 2007.

Based on:	Nanoparticles classification and examples
Physical and chemical characteristics	**Carbon-based** (such as graphene and carbon nanotubes)**, metallic** (such as gold and silver) **and polymeric** (such as chitosan and polymethylmethacrylate NPs).
Morphology	**High-aspect ratio** (nanotubes and nanowires) and **low-aspect ratio** NPs (spherical, oval and cubic morphologies).
Composition	**Single constituent material** or **composite** (such as when NPs are coated).
Uniformity and agglomeration	**Dispersed aerosols**, **suspensions/colloids**, or in **agglomerates.**
Applications	**Organic** (such as micelles, dendrimers, liposomes, and compact polymeric NPs) and **inorganic** NPs (gold, silver, platinum and silica NPs).

1.2.1.1. Gold Nanoparticles – a Brief Historical Perspective

Gold is one of the first metals been discovered (Daraee et al. 2016) and the study of gold and its applications spans at least several thousand years. The first data on colloidal gold can be found in treatises by Chinese, Arabian and Indian scientists, who obtained colloidal gold as early as in the V–IV centuries BC, using it for medicinal purposes. In Europe, during the Middle Ages, colloidal gold was

studied and used in alchemist laboratories. Paracelsus wrote "*Quinta Essentia Auri*" about the therapeutic properties of gold, using the "potable gold" for the treatment of several mental diseases and others (Daraee et al. 2016; Dykman and Khlebtsov 2011). The use of AuNPs is intimately related to the history of red-coloured glass (Louis 2017). The production of red glass colloidal gold was used to make ruby glass and for colouring ceramics, and these applications are continuing now.

The big development in NPs research, from a scientific point of view, was made by Michael Faraday in 1857. His systematic studies on the interaction of light with metal NPs can be regarded as the beginning of modern colloid chemistry and the emergence of Nanoscience and Nanotechnology. Faraday presented his work on 'Experimental Relations of Gold (and other Metals) to Light' to the Royal Society of London (Faraday 1857). He prepared his colloidal gold dispersions in a two-phase system consisting of an aqueous solution of a gold salt and a solution of phosphorus in carbon disulphide. After a short reaction time, the bright yellow colour of the $Na[AuCl_4]$ solution turned into a ruby colour characteristic of AuNPs (Das et al. 2011; Heiligtag and Niederberger 2013; Thompson 2007; Sharma, Park, and Srinivasarao 2009). Forty years later, based on Faraday's discovery, Zsigmondy introduced the procedure called "seed mediated method", which is still currently used for the synthesis of several NPs (Sharma, Park, and Srinivasarao 2009). At same time, new equipment and methodologies to characterise NPs were developed (Svedberg 1921; Svedberg, Pedersen, and Bauer 1940; Svedberg and Tiselius 1928; Zsigmondy and Alexander 1909) and Mie advanced his theory to explain the different colours of colloidal gold dispersions (Mie 1908). Bulk gold is yellow whereas gold at the nanoscale may appear red, blue, green or brown (Alkilany and Murphy 2010).

AuNPs present characteristics that make them attractive for human use such as their easy preparation, the variety of types that may be synthetized, size-related electronic, magnetic, catalytic and optical properties, their behaviour in different media and ability of several molecules of biological interest to attach to them (Daniel and Astruc 2004; Giasuddin, Jhuma, and Haq 2013; Sobczak-Kupiec et al. 2011).

Notwithstanding its centuries-old history, the “revolution in immunochemistry” associated with the use of AuNPs in biological studies happened in 1971, when the researchers Faulk and Taylor described a method of antibody conjugation with colloidal gold (Page and Taylor 1971). Over the past 50 years, there have been several investigations in the application of functionalized AuNPs conjugated with recognizing biological macromolecules like antibodies and enzymes (Dykman and Khlebtsov 2011).

1.2.1.2. Gold Nanoparticles – Synthesis, Characterisation and Applications

AuNPs may be manufactured into a variety of shapes including: nanospheres, nanorods, nanobelts, nanocages, nanoprisms and nanostars, with chemical, optical and electromagnetic properties of AuNPs strongly influenced by their size and shape (Thakor et al. 2011). AuNPs are synthesized, in general, by the chemical reduction of chloroauric acid ($HAuCl_4$) using various reducing agents (Low and Bansal 2010; Lu et al. 2008; Sharma, Park, and Srinivasarao 2009). The reduction process origins Au^{3+} to be reduced to neutral gold atoms which further become supersaturated and precipitated as more gold atoms aggregate to form sub-nanogold particles (Sardar et al. 2009). There are numerous methods employed in the synthesis of AuNPs that include modifications of the classic methods (Brust et al. 1994; Chauhan et al. 2011; Kimling et al. 2006; Martin et al. 2010; Perrault and Chan 2009; Srivastava et al. 2013; Vinodgopal et al. 2010). These methods are being adapted to produce AuNPs with different sizes and forms using various reducing agents (Balasubramanian et al. 2010; Low and Bansal 2010; Srivastava et al. 2013; Wu, Liu, and Huang 2006). The diverse applications of AuNPs and the need of great stability in high ionic strength media such as biological media, has driven researchers to explore alternative methods of synthesis (Min et al. 2009) and different coating agents (Jokerst et al. 2011; Mahl et al. 2010; Manson et al. 2011; Min et al. 2009; Nghiem et al. 2010; Nghiem et al. 2012; Pyshnaya et al. 2014) such as bovine serum albumin (BSA), polyvinylpyrrolidone (PVP) and polyethylene glycol (PEG). Furthermore, these ligands stabilized AuNPs can be modified by attaching other functional groups based on the application of AuNPs.

The most commonly used methodology to characterise NPs include Dynamic Light Scattering (DLS) which allow determine the hydrodynamic size and size distribution of NPs and Transmission Electron Microscopy (TEM) analysis which offers relevant data regarding the primary size, shape, the dimensional range and size distribution of NPs. Image analysis on the TEM micrographs gives the 'true radius' of the particles and DLS provides the hydrodynamic radius on an ensemble average. The hydrodynamic radius is the radius of a sphere that has the same diffusion coefficient within the same viscous environment of the particles being measured. It is directly related to the diffusive motion of the particles (Lim et al. 2013). Figure 1 shows two NPs coated with macromolecules and the major interactions involved between them. The equilibrium of the interactions is crucial to the colloidal stability of NPs suspension. Difference between "true radius" and hydrodynamic radius is also represented in the figure.

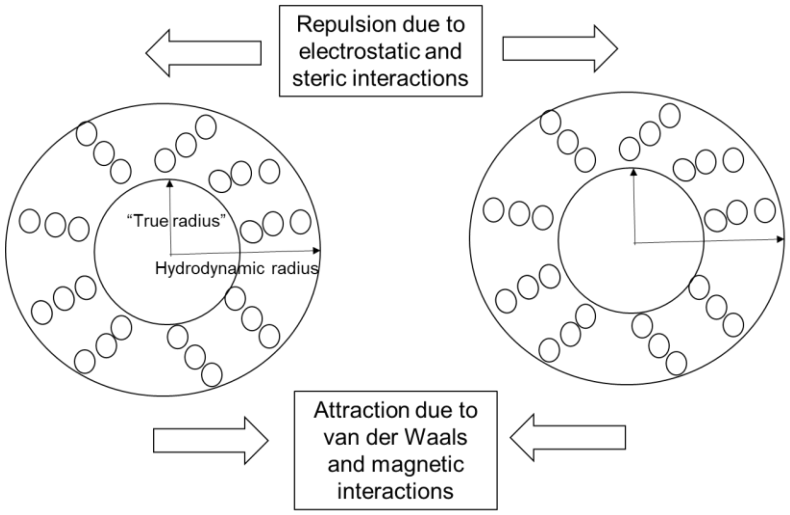

Figure 1. Representation of two nanoparticles and major interactions.

The characterisation of AuNPs is crucial as their characteristics are determinant in their applications as well as bioavailability, bioaccumulation and toxicity to biological systems (Sengani, Grumezescu, and Rajeswari 2017).

AuNPs surface exhibits a peculiar surface plasmon resonance (SPR) phenomenon, resulting in strong extinction of radiating light wavelength. This unique activity related to AuNPs optical properties – which is missing in bulk

material – is conferred by the collective oscillation of free conduction electrons within the metal after interaction with the concerned electromagnetic field. SPR can be localized in the broad region, from the visible to the infrared (IR) region, depending on the particle size, shape and structure (Dykman and Khlebtsov 2011; Pattnaik 2005; Sengani, Grumezescu, and Rajeswari 2017). The surface charge of AuNPs, estimated in terms of zeta potential, facilitates their physicochemical stability and further implementation in the cellular process and bioaccumulation (Sengani, Grumezescu, and Rajeswari 2017). The SPR peak and the zeta potential are frequently evaluated to characterise the NPs (Lin et al. 2014).

AuNPs are considered a good ‘‘model particle’’ to study the behaviour of NPs in different media because they may self-associate after many different treatments including heating (Xu et al. 2007), pH changes (Nam et al. 2009), addition of salts (Jans et al. 2009) or proteins (Lacerda et al. 2010) and are insoluble.

In terms of applications, AuNPs have been intensively applied for medical applications. They are considered as one of the most convenient carrier systems, given their reported enhanced biocompatibility, stability and oxidation resistance. Thus, AuNPs with different sizes and shapes are applicable in various medical-related research fields, including sensing and detection of microorganisms and cancer cells, catalysis and bioelectronics, drug/biomolecules delivery carriers and macromolecular carriers, bioimaging and photo hyperthermia (He et al. 2013; Murphy et al. 2008; Sengani, Grumezescu, and Rajeswari 2017). AuNPs have been used in many medical applications: diagnostics, therapy, prevention and hygiene. In addition to medical applications, AuNPs are used in electronics, cosmetic and food industries (Lapresta-Fernández, Fernández, and Blasco 2012). 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 increased use of AuNPs in various applications, the global AuNPs market is expected to reach USD 4.86 billion by 2020 and analysts have predicted that the global AuNPs market will grow at a compound annual growth rate of 19% during the period 2017-2021 (Kumar 2015).

1.2.1.3. Gold Nanoparticles – Toxicity

Nanomaterials, even when they are made from inert elements (such as gold) may become highly biologically active when they are present in nanometer dimensions (Ai et al. 2011; Boverhof et al. 2015). Nanotoxicological studies are used to determine whether and to what extent these properties may pose a threat to the environment and to human health (Buzea, Pacheco, and Robbie 2007).

Despite the development of nanotechnology and nanomaterials throughout the last 10–20 years, the potential toxicological effects of NPs on humans, animals and in the environment has only recently received some attention (Jahangirian et al. 2017).

The enormous range of potential applications of AuNPs and their increased future use could result in greater risk of environmental release and exposure at low concentrations, as is the case with many pharmaceutical products (Mahapatra et al. 2015; Miller et al. 2015; Ramirez et al. 2009). AuNPs have the potential to become a significant nanomaterial in the environment (Hull et al. 2011; Klaine et al. 2008). Information available on the current levels of AuNPs in aquatic media is limited to predicted concentrations arising from use in consumer products (0.1 $\mu g.L^{-1}$ in aquatic environments) (García-Negrete et al. 2013; Tiede et al. 2009).

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

There is clearly a need for increased research on the toxicological effects of AuNPs to non-target organisms, particularly to marine organisms, its behaviour and their characteristics' alterations (e.g., surface charge, size and shape) at high ionic strength media, such as in marine environments.

1.2.2. Pharmaceuticals

Pharmaceuticals are molecules designed to produce a therapeutic effect on the body, generally active at low concentrations. Pharmaceuticals may pass through biological membranes and persist in the body long enough to avoid being inactivated before having an effect (Bottoni, Caroli, and Caracciolo 2010). Pharmaceuticals are considered ECs due to their continuous environmental release (as parental compound and/or metabolites). These substances may appear in the environment due to inefficient wastewater treatment processes, high environmental persistence and low degradation rates (Gaw, Thomas, and Hutchinson 2014; Jelic et al. 2011).

The use and consumption of pharmaceuticals is continuously increasing due to the discovery of new drugs, expanding populations and the age structure in the general population, as well as due to expiration of patents with a resulting increased availability of less expensive generics (Jelic et al. 2011). Some of the most popular groups of pharmaceuticals are non-steroidal anti-inflammatory drugs, antibiotics, anti-epileptics, β-blockers and lipid regulators.

While the concentrations of pharmaceuticals in the aquatic environments are generally very low (ng.L^{-1} to µg.L^{-1}), many chemicals have been shown to affect aquatic organisms at these concentrations, particularly oxidative stress and damage responses (Aguirre-Martinez, DelValls, and Martin-Diaz 2015; Gonzalez-Rey and Bebianno 2014; Jones, Voulvoulis, and Lester 2001). Most of the pharmaceuticals are only partially transformed or retained in the body and they are excreted through faeces and urine as unchanged parent compound, a mixture of metabolites or conjugates (generally glucuronides) (Bottoni, Caroli, and Caracciolo 2010). Even when pharmaceuticals are extensively metabolised, their metabolites may continue to be biologically active, can be more persistent and more toxic than the parent compound or, in some cases, be easily transformed into the parent compound by hydrolysis or due to bacterial action (Gaw, Thomas, and Hutchinson 2014; Halling-Sørensen et al. 1998). Thus, significant amounts of the parent compound in the unmetabolised form or as metabolites are continuously released to sewage systems, reaching WWTPs. As a result of the inadequate removal in WWTPs, the discharge of effluents is considered the primary pathway of

pharmaceuticals release into the environment (Gaw, Thomas, and Hutchinson 2014; Jelic et al. 2011). However, these compounds may also reach the environment (aquatic and terrestrial) through direct release from production industries, irrigation with treated wastewater, veterinary treatments, disposal of sewage from intensive livestock farming site, intensive aquaculture systems and incorrect household disposal of unused pharmaceuticals via trash or sewage (Bottoni, Caroli, and Caracciolo 2010; Ebele, Abou-Elwafa Abdallah, and Harrad 2017; Gaw, Thomas, and Hutchinson 2014; Jelic et al. 2011). The half-life of a chemical in the environment is determined by a combination of chemical-specific characteristics and environmental conditions (Bu et al. 2016). Loffler et al. categorised 10 pharmaceuticals and pharmaceutical metabolites into low, moderate and high persistence compounds according to their dissipation time (DT_{50}) in water/sediment samples. Paracetamol and ibuprofen were classified as low persistent (DT_{50} < 20 d); ivermectin and oxazepam were moderately persistent with DT_{50} values of 15 and 54 d, respectively; and clofibric acid, diazepam and carbamazepine were rated highly persistent (DT_{50} > 365 d) (Ebele et al. 2017).

Over the last 20 years there has been an increasing effort to understand the presence and impacts of pharmaceuticals present in freshwater ecosystems. By contrast, significantly less attention has been paid to understand the potential impacts of pharmaceuticals in coastal environments (Gaw, Thomas, and Hutchinson 2014). There is currently minimal data on the toxicity of pharmaceuticals to marine organisms. Despite the limited number of studies, a variety of adverse effects have been reported for marine organisms with the effects being both test species and pharmaceutical specific (Gaw, Thomas, and Hutchinson 2014). Pharmaceuticals have demonstrated the ability to alter biochemical and behavioural endpoints (e.g. mussel *Mytilus galloprovincialis* exposed to diclofenac (Gonzalez-Rey and Bebianno 2014) and in the clams (*Venerupis decussata*, *Venerupis philippinarum* and *Ruditapes philippinarum*) after exposure to carbamazepine (Almeida et al. 2014, 2015).

1.2.2.1. Gemfibrozil

Lipid regulators are among the most commonly prescribed human pharmaceuticals worldwide. Gemfibrozil (GEM) is a lipid regulator drug, which is structurally an amphipathic carboxylic acid molecule (Roy and Pahan 2009). GEM was designed in 1968 at the Parke Davis Research Laboratories, in Detroit, to decrease serum lipid in patients (Roy and Pahan 2009). After three years of intense research, GEM was proposed as a new drug with lipid lowering ability and sent for clinical trial (Nash 1980). In 1976, GEM was successfully introduced in the market as a hypolipidemic drug with its ability to reduce plasma triglyceride level (Betteridge, Higgins, and Galton 1976). In 1992, Johan Auwerx proposed a mode of action that involved a class of nuclear hormone receptor known as peroxisome proliferator-activated receptor α (PPARα) as the target protein for fibrate drugs, including GEM. Nowadays, GEM is widely prescribed to reduce the levels of triglycerides, very low-density lipoprotein (VLDL, “bad cholesterol”) and low-density lipoprotein (LDL, “bad cholesterol”) and increase high-density lipoprotein (HDL, “good cholesterol”) (Kim et al. 2017).

The widespread use of GEM makes it one of the most frequently encountered pharmaceuticals in the aquatic environment, with reported levels in surface waters up to 1.5 $\mu g.L^{-1}$ (Fang et al. 2012) and between 1 and 758 $ng.L^{-1}$ in seawater. A previous study reported that GEM to be a persistent compound with half-lifes ranging from 119 to 288 d in surface waters (Araujo et al. 2011). However, there is limited knowledge concerning the mechanisms involved in the effects of GEM to marine organisms (Lyssimachou et al. 2014; Solé, Fortuny, and Mañanós 2014; Teles et al. 2016).

1.2.3. Mixtures of Emerging Contaminants of Concern

Environmental regulation within the EU, such as the regulatory framework for chemicals REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), the Water Framework Directive (WFD), and the Marine Strategy Framework Directive (MSFD) focus mainly on toxicity assessment of individual chemicals, although the effect of contaminant mixtures is a matter of increasing concern (Beyer et al. 2014).

In the environment, organisms are typically exposed to a complex mixture of contaminants. The mixtures may sometimes cause toxic effects even at concentrations lower than the no observable effect concentration (NOEC) for single exposures (Brian et al. 2007; Kortenkamp 2008; Silva, Rajapakse, and Kortenkamp 2002). As the assessment of chemical toxicity is normally done substance by substance, not taking potential mixture effects into account, it is probable that adverse effects of environmental pollutant mixtures are underestimated (Beyer et al. 2014). Contaminants with similar or different modes of action may influence each other's toxicity, resulting in an almost unlimited number of possible additive, synergistic and antagonistic effects (Beyer et al. 2014).

Over the last 20 years the relevance of mixture toxicity has reached an increasing acceptance in ecotoxicology (Kortenkamp, Backhaus, and Faust 2009; Schmidt et al. 2016; Vighi et al. 2003). This fact notwithstanding, contaminants are still regulated on a single substance basis and not as mixtures (Schmidt et al. 2016), NOEC and lowest observed effect concentration (LOEC) derived from single substance testing may not be enough for deriving safe environmental quality standards (Backhaus, Scholze, and Grimme 2000). Knowledge concerning the effects of contaminant mixtures is still limited (Relyea 2009).

1.2.4. Assessment of the Effects of Emerging Contaminants of Concern

To assess the effects of contaminants in aquatic organisms, several studies have focused on the use of biomarkers as early responses (Sanchez et al. 2008). The term 'biomarker' is generally used in a broad sense to include almost any measurement reflecting an interaction between a biological system and a potential hazard, which may be chemical, physical or biological (WHO, 1993). Biomarkers may be defined as observable or measurable modifications at the molecular, biochemical, cellular, physiological or behavioural levels revealing the exposure of an organism to xenobiotics (Sanchez et al. 2008). A xenobiotic is a general term referring to any chemical foreign to an organism or, in other words, any compound not occurring within the normal metabolic pathways of a biological system. The term may also include substances that are present in much higher concentrations

than are usual (Park, Lee, and Cho 2014). Effects at biochemical and molecular levels tend to occur first, followed by responses at the cellular, tissue/organ and organism levels. Responses that occur at individual, population and ecosystem levels are considered long-term responses with great ecological importance, being less reversible and more adverse than effects at lower levels. Much attention has been given towards identifying and understanding toxic effects initiated at the sub-organism level (molecular, biochemical or physiological changes). The use of biomarkers must be a multiparametric approach, using different and complementary biomarkers at different levels of organization to reflect the mechanisms of action of the contaminants.

1.2.4.1. Oxidative Stress

Oxidative stress is a mechanism of toxicity described for some environmental contaminants such as metals and pesticides, and components of the cellular defence against oxidative stress has been increasingly used as biomarker of pollution in aquatic environments to evaluate the exposure effects as well as mechanisms of action of the contaminants (Lushchak 2016).

Reactive oxygen species (ROS) are produced by living organisms from molecular oxygen as a result of normal cellular metabolism. At low to moderate concentrations (usually not exceeding 10^{-8} M), they have functions in physiological cell processes but at high concentrations they may produce adverse modifications to cell components such as lipids, proteins and DNA (Birben et al. 2012). ROS may be divided into two groups: free radicals and nonradicals. Molecules containing one or more unpaired electrons and thus giving reactivity to the molecule are called free radicals (Valko et al. 2006). When two free radicals share their unpaired electrons, nonradical forms are created. The three ROS of major physiological significance are superoxide anion (O_2^-), hydroxyl radical (OH) and hydrogen peroxide (H_2O_2) (Birben et al. 2012; Land 1990).

Under normal conditions, there is a balance between ROS production and elimination. ROS produced in biological systems are detoxified and purportedly held in check by antioxidant defences (enzymatic and non-enzymatic) (Livingstone 2001). However, ROS concentration may be changed (e.g. after exposure to

chemicals) leading to a disturbance in the balance between oxidant/antioxidant in favour of oxidants, a process called “oxidative stress” (Birben et al. 2012; Pisoschi and Pop 2015).

The antioxidant defence system consists of enzymatic and non-enzymatic antioxidants, that cooperatively protect the body from oxidative stress (Halliwell 2007).

1.2.4.1.1. Enzymatic Antioxidants

Among the enzymatic antioxidant defence enzymes such as superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPx) play an important role and are frequently assessed in toxicity studies – Figure 2 (Jeeva et al. 2015). SOD is a metalloenzyme which catalyses the reaction to decompose superoxide anion radicals (O_2^-) into H_2O_2 (Ighodaro and Akinloye 2017; Peng et al. 2014). H_2O_2 is then converted to water and oxygen by CAT or GPx. Otherwise, H_2O_2 may be converted to hydroxyl radical, one of the most active and harmful radicals to living cells. CAT, abundant in peroxisomes, is one of the most efficient antioxidant enzymes, present almost in all living tissues that utilize oxygen, capable to break down millions H_2O_2 molecules in one second (Ighodaro and Akinloye 2017). CAT exists as a tetramer composed of four identical monomers, each of which contains an iron at the active site. GPx, mostly present in the cytosol but also in the mitochondria, is a selenium-containing enzyme, using a low-molecular-weight thiol – reduced glutathione (GSH) – to reduce H_2O_2 and lipid peroxides (generated as a result of membrane lipid peroxidation) to their corresponding alcohols (Birben et al. 2012). Glutathione reductase (GR) is responsible for maintaining the supply of GSH, one of the most abundant reducing thiols in the majority of cells. GR is an essential enzyme that converts oxidized glutathione (GSSG) to the reduced form (GSH) (Couto, Wood, and Barber 2016; Peng et al. 2014) – Figure 2. Common to these enzymatic antioxidants is the requirement of reduced nicotinamide-adenine dinucleotide phosphate (NADPH). NADPH maintains CAT in the active form and is used as a cofactor by GR which converts to GSH, a co-substrate for the GPx (Valko et al. 2006).

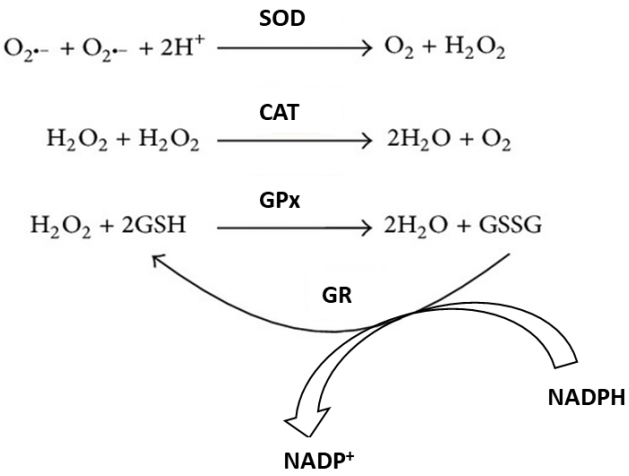

Figure 2. Reactions involved in the main enzymatic antioxidant defence system. SOD – Superoxide Dismutase; CAT – Catalase; GPx – Glutathione Peroxidase; GR – Glutathione Reductase; O_2^- – Superoxide Anion; H^+ – Hydrogen ion; O_2 – Oxygen molecule; H_2O_2 – Hydrogen Peroxide; H_2O – Water molecule; GSSG – Oxidized Glutathione; GSH – Reduced Glutathione; NADPH – Reduced Nicotinamide-Adenine Dinucleotide Phosphate; $NADP^+$ – Oxidized Nicotinamide-Adenine Dinucleotide Phosphate.

Glutathione *S*-transferases (GST), a family of phase-II isoenzymes, play a critical role in providing protection against electrophiles and products of oxidative stress. GST are primarily involved in the neutralization of harmful exogenous (e.g. polycyclic aromatic hydrocarbons) or endogenous (e.g. resultant of ROS activity) compounds by enzymatic conjugation with GSH and/or by direct binding of non-substrate ligands, i.e. in phase II reactions of xenobiotic metabolism. GST possess two binding sites: one for GSH and another for the substrate. GST have been described mainly in cytoplasm, but they are also present in the nucleus, endoplasmic reticulum (microsomes) and mitochondria (Sharma et al. 2016). In eukaryotes, GST are divided into three major families, namely cytosolic GST, mitochondrial GST (also known as kappa class GST) and microsomal GST (also called MAPEG GST – membrane-associated proteins in eicosanoid and glutathione metabolism) (Allocati et al. 2009). The cytosolic GST grouped into numerous classes based on their chemical, physical and structural properties, are the largest and diverse family of GST, having important roles in metabolism and defence against oxidative damage (Mashiyama et al. 2014; Sharma et al. 2016).

1.2.4.1.2. Non-Enzymatic Antioxidants

Non-enzymatic antioxidants can not only provide direct protection against oxidative damage, but they are crucial to the function of enzymatic antioxidants (Peng et al. 2014). Vitamins C, E and non-protein compounds that have sulfhydryl groups (–SH) called thiols are within the most important antioxidants in this category. The non-protein thiols (NPT) group is particularly important, playing an important role in defence against ROS (Chianeh and Prabhu 2014; Dirican et al. 2016). Measuring thiols compounds provides an indirect reflection of the antioxidative defence (Dirican et al. 2016; Taysi et al. 2002). Thiols can form disulphide bonds with oxidation reactions. The disulphide bonds can again be reduced to thiols, and thus, dynamic thiol–disulphide homeostasis. Dynamic thiol–disulphide homeostasis status has critical roles in antioxidant protection, detoxification, signal transduction, apoptosis, regulation of enzymatic activity and transcription factors, and cellular signalling mechanisms (Circu and Aw 2010; Dirican et al. 2016). GSH, which represents the bulk of NPT (Galano and Alvarez-Idaboy 2011), is capable of scavenging ROS, contributing to the control of redox homoeostasis. GSH tends to accumulate in cellular regions of high electron flux, where ROS are generated. In eukaryotes, GSH is found in the cytoplasm and within organelles including the nucleus and the mitochondria. Like many cysteine-containing molecules, GSH is readily oxidized; a disulphide bridge forms between two GSH molecules to yield the oxidized dimer (GSSG). On average, the cellular concentration in the cytosol can range from 1 to 10 mM with a ratio of GSH:GSSG in the range 30:1 to 100:1 (Couto, Wood, and Barber 2016).

1.2.4.2. Oxidative Damage

In normal situation, the production of ROS is thought to be held in check by antioxidant defence systems. However, sometimes the antioxidant defence is not sufficient to eliminate ROS and oxidative damage to key molecules like DNA, proteins and lipids may occur. In the aquatic organisms, it was already described that the antioxidant defence system activation may not be enough to avoid the oxidative damage in organisms exposed to contaminants (Livingstone 2001).

The evaluation of lipids oxidation is the most commonly used approach in free radical research field because many organisms, especially aquatic ones, contain high amounts of lipids with polyunsaturated fatty acid residues, a substrate for oxidation. Polyunsaturated fatty acids, the main component of cell membranes, are vulnerable to free radical attack because they contain multiple double bonds, which possess extremely reactive hydrogen atoms. As a result, the structure is susceptible to attacks by free radicals, especially hydroxyl radicals, which will lead to the destruction of cell membrane permeability, and, eventually, cellular dysfunction (Ighodaro and Akinloye 2017) – Figure 3. Since lipids will usually be oxidized through the formation of peroxides, the process of their formation has been called “lipid peroxidation” (Lushchak 2011).

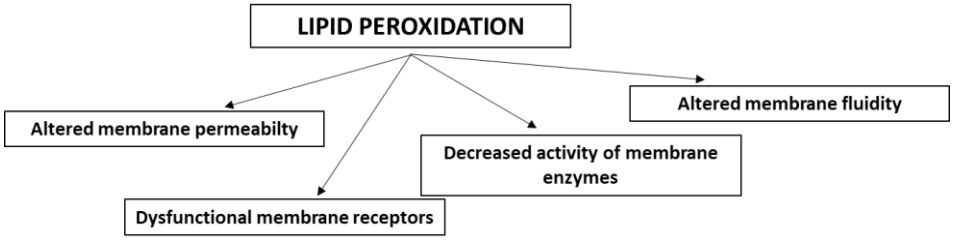

Figure 3. Consequences of lipid peroxidation in the cellular membranes.

The most frequently used methods to detect lipid peroxidation are based on measuring of the end products. Malonic dialdehyde (MDA) is one of the final products of peroxidation of unsaturated fatty acids in phospholipids and is responsible for cell membrane damage. The measurement of MDA levels is frequently performed with thiobarbituric acid (TBA). As TBA also reacts with many types of compounds, such as different aldehydes, amino acids and carbohydrates, it is not correct to refer to MDA measurement, but rather to TBA-reactive substances (TBARS) (Lushchak 2011).

ROS may also induce damage in the DNA. In fact, the major source of endogenous DNA damage is ROS generated from normal cellular metabolism. Exogenous sources of DNA damage include environmental agents such as ultraviolet light, toxins and pollutants (Maynard et al. 2009). ROS-induced DNA damage mainly comprises strand break, cross-linking, base hydroxylation and

base excision. The induction of such DNA damage may cause mutagenesis or cell transformation, especially if combined with a deficient apoptotic pathway. Unlike proteins, lipids and RNA, DNA cannot be replaced when damaged and must therefore be repaired. If the damage is not repaired, the cell may resort to induction of apoptosis or necrosis, so that the mutations are not passed on to progeny cells and do not result in disease (Maynard et al. 2009). The critical importance of DNA for the cell makes ROS-induced DNA modifications a relevant toxicity endpoint. Several techniques have been developed to address this issue such as comet assay, applying different enzymes that allow recognize the type of damage (for instance the formation of oxidized bases, particularly 8-oxoguanine) (Lushchak 2011). These methodologies have already been applied in aquatic organisms (Gielazyn et al. 2003; Oliveira, Ahmad, et al. 2010; Oliveira, Maria, et al. 2010).

ROS-induced modification of proteins has become an increasingly used measure of oxidative stress. The attack of ROS on proteins may occur directly, involving the modulation of a protein's activity and indirectly by conjugation with breakdown products of fatty acid peroxidation. As a consequence of excessive ROS production, site-specific amino acid modification, fragmentation of the peptide chain, altered electric charge among others may occur. The amino acids in a peptide differ in their susceptibility to attack by ROS, thiol groups and sulphur containing amino acids are very susceptible (Sharma et al. 2012).

Biomarkers of oxidative stress and damage are therefore very important to understand the effect of contaminants, alone or in a mixture, to non-target organisms, specially to marine top predators, in which studies are scarce.

1.2.4.3. Alterations of Behaviour

Behaviour is typically defined as a series of evident, observable and whole-body activities that operate through the central nervous system and allow an organism to survive, grow and reproduce (Calfee et al. 2016). Changes on behaviour due to exposure to a toxicant are among the most sensitive indicators of environmental stress often between 10 to 100 times more sensitive comparing with the survival endpoint (Gerhardt 2007). Toxicant exposure often completely eliminates the

performance of behaviours that are essential to fitness and survival in natural ecosystems, frequently after exposures of lesser magnitude than those causing significant mortality (Scott and Sloman 2004). Depending on the organism, several behavioural assessments may be performed: swimming performance, avoidance, feeding and reproductive behaviour.

Life histories of fish are intimately associated with numerous interspecific (e.g. predation) and intraspecific (both reproductive and non-reproductive) interactions, which depend on its behaviour. Behaviour is considered an important biomarker of effect at the individual level since it has obvious reflexes at the population level: it is determinant to capture preys, to escape from predators, to find a partner and mate, among others (Barbieri 2007; Lurman, Bock, and Poertner 2009; Oliveira, Gravato, and Guilhermino 2012). Changes in the normal behavioural patterns caused by the exposure to contaminants, such as NPs, pharmaceuticals and metals, as already reported (Almeida et al. 2009; Barbieri 2007; Barry 2013; Berntssen, Aatland, and Handy 2003; Brodin et al. 2017; Lurman, Bock, and Poertner 2009; Mattsson et al. 2015, 2017; Oliveira, Gravato, and Guilhermino 2012), may cause serious risks to the success of fish populations (Scott and Sloman 2004). However, the effects of many xenobiotics on fish behaviour is unknown.

Swimming performance of fish is one of the most frequently used sublethal endpoint to determine a behavioural change in response to a contaminant. Swimming is dependent on several physiological functions, being the most important the neurofunction and neuromuscular transmission for movement coordination, recognizing preys and predators and act accordingly, growth and development, among others (Oliveira, Gravato, and Guilhermino 2012).

The most commonly observed links with behavioural disruption include cholinesterases (ChE) inhibition, altered brain neurotransmitter levels, sensory deprivation and impaired gonadal or thyroid hormone levels (Scott and Sloman 2004). The activity of brain and muscle ChE is frequently used as sub-individual endpoint, being considered a biomarker of effect on cholinergic neurological and neuromuscular transmission, respectively. Acetylcholinesterase, one of the most important behind the family of ChE, plays a key role in the correct transmission of

nerve impulses both in vertebrates and invertebrates, being essential for the degradation of the neurotransmitter acetylcholine in cholinergic synapses (Sureda et al. 2018). Because behaviour links physiological function with ecological processes, behavioural indicators of toxicity appear ideal for assessing the effects of aquatic pollutants on fish populations.

1.3. *Sparus aurata* as a Model Organism

Long-living animal species at the top of the food chain may be under particular risk in contaminated environments, mainly if the compounds present are biomagnified along the food chain [112].

The gilthead seabream – *Sparus aurata* (Linnaeus, 1758) – is a perciform fish, belonging to the family *Sparidae* and to the genus *Sparus* (Arabaci et al. 2010). *S. aurata* has a colour silvery grey, a golden frontal band between eyes edged by two dark areas (not well defined in young individuals), dark longitudinal lines often present on sides of body and fork and tips of caudal fin edged with black (Jawad 2012). *S. aurata* is common in the Mediterranean Sea, present along the Eastern Atlantic coast from Great Britain to Senegal, and rare in the Black Sea. Due to its euryhaline and eurythermal habits, the species is found in both marine and brackish water environments such as coastal lagoons and estuarine areas, in particular during the initial stages of its life cycle (Arabaci et al. 2010). Born in the open sea during October-December, juveniles typically migrate in early spring towards protected coastal waters, where they can find abundant trophic resources and milder temperatures. Very sensitive to low temperatures (lower lethal limit is 4°C), in late autumn they return to the open sea, where the adult fish breed (Studer 2015). In the sea, gilthead seabream are usually found on rocky shore and seagrass (*Posidonia oceanica*) meadows, but is also frequently caught on sandy grounds. Young fish remain in relatively shallow areas (up to 30 m), whereas adults may reach deeper waters (maximum depth of 150 m) (Arabaci et al. 2010). They are manly carnivorous (feed on shellfish including mussels and oysters) but may be facultative herbivores (Studer 2015).

This species is a protandrous hermaphrodite. Sexual maturity develops in males at 2 years of age (20-30 cm) and in females at 2-3 years (33-40 cm).

Females are batch spawners that can lay 20 000-80 000 eggs every day for a period up to 4 months. In captivity, sex reversal is conditioned by social and hormonal factors (Studer 2015).

S. aurata is very suitable species for extensive aquaculture due to their good market price, high survival rate and feeding habits (which are relatively low in the food chain). Traditionally, gilthead seabream was cultured extensively in coastal lagoons and saltwater ponds, until intensive rearing systems were developed during the 1980s. The Italian “vallicoltura” or the Egyptian “hosha” are extensive fish rearing systems that act like natural fish traps, taking advantage of the natural trophic migration of juveniles from the sea into coastal lagoons (Studer 2015).

Artificial breeding was successfully achieved in Italy in 1981-82 and large-scale production of gilthead seabream juveniles was definitively achieved in 1988-1989 in Spain, Italy and Greece. The hatchery production and farming of this fish is one of the success stories of the aquaculture business. This species very quickly demonstrated a high adaptability to intensive rearing conditions, both in ponds and cages, and its annual production increased regularly until 2000, when it reached a peak of over 87 000 tons. In the Mediterranean, at the beginning of the 1990s, 20 seabream hatcheries were open, whereas, at 2006, over 65 hatcheries were operating in Croatia, Cyprus, France, Greece, Italy, Morocco, Portugal, Spain and Tunisia (Sola et al. 2018). Most of the production occurs in the Mediterranean, reaching over 160 000 tons in 2016, with Turkey and Greece being the largest producers, followed by Spain and Italy. In Portugal, in 2016, around 1500 tons of seabream were produced (EUMOFA 2016).

Portugal is one of the countries with the highest fish and seafood consumption per capita in the world (Almeida, Vaz, and Karadzic 2015). Among the EU Member States, Portugal registers the highest per capita consumption of fish and seafood products (55.3 kg per year), consuming 30 kg per capita more than the EU average, in 2014 (EUMOFA 2016).

Due to the widespread presence in Atlantic and Mediterranean coastal waters, high economic importance, high consumption in the Mediterranean area (may represent an important route of possible entry of contaminants into humans), the use of *S. aurata* as a model organism in ecotoxicological studies is high relevant.

Furthermore, *S. aurata* has already proved its suitability as a bioindicator in toxicity testing (Souid et al. 2015; Teles 2016a, 2016b; Zena et al. 2015).

2. AIMS AND CONCEPTUAL FRAMEWORK OF THE THESIS

As there is limited and controversial information about the toxic effects of AuNPs and how AuNPs characteristics influence the effects, particularly to marine fish, this thesis aimed to answer the following general question:

Will AuNPs affect molecular, biochemical and behavioural responses of *Sparus aurata*? To answer this question fish were *in vitro* and *in vivo* exposed to AuNPs, with different coatings and sizes, alone and combined with the human pharmaceutical GEM.

The specific objectives are:

- To determine whether AuNPs with different characteristics have different effects (e.g. genotoxicity, behavioural changes, oxidative stress and damage responses) in *S. aurata*;

- To clarify whether AuNPs will modulate the effects of other ECs, namely GEM;

- To compare effects from *in vitro* and *in vivo* exposures to AuNPs;

- To clarify mechanisms underlying the effects of different AuNPs and any links between the observed effects and their characteristics.

This thesis will be divided in 10 chapters: Chapter I, is the current General Introduction, Chapters II to IX constitute the description of the experimental component of this thesis and Chapter X comprises the General Discussion and Future Perspectives.

Chapter II entitled "*In vitro* effects after single and combined exposures to gold (nano versus ionic form) and gemfibrozil", describes the biological effects of gold nanoparticles (AuNPs), ionic gold and the human pharmaceutical gemfibrozil, alone and in a combined exposure, using as biological model, fish liver. Tested AuNPs presented two sizes (7 and 40 nm) and surface coatings (citrate and polyvinylpyrrolidone – PVP). Responses involved on biotransformation, oxidative stress/damage and DNA integrity were assessed after 24 h exposure.

Chapter III entitled "Biological effects and bioaccumulation of gold in gilthead seabream (*Sparus aurata*) – Nano versus ionic form", describes the effects of 96 h waterborne exposure to 7 nm gold nanoparticles (AuNPs) – (citrate coated (cAuNPs) or polyvinylpyrrolidone coated (PVP-AuNPs)) – and ionic gold on gilthead seabream (*Sparus aurata*). Effects at different levels of biological organization (behaviour, neurotransmission, biotransformation, oxidative stress/damage and genotoxicity) were assessed.

Chapter IV entitled "A multibiomarker approach highlights effects induced by the human pharmaceutical gemfibrozil to gilthead seabream *Sparus aurata*", describes the biochemical (neurotransmission, biotransformation and oxidative stress/damage) and behavioural effects on *Sparus aurata* after a 96-h waterborne exposure to gemfibrozil.

Chapter V entitled "Genotoxicity of gemfibrozil in the gilthead seabream (*Sparus aurata*)", describes the genotoxicity of gemfibrozil in the gilthead seabream (*Sparus aurata*) after 96 h waterborne exposure.

Chapter VI entitled "Effects and bioaccumulation of gold nanoparticles in the gilthead seabream (*Sparus aurata*) – single and combined exposures with gemfibrozil", describes the effects of 96 h waterborne exposure to 40 nm gold nanoparticles (AuNPs) – (citrate coated (cAuNPs) or polyvinylpyrrolidone coated (PVP-AuNPs)) – alone and combined with gemfibrozil on different biological

responses (behaviour, neurotransmission, biotransformation and oxidative stress/damage) of the seabream *Sparus aurata*.

Chapter VII entitled “Genotoxicity of gold nanoparticles in the gilthead seabream (*Sparus aurata*) after single exposure and combined with the pharmaceutical gemfibrozil”, describes the genotoxicity of 40 nm gold nanoparticles (AuNPs) – (citrate coated (cAuNPs) or polyvinylpyrrolidone coated (PVP-AuNPs)) – alone and combined with gemfibrozil on *Sparus aurata*, after 96 h waterborne exposure.

Chapter VIII entitled “Effects of gold (ionic and nano form) and gemfibrozil mixtures in gilthead seabream (*Sparus aurata*)”, describes the effects of 96 h combined waterborne exposures to 7 nm gold nanoparticles (AuNPs) – (citrate coated (cAuNPs) or polyvinylpyrrolidone coated (PVP-AuNPs)) – or ionic gold and gemfibrozil to the marine fish *Sparus aurata.* Effects at different levels of biological organization (behaviour, neurotransmission, biotransformation, oxidative stress/damage and genotoxicity) were assessed.

Chapter IX entitled “Effects of gold nanoparticles in gilthead seabream – a proteomic approach”, describes the effects of 96 h waterborne exposure to 7 and 40 nm gold nanoparticles (AuNPs) – citrate and polyvinylpyrrolidone (PVP) coated) – on the gilthead seabream (*Sparus aurata*) liver proteome.

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Chapter II

In vitro effects after single and combined exposures to gold (nano versus ionic form) and gemfibrozil

A. Barreto[1*], A. Carvalho[1], D. Silva[1], T. Trindade[2], A.M.V.M. Soares[1], K. Hylland[3], S. Loureiro[1], M. Oliveira[1]

[1] Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] Departamento de Química & CICECO - Aveiro Instituto de Materiais, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[3] Department of Biosciences, University of Oslo, PO Box 1066, N-0316 Oslo, Norway

*Corresponding author: E-mail: abarreto@ua.pt, Tel +351 234 370 350, Fax +351 234 372 587

Highlights

- Gold nanoparticles (AuNPs) led to damage in DNA and in cellular membranes of liver organ culture of *Sparus aurata*;
- Gemfibrozil caused DNA damage at an environmental relevant concentration (1.5 $\mu g.L^{-1}$);
- The observed effects in the combined exposures were in many endpoints higher than the predicted;
- Liver organ culture was sensitive to the tested xenobiotics, supporting its use as an alternative to *in vivo* testing.

Abstract

In vitro methodologies have gained an increasing importance in toxicological research emphasized by the concerns about animal welfare. Liver cell cultures is a well-established biological model, but organ culture allows the study of effects in a more physiologically relevant context. The aim of the present study was to assess the biological effects of gold nanoparticles (AuNPs), ionic gold and the human pharmaceutical gemfibrozil (GEM), alone and in a combined exposure, using liver organ culture as a model. Two sizes of AuNPs (7 and 40 nm) and two surface coatings (citrate and polyvinylpyrrolidone – PVP) were tested. Measured responses included biotransformation activity, oxidative stress/damage and DNA integrity, assessed after 24 h exposure. AuNPs exposures increased catalase and glutathione reductase activities, led to damage in DNA and in cellular membranes, with observed effects depending on the nanoparticles (NPs) size, coating and concentration. Single exposure to GEM caused DNA damage at an environmental relevant concentration (1.5 $\mu g.L^{-1}$). The observed percentages of effect in the combined exposures – gold and GEM – were in many cases higher than the predicted effects. Liver organ culture was sensitive to the tested xenobiotics, supporting its use as an alternative to *in vivo* testing.

Keywords: Liver organ culture, nanotoxicology; mixtures; oxidative stress; DNA damage

1. Introduction

The most widely used toxicological approach for evaluating chemical toxicity involves complex *in vivo* studies which are both time consuming and costly (Soldatow et al 2013). Due to concerns regarding animal welfare, time and cost constraints, establishing workable *in vitro* culture systems has become a priority for the toxicology community. In this perspective, the use of organ/cell cultures has the advantage of allowing a reduction of the number of animals per test, improved control of environmental conditions, reduction of the genetic heterogeneity and of needed test chemicals as well as a reduction in toxic waste (Oliveira et al. 2003;

Soldatow et al 2013). *In vitro* liver cell culture models have gained a high importance in toxicological research due to the function of this organ (Zeilinger et al. 2016). The liver is involved in (i) metabolism of endogenous substrates (e.g. products resultant of reactive oxygen species (ROS) activity) and exogenous compounds (e.g. drugs, chemicals); (ii) regulation of amino acids, carbohydrates and fatty acids; (iii) synthesis of proteins (e.g. albumin or transferrin); (iv) activation of inflammatory and immune reactions upon liver injury due to disease, drug or toxicant exposure (Zeilinger et al. 2016). The use of liver slices can be useful in toxicity assessment as they retain the 3D structure, contain all liver cell types and shows good *in vitro*/*in vivo* correlation for xenobiotic metabolism (Soldatow et al 2013). Despite the successful use of organ culture in toxicological research, fish organ culture has not been extensively used (Oliveira et al. 2003).

Thus, the purpose of the present study was to use an *in vitro* model, i.e. liver organ culture, to assess the single and combined effects of two environmental contaminants of concern: gold nanoparticles (AuNPs) and the lipid regulator gemfibrozil (GEM). Two sizes (7 and 40 nm) and two surface coatings (citrate and polyvinylpyrrolidone – PVP) of AuNPs were used in addition to ionic gold to differentiate the effects of nano versus ionic form. Despite their increasing production, use and disposal, the effects of AuNPs and GEM on marine fish remains largely unknown. Liver organ culture of *Sparus aurata* was selected as the experimental model because the liver is the main organ for xenobiotic accumulation and biotransformation, allowing excretion and elimination (Kunjiappan, Bhattacharjee, and Chowdhury 2015). Previous studies have reported that the liver is the main organ for AuNPs accumulation (Chen et al. 2013; Iswarya et al. 2016; Khan, Vishakante, and Siddaramaiah 2013; Mateo et al. 2014; Simpson et al. 2013;) and thus a potential target following *in vivo* exposure. Fish liver organ culture has earlier been successfully used to assess the effects of metals (Oliveira et al. 2003). In the present study, *in vitro* effects of AuNPs and GEM were assessed after 24 h of exposure, including enzymatic antioxidants, i.e. catalase (CAT), glutathione reductase (GR) and glutathione *S*-transferases (GST) activities, measurement of oxidative damage as lipid peroxidation (LPO) and DNA damage. This set of biomarkers was chosen to assess potential effects on the

liver, focusing on the ability to respond to oxidative challenge, maintain biotransformation and prevent damage.

2. Material and Methods

2.1. Test organisms

Juvenile gilthead seabream (*Sparus aurata*), length 100 ± 0.4 cm, acquired from a Spanish aquaculture facility, were acclimated for 4 weeks in aquaria containing aerated and filtered artificial seawater (ASW; prepared by dissolving the salt in reverse osmosis water to obtain a salinity of 30), in a controlled room temperature (20ºC) and natural photoperiod. During this period, animals were fed daily with commercial fish food (Sorgal, Portugal) at a ratio of 1% of body weight/day.

2.2. Synthesis and characterisation of gold nanoparticles (AuNPs)

Citrate-coated AuNPs (cAuNPs) of 7 nm diameter were synthesised using the pH-shifting method, with reduction of gold (III) chloride trihydrate by citric acid, followed by neutralization with NaOH (Shiba 2013). cAuNPs with 40 nm diameter were prepared, using 15 nm seeds, by sodium citrate reduction of gold (III) chloride trihydrate (Lekeufack et al. 2010). Part of cAuNPs were coated with PVP as described by Barreto et al. (2015). cAuNPs and polyvinylpyrrolidone coated gold nanoparticles (PVP-AuNPs) were centrifuged and the pellet resuspended in ultrapure water. AuNPs in ultrapure water and in the media used for the experiments – Dulbecco's Modified Eagle's medium with fetal bovine serum (DMEM+FBS) – were characterised by UV-Vis spectrophotometry (Cintra 303, GBC Scientific) to obtain the UV-Vis spectra; hydrodynamic size was assessed by dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern) and size/shape evaluated by transmission electron microscopy – TEM (Hitachi, H9000 NAR) or scanning electron microscopy – SEM (Hitachi, SU70). Zeta potential (ZP) was measured using Zetasizer (Nano ZS, Malvern). The measurements were performed at 0, 12 and 24 h, at concentrations higher than 80 $\mu g.L^{-1}$, considering that, for concentrations lower than 80 $\mu g.L^{-1}$ the detection limits of the used techniques did not allow the characterisation of AuNPs. The characterisation was also performed visually, assessing the colour of the AuNPs suspensions.

2.3. *In vitro* exposures

DMEM+FBS was prepared as follow: 50% DMEM, 40% ultrapure water, 1 mM of glutamine, 15 mM HEPES, 10% FBS and 100 µg.mL^{-1} antibiotics (penicillin and streptomycin). A stock solution of GEM (50 g.L^{-1}) was prepared in dimethyl sulfoxide (DMSO) and test solutions prepared by the dilution of the stock in DMEM+FBS. Test suspensions of AuNPs were prepared from cAuNPs (100 and 97 mg.L^{-1} for 7 and 40 nm, respectively) and PVP-AuNPs (78 and 58 mg.L^{-1} for 7 and 40 nm, respectively) stock suspensions. Test solutions of ionic gold were prepared by dilution of the stock (2.7 g.L^{-1}) in DMEM+FBS.

After the acclimatization period, fish (n=5) were randomly selected and anesthetized with tricaine methanesulfonate (MS-222) and subsequently euthanized by spinal section. Liver of each animal was removed, washed with fresh phosphate-buffered saline (PBS), cut into small cubes (2x2 mm) and cultured during 24 h as previously reported (Oliveira et al. 2003), in the following experimental conditions: 4, 80, 1600, 3200, 4200, 5200, 6200 and 7200 µg.L^{-1} of gold (ionic or nano form – 7 and 40 nm; citrate and PVP coating); 1.5, 15, 150, 1500 and 15000 µg.L^{-1} of GEM and mixture of 150 µg.L^{-1} GEM with 80 µg.L^{-1} of gold (ionic or nano form). A control (only DMEM+FBS) and a solvent control with DMSO (at 0.03%, the highest concentration of DMSO used in the GEM treatments) were also performed. Immediately after liver sampling and before organ culture initiation, a small number of liver pieces were stored at -80ºC until further processing. These samples were collected to determine the basal activities/levels of the liver for the assessed endpoints, being a control at 0 h. After 24 h exposure, samples were collected for biochemical analysis (stored at -80ºC until further processing) and DNA integrity assessment (were immediately processed).

2.4. Biochemical analysis

Portions of the liver were homogenized in potassium phosphate buffer (0.1 mM, pH 7.4), using an ultrasonic homogenizer (Branson Ultrasonics Sonifier S-250A). This homogenate was then divided in two aliquots: for determination of lipid peroxidation (LPO) and the other for post-mitochondrial supernatant (PMS)

isolation. 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 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 GST, CAT and GR activities determination.

Protein concentration was determined according to Bradford (1976), measuring the absorbance at 600 nm, adapted to microplate, using bovine γ – globuline as a standard.

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

GR activity was estimated according the method of Carlberg and Mannervik (1975) adapted to microplate (Lima et al. 2007), being spectrophotometrically determined by measuring reduced nicotinamide-adenine dinucleotide phosphate (NADPH) disappearance at 340 nm and expressed as nmol of oxidized Nicotinamide-Adenine Dinucleotide Phosphate ($NADP^+$) formed per min per mg of protein (ε=6.22×10^3 $M^{-1}.cm^{-1}$).

GST activity was determined spectrophotometrically by the method of Habig et al. (1974), adapted to microplate (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 activity calculated as nmol CDNB conjugate formed per min per mg of protein (ε=9.6×10^{-3} $M^{-1}.cm^{-1}$).

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). Absorbance was measured at 535 nm and LPO levels were expressed as nmol of TBARS formed per mg of protein (ε=1.56×10^5 $M^{-1}.cm^{-1}$).

2.5. DNA integrity assessment

The alkaline comet assay was performed according to method of Singh et al. (1988) with some modifications. All the steps were conducted under low light to prevent the occurrence of additional DNA damage. Each liver portion was

disrupted in PBS (pH 7.4) to obtain a suspension. This suspension was centrifuged, the supernatant was discarded, and the pellet was resuspended in fresh PBS. Then, cell suspension was added to of 1% (w/v) low melting point agarose (at 40ºC) and the mixture added to a microscope slide pre-coated with 1% (w/v) of normal melting point agarose. Solidification of agarose was allowed by keeping the slides on ice for 5 min. Positive controls (cell suspension treated with 25 µM of H_2O_2 for 10 min) were included for each electrophoresis run to verify that the electrophoresis conditions were adequate. To lyse the cells, the slides were subsequently immersed in prepared ice-cold lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0) containing freshly added 1% Triton X-100 for 1 h, at 4°C, in the dark. The slides were incubated in alkaline buffer (300 mM NaOH and 1 mM EDTA, pH>13) during 20 min for DNA unwinding. Electrophoresis was performed in the same buffer for 30 min by applying an electric field of 20 V and adjusting the current to 300 mA. After the electrophoresis, the slides were washed with 400 mM Tris-HCl buffer (pH 7.5). The slides were also dehydrated with absolute ethanol and left to dry in the dark. For analysis, slides were stained with ethidium bromide (20 $\mu L.mL^{-1}$), covered with a coverslip and then analysed using a fluorescence microscope (Olympus BX41TF) at 400X magnification.

Slides were analysed randomly, by counting one hundred cells from each slide, arbitrarily selected. Cells were scored visually, according to tail length, into 5 classes: class 0 – undamaged, without a tail; class 1 – with a tail shorter than the diameter of the head (nucleus); class 2 – with a tail length 1-2 times the diameter of the head; class 3 – with a tail longer than twice the diameter of the head; class 4 – comets with no heads (Collins 2004). A damage index (DI) expressed in arbitrary units was assigned to each replicate (for 100 cells) and consequently for each treatment, according to the damage classes, using the formula:

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

Where:

n = number of cells in each class analysed

2.6. Data analysis

Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test) prior to ANOVA, using the Sigma Plot 12.0 software package. Differences between controls (negative and solvent) were carried out using a Student t-test ($p<0.05$). Differences between treatments and controls were compared using one-way analysis of variance (ANOVA), followed by Dunnett's post-hoc test whenever applicable ($p<0.05$).

Observed percentages of effect in the combined exposures were compared with the corresponding 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 gold (nano or ionic form) and GEM was similar, lower or greater than the sum of each separately.

3. Results

3.1. Characterisation and behaviour of gold nanoparticles (AuNPs)

The synthesized cAuNPs displayed a round shape (Figure 1A and C), a well-defined absorption band and negative surface charge (Table 1) associated with the citrate layer. The analysis of the size, taking into account the results obtained by DLS and TEM images, revealed an expected average size around 7 and 40 nm. PVP coating led to an increased size due to the PVP layer. This PVP layer was detectable by SEM in some AuNPs (Figure 1B and D).

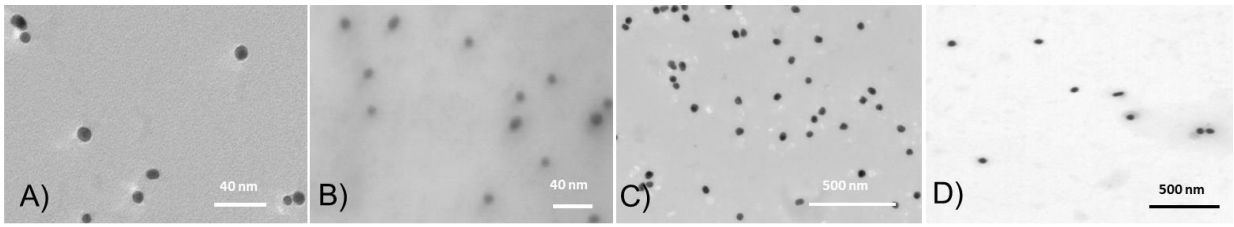

Figure 1. Electron microscopy images of 7 and 40 nm citrate (cAuNPs) and polyvinylpyrrolidone (PVP-AuNPs) gold nanoparticles in ultrapure water: **A)** 7 nm cAuNPs; **B)** 7 nm PVP-AuNPs; **C)** 40 nm cAuNPs; **D)** 40 nm PVP-AuNPs.

The UV-Vis spectra revealed a slight shift in surface plasmon resonance (SPR) peak to longer wavelength when compared with the original cAuNPs (Table 1). ZP shifted from -43 to around -13 mV and from -44 to -17 mV, for 7 and 40 nm AuNPs, respectively (Table 1).

Table 1. Characteristics of gold nanoparticles (AuNPs) in ultrapure water. cAuNPs – Citrate coated gold nanoparticles; PVP-AuNPs – Polyvinylpyrrolidone coated gold nanoparticles; PdI – Polydispersity Index; SPR – Surface Plasmon Resonance; ZP – Zeta Potential.

	Size (nm)	PdI	SPR (nm)	ZP (mV)	pH
7 nm cAuNPs	6.7	0.5	519	-43.3	6.4
7 nm PVP-AuNPs	7.6	0.5	521	-12.8	6.9
40 nm cAuNPs	37.0	0.3	534	-44.0	5.9
40 nm PVP-AuNPs	52.3	0.3	535	-17.0	6.4

In ultrapure water, AuNPs were stable, with no detectable agglomerates/aggregates (Figure 1). Size, ZP and UV-Vis spectra of each type of AuNPs were similar during the assessed periods 0, 12 and 24 h.

In DMEM+FBS, at 0 h, the characteristics of each type of AuNPs, were similar to those in ultrapure water, with a slight less negative ZP, slight 1-4 nm increased sizes and shifted SPR peaks toward higher wavelengths (increased about 2–4 nm). Within 12 h, for concentrations higher than 1600 $\mu g.L^{-1}$, AuNPs aggregated/agglomerated with sizes, assessed by DLS, bigger than 100 nm (Figure 2) and SPR peaks shifted toward higher wavelengths (Figure 3). Alterations in ZP were also found, with different peaks correspondent to different charges. After 24 h, no alterations in the size were found, comparing with 12 h (Figure 2) but the SPR peak disappeared (Figure 3).

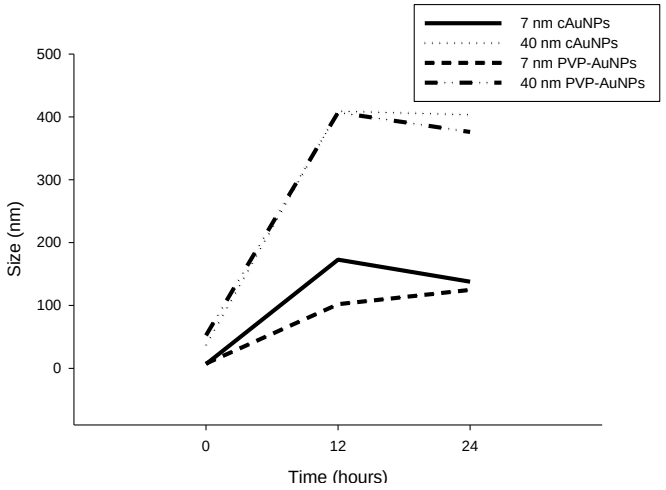

Figure 2. Size of gold nanoparticles (AuNPs), measured by dynamic light scattering (DLS), in Dulbecco's Modified Eagle's medium with fetal bovine serum (DMEM+FBS) at 0, 12 and 24 h. cAuNPs – Citrate coated gold nanoparticles; PVP-AuNPs – Polyvinylpyrrolidone coated gold nanoparticles.

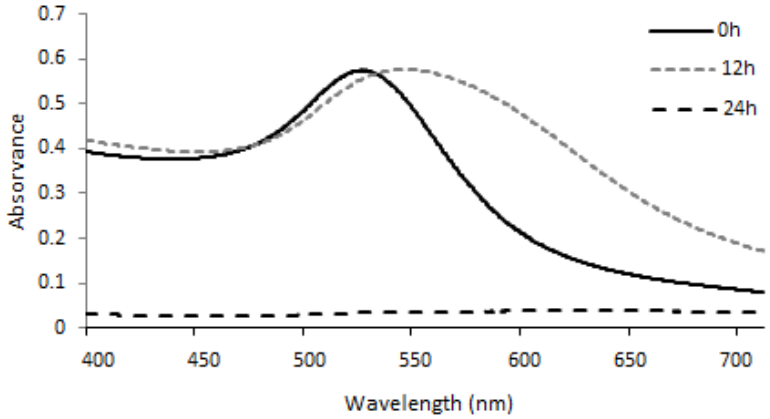

Figure 3. UV–Vis spectra of gold nanoparticles (AuNPs) in Dulbecco's Modified Eagle's medium with fetal bovine serum (DMEM+FBS) at 0, 12 and 24 h.

The colour of the AuNPs in DMEM+FBS, at 12 h, was between red and blue, being bluer in the highest concentrations. At 24 h, some dark sediment was found in the bottom of the wells. This sediment increased with the increase of AuNPs concentration. At the lower tested concentrations (4 and 80 $\mu g.L^{-1}$), the media did not present the typical colour of AuNPs agglomeration/aggregation.

3.2. Biological Effects

For all the tested endpoints, the results from the samples of liver collected at 0 h (control at 0 h) and from the liver pieces exposed during 24 h to DMEM+FBS (control) did not display significant differences ($p>0.05$; t-test). Additionally, solvent control (with DMSO) did not induce significant effects when compared to the control ($p>0.05$; t-test), for all the endpoints reported. Therefore, the treatments were compared to the control.

3.2.1. Gold nanoparticles (AuNPs)

3.2.1.1. 7 nm AuNPs

For the smallest tested AuNPs, a different response pattern was displayed between cAuNPs and PVP-AuNPs. The cAuNPs only affected CAT activity at the highest tested concentration, increasing the enzyme activity ($p<0.05$; Dunnett's test). However, for PVP-AuNPs, almost all the tested concentrations increased the activity of CAT ($p<0.05$; Dunnett's test) with exception to 4, 3200 and 4200 $\mu g.L^{-1}$ – Figure 4A.

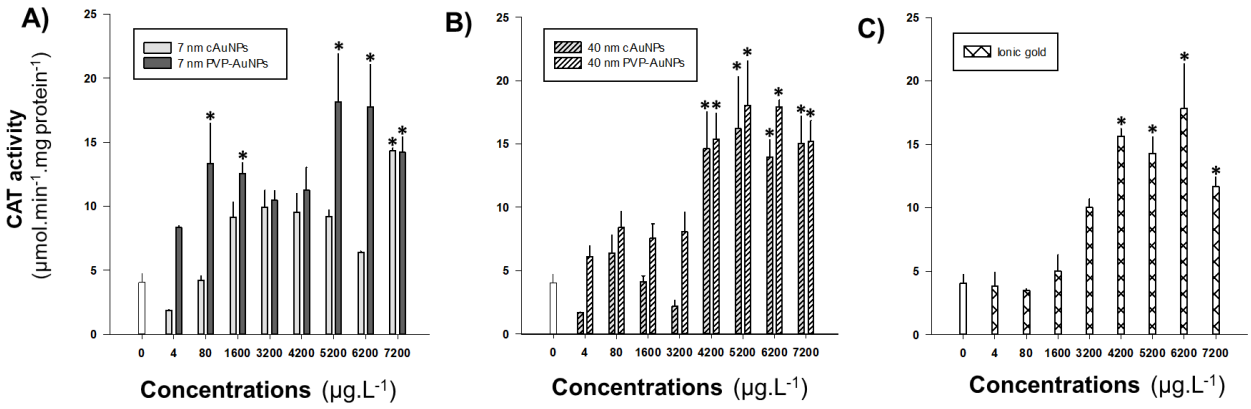

Figure 4. Liver catalase (CAT) activity of *Sparus aurata* after 24 h *in vitro* exposure to gold nanoparticles: 7 nm **(A)**, 40 nm **(B)** and ionic gold **(C)**. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). Citrate coated gold nanoparticles – cAuNPs; Polyvinylpyrrolidone coated gold nanoparticles – PVP-AuNPs.

In terms of GR, a significantly increased activity was found after exposure to cAuNPs, at 3200 and 5200 µg.L^{-1}, and PVP-AuNPs, at concentrations higher than 80 µg.L^{-1} ($p<0.05$; Dunnett's test; Figure 5A).

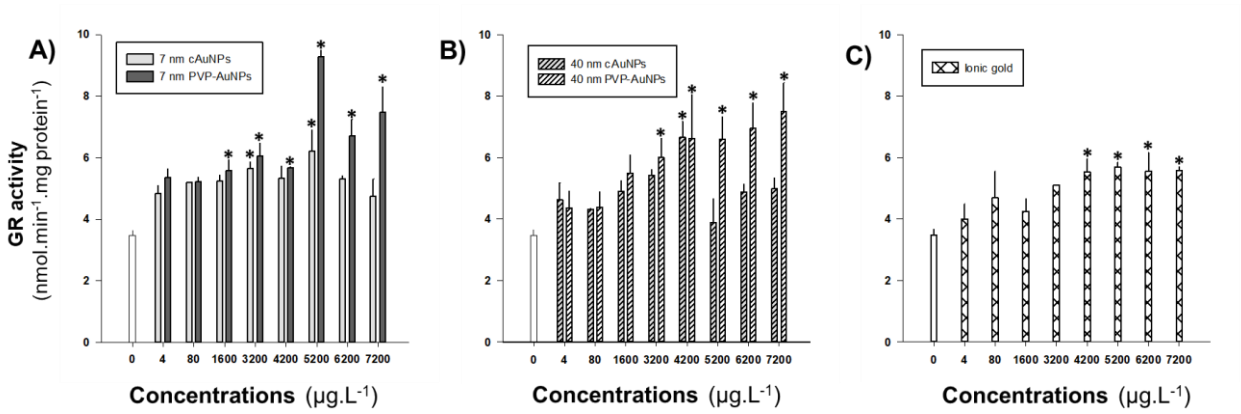

Figure 5. Liver glutathione reductase (GR) activity of *Sparus aurata* after 24 h *in vitro* exposure to gold nanoparticles: 7 nm **(A)**, 40 nm **(B)** and ionic gold **(C)**. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). Citrate coated gold nanoparticles – cAuNPs; Polyvinylpyrrolidone coated gold nanoparticles – PVP-AuNPs.

GST activity was not significantly affected by exposure to 7 nm AuNPs ($p>0.05$; ANOVA; Figure 6A).

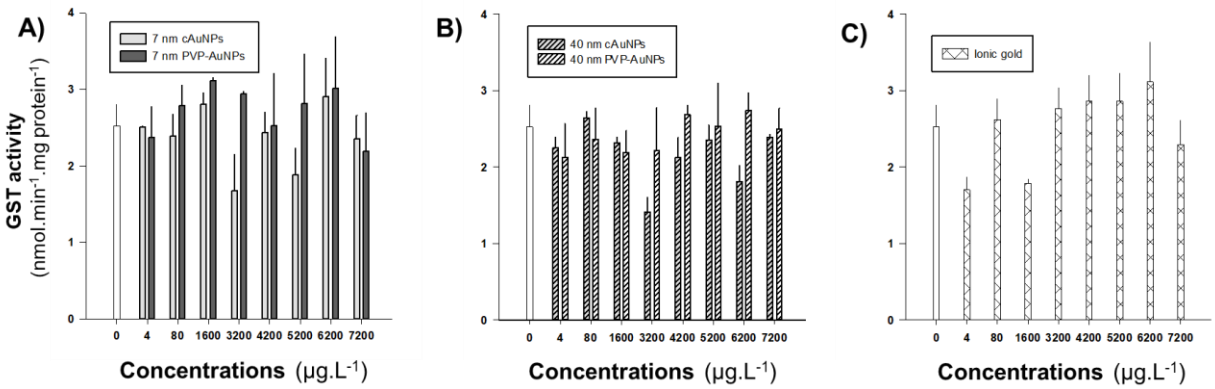

Figure 6. Liver glutathione *S*-transferases (GST) activity of *Sparus aurata* after 24 h *in vitro* exposure to gold nanoparticles: 7 nm **(A)**, 40 nm **(B)** and ionic gold **(C)**. Results are expressed as mean ± standard error. Citrate coated gold

nanoparticles – cAuNPs; Polyvinylpyrrolidone coated gold nanoparticles – PVP-AuNPs.

The 7 nm AuNPs displayed ability to induce peroxidative damage in membranes. This effect was more detectable after exposure to PVP-AuNPs, that induced increased TBARS to concentrations higher than 4200 µg.L^{-1} whereas for cAuNPs, effects were only found at 3200 µg.L^{-1} ($p<0.05$; Dunnett's test; Figure 7A).

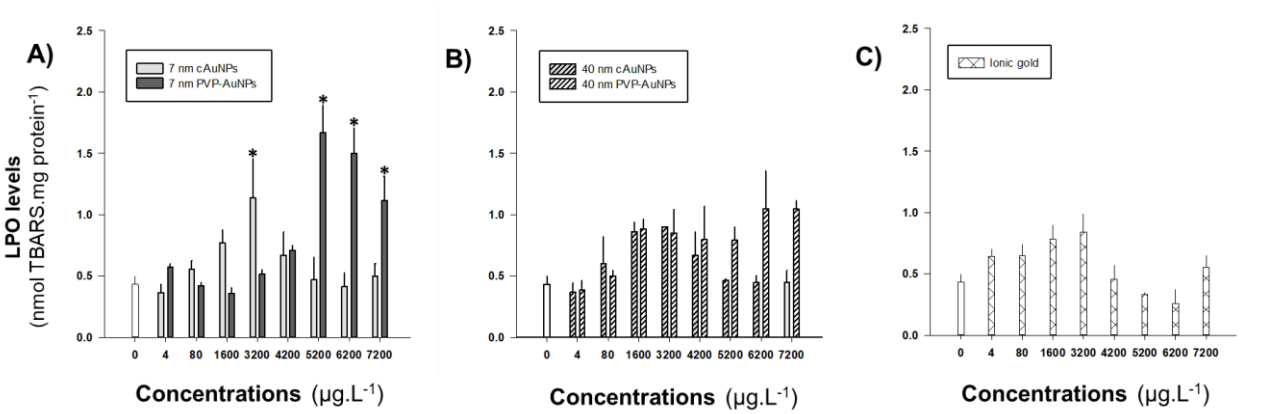

Figure 7. Liver lipid peroxidation (LPO) levels of *Sparus aurata* after 24 h *in vitro* exposure to gold nanoparticles: 7 nm **(A)**, 40 nm **(B)** and ionic gold **(C)**. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). Citrate coated gold nanoparticles – cAuNPs; Polyvinylpyrrolidone coated gold nanoparticles – PVP-AuNPs.

All the tested concentrations of 7 nm AuNPs induced significantly DNA damage ($p<0.05$; Dunnett's test; Figure 8A).

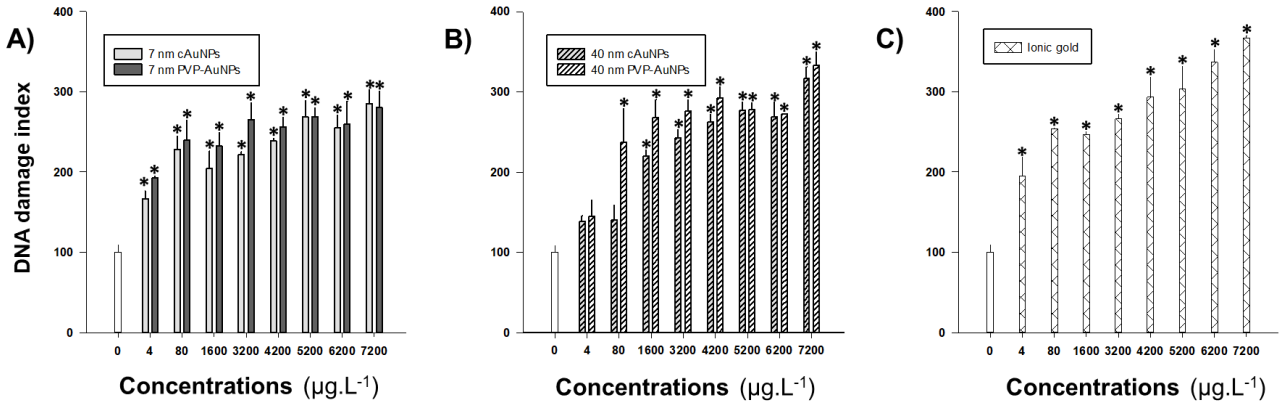

Figure 8. Liver DNA damage index (arbitrary units) of *Sparus aurata* after 24 h *in vitro* exposure to gold nanoparticles: 7 nm **(A)**, 40 nm **(B)** and ionic gold **(C)**. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). Citrate coated gold nanoparticles – cAuNPs; Polyvinylpyrrolidone coated gold nanoparticles – PVP-AuNPs.

3.2.1.1. 40 nm AuNPs

Effects of 40 nm AuNPs on CAT activity were found for both coatings at concentrations higher than 3200 µg.L^{-1}. This enzyme activity was significantly induced by AuNPs ($p<0.05$; Dunnett's test; Figure 4B). In terms of effects on GR activity, the particles induced increased activities at concentrations higher than 1600 µg.L^{-1} for PVP-AuNPs and 3200 µg.L^{-1} for cAuNPs ($p<0.05$; Dunnett's test; Figure 5B). As observed in liver portions exposed to 7 nm AuNPs, GST activity was not significantly affected by 40 nm AuNPs ($p>0.05$; ANOVA; Figure 6B).

No oxidative damage, assessed as LPO, was found after liver exposure to 40 nm AuNPs ($p>0.05$; ANOVA; Figure 7B). However DNA damage was found after exposure to concentrations higher than 4 µg.L^{-1} for PVP-AuNPs and 80 µg.L^{-1} for cAuNPs ($p<0.05$; Dunnett's test; Figure 8B).

3.2.2. Ionic gold

Ionic gold significantly increased the activities of CAT and GR at concentrations higher than 3200 µg.L^{-1} ($p<0.05$; Dunnett's test; Figures 4C and 5C). As observed for AuNPs, liver exposure to ionic gold did not induce significant alterations in GST

activity (p>0.05; ANOVA; Figure 6C). The LPO levels remained unchanged after the exposure to ionic gold (p>0.05; ANOVA; Figure 7C). However, DNA damage was found after all the tested concentrations (p<0.05; Dunnett's test; Figure 8C).

3.2.3. Gemfibrozil (GEM)

CAT activity was significantly increased after exposure to 15000 µg.L^{-1} GEM (p<0.05; Dunnett's test; Figure 9A). However, GR and GST activities were not significantly affected by exposure to GEM (p>0.05; ANOVA; Figure 9B and C).

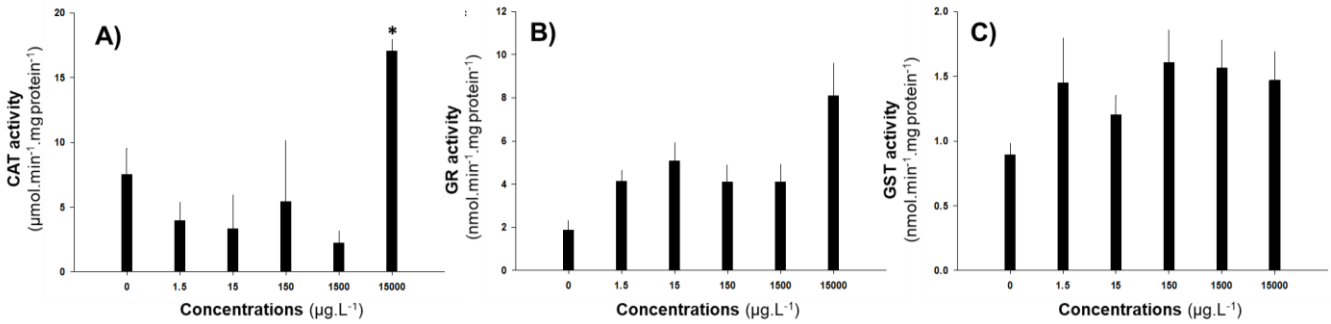

Figure 9. Liver catalase (CAT) **(A)**, glutathione reductase (GR) **(B)** and glutathione *S*-transferases (GST) **(C)** activities of *Sparus aurata* after 24 h *in vitro* exposure to gemfibrozil. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05).

LPO levels significantly increased after exposure to 15000 µg.L^{-1} (p<0.05; Dunnett's test; Figure 10A). In terms of DNA damage, GEM exposure led to a significant decrease in the DNA integrity, for all tested concentrations (p<0.05; Dunnett's test; Figure 10B).

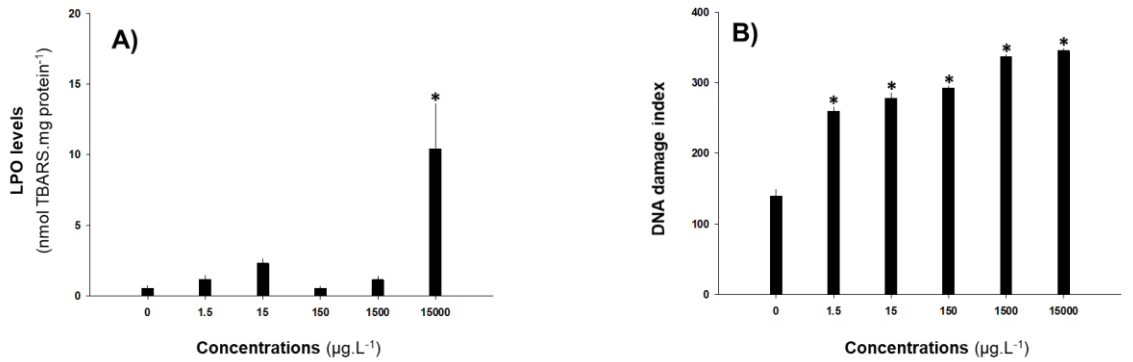

Figure 10. Liver lipid peroxidation (LPO) levels **(A)** and DNA damage index (arbitrary units) **(B)** of *Sparus aurata* after 24 h *in vitro* exposure to gemfibrozil. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05).

3.2.4. Combined exposures of gold and gemfibrozil (GEM)

In the combined exposure conditions, a significant increase of CAT activity was found in all tested experimental conditions (p<0.05; Dunnett's test; Figure 11A). In this case, the observed percentages of effect were higher than the predicted effects (Table 2).

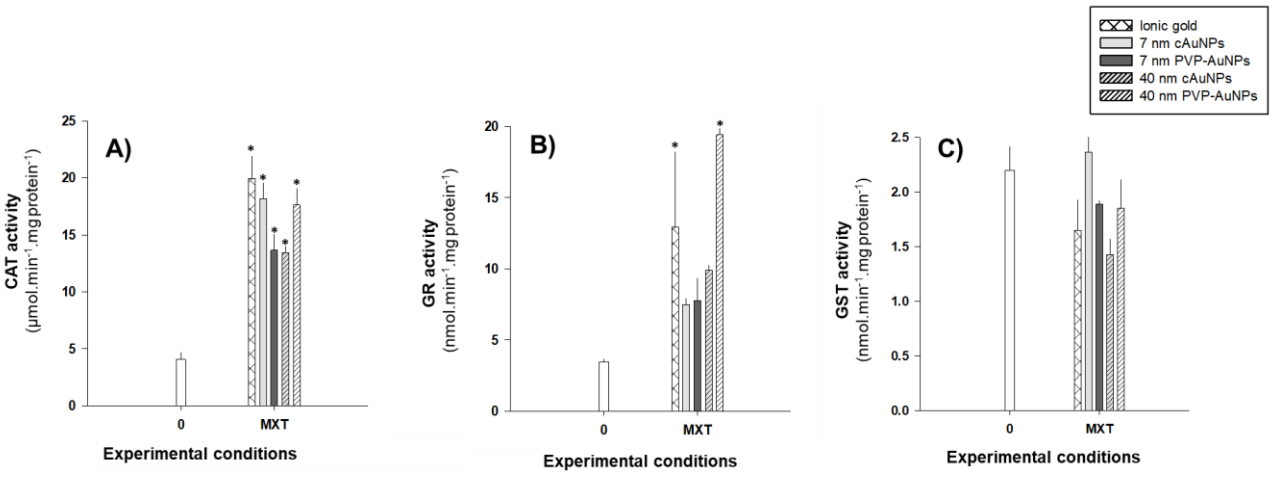

Figure 11. Liver catalase (CAT) **(A)** glutathione reductase (GR) **(B)** and glutathione *S*-transferases (GST) **(C)** activities of *Sparus aurata* after 24 h *in vitro* combined exposure to gold nanoparticles (AuNPs) or ionic gold with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05). Citrate coated gold nanoparticles – cAuNPs; Polyvinylpyrrolidone coated gold nanoparticles – PVP-AuNPs; MXT – 80 µg.L^{-1} ionic gold or AuNPs (cAuNPs or PVP-AuNPs) with 150 µg.L^{-1} GEM.

Regarding effects of GR, the combined exposures to ionic gold plus GEM and 40 nm PVP-AUNPs+GEM significantly increased GR activity (p<0.05; Dunnett's test; Figure 11B), with observed percentages of effect higher than the predicted (Table 2). GST activity was not significantly affected by the combined exposures

(p>0.05; ANOVA; Figure 11C), as observed in the single exposures. In this endpoint, the observed percentages of effect were similar than the predicted (Table 2). The combined exposures to PVP-AuNPs (7 and 40 nm) with GEM significantly increased the liver LPO levels (p<0.05; Dunnett's test; 12A), yielding observed percentages of effect higher than the predicted effects (Table 2). All the combined exposures induced DNA damage (p<0.05; Dunnett's test; Figure 12B), with the observed percentages of effect similar to those expected (Table 2).

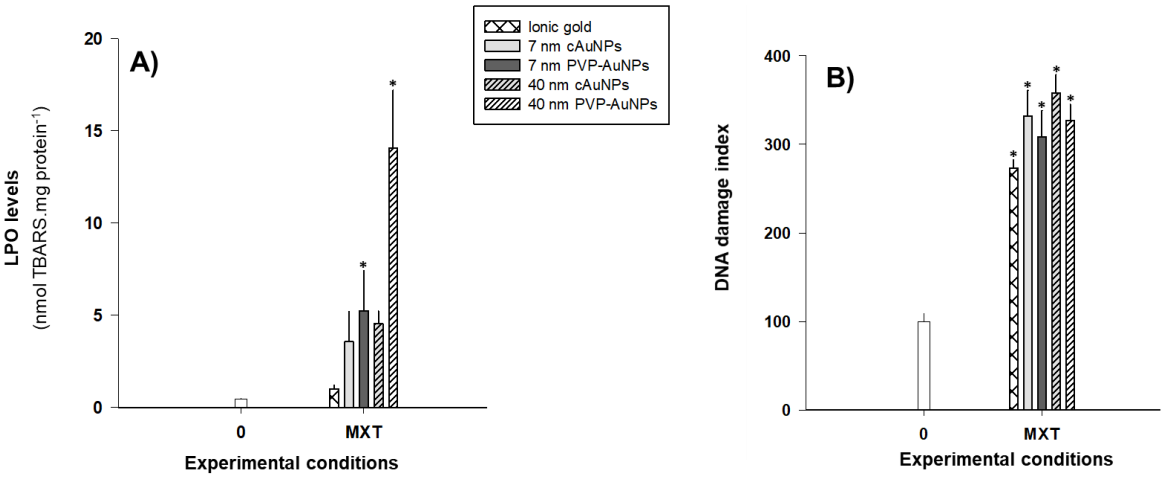

Figure 12. Liver lipid peroxidation (LPO) levels **(A)** and DNA damage index (arbitrary units) **(B)** of *Sparus aurata* after 24 h *in vitro* combined exposure to gold nanoparticles (AuNPs) or ionic gold with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05). Citrate coated gold nanoparticles – cAuNPs; Polyvinylpyrrolidone coated gold nanoparticles – PVP-AuNPs; MXT – 80 µg.L^{-1} ionic gold or AuNPs (cAuNPs or PVP-AuNPs) with 150 µg.L^{-1} GEM.

Table 2. The relative percentage of effect in the different assessed endpoints, after 24 h *in vitro* single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold (80 µg.L^{-1}) and gemfibrozil (GEM; 150 µg.L^{-1}) 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).

Assessed Endpoints	% of effect related to control										
	Ionic gold	7 nm cAuNPs	7 nm PVP-AuNPs	40 nm cAuNPs	40 nm PVP-AuNPs	GEM	Ionic gold + GEM	7 nm cAuNPs + GEM	7 nm PVP-AuNPs + GEM	40 nm cAuNPs + GEM	40 nm PVP-AuNPs + GEM
Catalase Activity	40	27	- 131 *	-11	-45	6	P: 46 O: - 244 *	P: 33 O: - 213 *	P: - 124 O: - 136 *	P: - 5 O: - 132 *	P: - 39 O: - 204 *
Glutathione Reductase Activity	- 57	- 73	- 74	- 44	- 47	- 37	P: - 94 O: - 333 *	P: - 110 O: - 150	P: - 111 O: - 159	P: - 81 O: - 231	P: - 84 O: - 549 *
Glutathione *S*-Transferases Activity	- 69	- 54	- 80	- 71	- 53	- 4	P: - 73 O: - 7	P: - 58 O: - 53	P: - 84 O: - 22	P: - 74 O: - 8	P: - 57 O: -20
Lipid Peroxidation Levels	- 42	- 22	8	- 31	- 10	- 14	P: - 57 O: - 114	P: - 36 O: - 679	P: - 7 O: - 1048 *	P: - 45 O: - 896	P: - 24 O: - 2988 *
DNA Damage Index	- 128 *	- 104 *	- 115 *	- 26	- 112 *	- 162 *	P: - 290 O: - 145 *	P: -266 O: - 197 *	P: - 277 O: - 177 *	P: - 188 O: - 221 *	P: - 274 O: - 193 *

4. Discussion

The agglomeration/aggregation of AuNPs within 12 h in DMEM+FBS is an important aspect to consider because this may influence the NPs toxicity. Several groups already reported that aggregation of NPs in cell culture media or PBS might be prevented by adding serum, presumably due to proteins adsorbing onto the particle surface (Allouni et al. 2009; Balog et al. 2015; Bihari et al. 2008; Mahl et al. 2010, 2012). A previous study reported that cAuNPs immediately aggregated/agglomerated in DMEM whereas in DMEM+FBS they were stable for 12 hours (Barreto et al. 2015). In the present study, at 0 h, in DMEM+FBS, the non-significantly increased sizes of AuNPs, the displayed SPR peaks shifted toward higher wavelengths and ZP slightly less negative as previously reported (Barreto et al. 2015), suggests that FBS was bound to AuNPs. This is a relevant feature to take into consideration because, as previously reported, the attachment of FBS with NPs may prevent its incorporation into the cells/organs and consequently reduce NPs toxic effects (Durán et al. 2015).

Considering the effect of AuNPs concentration, it was observed that the time needed for AuNPs to aggregate/agglomerate in the DMEM+FBS decreased with increase of AuNPs concentration. This is an expected output because the probability of NPs collisions will increase, increasing the number of particles per volume (Barreto et al. 2015). At 12 h, media (DMEM+FBS with AuNPs) were bluer in the highest tested concentrations of AuNPs than in the lowest, corroborating with the previously described. According to Zeng et al. (2012), the surface energy of AuNPs increases with the decrease of the diameter. Therefore, smaller AuNPs interact more strongly with the compounds present in the solution, leading to size-dependent aggregation of AuNPs (Zeng et al. 2012). In the present study, this was not visually observed, as detected to the highest versus lowest concentrations. Additionally, at 12 h, all 7 and 40 nm AuNPs already had aggregated/agglomerated. Thus, for the same concentration, the tested AuNPs sizes displayed similar behaviour in the test media, in terms of aggregation/agglomeration and stability period. However, different sizes of aggregates/agglomerates were detected, depending on the initial size of AuNPs,

with aggregates/agglomerates resultant from 40 nm AuNPs being bigger than those resultant from 7 nm AuNPs.

In the available literature, AuNPs toxicity data are often conflicting due to the variability of the used toxicity assays, in terms of: cell lines, exposure times, assessed endpoints, NPs concentrations and chemical/physical properties. AuNPs have been reported as “nontoxic” according to some *in vitro* tests (Alkilany and Murphy 2010; Connor et al. 2005; Luis et al. 2016; Shukla et al. 2005). Shukla et al. (2005), using RAW264.7 macrophage murine cell line, reported that AuNPs are not cytotoxic, reducing the production of reactive oxygen and nitrite species and not eliciting secretion of proinflammatory cytokines, making them suitable candidates for nanomedicine. Connor et al. (2005) reported that AuNPs exposure did not cause acute cytotoxicity in human K562 cells. Luis et al. (2016) also demonstrated that AuNPs did not have effect on the haemolymph' acetylcholinesterase and gills' GST activities of mussel *Mytilus galloprovincialis*. However, other authors have been reported that AuNPs may present toxicity (Baharara et al. 2016; Goodman et al. 2004; Li et al. 2010; Pan et al. 2009; Tkachenko et al. 2004). AuNPs, which were taken up by MRC-5 human lung fibroblasts, induced autophagy with oxidative stress (Li et al. 2010). The investigation of Baharara et al. (2016) demonstrated the induction of apoptosis in human HeLa cell line treated with AuNPs. AuNPs exposure on HeLa and 3T3/NIH mouse embryo fibroblast cell lines resulted in cell viability reduction (Tkachenko et al. 2004).

In the current study, the effects showed be dependent on the AuNPs size, coating and concentration. In general, AuNPs significantly increased CAT and GR activities, mostly at the highest concentrations (> 3200 $\mu g.L^{-1}$). Also, AuNPs induced damage to different cellular components (DNA strand breaks and lipid membrane peroxidation), even at low concentrations (4 $\mu g.L^{-1}$). Previous studies also revealed that AuNPs may induce the formation of reactive oxygen species (ROS) and increase the levels of LPO (Gao et al. 2011; Li et al. 2010; Tedesco et al. 2010). An *in vivo* genotoxic effect of different sizes (2, 20 and 200 nm) of AuNPs was observed by Schulz et al. (2012) in the lungs of rats, showing that DNA damage had a weak size-related increase of the mean tail intensity (Schulz

et al. 2012). As reported by others, the possible adverse effects of AuNPs may be attributed to: 1) their interaction with the cell membrane (Goodman et al. 2004); 2) oxidative stress leading to cytotoxicity effects (Pan et al. 2009); 3) the inhibition of metabolic activity, e.g., leading to mitochondrial damage (Panessa-Warren et al. 2008); 4) possible damage or alteration in the nuclear DNA (Panessa-Warren et al. 2008; Schulz et al. 2012).

The 7 nm AuNPs induced more effects than 40 nm AuNPs. Only 7 nm AuNPs increased LPO levels. Lower concentrations of 7 nm AuNPs (80 and 1600 $\mu g.L^{-1}$) increased the activities of GR and CAT whereas with 40 nm AuNPs these increases only occurred after exposure to highest concentrations (> 1600 $\mu g.L^{-1}$). At 4 $\mu g.L^{-1}$, only 7 nm AuNPs caused DNA damage. This may be explained by the sizes of AuNPs (7 versus 40 nm). As already previous described the *in vitro* permeation on rat skin of smaller AuNPs (15 nm) was higher and more rapid than the bigger ones (102 and 198 nm) (Sonavane et al. 2008) and consequently may induce more effects. 7 nm PVP-AuNPs, which presented the smallest sizes during the experimental test comparing with the other tested AuNPs, induced more effects in the liver organ culture. They induced effects at lower concentrations (between 4 to 1600 $\mu g.L^{-1}$) while the other AuNPs only induced at higher concentrations (> 1600 $\mu g.L^{-1}$). Additionally, oxidative damage was mostly detected after the exposure to 7 nm PVP-AuNPs. Comparing 40 nm cAuNPs and PVP-AuNPs, PVP-AuNPs also induced effects (increased GR activity and led to DNA integrity loss) at concentrations lower than those induced by cAuNPs. Previous studies also reported different effects of AuNPs with different coatings (Iswarya et al. 2016; Fraga et al. 2013; Paino et al. 2012). In a mice model, 96 h exposure to 65 nm PVP-AuNPs was found to have more effects in the DNA of liver cells (assessed as DNA strand breaks) than 29 nm cAuNPs (Iswarya et al. 2016).

In the present work, gold in nano form induced more effects in the liver organ culture of *S. aurata* than ionic form. Oxidative damage was only detected after the exposure to AuNPs. Additionally, for instance, 80 and 1600 $\mu g.L^{-1}$ of AuNPs increased CAT activity whereas for ionic gold only concentrations higher than 3200 $\mu g.L^{-1}$ had effects. The study of Barbasz and Oćwieja (2016), using two types of human cell lines, showed a higher cytotoxicity of AuNPs than ionic gold

(Barbasz and Oćwieja 2016), in agreement with the results of the present study. However, other studies reported a higher toxicity of ionic gold comparing with nano form (Farkas et al. 2010; Luis et al. 2016). Farkas et al. (2010) reported that, in rainbow trout (*Oncorhynchus mykiss*) hepatocyte cells, the *in vitro* exposure to 17.4 $mg.L^{-1}$ ionic gold significantly increased ROS levels. At the same concentration, nano form did not have any effect (Farkas et al. 2010). Luis et al. (2016) in another *in vitro* test also showed that ionic gold significantly decreased the haemolymph' acetylcholinesterase and gills' GST activities of mussel *Mytilus galloprovincialis*. However, no significant alterations were found after *in vitro* exposure to AuNPs, regardless of their coating (Luis et al. 2016). There are few available studies about the possible mechanisms of Au^{+} toxic action. Nonetheless, the ionic gold ability to undergo redox reactions with peptides and proteins, particularly involving sulfur amino acids, to deprotonate and bind to peptide amide bonds and cross-link histidine imidazole rings has been already reported (Best and Sadler 1996; Luis et al. 2016).

Concerning GEM, despite increased the CAT activity and LPO levels were only found for the highest tested concentration (15000 $\mu g.L^{-1}$), all the tested concentrations led to DNA integrity loss. The *in vitro* toxic effects of GEM were also already reported (Zurita et al. 2007). Zurita et al. (2007) described that GEM reduced protein content, neutral red uptake, methylthiazol metabolization and lysosomal function in the hepatoma fish cell line PLHC-1.

The effects of the combined exposures of AuNPs and GEM were, for many endpoints, higher than the predicted (the sum of the effects of each contaminant alone). The occurrence of synergistic effects between AuNPs and GEM detected in the present study must be taking into consideration considering that, in the environment, there is a variety of contaminants that may interact between them. To our best knowledge, a single study has so far assessed the combined effects of NPs and pharmaceuticals in aquatic organisms (Luis et al. 2016). Luis et al. (2016) demonstrated that AuNPs, in combined exposures, may significantly alter the effects of the pharmaceuticals carbamazepine and fluoxetine, even at concentrations that may be considered environmentally relevant, with these effects dependent on the coating of NPs and tested endpoint. In the present study, the

detected effects of the combined exposures were also dependent on the characteristics of AuNPs, with 40 nm PVP-AuNPs with GEM inducing more synergistic effects than 40 nm cAuNPs combined with GEM and 7 nm AuNPs plus GEM.

The liver organ culture of *Sparus aurata* were sensible to low concentrations of the tested contaminants and allowed to differentiate responses to NPs with different characteristics: size and coating. They also allowed the study of combined exposures, proving sensitive to distinguish experimental conditions. Taking into account that the organ cultures involve "the maintenance or growth of tissues, organ primordia or the whole or parts of an organ *in vitro* for a period of 24 h or longer, in a way which may allow differentiation and/or preservation of architecture and/or function" (Oliveira et al. 2003), this approach showed be very useful, allowing to understand better the effects of the contaminants to the organism than using cell lines.

5. Conclusions

The *in vitro* system used in the present study proved to be a valuable approach to evaluate the single and combined effects of contaminants, such as nanoparticles and pharmaceuticals, to aquatic organisms. Gold nanoparticles (AuNPs) increased the enzymatic activities (catalase and glutathione reductase) and induced damage in DNA and cellular membranes, even at lower concentrations (4 $\mu g.L^{-1}$), in the liver organ culture of *Sparus aurata*. The effects showed be dependent on the size, coating and concentration of AuNPs, being the 7 nm PVP-AuNPs that induced more effects. Additionally, GEM also induced DNA damage at an environmental relevant concentration (1.5 $\mu g.L^{-1}$). In many endpoints, the combined exposures of AuNPs and GEM induced more effects than the predicted. *Sparus aurata* liver organ culture proved to be a successful alternative to *in vivo* studies.

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Chapter III

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

A. Barreto[1*], A. Dias[1], B. Duarte[1], E. Pinto[2], A. Almeida[2], T. Trindade[3], A.M.V.M. Soares[1], K. Hylland[4], S. Loureiro[1], M. Oliveira[1]

[1] Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] LAQV/REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

[3] Departamento de Química & CICECO - Aveiro Instituto de Materiais, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[4] Department of Biosciences, University of Oslo, PO Box 1066, N-0316 Oslo, Norway

*Corresponding author: E-mail: abarreto@ua.pt, Tel +351 234 370 350, Fax +351 234 372 587

Highlights

- Gold (nanoparticles or ionic form), at 4 μg.L^{-1}, induced lipid peroxidation and genotoxicity in *Sparus aurata*;
- Ionic gold induced more effects on *Sparus aurata* than a nano form of the metal;
- Despite being less stable in seawater, citrate coated nanoparticles induced more effects than polyvinylpyrrolidone coated nanoparticles.

Abstract

The question of whether gold (Au) is more toxic as nanoparticles or in its ionic form remains unclear and controversial. The present work aimed to clarify the effects of 96 h exposure to 4, 80 and 1600 $\mu g.L^{-1}$ 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 $\mu g.L^{-1}$, and reduced the ability of *S. aurata* to swim against a water flow at 1600 $\mu g.L^{-1}$. Exposure to cAuNPs induced more 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 on the 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 $\mu g.L^{-1}$ of iAu.

Keywords: nanotoxicity; gold; marine fish; seawater

1. Introduction

Throughout its history, gold (Au) has been recognized as an inert, non-toxic and biocompatible noble metal with therapeutic properties (Daniel and Astruc 2004; Fratoddi et al. 2015). However, when Au decreases to nanometer dimensions, the safety of the resulting nanomaterials has been questioned (Boverhof et al. 2015). Gold nanoparticles (AuNPs) have been widely used in medicine and biological research (Fratoddi et al. 2015), including targeted delivery of drugs (Ghosh et al. 2008), imaging and diagnosis (Bhattacharya and Mukherjee 2008). Its application in aquaculture as antimicrobial agent (Saleh et al. 2016) and to detect contaminants (Loganathan and John 2017) has also been investigated. Despite the widespread use of AuNPs and consequent release to the environment, there is

limited understanding of their consequences for environmental health (Iswarya et al. 2016; Klaine et al. 2008; Teles et al. 2016). In addition, the question of whether AuNPs are more toxic than ionic Au (iAu) remains unresolved (Barbasz and Oćwieja 2016; Botha, James, and Wepener 2015; Dedeh et al. 2015; Farkas et al. 2010; Luis et al. 2016) – Table 1.

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

Test type	Cells/ Organisms	Exposure times	Endpoints/ Parameters	Coatings	Sizes/Shapes (nm)	Doses	Ionic or nano form more toxic?	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	Citrate	Spherical 21	0.75 to 25 ppm	Nano	1
In vitro	*Mytilus galloprovincialis* hemolymph and subcellular fraction of gills	10 min	Enzymatic activities	Citrate, PVP and BSA	Spherical 7	54 $ng \cdot L^{-1}$ to 2.5 $mg \cdot L^{-1}$	Ionic	2
In vivo	*Daphnia pulex, D. magna, Danio rerio, Poecilia reticulata, Labeobarbus aeneus, Pseudocrenilabrus philander, Tilapia sparrmanii, Oreochromis mossambicus*	48 and 96 h	Species sensitivity distributions	Citrate	Spherical 14	0.0005 to 200 $mg.L^{-1}$	Ionic	3
In vivo	*Danio rerio*	20 d	Gene expression	Citrate	Spherical 14	0.25 and 0.8 $\mu g.L^{-1}$	Nano	4
In vitro	Hepatocyte cell culture of *Oncorhynchus mykiss*	2 and 48 h	Cytotoxicity and ROS formation	Citrate	Spherical 5-10	0.063 to 19 $mg.L^{-1}$	Ionic	5

Thus, the aim of the present study was to 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. 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 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)

2.1.1. Synthesis

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 known non-toxicity of citrate, the use of water as solvent and the fact that cAuNPs have been widely used in diverse applications (Hanžić et al. 2015; Li et al. 2011; Turkevich, Stevenson, and Hillier 1951). 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). cAuNPs and PVP-AuNPs were

centrifuged and the pellets resulting from the centrifugation were resuspended in ultrapure water.

2.1.2. Characterisation

AuNPs stock suspensions and AuNPs in the experimental media (artificial seawater – ASW) and in ultrapure water were characterised at 0, 24 and 96 h. Characterisation was performed by UV–Vis spectra (Cintra 303, GBC Scientific), dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern), transmission electron microscopy – TEM (Hitachi, H9000 NAR) and scanning electron microscopy – SEM (Hitachi, SU70).

2.2. Bioassay

2.2.1. Fish

Juvenile gilthead seabream (*Sparus aurata*) with length 7.6 ± 0.1 cm, acquired from an aquaculture facility in Spain (Santander), were acclimated for 1 month in aquaria containing aerated and filtered artificial seawater (ASW, prepared by dissolving the salt in reverse osmosis water to obtain a salinity of 30), under controlled temperature (17ºC) and natural photoperiod. During this period, the fish were fed daily at a ratio of 1 g per 100 g of fish with commercial fish food (Sorgal, Portugal).

2.2.2. Experimental design

The ASW used to maintain fish during the acclimation was used to perform the toxicity tests. During the bioassay, temperature, salinity, conductivity, pH, dissolved oxygen and aeration conditions were similar to conditions during 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 near to the predicted values of AuNPs for the environment: water (0.14 $\mu g.L^{-1}$) and soil (5.99 $\mu g.kg^{-1}$) (García-Negrete et

al. 2013; Tiede et al. 2009). The other concentrations tested were 20-fold increases.

Part of the experimental media (around 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.

2.3. Assessment of swimming performance

After 96 h exposure, fish were individually introduced into a 1.2 m long flume with 6.7 cm diameter and induced to swim against a water flow of 19 $L.min^{-1}$, generally following the procedure described by Oliveira et al. (2012). The time that fish spent swimming against the water flow was recorded and presented in seconds. After this behavioural assessment, fish were transferred back into their original test aquaria where they remained for 2 h prior to sampling.

2.4. Collection of biological material

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

2.4.1. Biochemical biomarkers analysis

Liver and gills were homogenized in potassium phosphate buffer (0.1 mM, pH 7.4) using an ultrasonic homogenizer. The homogenate was then divided into three aliquots for: lipid peroxidation (LPO) assay, NPT quantification and post-mitochondrial supernatant (PMS) preparation. To prevent oxidation, the aliquot of

homogenate for LPO evaluation was transferred to a microtube with 4% BHT (2,6-di-tert-butyl-4-methylphenol) in methanol. The aliquots for LPO and NPT levels determination were stored at -80ºC until analysis. PMS was accomplished by centrifugation 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, and the obtained supernatant was collected and stored at -80ºC until ChE activity determination.

Protein concentration 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).

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 modifications, as previously described (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 previously reported procedures (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 (blood from fish 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). For the enzyme treatment, the correspondent slides were removed from lysis buffer and were washed 3 times in cold (4°C) enzymatic buffer solution (40 mM HEPES; 0.1 M KCl; 0.5 mM EDTA; 0.2 $mg.mL^{-1}$ bovine serum albumin, pH 8.0). Fpg (45 µL, 1:60 diluted in enzymatic buffer solution) was added to the slides, which were individually sealed with a coverslip and incubated during 30 min at 37°C. The other steps involved in the comet assay were common to slides with or without Fpg. Cells were classified according to tail length, into five classes (Collins 2004): class 0 – undamaged, without a tail; class 1 – with a tail shorter than the diameter of the nucleus; class 2 – with a tail length 1–2 times the diameter of the nucleus; class 3 – with a tail longer than twice the diameter of the nucleus; class 4 – comets with no nucleus. A damage index (DI), in arbitrary units, was assigned to each slide (for 100 cells) and consequently for each treatment, using the formula:

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

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

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

The ENAs assay was carried out in mature peripheral erythrocytes according previous procedures and nuclear lesions were scored as micronuclei, lobed, segmented, kidney-shaped and vacuolated nuclei (Barreto et al. 2017; Pacheco

and Santos 1996). Results were expressed as the ENAs frequency (‰) to each replicate (for 1000 cells) and consequently for each treatment using the formula:

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

2.5. Gold (Au) quantification

The number of nanoparticles (NPs) and theoretical concentrations of stock suspensions were estimated based on their absorption spectra and sizes (Barreto et al. 2015; Liu et al. 2007; Paramelle et al. 2014).

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

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

Total Au content ($[Au]_{total}$), in µg.g^{-1}, was calculated as the sum of the Au content in each assessed tissue of the fish according to the formula:

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

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

The bioaccumulation factor (BAF), in L.g^{-1}, was calculated according a previous study (Yoo-Iam, Chaichana, and Satapanajaru 2014), dividing the Au content

(µg.g^{-1}) in each tissue of the fish (gills, liver, spleen or muscle) by the initial concentration of Au in the exposure media (µg.L^{-1}):

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

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

As *Sparus aurata* is a fish for human consumption an extrapolation of Au intake for humans was calculated, using the following 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). Au content in the ingested fish corresponds to the content of Au determined in the fish muscle (µg.g^{-1}). The calculated Au intake values were compared with the maximum amount of Au that each person may be exposed daily over their lifetimes without considerable health risk – “tolerable daily intake” (TDI) 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 (Ahmed et al. 2012). The NOAEL was then divided by a safety factor (100), which resulted in a large margin of safety (FDA 2015):

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

TDI is expressed in µg per kg body weight per day.

2.7. 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 treatments and control and between all the treatments were analysed using one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey´s test whenever applicable. Significant differences were assumed for $p<0.05$.

3. Results

3.1. Gold nanoparticles (AuNPs) – Characterisation and behaviour

The UV-Vis spectra and characteristics of cAuNPs and PVP-AuNPs are presented in Figure 1A and Table 2.

Table 2. Characteristics of gold nanoparticles (AuNPs) in ultrapure water and artificial seawater after 96 h. cAuNPs – Citrate coated gold nanoparticles; PVP-AuNPs – Polyvinylpyrrolidone coated gold nanoparticles; PdI – Polydispersity Index; SPR – Surface Plasmon Resonance; ZP – Zeta Potential; N. D. – Not detected

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

Electron microscopy analysis confirmed that NPs were spherical and had similar sizes (Figure 1B and C).

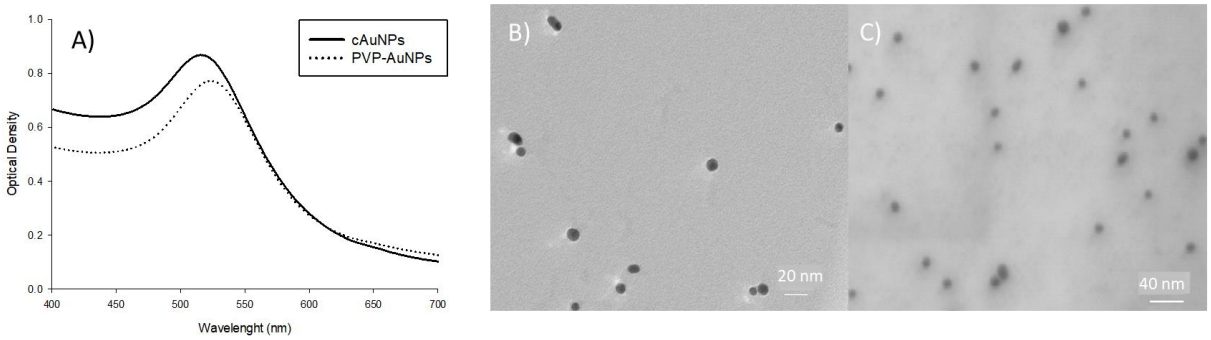

Figure 1. UV–Vis spectra **(A)** and electron microscopy images of citrate coated gold nanoparticles – cAuNPs **(B)** and polyvinylpyrrolidone coated – PVP-AuNPs **(C)**.

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. The hydrodynamic size of cAuNPs (1600 µg.L^{-1}) increased to around 160 nm, maintaining this size till the end of the exposure (96 h). The characteristic SPR peak detected in ultrapure water was not detected in ASW (Table 2). Different peaks corresponding to different charges were found in the ZP analysis. 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 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.

3.2. Gold (Au) quantification in experimental media

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

Table 3. Nominal and measured concentrations (μg.L^{-1}) of gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold in experimental media (artificial seawater) at 0 and 24 h. Results are expressed as mean ± standard error.

Nominal concentrations (μg.L^{-1})	Time (h)	Measured concentrations (μg.L^{-1})		
		cAuNPs	PVP-AuNPs	Ionic gold
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
	2 4	7.0 ± 0.6	38.4 ± 1.6	89.1 ± 1.3
1600	**0**	88.9 ± 7.0	1341.1 ± 51.7	1370.2 ± 36.0
	24	34.6 ± 5.8	1140.7 ± 19.9	1285.1 ± 81.8

At 0 h, the Au quantified in ASW, in general, was lower than the nominal concentrations, with exception to 4 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 cAuNPs (Table 3). This difference increased with the increasing in cAuNPs concentration. 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. Comparing the Au quantification at 0 and 24 h, the concentration of cAuNPs in suspension decreased more than the concentration of PVP-AuNPs (Table 3). In the nominal concentration 4 μg.L^{-1}, a 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 in suspension after 24 h was also observed with 61% for cAuNPs, 15% for PVP-AuNPs and 6% for iAu.

3.3. Biological responses

3.3.1. Nano form

3.3.1.1. Citrate coated gold nanoparticles (cAuNPs)

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

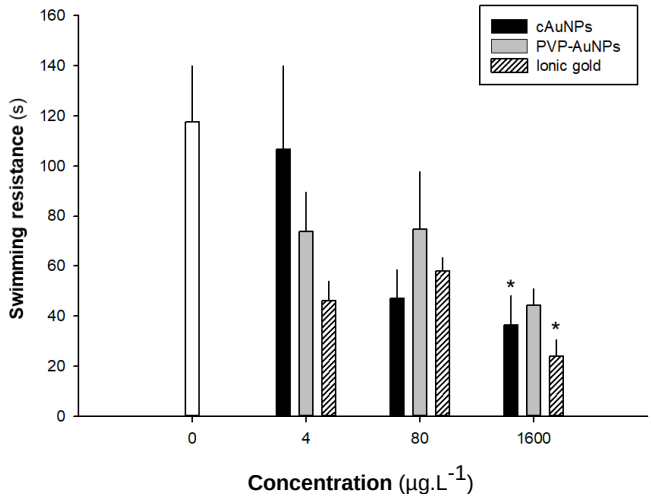

Figure 2. Resistance of *Sparus aurata* to withstand swimming against a water flow after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s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

ChE activity in brain and muscle was not significantly altered ($p>0.05$; ANOVA; Figure 3).

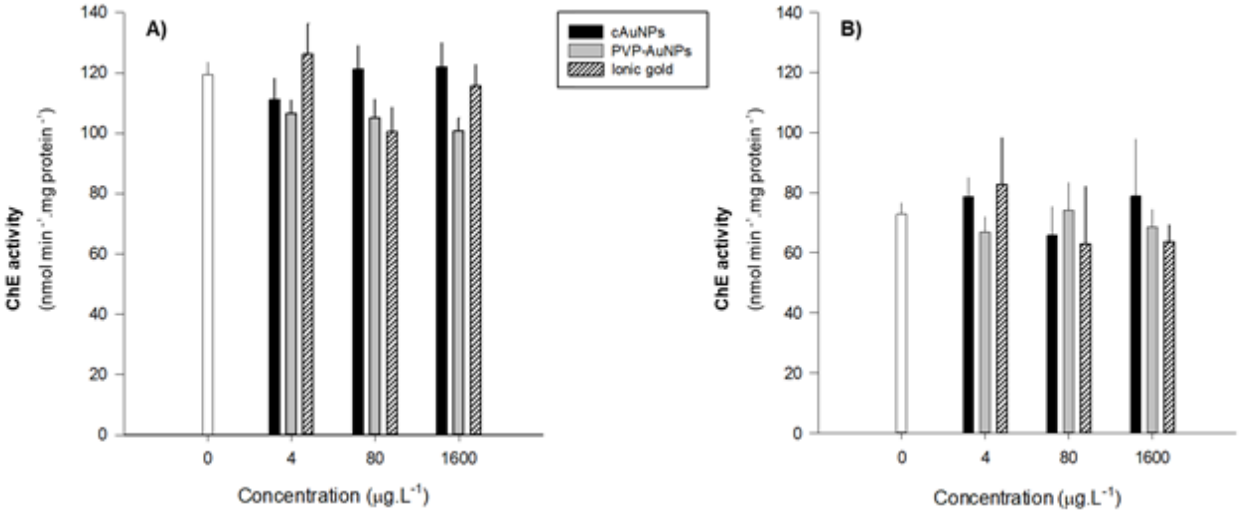

Figure 3. Brain **(A)** and muscle **(B)** cholinesterases (ChE) activity of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error.

Also, CAT activity, in gills and liver, was not significantly altered ($p>0.05$; ANOVA; Figure 4).

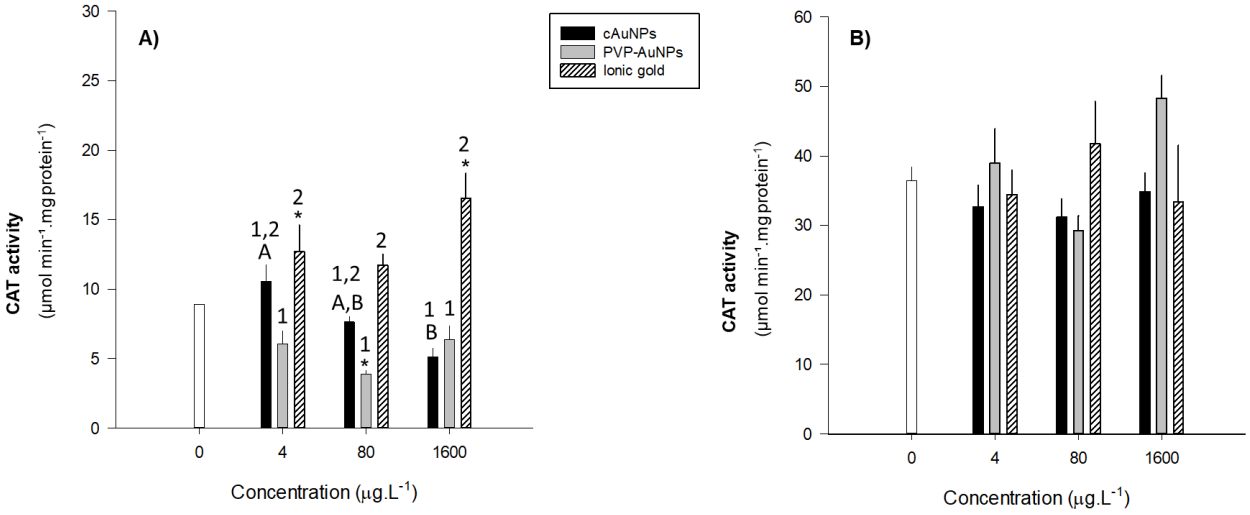

Figure 4. Gills **(A)** and liver **(B)** catalase (CAT) activity of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s 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} (p<0.05; Dunnett's test; Figure 5B).

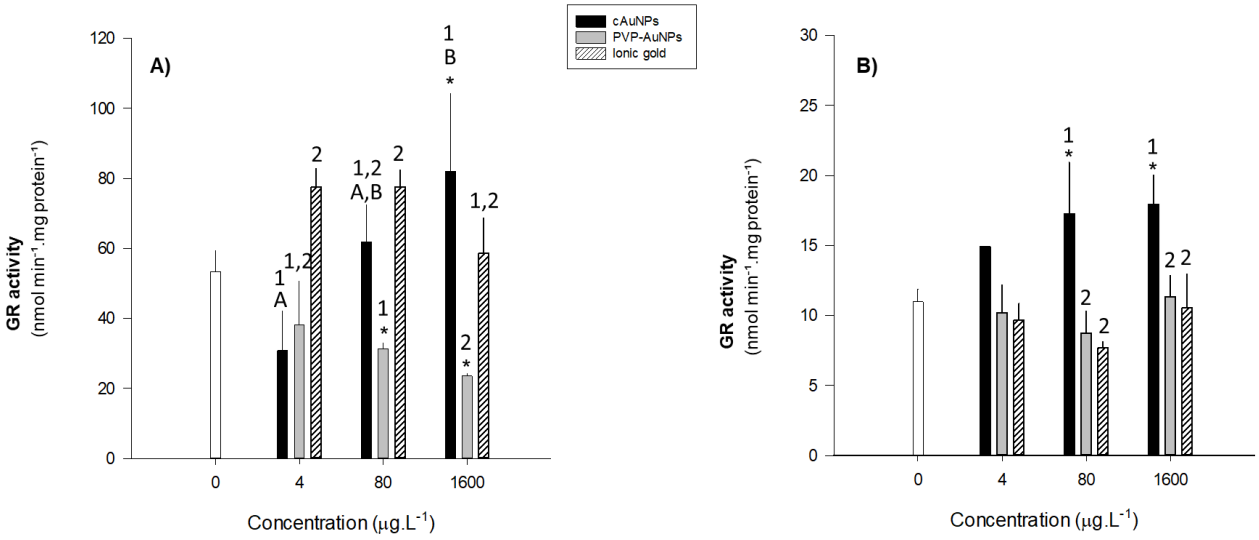

Figure 5. Gills **(A)** and liver **(B)** glutathione reductase (GR) activity of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to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

cAuNPs at 4 µg.L^{-1} significantly increased gills GPx activity (p<0.05; Dunnett's test; Figure 6A), but did not induce significant alterations in the liver (p>0.05; ANOVA; Figure 6B).

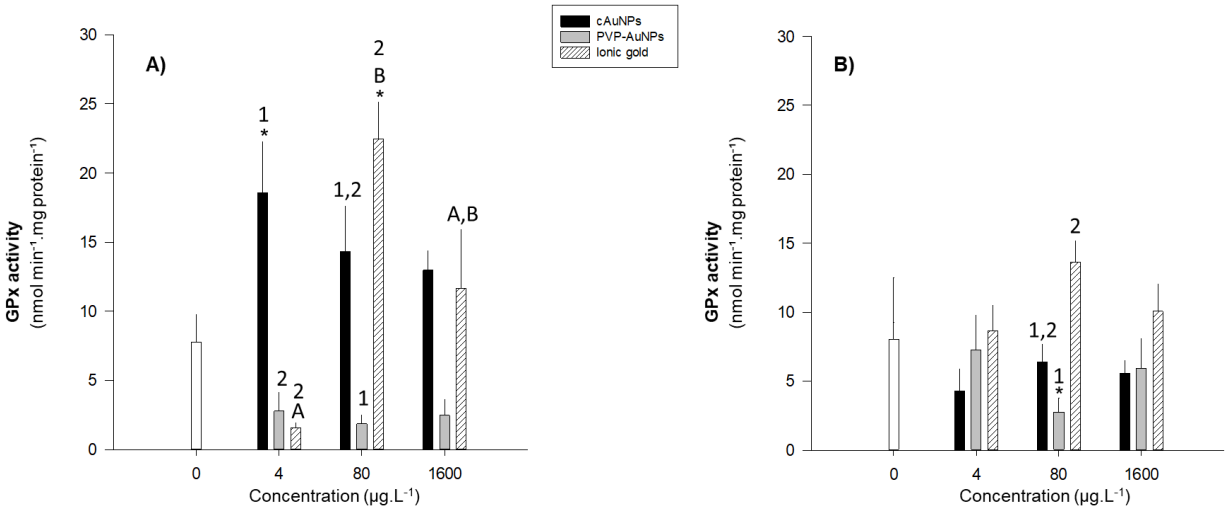

Figure 6. Gills **(A)** and liver **(B)** glutathione peroxidase (GPx) activity of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to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

All tested concentrations of cAuNPs significantly increased gills NPT levels ($p<0.05$; Dunnett's test; Figure 7A). In liver, only 1600 µg.L^{-1} significantly increased the levels of NPT ($p<0.05$; Dunnett's test; Figure 7B).

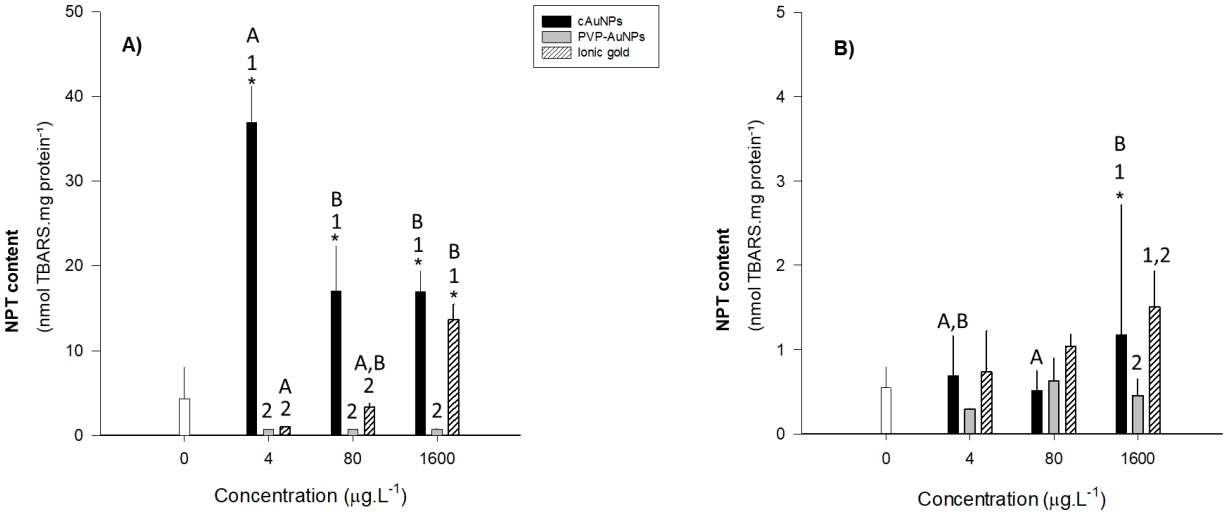

Figure 7. Gills **(A)** and liver **(B)** non-protein thiols (NPT) levels of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control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

cAuNPs at 4 µg.L^{-1} significantly increased gills GST activity (p<0.05; Dunnett's test; Figure 8A). In liver, cAuNPs did not have a significant effect in the activity of this enzyme (p>0.05; ANOVA; Figure 8B).

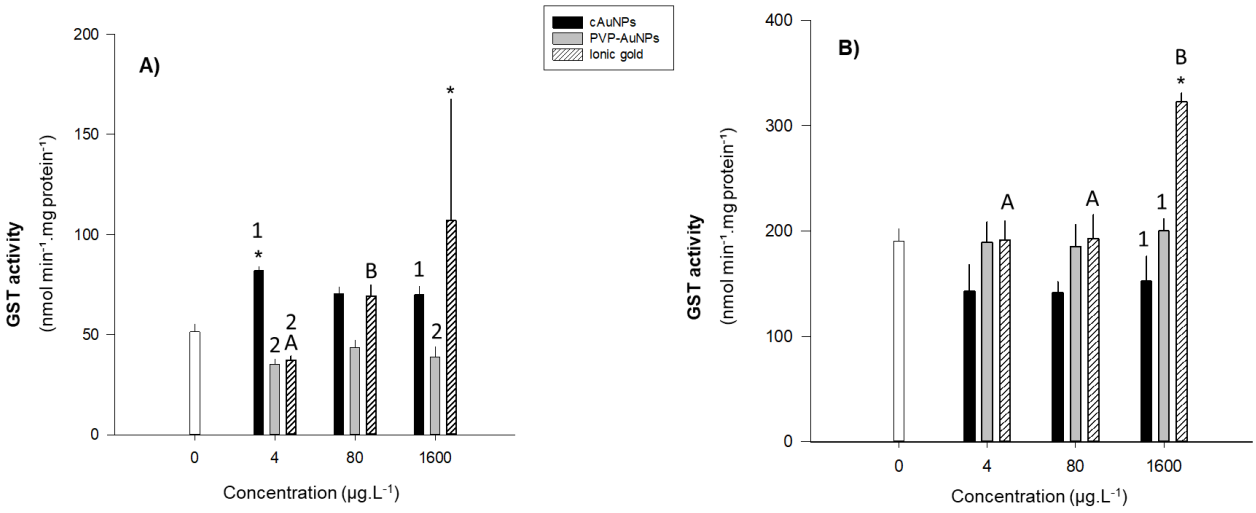

Figure 8. Gills **(A)** and liver **(B)** glutathione *S*-transferases (GST) activity of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences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

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

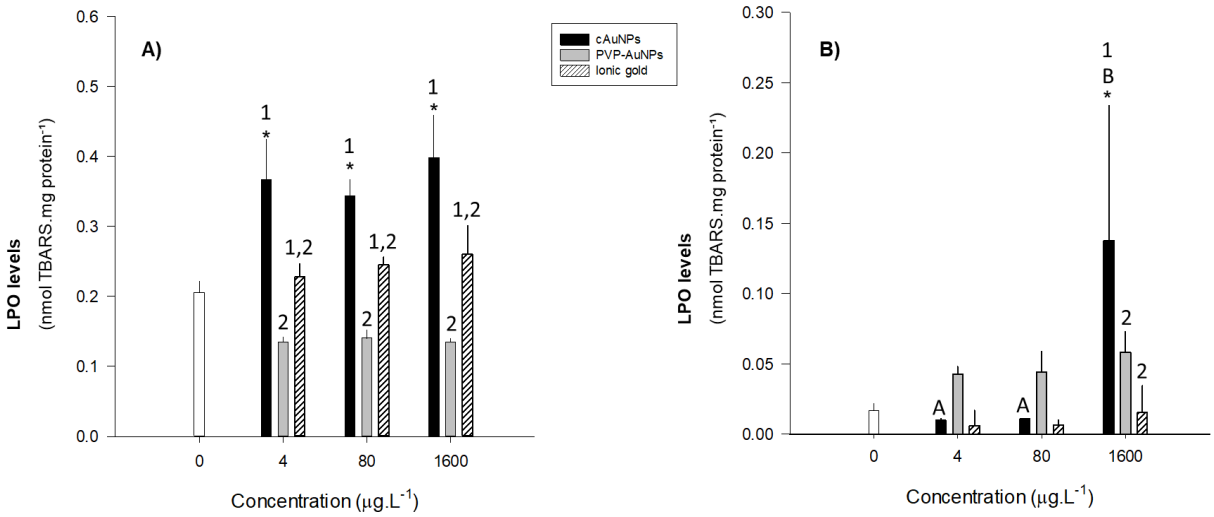

Figure 9. Gills **(A)** and liver **(B)** lipid peroxidation (LPO) levels of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control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

In brain and muscle, Figure 10, oxidative damage was not found after exposure to cAuNPs ($p>0.05$; ANOVA).

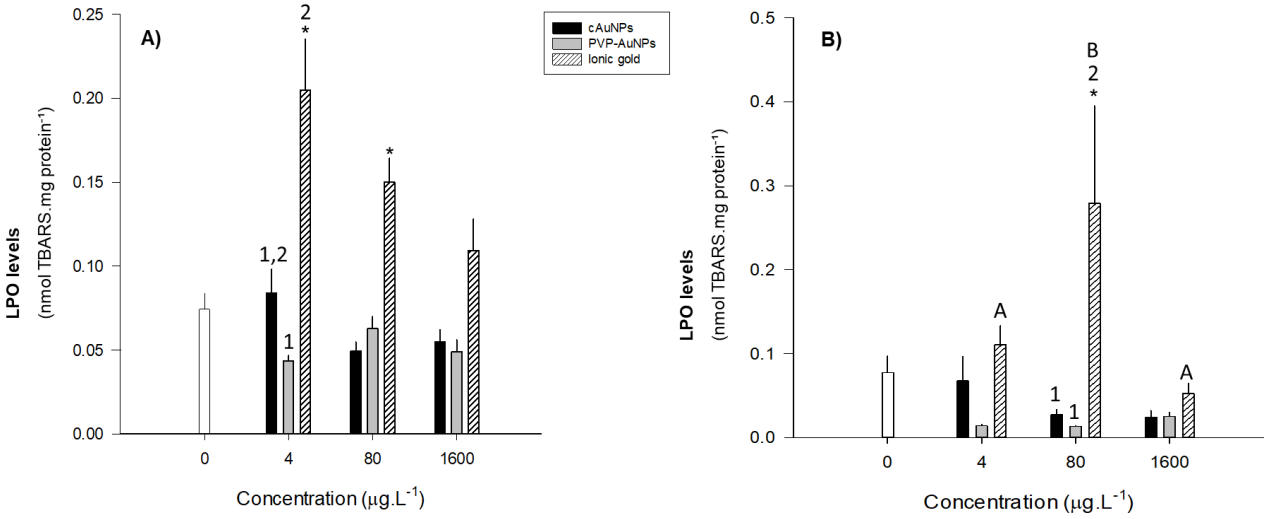

Figure 10. Brain **(A)** and muscle **(B)** lipid peroxidation (LPO) levels of *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (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 of cAuNPs 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 cAuNPs concentration. 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 $\mu g.L^{-1}$ of cAuNPs and classes 2 and 3 in the exposures to 80 $\mu g.L^{-1}$ ($p<0.05$; Dunnett's test). At 1600 $\mu g.L^{-1}$, cAuNPs 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}, led to significantly higher ENAs frequency ($p<0.05$; Dunnett's test), as shown in Figure 11.

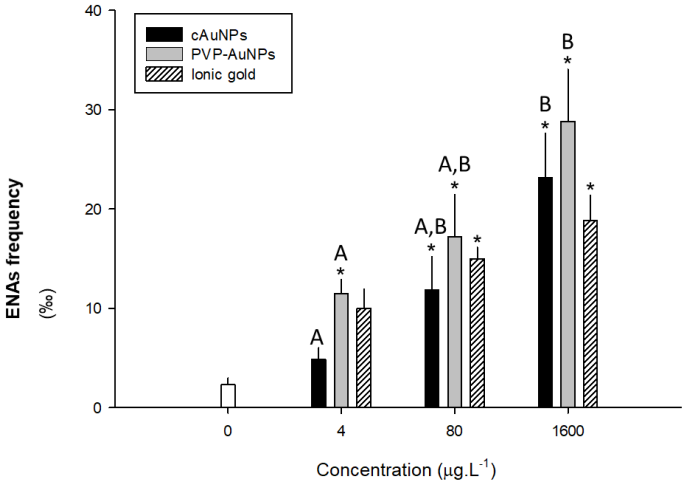

Figure 11. Erythrocytic nuclear abnormalities (ENAs) frequency in *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s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

The frequency of ENAs increased with the increase of cAuNPs concentration. As shown in Table 5, lobed nuclei was the abnormality most commonly detected in all cAuNPs treatments, followed by kidney-shaped nuclei, and were significantly different from control for all treatments with the exception to 4 $\mu g.L^{-1}$ ($p<0.05$; Dunnett's test). The segmented and vacuolated nuclei were the abnormalities less detected. Micronuclei abnormality was not found – 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	-
4 µg.L^{-1} cAuNPs	2.0±0.8	0.3±0.2	2.3±0.4	0.3±0.2	-
80 µg.L^{-1} cAuNPs	4.0±1.1*	0.2±0.2	7.7±2.5*	-	-
1600 µg.L^{-1} cAuNPs	7.6±1.3*	0.1±0.1	15.4±3.7*	-	-
4 µg.L^{-1} PVP-AuNPs	4.3±0.7*	-	7.2±1.1*	-	-
80 µg.L^{-1} PVP-AuNPs	4.6±0.8*	0.4±0.2	12.2±3.8*	-	-
1600 µg.L^{-1} PVP-AuNPs	10.3±1.6*	-	18.3±4.1*	-	0.2±0.2
4 µg.L^{-1} ionic gold	3.3±0.5*	0.4±0.2*	6.3±1.4*	-	-
80 µg.L^{-1} ionic gold	4.7±8*	0.3±0.2	9.7±0.9*	0.3±0.2	-
1600 µg.L^{-1} ionic gold	4.5±1.0*	0.3±0.3	14.2±1.2*	0.5±0.3	-

3.3.1.2. Polyvinylpyrrolidone coated gold nanoparticles (PVP-AuNPs)

PVP-AuNPs did not have a significant effect on the swimming performance of *Sparus aurata* ($p>0.05$; ANOVA; Figure 2) or on ChE activity in brain and muscle ($p>0.05$; ANOVA; Figure 3). Concerning the enzymatic antioxidant defence, 80 µg.L^{-1} PVP-AuNPs significantly decreased gills CAT activity ($p<0.05$; Dunnett's test; Figure 4A), whereas, liver CAT activity was not significantly affected by the PVP-AuNPs exposure ($p>0.05$; ANOVA; Figure 4B). PVP-AuNPs, 80 and 1600 µg.L^{-1}, significantly decreased gills GR activity ($p<0.05$; Dunnett's test; Figure 5A), whereas, in liver, they did not induce significantly alterations ($p>0.05$; ANOVA;

Figure 5B). PVP-AuNPs did not induce significant alterations in gills GPx activity ($p>0.05$; ANOVA; Figure 6A). In liver, 80 $\mu g.L^{-1}$ significantly decreased the activity of this enzyme ($p<0.05$; Dunnett's test; Figure 6B). Concerning non-enzymatic defence, NPT levels, both in gills and liver, were not significantly altered ($p>0.05$; ANOVA; Figure 7). PVP-AuNPs did not affect gills or liver GST activity ($p>0.05$; ANOVA; Figure 8). As shown in Figure 9 and 10, oxidative damage, assessed as TBARS, was not found after exposure to PVP-AuNPs ($p>0.05$; ANOVA). All the treatments of PVP-AuNPs induced significant genotoxic effects ($p<0.05$; Dunnett's test), assessed as DNA strand breakage – Table 4. A dose response pattern was found with damage index increasing with the increase of PVP-AuNPs concentration. In terms of damage classes, as shown in Table 4, the results were similar of those detected to cAuNPs. Also, as for cAuNPs, no significant oxidative DNA damage was found after exposure to PVP-AuNPs ($p>0.05$; ANOVA; Table 4). All the treatments of PVP-AuNPs led to significantly higher ENAs frequency ($p<0.05$; Dunnett's test; Figure 11). The frequency of ENAs increased with the increase of PVP-AuNPs concentration. As shown in Table 5, as detected for cAuNPs, the lobed nuclei abnormality was the most commonly detected in all PVP-AuNPs treatments, followed by kidney-shaped nuclei ($p<0.05$; Dunnett's test). The segmented and micronuclei abnormalities were the less detected. The vacuolated nuclei abnormality was not observed – Table 5.

3.3.2. Ionic form

After 24 h of exposure, one fish died in the 1600 $\mu g.L^{-1}$ iAu treatment. Also, exposure to 1600 $\mu g.L^{-1}$ caused significantly decreased ability of *Sparus aurata* to continue swimming against a water flow ($p<0.05$; Dunnett's test; Figure 2). As for AuNPs exposure, iAu did not significantly alter the activity of ChE ($p>0.05$; ANOVA; Figure 3). Exposure to 4 and 1600 $\mu g.L^{-1}$ significantly increased gills CAT activity ($p<0.05$; Dunnett's test; Figure 4A). Liver CAT ($p>0.05$; ANOVA; Figure 4B), gills and liver GR activities ($p>0.05$; ANOVA; Figure 5) were apparently not affected by exposure to iAu. At 80 $\mu g.L^{-1}$, iAu significantly increased gills GPx activity ($p<0.05$; Dunnett's test; Figure 6A). However, in liver, iAu did not have a significant effect on the activity of this enzyme ($p>0.05$; ANOVA; Figure 6B).

Exposure to 1600 µg.L^{-1} of iAu significantly increased the gills NPT levels ($p<0.05$; Dunnett's test; Figure 7A). Again, iAu did not have a significant effect on the NPT levels in liver ($p>0.05$; ANOVA; Figure 7B). Exposure to 1600 µg.L^{-1} significantly increased gills and liver GST activity ($p<0.05$; Dunnett's test; Figure 8). Oxidative damage in gills and liver was not found after exposure to iAu ($p>0.05$; ANOVA; Figure 9). However, oxidative damage, i.e. increase of LPO levels, was detected following exposure to 4 and 80 µg.L^{-1} in brain and following exposure to 80 µg.L^{-1} in muscle ($p<0.05$; Dunnett's test; Figure 10). All the treatments with iAu significantly induced genotoxic effects ($p<0.05$; Dunnett's test; Table 4). A DNA damage index around 319 was detected in animals exposed to 1600 µg.L^{-1} of iAu, the highest value detected considering all the treatments (Table 4). In terms of damage classes, as shown in Table 4, similar results were found when comparing with the nano form. No significant oxidative DNA damage was found after the exposure to iAu as detected for AuNPs ($p>0.05$; ANOVA; Table 4). All the iAu treatments, with the exception to 4 µg.L^{-1}, led to significantly higher ENAs frequency ($p<0.05$; Dunnett's test; Figure 11). The frequency of ENAs increased with the increase of iAu concentration. As detected for AuNPs, the lobed nuclei abnormality was the most detected in all iAu treatments, followed by kidney-shaped nuclei ($p<0.05$; Dunnett's test; Table 5). The segmented and vacuolated were the abnormalities less detected and micronuclei were not found – Table 5.

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

3.4.1. Nano form

AuNPs did not accumulate significantly in the assessed tissues of *S. aurata* ($p>0.05$; ANOVA; Table 6). The highest calculated BAF value (2 L.g^{-1}) was for the nominal concentration 4 µg.L^{-1} of cAuNPs, in the spleen (Table 6).

Table 6. Gold content in tissues (gills, liver, spleen and muscle) of *Sparus aurata* exposed to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold for 96 h and respective estimated bioaccumulation factor (BAF). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). b.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**	b.d.l.	b.d.l.	b.d.l.	-	-	-
	Liver	b.d.l.	b.d.l.	b.d.l.	-	-	-
	Spleen	b.d.l.	b.d.l.	b.d.l.	-	-	-
	Muscle	b.d.l.	b.d.l.	b.d.l.	-	-	-
4	**Gills**	b.d.l.	0.2 ± 0.0	0.1 ± 0.0	-	0.0	0.0
	Liver	0.1 ± 0.0	b.d.l.	0.1 ± 0.0	0.0	-	0.0
	Spleen	4.8 ± 0.4	b.d.l.	0.4 ± 0.0	2.0	-	0.1
	Muscle	b.d.l.	b.d.l.	b.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	b.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	b.d.l.	1.1 ± 0.6	0.0	-	0.0

3.4.2. Ionic form

As shown in Table 6, iAu significantly accumulated in gills, liver and spleen of *S. aurata* after the exposure to 1600 $\mu g.L^{-1}$ iAu ($p<0.05$; Dunnett's test). The highest $[Au]_{total}$ value (around 24 $\mu g.g^{-1}$) was detected after exposure to 1600 $\mu g.L^{-1}$ iAu ($p<0.05$; Dunnett's test; Figure 12).

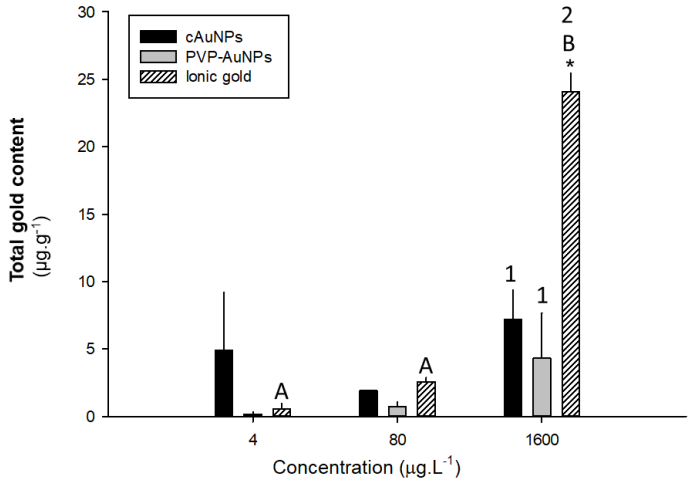

Figure 12. Total gold content on *Sparus aurata* after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). Different letters 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, $p<0.05$).

The highest estimated value for Au intake by each Portuguese person would be for the condition 1600 µg.L^{-1} of iAu (Table 7). 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 µg.kg^{-1} body weight.

Table 7. Estimated gold intake (µg per kg body weight per year), by each Portuguese person, after the ingestion of *Sparus aurata*, taking into account the total content of gold detected in muscle of fish after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold.

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

4. Discussion

The coating of 7 nm AuNPs with PVP resulted in a slight shift in the SPR peak to a longer wavelength when compared with the original cAuNPs as previously observed for the same AuNPs (Barreto et al. 2015). DLS measurements showed an increased size of PVP-AuNPs and a less negative ZP value when compared with cAuNPs, also in agreement with a previous study (Barreto et al. 2015). The detected size difference may be explained by the fact that PVP presenting a larger size than citrate (Iswarya et al. 2016; Tejamaya et al. 2012). In terms of ZP, the observed difference between cAuNPs and PVP-AuNPs may be explained by the fact that PVP is an uncharged molecule thus making the PVP-AUNPs less negative than cAuNPs (Mahl et al. 2010). In the experimental media (ASW), 80 and 1600 $\mu g.L^{-1}$ cAuNPs changed the colour, as a result of NPs agglomeration/aggregation. PVP-AuNPs, at 80 and 1600 $\mu g.L^{-1}$, did not show colour alteration in ASW. These results are in agreement with the previous study of Barreto et al. (2015) which demonstrated that 7 nm PVP-AuNPs were stable in ASW for more than 30 days. Thus, the present study confirmed that PVP-AuNPs may remain stable in suspension in a nano size range in ASW, whereas cAuNPs immediately alter their characteristics and aggregate/agglomerate, increasing their size. These characteristics and behaviour of different AuNPs may influence their accumulation and effects to the organisms. NPs size may affect its bioavailability to the organisms. When aggregates become too large for direct transport across the cell membrane, uptake may be prevented (Vale et al. 2016).

Although the stability of the tested AuNPs was different in ASW, no significant differences were found in terms of Au accumulation in the tissues of *S. aurata* after the exposure to cAuNPs and PVP-AuNPs. No significant Au accumulation in the fish was detected after the exposure to AuNPs. However, concerning the effects to fish, 7 nm cAuNPs induced more pronounced effects, in terms of oxidative stress and damage responses, than PVP-AuNPs. This result was unexpected when considering the stability of the particles. The 7 nm PVP-AuNPs remained stable in ASW, dispersible in the water column and, therefore, more available for the uptake by fish. The tested concentrations of cAuNPs were able to induce effects in fish, decreasing the swimming resistance, inducing enzymatic and non-enzymatic responses involved in the oxidative defence, oxidative damage and genotoxicity. Therefore, it seems that the formed agglomerates/aggregates in the ASW (less than 200 nm) may be incorporated through the cellular membranes.

Comparing the ionic with nano form, Au significantly accumulated in almost all assessed tissues of *S. aurata* after the exposure to 1600 $\mu g.L^{-1}$ iAu, whereas after the exposure to AuNPs, Au did not significantly accumulate. Despite the significantly higher effects of cAuNPs in some endpoints (such as gills GPx and liver GR activities, gills NPT and liver LPO levels), even at the lowest tested concentration (i.e., 4 $\mu g\ L^{-1}$), iAu induced, in general, more effects on the fish (gills CAT, GR and liver GST activities and muscle LPO levels). Additionally, no fish mortality was detected after the exposures with AuNPs, whereas one fish died after the exposure to 1600 $\mu g.L^{-1}$ of iAu. The results demonstrated a tissue specificity, cAuNPs induced LPO in gills and liver, while iAu induced LPO in brain and muscle. There are few available studies about the mechanisms involved in the toxicity of iAu. Nonetheless, the iAu ability to undergo redox reactions with peptides and proteins, particularly involving sulphur amino acids, to deprotonate and bind to peptide amide bonds and cross-link histidine imidazole rings, has been already reported (Best and Sadler 1996; Luis et al. 2016). Some authors, using *in vitro* tests, reported that iAu induced effects to mussel (*Mytilus galloprovincialis*) and rainbow trout (*Oncorhynchus mykiss*) whereas the nano form did not have any effect (Farkas et al. 2010; Luis et al. 2016). Botha el al. (2015), using different aquatic species (daphnia and fish), also showed that iAu was more toxic than

nano form. However, Barbasz et al. (2016), using two types of human cell lines, showed a higher cytotoxicity of AuNPs than iAu. Dedeh et al. (2014) described that, in spite of iAu having accumulated more in the tissues of zebrafish (*Danio rerio*) than the AuNPs, the latter had more effects on the fish, in terms of gene expression and neurotransmission. Nano form also induced more effects in the liver organ cultures of *S. aurata* than iAu (Chapter II).

In terms of genotoxicity, all the treatments induced DNA strand breaks, assessed by comet assay, in *S. aurata* peripheral blood cells. Concerning cytogenetic damage, ENA frequency increased with the increase of Au concentration (nano or ionic form). Comet assay is a rapid method to detect low levels of DNA damage. However, this technique gives limited information about the kind of DNA damage, if it is a direct consequence of the damaging agent or indirect effects, such as oxidative damage, apurinic/pyrimidinic sites or DNA repair (Smith, O'Donovan, and Martin 2006). As previously described, the genotoxic effects of AuNPs may be caused directly following the entry of NPs into the nuclei, binding to DNA; or indirectly, through oxidative stress, which may consequently induce DNA oxidative damage (Auffan et al. 2009; Cardoso et al. 2014). The production of reactive oxygen species (ROS) following AuNPs exposure has been demonstrated in studies involving aquatic organisms (Farkas et al. 2010; Tedesco et al. 2008, 2010; Pan et al. 2012). Modification of the comet assay with the incorporation of lesion specific endonucleases, such as Fpg, increases its sensitivity and specificity through the recognition of damaged bases and introduction of additional breaks (Azqueta et al. 2013; Smith, O'Donovan, and Martin 2006; Speit et al. 2004). The present study showed that Au (nano and ionic form) induced DNA breaks, but oxidative DNA damage was not observed. This result was previously described in studies with different types of NPs (Ag, CeO_2, Co_3O_4 and SiO_2) and metal ions (Al^{3+} , Ni^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+} and Zn^{2+}) (Grin et al. 2009; Kain, Karlsson, and Möller 2012) and may be due to: 1) the low potential of the tested conditions to induce oxidative damage on the erythrocyte DNA of *S. aurata*, which is not supported by the LPO data; 2) the oxidative DNA lesions caused by the exposure to Au may have been already been repaired by cellular DNA repair systems (Catalán et al. 2014); 3) NPs and ionic forms may interact

with Fpg, not allowing the binding of the enzyme with DNA (Asmuss et al. 2000; Kain, Karlsson, and Möller 2012).

Overall, after Au exposures, enzymatic and non-enzymatic responses involved in the defence of *S. aurata* against oxidative damage were more activated in the gills than in the liver. Additionally, oxidative damage (LPO increase) was more clearly expressed in gills than in liver. For instance, gills NPT and LPO levels were significantly increased by all the tested concentrations of cAuNPs, whereas, in the liver, only 1600 $\mu g.L^{-1}$ caused increased levels. Gills are the first organ to be exposed and provide a large surface area for contaminants such as AuNPs, being considered a good candidate to an early assessment of the effects of waterborne contaminants (Oliveira, Pacheco, and Santos 2008).

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

5. Conclusions

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

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

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Supplementary Information

Table S1. Measured versus theoretical concentrations and number of particles present in 7 nm gold nanoparticles stock suspensions (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs). NPs – Nanoparticles.

	Measured concentrations ($mg.L^{-1}$)	**Theoretical concentrations** ($mg.L^{-1}$)	**Number of particles** ($NPs.L^{-1}$)
cAuNPs	98	67	2.18×10^{16}
PVP-AuNPs	51	57	$1.06x10^{16}$

Chapter IV

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A multibiomarker approach highlights effects induced by the human pharmaceutical gemfibrozil to gilthead seabream *Sparus aurata*

A. Barreto[1*], L.G. Luis[1], P. Paíga[2], L.H.M.L.M. Santos[2,3], C. Delerue-Matos[2], A.M.V.M. Soares[1], K. Hylland[4], S. Loureiro[1], M. Oliveira[1]

[1] Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] REQUIMTE/LAQV, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida, 431, 4200-072 Porto, Portugal

[3] Present affiliation: Catalan Institute for Water Research (ICRA), Carrer Emili Grahit 101, 17003 Girona, Spain

[4] Department of Biosciences, University of Oslo, PO Box 1066, N-0316 Oslo, Norway

*Corresponding author: E-mail: abarreto@ua.pt, Tel +351 234 370 350, Fax +351 234 372 587

Highlights

- Gemfibrozil affected *Sparus aurata* even at an environmentally relevant concentration;
- Gemfibrozil decreased the swimming resistance of *Sparus aurata* against a water flow;
- Gemfibrozil induced hepatic oxidative damage in gilthead seabream.

Abstract

Lipid regulators are among the most prescribed human pharmaceuticals worldwide. Gemfibrozil (GEM), which belongs to this class of pharmaceuticals, is one of the most frequently encountered in the aquatic environment. However, there is limited information concerning the mechanism involved in GEM effects to aquatic organisms, particularly to marine organisms. Based on this knowledge gap, the current study aimed to assess biochemical and behavioural effects following a sublethal exposure to GEM (1.5, 15, 150, 1500 and 15000 $\mu g.L^{-1}$) in the estuarine/marine fish *Sparus aurata*. After the exposure to 1.5 $\mu g.L^{-1}$ of GEM, fish had reduced ability to swim against a water flow and increased lipid peroxidation in the liver. At concentrations between 15 to 15000 $\mu g.L^{-1}$, the activities of some enzymes involved in antioxidant defence were induced, appearing to be sufficient to prevent oxidative damage. Depending on the organ, different responses to GEM were displayed, with enzymes like catalase being more stimulated in gills, whereas glutathione peroxidase was more activated in liver. Although there were no obvious dose-response relationships, the integrated biomarker response version 2 (IBRv2) analysis revealed that the highest concentrations of GEM (between 150 to 15000 $\mu g.L^{-1}$) caused more alterations. All the tested concentrations of GEM induced effects in *S. aurata*, in terms of behaviour and oxidative stress responses. Oxidative damage was found at a concentration that is considered environmentally relevant, suggesting a potential of this pharmaceutical to impact fish populations.

Keywords: fibrates; seabream; behaviour; biomarkers; oxidative damage

1. Introduction

Pharmaceuticals are considered emerging contaminants of concern due to their high consumption and continuous environmental release (as parental compound and/or metabolites). This is both due to inefficient wastewater treatment processes and, for some substances, high environmental persistence and low degradation rates (Andreozzi et al. 2003; Fent et al. 2006, Schmidt et al. 2011). The

prescription rates of lipid regulators are continually increasing and gemfibrozil (GEM) is among the most widely used (Al-Habsi et al. 2016; Prindiville et al. 2011). GEM was approved by the Food and Drug Administration (FDA) in 1976 for use by humans to reduce serum lipids. It reduces the levels of triglycerides, very low-density lipoprotein (VLDL, “bad cholesterol”) and low-density lipoprotein (LDL, “bad cholesterol”) and increases high-density lipoprotein (HDL, “good cholesterol”) (Kim et al. 2017). In North America and Europe these drugs are widely used to control hyperlipidaemia resulting from the western diet (Ido et al. 2017). In the United States, in 2009, GEM was prescribed over 500 000 times (Jackevicius et al. 2011; Bulloch et al. 2012). Being among the most prescribed human pharmaceuticals, lipid regulators are frequently reported in wastewater and surface waters (Andreozzi et al. 2003; Gros et al. 2006; Lin and Reinhard 2005; Sanderson et al. 2003; Schmidt et al. 2011; Togola and Budzinski 2007). In Europe, GEM has been found at concentrations up to 4.76 $\mu g.L^{-1}$ in wastewater treatment plant effluents (Andreozzi et al. 2003) and up to 1.5 $\mu g.L^{-1}$ in surface waters (Fang et al. 2012). In marine ecosystems, GEM is also among the most frequently detected compounds, with concentrations between 1 and 758 $ng.L^{-1}$ in seawater (Gaw et al. 2014; Vidal-Dorsch et al. 2012). Despite its presence in aquatic ecosystems, there is still limited information concerning mechanisms of toxicity for GEM to aquatic organisms, particularly for marine fish (Teles et al. 2016).

Earlier studies on GEM exposure to aquatic organisms have revealed a potential of this pharmaceutical to induce alterations of biochemical and behavioural endpoints (Al-Habsi et al. 2016; Fraz et al. 2018; Henriques et al. 2016; Mimeault et al. 2006; Prindiville et al. 2011; Quinn et al. 2011, 2008; Schmidt et al. 2011; Skolness et al. 2012; Zurita et al. 2007). In zebrafish (*Danio rerio*), GEM was found to impair hatching success and embryonal development, change locomotor activity and reduce survival, with a reported 96-h LC_{50} (50% lethal effect concentration) of 11.01 $mg.L^{-1}$ (Henriques et al. 2016). GEM activated cholinesterases (ChE) in the PLHC-1 cell lines of the fish clearfin livebearer (*Poeciliopsis lucida*) (Zurita et al. 2007) and enzymes involved in oxidative stress of goldfish (*Carassius auratus*), as well as increased lipid peroxidation – LPO

(Mimeault et al. 2006). GEM has furthermore been reported to decrease plasma testosterone levels in freshwater goldfish (*Carassius auratus*) (Mimeault et al. 2005) and to activate antioxidants enzymes and modulate metallothionein expression in blue mussel (*Mytilus* spp.) (Schmidt et al. 2011). The ability to induce behavioural alterations has also been reported for the freshwater cnidarian *Hydra attenuata* (Quinn et al. 2008). GEM exposure increased growth and reproduction of *Daphnia magna* (Salesa et al. 2017, Steinkey et al. 2018). The effect of GEM on lipid metabolism was previously reported for the freshwater fish fathead minnow (*Pimephales promelas*) (Skolness et al. 2012) and rainbow trout (*Oncorhynchus mykiss*) (Prindiville et al. 2011). In marine fish, GEM has been reported to affect antioxidant defences in sole (*Solea senegalensis*) (Solé et al. 2014) and to inhibit the activity of P450-catalysed pathways of yellow European eel (*Anguilla anguilla*) (Lyssimachou et al. 2014). In these studies fish were exposed through intraperitoneal injection. In the gilthead seabream (*Sparus aurata*), GEM has been reported to induce genotoxic effects at a concentration frequently detected in the environment (1.5 $\mu g.L^{-1}$) (Barreto et al. 2017), caused transcriptional levels of key genes involved in lipid homeostasis and was characterised as a stress-inducing agent (Teles et al. 2016). It is however not known if GEM alters enzymatic activities associated with oxidative stress and biotransformation and whether exposure to GEM would also affects behaviour.

Considering the existing knowledge gaps concerning the mechanistic effects of GEM exposure to marine fish, the gilthead seabream (*Sparus aurata*) was selected as a model species and several biomarkers were included in an integrated assessment of possible effects. This top predator is widespread in Atlantic and Mediterranean coastal waters, with a high economic importance for both fishery and aquaculture, being one of the most consumed fish in the Mediterranean area (Teles et al. 2016). Furthermore, *S. aurata* has previously been shown to be sensitive to short-term exposure to GEM as demonstrated by increased DNA damage and increased cortisol levels (Barreto et al. 2017; Teles et al. 2016). Effects of GEM were determined following 96-h waterborne exposure by assessing swimming ability, which may provide information on the ability of fish to escape predators, to chase prey and escape pernicious conditions, and

biomarkers involved in neurotransmission (ChE), biotransformation and antioxidant defences (catalase (CAT), glutathione *S*-transferases (GST), glutathione peroxidase (GPx) and glutathione reductase (GR)) as well as oxidative damage, i.e. LPO. This set of biomarkers was chosen to assess fish general health status focusing on the ability to respond to oxidative challenge, maintain biotransformation and prevent damage in order to maintain their fitness. This approach has been previously adopted by other authors that used a battery of behavioural and biochemical biomarkers, such as ChE, GST, CAT activities and LPO levels, to assess the effects of heavy metals and bisphenol A to *S. aurata* (Souid et al. 2013, 2015). The purpose of the present study was thus to understand the potential effects of GEM to the marine fish *S. aurata* and the mechanisms of toxicity involved.

2. Material and Methods

2.1. Chemicals

All reagents used were analytical grade obtained from Sigma-Aldrich (Germany), Bio-Rad (Germany) and Merck (Germany). GEM was acquired from Tokyo Chemical Industry Co., Ltd. (TCI) and the isotopically labelled standard gemfibozil-d6 was purchased from Santa Cruz Biotechnology (USA).

2.2. Test organisms and acclimation

Juvenile gilthead seabream (*Sparus aurata*), with a length of 9 ± 0.5 cm and a weight of 8.1±0.6 g, from an aquaculture facility (Santander, Spain), were acclimated for 4 weeks in aquaria with aerated and filtered (Eheim filters) artificial seawater (ASW, Ocean Fish, Prodac). This water was prepared by dissolving the salt in reverse osmosis purified water to obtain a salinity of 35, in a controlled room temperature (20ºC) and natural photoperiod. During the acclimation 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 used during the toxicity test.

2.3. Experimental design

All experimental procedures were carried out following the Portuguese and European legislation (authorization N421/2013 of the Portuguese legal authorities). Animal handling was performed by an accredited researcher. The bioassay followed, in general, the OECD guidelines for fish acute bioassays (OECD 1992). A stock solution of GEM (50 $g.L^{-1}$) was prepared, daily, in dimethyl sulfoxide (DMSO) due to its limited water solubility. DMSO was selected as a solvent due to its widespread use in several toxicological studies (Mimeault et al. 2006; Zurita et al. 2007; Quinn et al. 2008; Schmidt et al. 2011). Test solutions of GEM were prepared by dilution of the stock solution in ASW.

After the acclimation period, 70 fish were randomly distributed in the experimental aquaria, with ten fish per condition (n=10) in the ratio 1 g of fish per 1 L of ASW. The experimental design included a negative control (ASW only), a solvent control (0.03% DMSO, the maximal concentration of DMSO used in the GEM treatments) and five GEM concentrations: 1.5, 15, 150, 1500 and 15000 $\mu g.L^{-1}$. Fish were exposed for 96 h as recommended by the OECD guideline for fish acute toxicity testing (203), without feeding. The lowest tested concentration of GEM was chosen because it is considered a concentration environmentally relevant, based on levels detected in surface waters (Fang et al. 2012). The concentration range used was based on 10-fold increases.

Daily, after checking fish mortality, behaviour alterations and assessing the water parameters (temperature, salinity, conductivity, pH and dissolved oxygen), approximately 80% of the experimental media was renewed to circumvent GEM degradation and to reduce the build-up of excretion products. During the exposure time, photoperiod, temperature and aeration conditions were similar to those used in the acclimation period.

2.4. Quantification of gemfibrozil (GEM) in the experimental media

Water samples were collected daily (at 0 and 24 h) from each aquarium. GEM was extracted using solid phase extraction (SPE). Briefly, Strata X cartridges (200 mg, 3 mL) (Phenomenex, USA) were conditioned with 5 mL methanol and 5 mL ultrapure water. Then, 10 mL of water sample was percolated through the

cartridge (3-5 $mL.min^{-1}$), rinsed with 5 mL ultrapure water and dried under vacuum (20 min). Finally, GEM was eluted from the cartridges with methanol (10 mL). Extracts were evaporated until dryness under a gentle stream of nitrogen and reconstituted with 1 mL acetonitrile/ultrapure water (30:70, v/v). Gemfibrozil-d6 (10 µL of 5 $mg.L^{-1}$ in methanol) was added to the extract as internal standard. GEM analysis was performed on a Nexera UHPLC (ultra-high performance liquid chromatography) system with a triple-quadrupole mass spectrometer detector LCMS-8030 (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was achieved using a Kinetex C18 column (2.1 x 150 mm i.d., 1.7 µm particle size) from Phenomenex (USA) using 5 mM ammonium acetate/ammonia buffer (pH 8) as solvent A and acetonitrile as solvent B at a flow rate of 0.22 $mL.min^{-1}$. The gradient elution was performed as follows: initial conditions: 30% B; 0-2.0 min, 30-100% B; 2.0-4.5 min maintained at 100% B, 4.5-5.5 min return to initial conditions; and from 5.5-9.5 min, re-equilibration of the column. Column oven was set at 30ºC and the autosampler was operated at 4ºC. The injection volume was 5 µL.

GEM was analysed in the negative ionization mode and quantification was performed in multiple reaction monitoring mode (MRM) using two transitions between the precursor ion and the most abundant fragment ions (MRM1: 249.00>121.15 and MRM2: 249.00>127.05). Quantification was performed by the internal standard calibration method. The method detection limit (MDL) for GEM in water was 4.0 $ng.L^{-1}$.

2.5. Assessment of swimming performance

After 96 h exposure, each fish was gently transferred to a 1.5 m long track race flume with 7 cm diameter with a running water flow of 20 $L.min^{-1}$ and induced to swim, generally following the procedure described by Oliveira et al. (2012). The time that animals were able to swim against the water flow was recorded as swimming resistance. After this test, fish were put back into their original test aquaria where they stood for 2 h before being used to determine biochemical endpoints.

2.6. Preparation of biological material for biomarkers determination

After the recovery period, animals were anesthetized with tricaine methanesulfonate (MS-222), their length measured, weighed and euthanized by spinal section. Liver, gills, brain and muscle were taken from each animal, snap frozen in liquid nitrogen to prevent enzyme degradation and stored at -80ºC until further processing.

2.6.1. Liver and gills

Liver and gills were homogenized in potassium phosphate buffer (0.1 mM, pH 7.4), using an ultrasonic homogenizer (Branson Ultrasonics Sonifier S-250A). One aliquot of homogenate, for LPO determination, was transferred to a microtube with 4% BHT (2,6-dieter-butyl-4-metylphenol) in methanol, to prevent oxidation and stored at -80ºC until analysis. The remaining homogenate was used for post-mitochondrial supernatant (PMS) isolation. PMS was accomplished by centrifugation at 12 000 g for 20 min at 4ºC. PMS aliquots were stored at -80ºC until CAT, GST, GPx and GR activities determination.

2.6.2. Muscle and brain

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 x g for 3 min at 4ºC, supernatant was collected and stored at -80ºC.

2.7. Biochemical biomarkers analysis

Protein concentration was determined according to Bradford (1976), adapted to microplate, using bovine γ - globuline as a standard.

2.7.1. Cholinesterases (ChE) activity

ChE activity was determined according to the Ellman's method (Ellman et al. 1961) adapted to microplate (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 using acetylthiocholine as substrate.

2.7.2. Catalase (CAT) activity

CAT activity was assayed as described by Claiborne (1985) and the variations in absorbance at 240 nm, caused by the dismutation of hydrogen peroxide (H_2O_2), were recorded. CAT activity was calculated as µmol H_2O_2 consumed per min per mg of protein.

2.7.3. Glutathione *S*-transferases (GST) activity

GST activity was determined spectrophotometrically by the method of Habig et al. (1974) adapted to microplate (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 (25ºC) and activity expressed as nmol CDNB conjugate formed per min per mg of protein.

2.7.4. Glutathione peroxidase (GPx) activity

GPx activity was measured according to the method described by Mohandas et al. (1984), modified by Athar and Iqbal (1998). Oxidation of reduced nicotinamide-adenine dinucleotide phosphate (NADPH) was recorded spectrophotometrically at 340 nm and the enzyme activity results expressed as nmol of oxidized nicotinamide-adenine dinucleotide phosphate ($NADP^+$) per min per mg of protein.

2.7.5. Glutathione reductase (GR) activity

GR activity was estimated according the method of Carlberg and Mannervik (1975) adapted to microplate (Lima et al. 2007), measuring NADPH disappearance at 340 nm. GR activity was expressed as nmol of $NADP^+$ formed per min per mg of protein.

2.7.6. Lipid peroxidation (LPO) levels

LPO levels were estimated by the formation of thiobarbituric acid reactive substances (TBARS) based on Ohkawa et al. (1979), adapted by Filho et al. (2001). Absorbance was measured at 535 nm and LPO levels were expressed as nmol of TBARS formed per mg of protein.

2.8. Integrated biomarker response (IBR)

To integrate all the results from the different tested biomarkers and understand global responses, the IBR index was calculated according to Sanchez et al. (2013), using IBR version 2 (IBRv2). IVRv2 was designed to modify the IBR previously developed by Beliaeff and Burgeot (2002). The IBR was chosen to integrate the different biomarker responses into a numeric value (Devin et al. 2014). The assessed endpoints were combined into one general ‘‘stress index’’ to integrate biomarker data into a value representing the stress level at each tested concentration, based on the principle of reference deviation. Overall, data were log-transformed (Yi) and the overall mean (μ) and standard deviation (s) calculated. Data was further standardized by subtracting the overall mean and dividing by the standard deviation as presented in the following equation:

$$Zi = \left(\frac{yi - \mu}{s}\right)$$

The difference between Zi and Z0 (control) was calculated in order to determine A values. Representative results are shown as star plot charts indicating the deviation of all biomarkers in relation to the control (0) (Sanchez et al. 2013). In addition, data was analysed using a weighing procedure for endpoints as previously described (Liu el al. 2013, 2015), assuming that a biochemical alteration has lower impact on the organism health than changes at an individual level. Behavior is considered as the outcome of many biological processes resultant from interactions between the organisms and the surrounding environment (Oliveira et al. 2015). Thus, biochemical biomarkers were weighted with a factor of one and behaviour with a factor of three. More information about IBRv2 and the difference between this version and version 1 can be found in the supplementary information.

2.9. Data analysis

Data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene’s test), using Sigma Plot 12.0 software package. Differences between controls (negative and solvent) were carried out using a Student t-test ($p<0.05$).

Differences between treatments and controls were compared using one-way analysis of variance (ANOVA), followed by Dunnett's test whenever applicable ($p<0.05$).

3. Results and Discussion

3.1. Quantification of gemfibrozil (GEM) in the experimental media

The analysis of GEM concentrations revealed that nominal concentrations of GEM differed 6 to 37% from the results obtained by chemical analyses (Table S1). After 24 h, GEM degradation was higher in the aquaria with the lowest concentrations, as previously presented (Barreto et al. 2017). Other authors also reported a decrease of GEM concentrations more evident in the lowest concentrations after goldfish 14 d exposure (Mimeault et al. 2005). In that study, the initial concentrations 1.5 and 1500 $\mu g.L^{-1}$ after 14 d derived concentrations of 0.34 and 851.9 $\mu g.L^{-1}$, respectively. The observed decrease of GEM concentrations in the water can be also explained by the incorporation of GEM in the fish. In the study of Mimeault et al. (2005), the quantification of GEM in the plasma of goldfish exposed to 1500 and 10000 $\mu g.L^{-1}$ revealed that, after 96 h, GEM was present in concentrations higher than 75000 $\mu g.L^{-1}$ for both treatments. After 14 d exposure, plasma concentrations of animals exposed to nominal concentrations of 1.5 and 1500 $\mu g.L^{-1}$ were 170 and 78000 $\mu g.L^{-1}$, respectively (Mimeault et al. 2005).

3.2. Biological responses

For all endpoints reported, the solvent DMSO did not induce significant effects when compared to the negative control ($p>0.05$; t-test). Therefore, all GEM exposure data were compared to the negative control.

The ability of *S. aurata* to continue swimming against a water flow was significantly decreased, between 50 and 65%, in individuals exposed to GEM ($p<0.05$; Dunnett's test; Figure 1), highlighting behaviour as a sensitive endpoint.

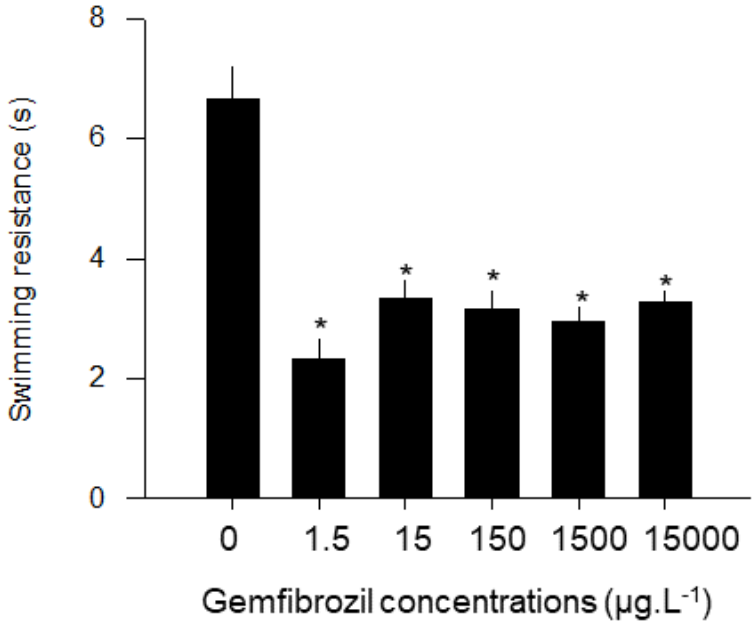

Figure 1. Gemfibrozil effects on the swimming resistance of *Sparus aurata* against a water flow, after 96 h exposure. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$).

The detected swimming performance impairment may have serious environmental consequences (Wolter and Arlinghaus 2003). Fish basic activities, such as predator–prey interactions, reproduction and migration, are completely dependent on the individuals' capacity for locomotion (Svendsen et al. 2015; Vieira et al. 2009). A decrease in locomotion was also reported for zebrafish larvae exposed to GEM concentrations equal to or higher than 1500 $\mu g.L^{-1}$ (Henriques et al. 2016). However, unlike the present study, the locomotor activity decreased when GEM concentration increased (Henriques et al. 2016).

Altered swimming behaviour may be associated with effects on neurotransmission. Acetylcholinesterase, one of the most important behind the family of ChE, is essential for the degradation of the neurotransmitter acetylcholine in cholinergic synapses and thus involved in a correct transmission of nerve impulses both in vertebrates and invertebrates (Pan et al. 2012; Sureda et al. 2018). Thus, it could be hypothesized that a decrease in ChE might be a possible explanation for the observed decrease in the swimming performance (Hernández-Moreno et al.

2011). However, in the present study, ChE activity was not significantly altered at the tested concentrations ($p>0.05$; ANOVA; Figure 2), suggesting that other factors (e.g. decreased available energy associated with the need to metabolize GEM and activate enzymatic processes or incapacity to supply oxygen to tissues (Kennedy and Farrell 2006)) may be involved in the detected behavioural alteration.

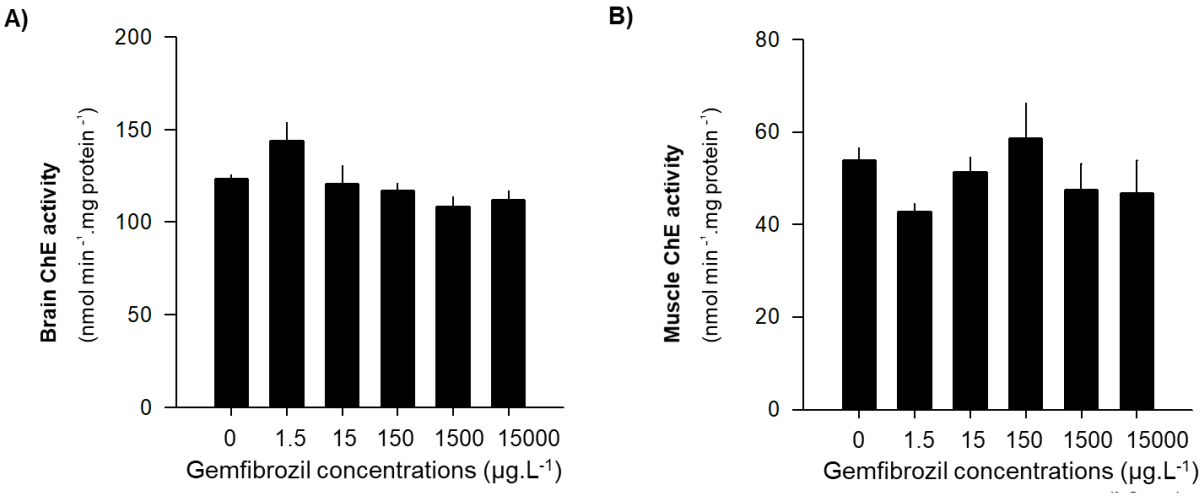

Figure 2. Gemfibrozil effects on the brain **(A)** and muscle **(B)** cholinesterases (ChE) activity of *Sparus aurata* after 96 h exposure. Results are expressed as mean ± standard error.

Oxidative stress is a mechanism of toxicity described for several environmental contaminants such as metals and pesticides (Lushchak 2016). In the present study, CAT activity significantly increased (between 50 and 93%) in the gills of fish exposed to concentrations higher than 1.5 $\mu g.L^{-1}$ ($p<0.05$; Dunnett´s test; Figure 3A). GR activity also significantly increased between 46 and 72% in gills in individuals exposed to concentrations of 15, 150 and 1500 $\mu g.L^{-1}$ ($p<0.05$; Dunnett´s test; Figure 3D), but not under the highest exposure concentration. However, no significant alterations were found in terms of gills´ GST and GPx activities ($p>0.05$; ANOVA; Fig. 3B and 3C, respectively).

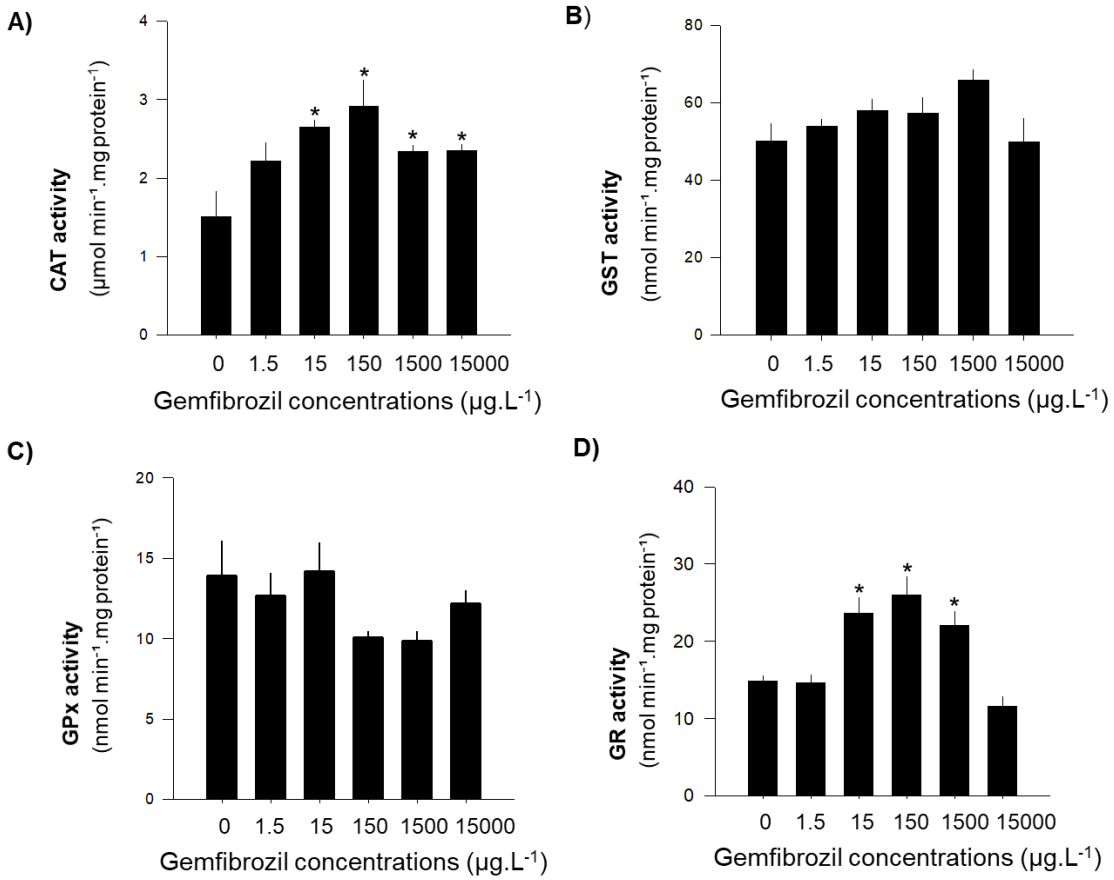

Figure 3. Gemfibrozil effects on the gills of *Sparus aurata* after 96 h exposure: **A)** Catalase (CAT) activity; **B)** Glutathione *S*-transferases (GST) activity; **C)** Glutathione peroxidase (GPx) activity; **D)** Glutathione reductase (GR) activity. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$).

In the liver, CAT activity was significantly increased (150%) ($p<0.05$; Dunnett´s test; Figure 4A) in animals exposed to 15000 µg.L^{-1} whereas no significant alterations were found in GST activity ($p>0.05$; ANOVA; Figure 4B). GPx and GR activities significantly increased ($p<0.05$; Dunnett´s test), between 156 and 243% (Figure 4C) and 42-75% (Figure 4D), respectively, in concentrations higher than 1.5 µg.L^{-1} of GEM.

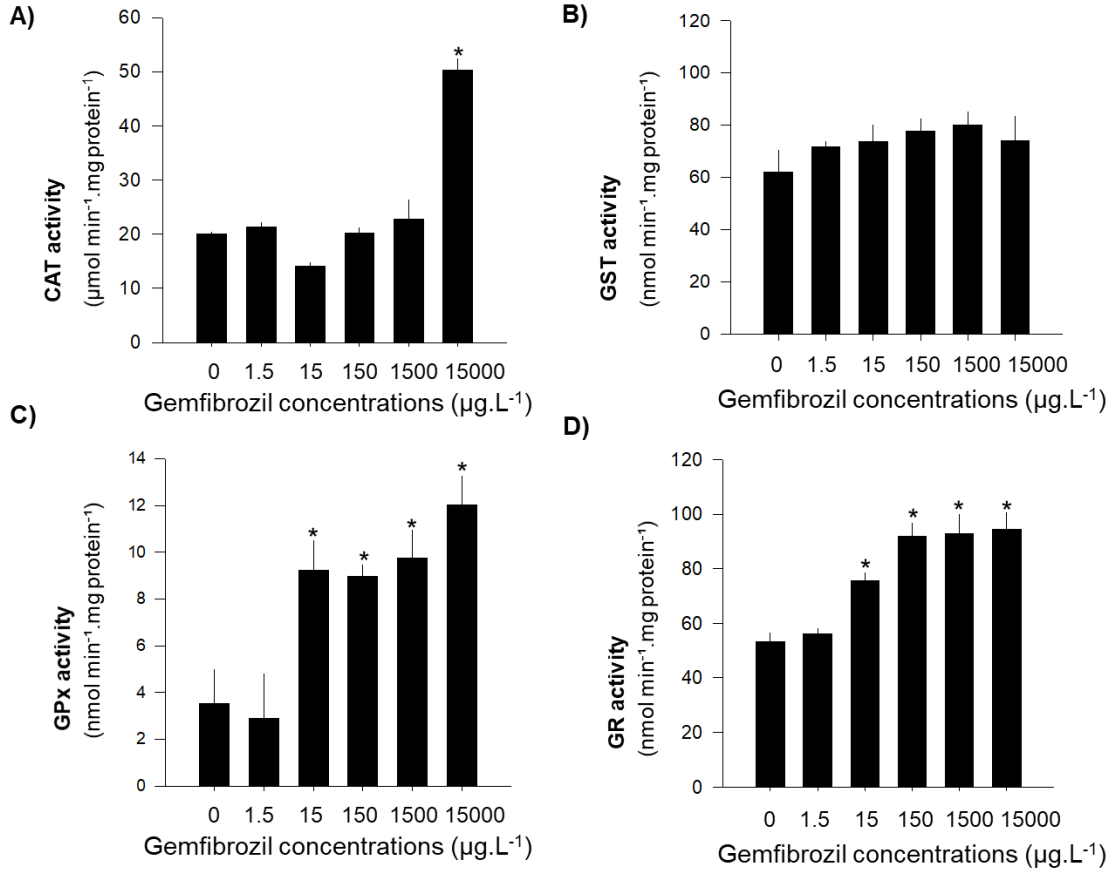

Figure 4. Gemfibrozil effects on the liver of *Sparus aurata* after 96 h exposure**: A)** Catalase (CAT) activity; **B)** Glutathione *S*-transferases (GST) activity; **C)** Glutathione peroxidase (GPx) activity; **D)** Glutathione reductase (GR) activity. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$).

The activity of GST, involved in the detoxification of many xenobiotics and playing an important role in protecting tissues from oxidative stress, was not affected by exposure to GEM. However, the assessed enzymes involved in antioxidant defence (CAT, GPx and GR) were activated both in gills and liver in a tissue- and concentration-dependent manner. At concentrations of GEM higher than 15 µg.L^{-1}, some enzymatic activities were maintained (gills CAT and liver GR) or decreased (gills GR). These observed responses may be due to the negative feedback from excess of substrate or direct damage by oxidative modifications (Ceyhun et al. 2010; Rodrigues et al. 2016). On other hand, at concentrations

between 150 and 15000 µg.L^{-1}, fish may have also cope to this xenobiotic compound, resulting in similar responses at these three concentrations.

As in the present study, previous studies with aquatic organisms have demonstrated the induction of oxidative stress by GEM (Mimeault et al. 2006; Schmidt et al. 2014, 2011), but a direct comparison between results is not straightforward due to differences in exposure duration, test organisms (species and their natural environment, including freshwater versus seawater), and *in vivo* versus *in vitro* studies. Teles et al. (2016) reported that the *S. aurata* hepatic transcription of CAT, GPx and GST was not altered following 96 h exposure to GEM. However, the present study demonstrated that antioxidant enzymes (CAT, GPx and GR) were responsive to GEM exposure showing that evaluation of enzyme activity is key considering the complex regulatory mechanisms for gene expression that occurs at both post-transcriptional and post-translational levels.

As shown in Figure 5B, peroxidative damage (assessed as TBARS levels) was only found in liver at 1.5 µg.L^{-1} ($p<0.05$; Dunnett´s test). This concentration led to a 57% increase in TBARS levels compared to control group. In gills from fish exposed to 15 and 150 µg.L^{-1} GEM there was a significant decrease in LPO levels ($p<0.05$; Dunnett´s test), corresponding to 24 and 30% when compared to the control, respectively (Figure 5A).

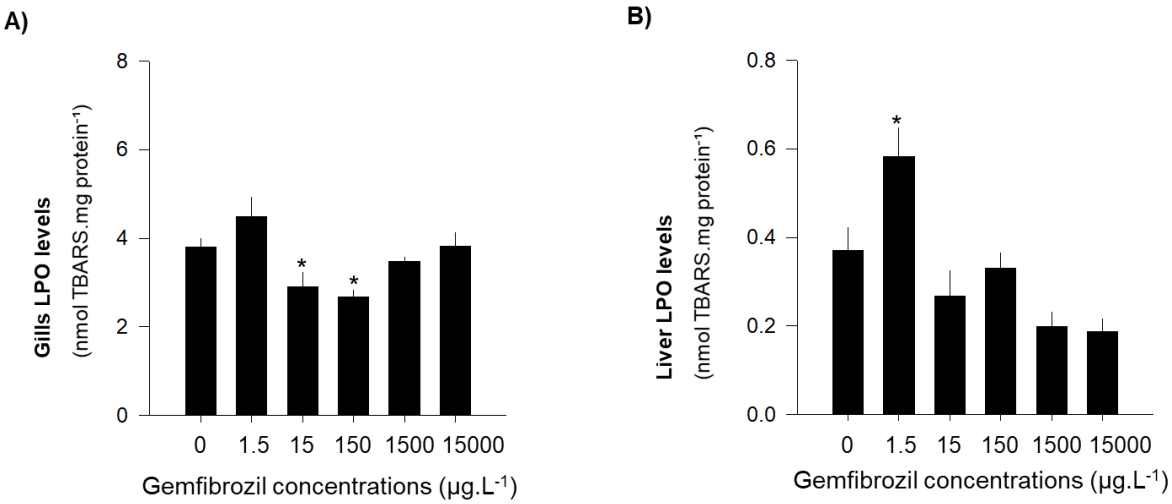

Figure 5. Gemfibrozil effects on the gills **(A)** and liver **(B)** lipid peroxidation (LPO) of *Sparus aurata* after 96 h exposure. Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$).

The decreased LPO levels observed after exposure to GEM have earlier been reported for the digestive gland of marine mussels (*Mytilus* spp.) and shown to depend on exposure time (Schmidt et al. 2014). Increased (Mimeault et al. 2006) or lack alterations (Quinn et al. 2011) in LPO levels after the exposure to GEM were also previous reported for freshwater organisms, suggesting that the mechanisms of GEM toxicity are to a large extent species-specific.

In the present study, 1.5 $\mu g.L^{-1}$ of GEM was able to induce oxidative damage in *S. aurata* without leading to significant alteration of antioxidant enzyme activity. At GEM concentrations higher than 1.5 $\mu g.L^{-1}$, activation of antioxidant defences appeared to be sufficient to prevent oxidative damage. Previous data involving GEM and other lipid regulators showed a high prevalence of peroxisome proliferation (even as an acute effect), indicating the possibility of occurrence of oxidative stress, which may lead to irreversible damage by LPO (Quinn et al. 2011; Nunes, Carvalho, and Guilhermino 2004; Qu et al. 2001). On other hand, the reported ability of GEM to reduce lipids may have also contributed to the observed LPO decreased (Roy and Pahan 2009; Sutken, Inal, and Ozdemir 2006; Ozansoy et al. 2001).

The analysis of antioxidant status and other stress responses in different tissues of organisms exposed to pollutants help to understand the associated mechanisms of toxicity and predict the degree of effects at different levels of biological organization (Franco et al. 2006; Oliveira et al. 2008). In the present study, responses in gills and liver were very different following exposure to GEM. CAT appeared as more responsive in gills than in the liver whereas GST and GR displayed overall similar profiles of response in both tissues. GPx, however, was more responsive in liver. These detected differences may be explained by the enzymatic basal activities. CAT basal activity was lower in gills than in the liver and GPx basal activity was lower in liver than in gills. Oxidative damage was only detected in liver at a concentration unable to activate enzymatic defences.

Although the mechanisms responsible for the effects of GEM is not known in detail, it is considered that many of the above-mentioned effects are mediated by GEM interaction with peroxisome proliferator-activated receptor α (PPARα) (Al-Habsi et al. 2016; Marija et al. 2011; Staels et al. 1992), which is involved in the

regulation of lipid metabolism in liver, heart, kidney and muscle (Marija et al. 2011; Pyper et al. 2010; Schoonjans et al. 1996). PPARα may be activated by natural ligands and synthetic agents, including fibrates (such as GEM) (Marija et al. 2011; Touyz and Schiffrin 2006). Fibrates are known to induce proliferation of peroxisomes in liver cells with associated coordinated transcriptional activation of peroxisomal fatty acid β-oxidation system and production of reactive oxygen species (ROS) (Lores Arnaiz et al. 1997, 1995; Marija et al. 2011; Moody et al. 1991; Palma et al. 1991; Pyper et al. 2010; Schoonjans et al. 1996). Elevated concentrations of H_2O_2 stimulate LPO, which this may explain the increase of LPO levels in the liver and the absence in the gills. On the other hand, gills are key organs for the direct action of waterborne pollutants since they are involved in a range of processes critical to survival (e.g. respiration, osmoregulation, excretion of nitrogenous residual products and regulation of the acid-base balance) (Evans 1987; Oliveira et al. 2008, 2012), and also in immune functions involving oxidative processes (Rodrigues et al. 2016; Tkachenko et al. 2014). Gills are highly vulnerable to toxic chemicals because their large surface area facilitates greater toxicant interaction and absorption (Evans 1987; Oliveira 2008), so it is expected that some enzymatic responses are activated more and primarily in gills than in the liver.

The integration of the data using IBR allows to visualize more clearly the specific responses of biomarkers for each tested condition (Beliaeff and Burgeot 2002). The IBR provides a combination of a graphical synthesis of the different biomarker responses and a numeric value which integrates all these responses at once (Devin et al. 2014). Based on the IBRv2 values, the effects of the different concentrations of GEM would be ordered as follows: 150 $\mu g.L^{-1} \approx 15000\ \mu g.L^{-1} \approx 1500\ \mu g.L^{-1} > 15\ \mu g.L^{-1} > 1.5\ \mu g.L^{-1}$ (Figure 6A). The similar IBRv2 values observed for 150, 1500 and 15000 $\mu g.L^{-1}$ may be explained by the similarity of the fish responses independent of the GEM concentration due to reasons described above. Although there was no dose-response relationship for the tested biomarkers (Figure 6A), the results showed that exposure to GEM at concentrations between 150 and 15000 $\mu g.L^{-1}$ caused more effects than exposure 1.5 and 15 $\mu g.L^{-1}$. In general, analysing the assessed endpoints star plots obtained

with IBRv2, for each experimental condition (Figure 6B1-B5), it seems clear that GEM had more effects in terms of swimming resistance of *S. aurata*, CAT and GR activities in gills and GPx and GR activities in liver.

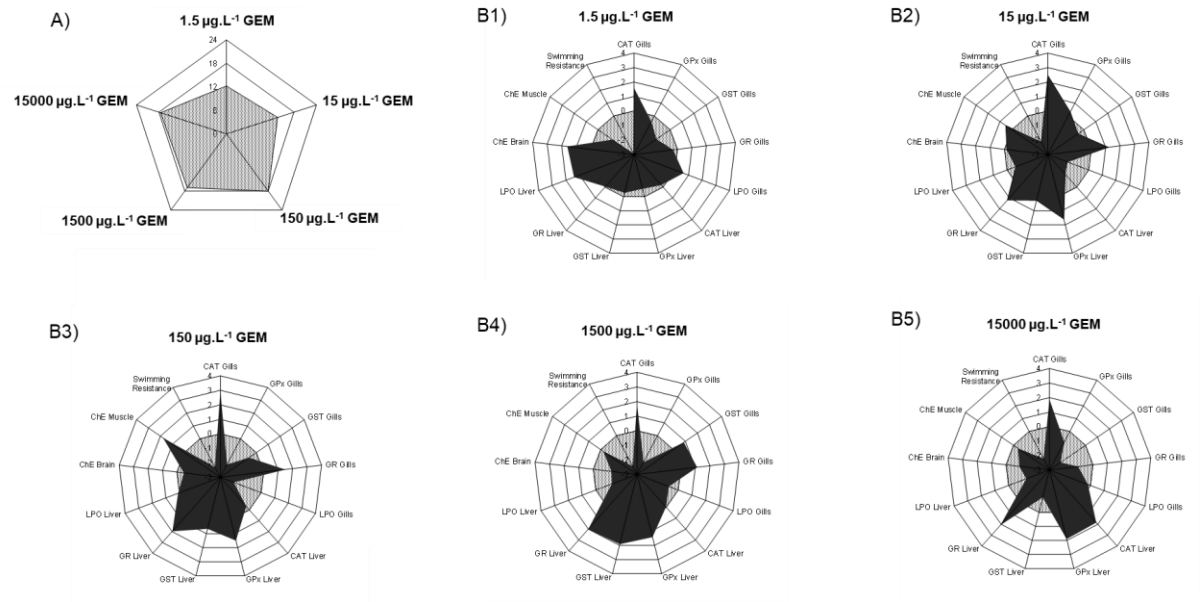

Figure 6. Integrated biomarker response version 2 (IBRv2) (**A**) and assessed endpoints star plots for each experimental condition (**B1-B5**). Gemfibrozil (GEM); Cholinesterases (ChE); Catalase (CAT); Glutathione *S*-transferases (GST); Glutathione peroxidase (GPx); Glutathione reductase (GR); Lipid peroxidation (LPO).

If the data analyses takes into account a weighing factor attributed to different biological levels of organization as suggested by Liu et al. (2013, 2015), the effects of the different concentrations of GEM would be ordered as follows: 150 $\mu g.L^{-1} \approx 15000\ \mu g.L^{-1} \approx 1500\ \mu g.L^{-1} > 1.5\ \mu g.L^{-1} \approx 15\ \mu g.L^{-1}$ (Table S2). This data analysis, attributing a higher weighing factor to behaviour, did however not alter the ranking of GEM impact. Considering the integration of the data from biochemical endpoints (CAT, GST, GPx and GR activities and LPO levels) per tissue (gills versus liver) – Table S3 – the IBRv2 values were higher in gills than in liver for 1.5, 15 and 150 $\mu g.L^{-1}$ of GEM. However, the IBRv2 values were similar between the two tissues for 1500 $\mu g.L^{-1}$ of GEM and for 15000 $\mu g.L^{-1}$ the IBRv2 value was higher in liver than in gills.

The detection of GEM toxicity at an environmentally relevant concentration may be of concern, taking into account that fish are exposed to a variety of contaminants in their natural habitat, including pharmaceuticals sharing the toxicological properties of GEM. Further studies assessing effects of low GEM concentrations and longer exposure periods are encouraged to improve the knowledge about the mechanisms involved on the toxicity of fibrates to non-target organisms like marine fish and its ability to adapt to these compounds.

4. Conclusions

A multibiomarker approach showed that short-term exposure to an environmentally relevant concentration of gemfibrozil (1.5 $\mu g.L^{-1}$) induced behavioural alterations and oxidative damage in the liver of the marine fish *Sparus aurata*. At higher concentrations the activities of some enzymes involved in antioxidant defence (catalase, glutathione peroxidase and glutathione reductase) were induced. Although there was no dose-response relationship for responses, it was clear that higher concentrations (150, 1500 and 15000 $\mu g.L^{-1}$) had more effects on fish than lower concentrations (1.5 and 15 $\mu g.L^{-1}$). The integrated biomarker response version 2 (IBRv2) was found to be a useful tool to combine the results from many biomarkers.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

Acknowledgments

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Supplementary Information

Integrated biomarker response (IBR)

The IBR for the tested biomarkers was calculated according to Sanchez et al. 2013, using IBR version 2 (IBRv2). Based on the limitations of IBRv1, the IBRv2 was developed with two major aims: 1) to remove the IBR result dependency on arrangement of the biomarkers on the star plot; 2) to discriminate induction and inhibition for each biomarker.

For the IBRv2 calculation, individual biomarker data (X_i) was compared to a mean reference data (X_0), in this case, the control group, and a log transformation was applied to reduce variance:

$$Y_i = \log (X_i / X_0)$$

Then, the general mean (μ) and deviation (σ) of Y_i were computed, as previously described by Beliaeff and Burgeot (2002), and Y_i was standardized:

$$Z_i = (Y_i - \mu) / \sigma$$

To create a basal line centered on 0 and to represent biomarker variation according to this basal line, the mean of standardized biomarker response (Z_i) and mean of reference biomarker data (Z_0) were used to define a biomarker deviation index (A):

$$A = Z_i - Z_0$$

To obtain an IBRv2 index, the absolute value of A parameters calculated for each biomarker in each experimental condition were summed:

$$IBR = \sum |A|$$

For each experimental condition, A parameters were reported in a star plot to represent the reference deviation of each tested biomarker. The area up to 0 reflects biomarker induction, and the area down to 0 indicates a biomarker inhibition.

Table S1. Nominal and measured concentrations ($\mu g.L^{-1}$) of gemfibrozil in experimental aquaria at 0 and 24 h.

Nominal concentrations ($\mu g.L^{-1}$)	**Measured concentrations** ($\mu g.L^{-1}$)	
	0h	**24h**
1.5	1.6	0.6
15	23.7	19.9
150	237.7	234.8
1500	1973.5	1958.8
15000	18824.6	18530.9

Table S2. Integrated biomarker response version 2 (IBRv2) values for each experimental condition taking into account a multiplication factor of one to biochemical biomarkers (cholinesterases, catalase, glutathione *S*-transferases, glutathione peroxidase and glutathione reductase activities and lipid peroxidation levels) and three to swimming resistance.

Concentrations ($\mu g.L^{-1}$)	**IBRv2 index**
1.5	18.1
15	17.7
150	22.4
1500	21.6
15000	21.7

Table S3. Integrated biomarker response version 2 (IBRv2) values for the tested biochemical endpoints (catalase, glutathione *S*-transferases, glutathione peroxidase and glutathione reductase activities and lipid peroxidation levels) per tissue (gills and liver) for each experimental condition.

Concentrations (µg.L^{-1})	**IBRv2 index**	
	Gills	**Liver**
1.5	4.1	2.5
15	6.0	4.9
150	9.0	4.5
1500	6.6	6.5
15000	6.0	8.2

Chapter V

Mutation Research/Genetic Toxicology and Environmental Mutagenesis, 2017. 821:36-42

Genotoxicity of gemfibrozil in the gilthead seabream (*Sparus aurata*)

A. Barreto[1*], L.G. Luis[1], P. Paíga[2], L.H.M.L.M. Santos[2,3], C. Delerue-Matos[2], A.M.V.M. Soares[1], K. Hylland[4], S. Loureiro[1], M. Oliveira[1]

[1] Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] REQUIMTE/LAQV, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida, 431, 4200-072 Porto, Portugal

[3] Present affiliation: Catalan Institute for Water Research (ICRA), Carrer Emili Grahit 101, 17003 Girona, Spain

[4] Department of Biosciences, University of Oslo, PO Box 1066, N-0316 Oslo, Norway

*Corresponding author: E-mail: abarreto@ua.pt, Tel +351 234 370 350, Fax +351 234 372 587

Highlights

- Gemfibrozil induced genotoxicity on fish at environmentally relevant concentrations;
- DNA integrity decreased following gemfibrozil short-term exposure;
- Cytogenetic damage increased after 96-h of gemfibrozil exposure.

Abstract

Widespread use of pharmaceuticals and suboptimal wastewater treatment have led to increased levels of these substances in aquatic ecosystems. Lipid-lowering drugs such as gemfibrozil (GEM), which are among the most abundant human pharmaceuticals in the environment, may have deleterious effects on aquatic organisms. We examined the genotoxicity of GEM in a fish species, the gilthead seabream (*Sparus aurata*), which is commercially important in southern Europe. Following 96-h waterborne exposure, molecular (erythrocyte DNA strand breaks) and cytogenetic (micronuclei and other nuclear abnormalities in cells) endpoints were measured. GEM was positive in both endpoints, at environmentally relevant concentrations, a result that raises concerns about the potential genotoxic effects of the drug in recipient waters.

Keywords: gemfibrozil; seabream; DNA damage; comet assay; erythrocyte abnormalities; micronucleus assay

1. Introduction

The presence of human and veterinary pharmaceuticals in the environment is of increasing concern (Heberer 2002, Andreozzi et al. 2003, Fent et al. 2006, Schmidt et al. 2011). The environmental release of these substances and their metabolites, their persistence, and their bioactivities have led to their classification as emerging contaminants of concern (Andreozzi et al. 2003, Fent et al. 2006, Schmidt et al. 2011). Lipid regulators, a group of human pharmaceuticals, are frequently 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, Gros et al. 2006, Togola and Budzinski 2007, Schmidt et al. 2011). The lipid regulator gemfibrozil (GEM) has been found in wastewater treatment plant effluents at levels as high as 2.1 $\mu g.L^{-1}$ in Canada (Lin and Reinhard 2005) and 4.76 $\mu g.L^{-1}$ in Europe (Andreozzi et al. 2003). In surface waters, the highest concentrations of GEM were detected in North America and Europe, around 0.75 and 1.5 $\mu g.L^{-1}$, respectively (Sanderson et al. 2003).

The risks to aquatic organisms associated with the presence of pharmaceuticals in the environment include behavioural alterations, genotoxicity, reduced pathogen resistance and endocrine disruption (Halling-Sørensen et al. 1998, Kümmerer 2004, Tambosi et al. 2010). Studies with GEM have shown that it affects feeding and attachment of the cnidarian *Hydra attenuata* (Quinn et al. 2008); growth of the alga *Chlorella vulgaris* (Zurita et al. 2007); decreases plasma testosterone levels in the goldfish (*Carassius auratus*) (Mimeault et al. 2005); and activates antioxidant enzymes and interferes with metallothionein expression in the blue mussel (*Mytilus edulis*) (Schmidt et al. 2011) and zebra mussel (*Dreissena polymorpha*) (Quinn et al. 2011). Henriques et al. (2016) showed that exposure to GEM affects the development and locomotor activity of zebrafish (*Danio rerio*) larvae. Only a few studies have evaluated its aquatic genotoxicity (Quinn et al. 2011, Schmidt et al. 2011; Rocco et al. 2012). GEM can damage DNA in the zebrafish (after 7-d exposure; Rocco et al. 2012) and in marine (*Mytilus* spp.) and freshwater mussels (*Dreissena polymorpha*) (after 96-h exposure; Quinn et al. 2011, Schmidt et al. 2011). However, to our knowledge, only one study has reported GEM effects on an estuarine/marine top-predator fish species (Teles et al. 2016).

Contaminants may interact with DNA directly or they may disrupt normal cellular processes, e.g. inducing oxidative stress (Oliveira et al. 2010). Elevated levels of reactive oxygen species (ROS) and/or depressed antioxidant defences may result in DNA oxidation and increased steady-state levels of unrepaired DNA leading to genotoxicity (Collins 2004, Azqueta et al. 2009, Guilherme et al. 2012).

The comet assay is widely used in environmental toxicology for assessing DNA damage (Azqueta et al. 2014, Amaeze et al. 2015, Martins and Costa 2015, Imanikia et al. 2016); it combines the simplicity of biochemical techniques for detecting DNA single-strand breaks (strand breaks and incomplete excision-repair sites), alkali-labile sites and cross-linking, by measuring the migration of DNA from immobilized nuclear DNA, using the single-cell approach typical of cytogenetic assays (Lee and Steinert 2003, de Andrade et al. 2004a, Kumaravel and Jha 2006, Kumaravel et al. 2009, Guilherme et al. 2010). The micronucleus (MN) assay, one of the most popular tests of environmental genotoxicity, is based on

chromatid/chromosome fragments or whole chromatids/chromosomes resulting from DNA strand damage, which are not reincorporated into the daughter nucleus and are transformed into a MN (Jenssen and Ramel 1980, Grisolia 2002, Fenech et al. 2003, Luzhna et al. 2013, Furnus et al. 2014). MN may be induced by oxidative stress, by exposure to clastogens or aneugens, or by defects in cell-cycle checkpoints or DNA repair (Fenech et al. 2003). The simultaneous expression of other morphological nuclear abnormalities in addition to MN has proven to be a valuable tool in detecting genotoxicity of several contaminants at low concentrations (Çavaş and Ergene-Gözükara 2003, Grisolia et al. 2009, da Rocha et al. 2011, Baršienė et al. 2014, Carrola et al. 2014, Corredor-Santamaría et al. 2016, Stankevičiūtė et al. 2016). We have tested the genotoxicity of waterborne GEM to a predatory fish species, *Sparus aurata*, following a 96-h exposure, by assessing damage with the comet assay and erythrocytic nuclear abnormalities (ENAs) assay.

2. Material and Methods

2.1. Chemicals

All reagents used were analytical grade and acquired from Sigma-Aldrich. GEM (≥98%) was purchased from TCI and isotopically labelled d_6-GEM was purchased from Santa Cruz Biotechnology (Dallas, USA). A stock solution (50 $g.L^{-1}$) was prepared in dimethyl sulfoxide (DMSO). Exposure solutions of GEM (1.5; 15; 150; 1500; and 15000 $\mu g.L^{-1}$) were prepared by serial dilutions in artificial seawater.

2.2. Test animals and experimental design

All experimental procedures were carried out following the Portuguese and European legislation (authorization N421/2013 of the Portuguese legal authorities). Animal handling was performed by an accredited researcher.

Juvenile gilthead seabream (*Sparus aurata*), length 9 ± 0.9 cm, acquired from an aquaculture facility (Spain), were acclimated for 4 weeks in 220 L aquaria containing aerated and filtered artificial seawater (salinity, 35), under a controlled room temperature (20ºC) and natural photoperiod. During this period, the experimental fish (n=75) were fed daily with commercial fish food (Sorgal,

Portugal) at a ratio of 1 g per 100 g of fish and the water in the aquarium was renewed daily.

The procedures generally followed the OECD guidelines for fish acute bioassays (OECD 1992). The experiment was carried out in 80 L aquaria, under the conditions described for the acclimation period. Following acclimation, fish were randomly distributed into seven aquaria, with ten fish per aquarium. The experimental design included a negative control (seawater only), a solvent control (0.03% DMSO, the DMSO concentration used for the highest concentration of GEM) and five GEM concentrations: 1.5; 15; 150; 1500; and 15000 $\mu g.L^{-1}$. Fish were exposed for 96 h as recommended by the OECD guideline for fish acute toxicity testing (203), without feeding, with 80% medium renewal every 24 h, to prevent significant GEM degradation and to reduce the build-up of metabolic residues. Fish mortality, behavioural alterations and water parameters (such as temperature, salinity, conductivity, pH and dissolved oxygen) were monitored daily.

After 96 h exposure, the animals were anesthetized with tricaine methanesulfonate (MS-222) and a blood sample was collected from the posterior cardinal vein of each fish. For the comet assay, blood samples were diluted with saline phosphate buffer (2:2000, v/v) and used immediately. Blood smears were prepared for the assessment of MN and other erythrocytic nuclear abnormalities.

2.3. Quantification of gemfibrozil (GEM) in the test media

Water samples (10 mL) were collected each day (at 0 and 24 h) from each aquarium and GEM was analysed by solid-phase extraction (SPE). Briefly, Strata X cartridges (200 mg, 3 mL) (Phenomenex, USA) were conditioned with 5 mL methanol and 5 mL ultrapure water. Then, the water sample (10 mL) was percolated through the cartridge at a flow rate of 3-5 $mL.min^{-1}$; the cartridge was rinsed with ultrapure water (5 mL) and dried under vacuum for 20 min. Finally, GEM was eluted with methanol, 10 mL. Extracts were evaporated until dryness under a gentle stream of nitrogen and reconstituted with acetonitrile/ultrapure water (30:70, v/v, 1 mL). An aliquot (10 µL) of gemfibrozil-d_6 (5 $mg.L^{-1}$) was added to the extract as internal standard before ultra-high performance liquid

chromatography tandem-mass spectrometry (UHPLC-MS/MS) analysis. GEM analysis was performed on a Nexera UHPLC system with a triple-quadrupole mass spectrometer detector LCMS-8030 (Shimadzu Corporation, Kyoto, Japan). Chromatographic separation was achieved on a Kinetex C18 column (2.1 x 150 mm i.d., 1.7 µm particle size) from Phenomenex (USA); column temperature, 30ºC; autosampler temperature, 4ºC; injection volume, 5 µL. Elution conditions were: solvent A, 5 mM ammonium acetate/ammonia buffer (pH 8); solvent B, acetonitrile; flow rate, 0.22 $mL.min^{-1}$. Gradient program was as follows: initial conditions: 30% B; 0-2.0 min, 30-100% B; 2.0-4.5 min, maintained at 100% B; 4.5-5.5 min, return to initial conditions; from 5.5-9.5 min, re-equilibration of the column.

GEM was analysed in the negative ionization mode and quantification was performed in multiple reaction monitoring mode (MRM) using two transitions between the precursor ion and the most abundant fragment ions. A summary of individual MS/MS parameters is shown in Table S1 (Supplementary Information). Quantification was performed by the internal standard calibration method. The method detection limit (MDL) for GEM in water was 4.0 $ng.L^{-1}$. Detailed QA/QC information is given in the Supplementary Information (Table S2).

2.4. Evaluation of genetic damage

2.4.1. Comet assay

The alkaline comet assay was conducted according to the method of Singh et al. (1988) with some modifications. To prevent UV-induced DNA damage, the procedure was conducted under yellow light. Briefly, diluted blood samples (20 µL) were added to 1% (w/v) low-melting-point agarose, 140 µL (at 40ºC) and the mixtures applied to microscope slides pre-coated with 1% (w/v) normal-melting-point agarose. A coverslip was added to each slide, which was then placed on ice for agarose solidification; then, the coverslips were carefully removed, and the slides immersed, for 1 h at 4ºC, in lysis solution (2.5 M NaCl, 100 mM EDTA and 10 mM Tris, pH 10.0), containing freshly added 1% Triton X-100. Slides were incubated in alkaline buffer (300 mM NaOH and 1 mM EDTA, pH > 13) for 10 min for DNA denaturation and unwinding. Electrophoresis was performed using the same buffer, for 30 min at 300 mA and 20 V. Note: State the field strength in 0.83

V.cm^{-1}. After electrophoresis, slides were neutralized in 400 mM Tris buffer (pH 7.5), dehydrated with absolute ethanol for 10 sec and left to dry for 1 day in the dark. Slides were stained with ethidium bromide (20 µL.mL^{-1}, 100 µL), covered with a coverslip and analysed using a fluorescence microscope (Olympus BX41TF) at 400X magnification. To verify that the electrophoresis conditions were adequate, negative (blood from fish maintained in an aquarium with seawater only) and positive (blood from fish treated with 25 µM hydrogen peroxide (H_2O_2) for 10 min) controls were included in each electrophoresis run. H_2O_2 was used as a model genotoxic agent since it produces both single-strand breaks and oxidative DNA damage (Termini 2000) and has been used routinely as a positive control in the comet assay (Singh et al. 1988, Gielazyn et al. 2003). To avoid bias, slides were randomly analysed, counting one hundred randomly selected cells from each slide. Cells were scored visually, according to tail length, into five classes: class 0 – undamaged, without a tail; class 1 – with a tail shorter than the diameter of the head (nucleus); class 2 – with a tail length 1-2 times the diameter of the head; class 3 – with a tail longer than twice the diameter of the head; class 4 – comets with no heads (Collins 2004). A damage index (DI) expressed in arbitrary units was assigned to each replicate (for 100 cells) and consequently for each treatment, using the formula:

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

where: n = number of cells in each class.

DI can range from 0 to 400 (de Andrade et al. 2004b). The percentage of DNA damage relative to the control was calculated.

2.4.2. Erythrocytic nuclear abnormalities (ENAs) assay

This assay was carried out in mature peripheral erythrocytes according to the procedure of Pacheco and Santos (1996). Blood smears were fixed in methanol during 10 min and stained with Giemsa (5%) for 30 min. The nuclear abnormalities were randomly scored under a light microscope in 1000 intact erythrocytes per fish.

Nuclear lesions were scored as: micronuclei, lobed nuclei that display a nucleus with a small evagination of the nuclear membrane with euchromatin; segmented nuclei, symmetrical or asymmetrical hourglass-shaped nuclei; kidney-shaped nuclei, nuclei with a kidney-shaped profile and vacuolated nuclei showing central vacuoles in the nucleus without nuclear material. Blebbed, lobed, and notched nuclei were considered in a single category – lobed nuclei – and not scored differentially, as suggested by other authors, due to some ambiguity in their distinction (Guilherme et al. 2008, Carrola et al. 2014). In general, blebbed nuclei have a relatively small evagination of the nuclear envelope, lobed nuclei present evaginations largest than the blebbed nuclei and the notched nuclei have an appreciable invagination (Carrola et al. 2014). Results were expressed as the percentage mean value for erythrocytic nuclear abnormalities (ENAs) using the equation:

$$ENAs(\%) = \frac{\text{Number of cells containing ENAs}}{\text{Total number of cells counted}} \times 100$$

2.5. Statistical Analysis

Prior to parametric analyses, data were tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test), using the SPSS 21 software package. Differences between controls (negative and solvent) were evaluated using the Student t-test ($p<0.05$). Data from treatments and controls were compared using one-way analysis of variance (ANOVA), followed by Dunnett's comparison post-hoc test whenever applicable ($p<0.05$).

3. Results and Discussion

We assessed the genotoxic effects of waterborne GEM in an important commercial species (*S. aurata*), at a wide range of concentrations, including environmentally relevant concentrations.

3.1. Quantification of GEM in the test media

Spiked nominal concentrations of GEM were generally lower than the observed concentrations; this difference varied from 6 to 37% (Table 1).

Table 1. Nominal and measured concentrations (µg.L^{-1}) of gemfibrozil from water samples collected at time 0 and after 24-h of spiking.

Nominal concentrations (µg.L^{-1})		**1.5**	**15**	**150**	**1500**	**15000**
Measured concentrations (µg.L^{-1})	**0 h**	1.6	23.7	237.7	1973.5	18824.6
	24 h	0.6	19.9	234.8	1958.8	18530.9

In a study with zebrafish, Henriques et al. (2016) similarly reported that nominal concentrations of GEM differed from 3-30% of the added amount, as detected by chemical analysis. After 24 h, the decrease of GEM was most evident at the lowest concentrations (about 60% at 1.5 µg.L^{-1} and 16% at 15 µg.L^{-1}). At the highest concentrations, the decrease of GEM amount was <2% (Table 1). In a study with goldfish (*Carassius auratus*), a decrease of GEM concentration was detected in water at the end of the exposure (14 d), also at the lowest concentrations (Mimeault et al. 2005).

3.2. Evaluation of genetic damage

DNA integrity loss was measured with the comet assay in *S. aurata* erythrocytes after DMSO exposure ($p<0.05$; t-test). The negative control displayed a damage index around 77, corresponding to 19.25% DNA damage, whereas the solvent control showed a damage index around 150, corresponding to 37.5% damage. There is limited and contradictory information available on the possible genotoxic effects of DMSO and no study was found concerning the genotoxicity of this solvent to fish. Although some studies have described DMSO as not genotoxic, other studies have shown the importance of studying possible genotoxic effects of DMSO in various organisms (Valencia-Quintana et al. 2012). A decrease in DNA integrity was reported for the digestive gland of blue mussel *Mytilus* spp. (Schmidt et al. 2011) and for the visceral mass of zebra mussel *Dreissena polymorpha* (Quinn et al. 2011) following exposure to 0.2% DMSO. The concentration of DMSO used in the current study (0.03%) was nearly one order of magnitude lower than the concentrations used in earlier studies with GEM (i.e., 0.1

and 0.2% DMSO) (Zurita et al. 2007, Quinn et al. 2008, Quinn et al. 2011, Schmidt et al. 2011). The concentration chosen here was a compromise, considering the limits recommended in the guidelines (OECD 1998, 2002, 2013), the results of previous studies (Mimeault et al. 2005, Hutchinson et al. 2006, Zurita et al. 2007, Quinn et al. 2008, Quinn et al. 2011, Schmidt et al. 2011, Maes et al. 2012), and the aim of the present work.

The differences between the negative and solvent control groups seen in the comet assay were not seen in the ENAs assay. DNA damage corresponds to a very early signal of stress in the cell. Some authors also reported loss of DNA integrity in marine mussels (*Mytilus* spp.) after exposure to 0.2% DMSO, but other biomarkers, such as lipid peroxidation levels and glutathione transferases activity, were not affected (Schmidt et al. 2011).

Taking into account the observed differences between the seawater control and the solvent control, the effects of GEM treatments were compared to the solvent control. Overall, GEM displayed genotoxic potential, assessed by DNA strand breakage at an environmentally relevant concentration (Figure 1). The damage index increased with GEM concentration, with a maximum of 316 in organisms exposed to 15000 µg.L^{-1}.

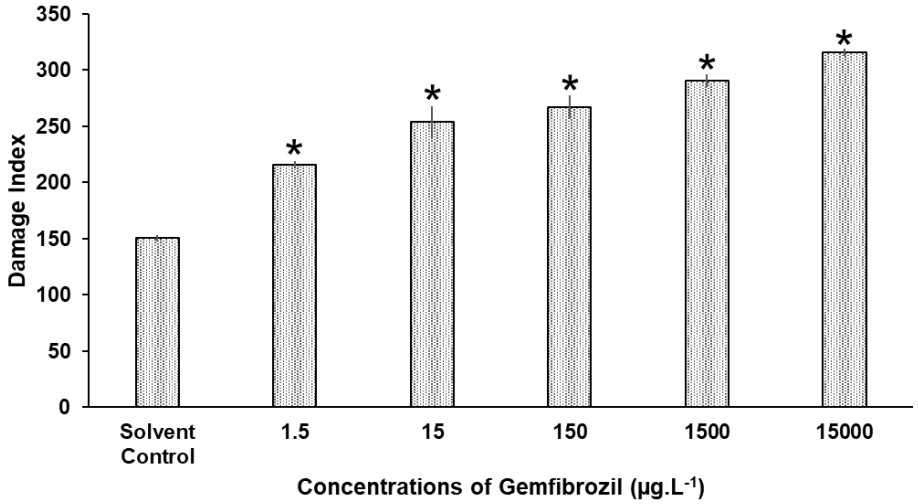

Figure 1. DNA damage index, in arbitrary units, of peripheral blood cells from *Sparus aurata* exposed for 96-h to gemfibrozil. *Significant differences to solvent control (Dunnett's test, $p<0.05$); data are presented as mean ± standard error.

In terms of damage classes, the two most abundant classes in the negative control group (seawater only), which displayed little or no DNA migration, were class 0 and 1 damage, unlike the solvent control, where classes 1 and 2 were the most abundant (Table 2). At all GEM treatment exposures, when compared to the solvent control, significantly lower class 0 and 1 cells were seen, with increased class 2 at 1.5 $\mu g.L^{-1}$, class 3 at 15 $\mu g.L^{-1}$ and classes 3 and 4 at 150, 1500, and 15000 $\mu g.L^{-1}$.

Table 2. DNA damage classes, measured by the comet assay, of peripheral blood cells of *Sparus aurata* exposure to gemfibrozil for 96 h. *Significant differences to solvent control (Dunnett's test, $p<0.05$); data are presented as mean ± standard error.

Treatment group ($\mu g.L^{-1}$)	Damage classes (%)					Damage index
	0	1	2	3	4	
Negative Control	40.8±3.1	44.8±6.0	11.8±4.0	1.8±2.2	0.8±1.3	77.0±3.0
Solvent Control	9.8±0.9	43.2±3.4	34.6±3.0	10.0±0.5	2.0±0.9	150.4±3.2
1.5	1.6±1.2*****	9.0±1.3*****	65.2±9.2*****	21.2±9.9	3.2±2.5	215.8±14.2*****
15	1.2±0.8*****	5.0±1.4*****	43.0±8.2	40.2±5.3*****	10.6±3.7	254.0±10.7*****
150	0.4±0.2*****	4.2±1.6*****	42.4±6.6	41.8±3.3*****	13.2±2.9*****	267.2±5.9*****
1500	0.6±0.6*****	1.4±0.9*****	43.2±3.9	41.8±4.9*****	13.0±3.2*****	290.6±3.4*****
15000	0.2±0.2*****	0.8±0.4*****	8.8±3.2*****	63.2±5.8*****	27.0±3.5*****	316.0±13.0*****

The ability of GEM to decrease DNA integrity has been previously reported in studies with aquatic organisms: in *Danio rerio* erythrocytes after 5 d exposure to 380 $ng.L^{-1}$ (Rocco et al. 2012), in *Mytilus* spp. digestive gland (Schmidt et al. 2011), and in *Dreissena polymorpha* visceral mass (Quinn et al. 2011) after 24 and 96-h exposure to 1 and 1000 $\mu g.L^{-1}$. The mechanism of GEM-dependent DNA integrity loss in aquatic organisms is not yet clear. GEM, classified under the generic designation of fibrates, is a potent peroxisome proliferator (PPs) (Nunes et al. 2008). Fibrates are characterised by the pronounced induction of hepatic peroxisome proliferation, mediated via peroxisome proliferator-activated receptor alpha (PPAR**α**), increasing the number and size of peroxisomes in the liver (O'Brien et al. 2001). PPAR**α**-induced oxidative stress may contribute to cell

proliferation via increased signalling or may damage DNA, initiating carcinogenesis. However, data for peroxisome proliferator-induced DNA damage are conflicting (Cheung et al. 2004, Shah et al. 2007). In mammals, GEM may be metabolized to reactive acyl glucuronide metabolites which may react with nucleophilic centres in DNA via a Schiff base mechanism (Sallustio et al. 1997). GEM may also cause DNA strand breaks via oxygen-radical generation. In addition, perturbed DNA repair may lead to formation of mutagenic and clastogenic lesions (Marsman et al. 1992).

In the current study, no micronuclei were detected in control fish (both seawater and solvent controls), in agreement with the study of Bolognesi et al. (2006), where micronuclei baseline frequencies of 0.012% were reported for *S. aurata* captured in a reference area. The 96-h exposure of *S. aurata* to 1500 and 15000 µg.L^{-1} GEM led to significantly higher MN frequencies (Figure 2).

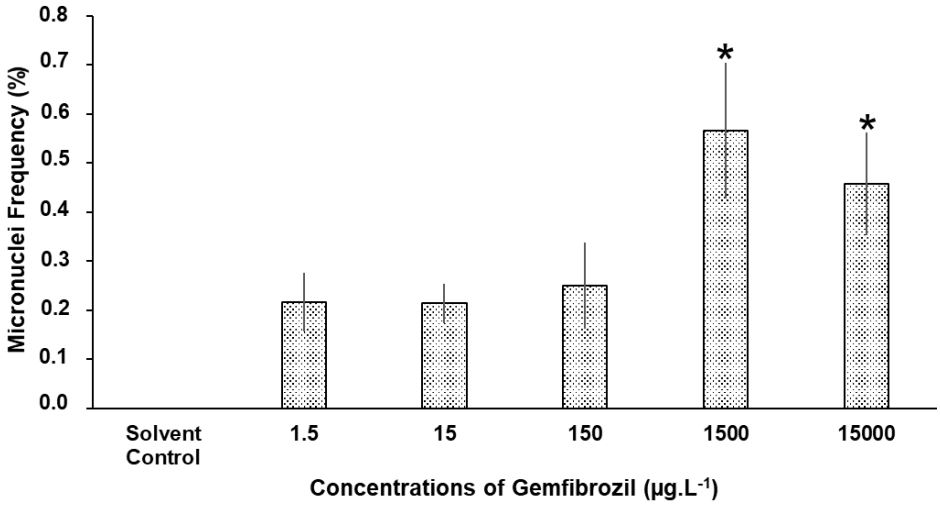

Figure 2. Micronuclei in mature peripheral erythrocytes of *Sparus aurata* after 96-h exposure to gemfibrozil. *Significant differences to solvent control (Dunnett's test, $p<0.05$); data are presented as mean percentage ± standard error.

However, if all ENAs were considered, significant differences to control would have been noted for all GEM concentrations (Figure 3).

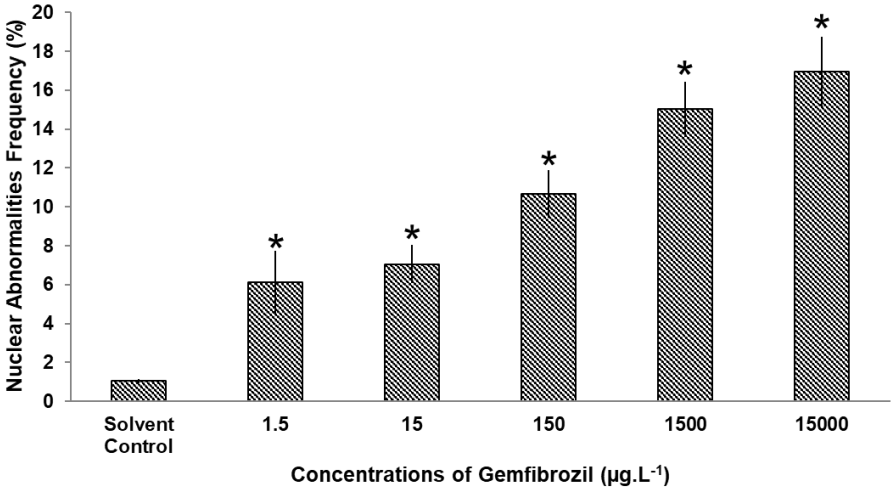

Figure 3. Erythrocytic nuclear abnormalities in *Sparus aurata* after 96-h exposure to gemfibrozil. *Significant differences to solvent control (Dunnett's test, $p<0.05$); data are presented as mean percentage ± standard error.

An analysis of the different types of anomalies suggested that events leading to segmented and vacuolated nuclei occurred both at lower and higher concentrations of GEM, with differences to control detected even at the lowest tested concentration (1.5 µg.L^{-1}). Kidney-shaped and lobed nuclei were mostly detected in fish exposed to concentrations >150 µg.L^{-1} (Table 3).

Table 3. Erythrocytic nuclear abnormalities detected in *Sparus aurata* after 96-h exposure to gemfibrozil. *Statistically significant differences to solvent 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 (µg.L^{-1})	Frequency (%)				
	K	S	L	V	MN
Solvent Control	0.7±0.1	0.4±0.1	0.0±0.0	0.0±0.0	0.0±0.0
1.5	3.1±0.9	1.7±0.5*****	0.3±0.1	0.9±0.2*****	0.2±0.1
15	3.0±0.7	2.3±0.4*****	0.5±0.1	1.0±0.2*****	0.2±0.0
150	6.3±0.9*****	2.5±0.4*****	0.8±0.2*****	1.0±0.3*****	0.3±0.1
1500	9.0±1.2*****	3.3±0.6*****	0.8±0.2*****	1.1±0.2*****	0.6±0.3*****
15000	9.4±1.1*****	3.3±0.8*****	2.5±0.4*****	1.3±0.4*****	0.5±0.1*****

To our knowledge, GEM induction of ENAs in aquatic organisms has not been reported previously. However, quantification of ENAs has been used to detect genotoxicity in fish species such as golden grey mullet (*Liza aurata*) (Oliveira et al. 2007, Guilherme et al. 2008), European seabass (*Dicentrarchus labrax*) (Gravato and Santos 2003a, b), Nile tilapia (*Oreochromis niloticus*) (Sponchiado et al. 2011), European minnow (*Phoxinus phoxinus*) (Ayllon and Garcia-Vazquez 2000) and European eel (*Anguilla anguilla*) (Maria et al. 2002a, Maria et al. 2002b, Teles et al. 2003). Although the mechanisms responsible for nuclear abnormalities are not completely understood, some nuclear abnormalities (such as lobed and segmented nuclei) may be interpreted as nuclear lesions analogous to MN that may be induced by genotoxic compounds even if MN *per se* are not induced (Ayllon and Garcia-Vazquez 2000, Guilherme et al. 2008, Harabawy et al. 2014, Stankevičiūtė et al. 2016). According to some authors, nuclear buds (lobed, blebbed and notched nuclei) may be caused by problems in segregating tangled and attached chromosomes or by gene amplification via the breakage-fusion-bridge cycle, during the elimination of amplified DNA from the nucleus (Shimizu et al. 1998, Guilherme et al. 2008). Segmented cells contain two nuclei, possibly due to blocking of cytokinesis or cell fusion (Rodilla 1993). With respect to vacuolated nuclei, these have been proposed to be a result of aneuploidy leading to MN formation (Carrola et al. 2014, Harabawy et al. 2014, Stankevičiūtė et al. 2016). Concerning kidney-shaped nuclei, such as nuclear invagination, for some authors, these are considered to have a cytological cause (Bolognesi et al. 2006), whereas by others, they are ascribed to genotoxic origin (Carrola et al. 2014, Harabawy et al. 2014). Although, at lower concentrations, GEM did not significantly induce the formation of MN, other nuclear abnormalities related to genotoxicity events were induced, namely high frequency of vacuolated and segmented nuclei. At the highest concentrations, vacuolated and segmented nuclei continued to be present, but GEM also induced the formation of MN, kidney-shaped and lobed nuclei.

The ENAs and comet assay proved to be sensitive tools for detection of GEM genotoxicity, supporting their use as biomarkers in water quality monitoring and risk assessment (Jindal and Verma 2015). As with the present study, several studies already used these assays simultaneously to understand the genotoxic

effects of contaminants in fish species (Bombail et al. 2001, Buschini et al. 2004, de Andrade et al. 2004a, Deguchi et al. 2007, de Campos Ventura et al. 2008, Fatima et al. 2014, Jindal and Verma 2015).

Overall, our data suggest that GEM may represent a hazard to aquatic organisms. The detected genotoxicity is cause for concern, taking into account that the concentrations were environmentally relevant and that, in the environment, fish are exposed to a variety of contaminants, including pharmaceuticals sharing the toxicological properties of GEM (e.g. other peroxisomal proliferators). The mechanism of GEM genotoxicity is probably multifactorial, based on the available studies with mammals; the effects may be due to oxidative stress or formation of reactive metabolites binding to DNA and causing adduct formation (Schwerdtle et al. 2003). Considering that the responses of fish to contaminants, in terms of genetic damage, are similar to those of other vertebrates, including humans (Melo et al. 2013), fish species may be useful model organisms for risk-assessment studies.

4. Conclusions

Our study highlights the potential consequences of the release of pharmaceuticals in the environment. Gemfibrozil (GEM) caused both molecular and cytogenetic effects in *Sparus aurata* erythrocytes after 96-h waterborne exposure. The findings are ecologically relevant as GEM induced genotoxic effects at a concentration observed in the environment (1.5 $\mu g.L^{-1}$). Our results should be considered in the management of aquatic environments and in regulation of pharmaceuticals.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Supplementary Information

Table S1. Ultra-high performance liquid chromatography tandem-mass spectrometry (UHPLC-ESI-MS/MS) conditions for the analysis of gemfibrozil (GEM).

Compound	Rt (min)	Precursor ion (m/z)	Product ion (Quantifier)				Product ion (Qualifier)				Ion ratio (± SD)
			Q3	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)	Q3	Q1 Pre Bias (V)	CE (V)	Q3 Pre Bias (V)	
GEM	3.545	249.00	121.15	16	14	24	127.05	16	10	27	14.8 (±0.2)
GEM-d6	3.550	255.30	121.90	16	14	25	—	—	—	—	—

Table S2. Validation parameters for the analysis of gemfibrozil in water by solid phase extraction-ultra-high performance liquid chromatography tandem-mass spectrometry (SPE-UHPLC-MS/MS). MDL – Method detection limit; MQL – Method quantification limit; RSD – Relative standard deviation; GEM – Gemfibrozil.

Pharmaceutical	Linearity range ($\mu g.L^{-1}$)	r^2	MDL ($ng.L^{-1}$)	MQL ($ng.L^{-1}$)	Recovery (%) ± RSD	Intra-day (RSD, %)	Inter-day (RSD, %)
GEM	1-250	0.9999	4.0	13.5	87.6 ± 1.6	1.43	4.77

Chapter VI

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

A. Barreto[1*], L.G. Luis[1], E. Pinto[2], A. Almeida[2], P. Paíga[3], L.H.M.L.M. Santos[3,4], C. Delerue-Matos[3], T. Trindade[5], A.M.V.M. Soares[1], K. Hylland[6], S. Loureiro[1], M. Oliveira[1]

[1] Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] LAQV/REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

[3] LAQV/REQUIMTE, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida, 431, 4200-072 Porto, Portugal

[4] Catalan Institute for Water Research (ICRA), Carrer Emili Grahit 101, 17003 Girona, Spain

[5] Departamento de Química & CICECO - Aveiro Instituto de Materiais, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[6] Department of Biosciences, University of Oslo, PO Box 1066, N-0316 Oslo, Norway

*Corresponding author: E-mail: abarreto@ua.pt, Tel +351 234 370 350, Fax +351 234 372 587

Highlights

- Antioxidant defences of *Sparus aurata* were induced after the exposure to gold nanoparticles (AuNPs);
- Polyvinylpyrrolidone coated AuNPs (PVP-AuNPs) accumulated more in the tissues and induced more effects in the fish than citrate coated AuNPs (cAuNPs);
- Decreased swimming performance and oxidative damage were detected after the exposure to PVP-AuNPs;
- The bioaccumulation and effects of AuNPs were altered when fish were simultaneously exposed to AuNPs and gemfibrozil.

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 specimens were exposed for 96 h to waterborne 40 nm AuNPs with two different 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 (at 1600 $\mu g.L^{-1}$) and oxidative damage in gills (at 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 citrate coated gold nanoparticles (cAuNPs) exposures. Although AuNPs and GEM combined effects in gills were generally low, in liver, they were higher than the predicted. The accumulation and the effects of AuNPs showed be dependent on the size, coating, surface charge and aggregation state of nanoparticles. Additionally, it was tissues' 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 their 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, 2018). 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 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 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 (20ºC) 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 used during toxicity tests.

2.2. Synthesis and characterisation of gold nanoparticles

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 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 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 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 in 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 potential (ZP; Zetasizer Nano ZS, Malvern).

2.3. Fish bioassay

The bioassay followed, in general, 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 used as negative control. The AuNPs lowest tested concentration (4 $\mu g.L^{-1}$) was near to the predicted values for water (0.14 $\mu g.L^{-1}$) and soil (5.99 $\mu g.kg^{-1}$) (García-Negrete et al. 2013; Tiede et al. 2009). The other tested concentrations 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 $\mu g.L^{-1}$) were prepared by appropriate dilution 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 $\mu g.L^{-1}$) 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) – 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 -80ºC until further processing. Liver, gills, muscle and spleen were collected from three animals and stored at -20ºC until further quantification of gold.

2.6. Quantification of gold and gemfibrozil (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^{TM} 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 ^{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 LCMS-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 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 concentration of gold in muscle.

The bioaccumulation factor (BAF), in $L.g^{-1}$, was determined according to Yoo-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 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, 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 -80ºC until analysis. PMS was accomplished by centrifugation at 12 000 g for 20 min at 4ºC. PMS aliquots were stored at -80ºC 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 4ºC and the obtained supernatant was collected and stored at -80ºC.

Protein content was determined for all samples according to Bradford (1976), by measuring the absorbance at 600 nm, using a microplate-adapted procedure, with bovine γ-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), modified by Athar and Iqbal (1998). Oxidation of NADPH was recorded

spectrophotometrically at 340 nm and the enzyme activity was calculated as nmol $NADP^+$ formed per min per mg of protein ($\varepsilon=6.22\times10^3\ M^{-1}.cm^{-1}$).

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), 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 daily intake” (TDI) (IPCS 2004), the following formula was used:

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

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 ($p<0.05$). 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 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 gold nanoparticles

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).

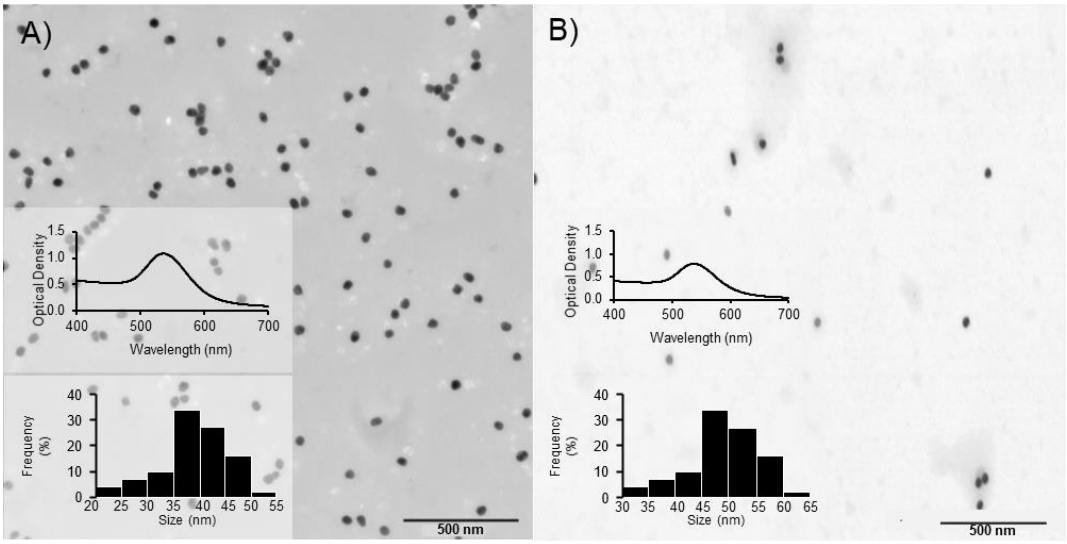

Figure 1. UV-Vis spectrum, size distribution histogram and electron microscopy image of **(A)** citrate coated gold nanoparticles – cAuNPs and **(B)** polyvinylpyrrolidone coated gold nanoparticles – PVP-AuNPs.

Charged species in the 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 µg.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 AuNPs 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. The same was found to 7 nm AuNPs (Chapter III).

A study of the interaction of GEM and AuNPs was not possible at the tested concentrations of 80 and 150 $\mu g.L^{-1}$ (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 $\mu g.L^{-1}$, 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 gemfibrozil (GEM) in the experimental media

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 $\mu g.L^{-1}$. The difference between the nominal and measured concentrations was more evident for cAuNPs. For the nominal concentration of 4 $\mu g.L^{-1}$ cAuNPs, the measured concentration of gold was 32% lower than the predicted. For the 80 $\mu g.L^{-1}$, 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 $\mu g.L^{-1}$), 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 expected for cAuNPs and PVP-AuNPs, respectively.

After 24 h of exposure, comparing with the gold quantified at 0 h, the concentration of cAuNPs in suspension decreased more than the concentration of PVP-AuNPs. In the nominal concentration 4 $\mu g.L^{-1}$, this decrease was 51 and 19% for cAuNPs and PVP-AuNPs, respectively. In the nominal concentration 80 $\mu g.L^{-1}$, 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 $\mu g.L^{-1}$, a decrease of gold in suspension 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.

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 PVP-AuNPs (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/agglomeration 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 $\mu g.L^{-1}$), PVP-AuNPs significantly accumulated ($p<0.05$; Dunnett's test) in the liver. However, at 80 and 1600 $\mu g.L^{-1}$ PVP-AuNPs significantly accumulated in the gills ($p<0.05$; Dunnett's test). cAuNPs also significantly accumulated in the gills following exposure to 1600 $\mu g.L^{-1}$ ($p<0.05$; Dunnett's test). In the single exposures to AuNPs, gills 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 PVP-AuNPs) (Table 2). The calculated BAF showed that bioaccumulation generally was higher for PVP-AuNPs than for cAuNPs and the highest value was observed for the nominal concentration 4 $\mu g.L^{-1}$ 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 mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05). b.d.l. – Bellow the detection limit.

Nominal Concentrations ($\mu g.L^{-1}$)	Tissues	Gold Content ($\mu g.g^{-1}$)		BAF ($L.g^{-1}$)	
		cAuNPs	PVP-AuNPs	cAuNPs	PVP-AuNPs
0 AuNPs	**Gills**	b.d.l.	b.d.l.	-	-
	Liver	b.d.l.	b.d.l.	-	-
	Spleen	b.d.l.	b.d.l.	-	-
	Muscle	b.d.l.	b.d.l.	-	-
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	0.01	0.00
	Spleen	b.d.l.	0.2 ± 0.1	-	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	0.00	0.00
	Spleen	0.4 ± 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).

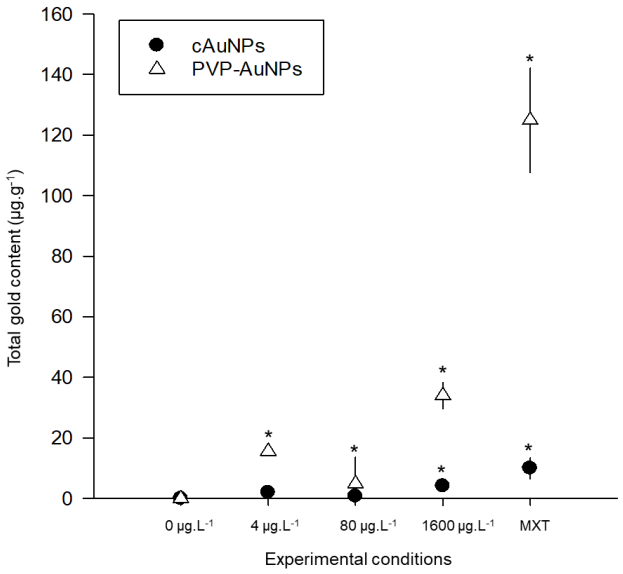

Figure 2. Total gold content on *Sparus aurata* after 96 h of single or combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s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

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

concentrations of 4.6 and 3.0 $\mu g.g^{-1}$, 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 $\mu g.g^{-1}$, gold was detected in the gills (between 0.01 and 0.03 $\mu g.g^{-1}$), digestive tract (between 0.22 and 1.40 $\mu g.g^{-1}$) 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*).

The greater accumulation of gold in tissues when fish were exposed to PVP-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 characterisation for both AuNPs indicated no interaction in ASW with GEM.

3.4. Effects of gold nanoparticles on *Sparus 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.

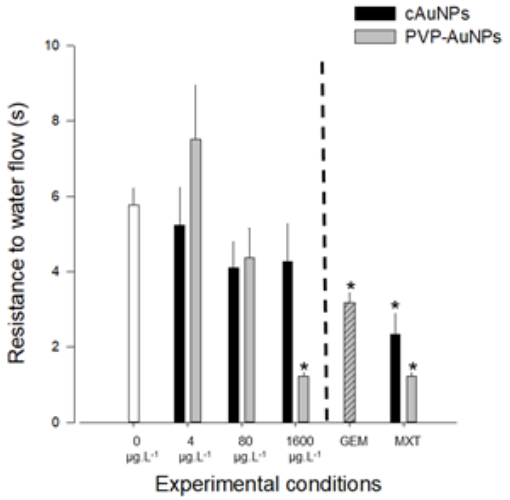

Figure 3. Resistance of *Sparus aurata* to withstand swimming against a water flow after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences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

Previous studies have shown that nanosized materials may affect the behaviour of fish: erratic swimming and slow opercular movements of cichlid *Etroplus maculatus* after 96 h exposure to 100 $\mu g.L^{-1}$ 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 $\mu g.L^{-1}$) (McNeil et al. 2014); significantly greater disruption of the olfactory-mediated behavioural response of *O. mykiss* after 12 h exposure to 50 $\mu g.L^{-1}$ copper NPs (Sovová et al. 2014). In terms of AuNPs, no study has so far reported alterations on the swimming behaviour of fish 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.

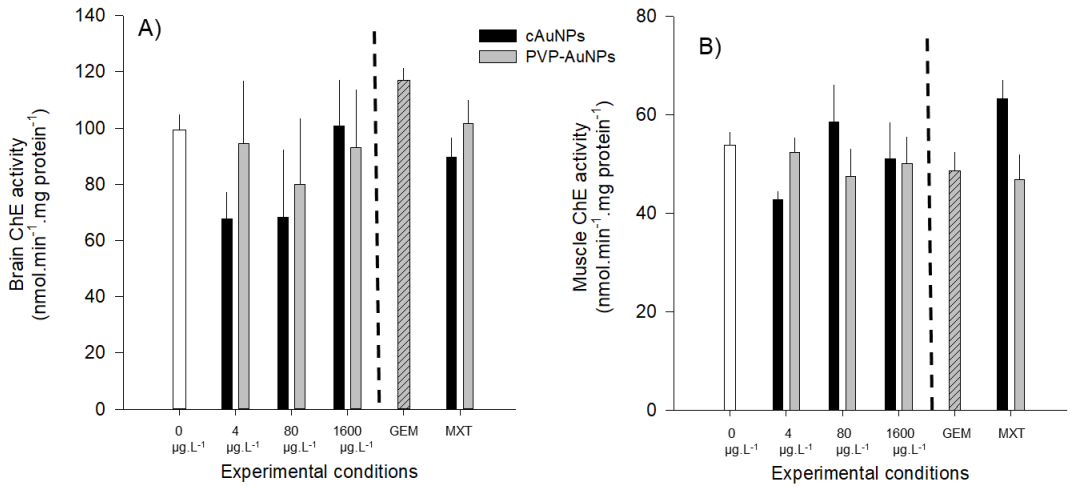

Figure 4. Brain **(A)** and muscle **(B)** cholinesterases (ChE) activity of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM.

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, 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 enzyme (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 $\mu g.L^{-1}$ AuNPs (both citrate and PVP coated NPs) – Figure 5B. In the case of PVP-AuNPs, a dose-response relationship was apparent.

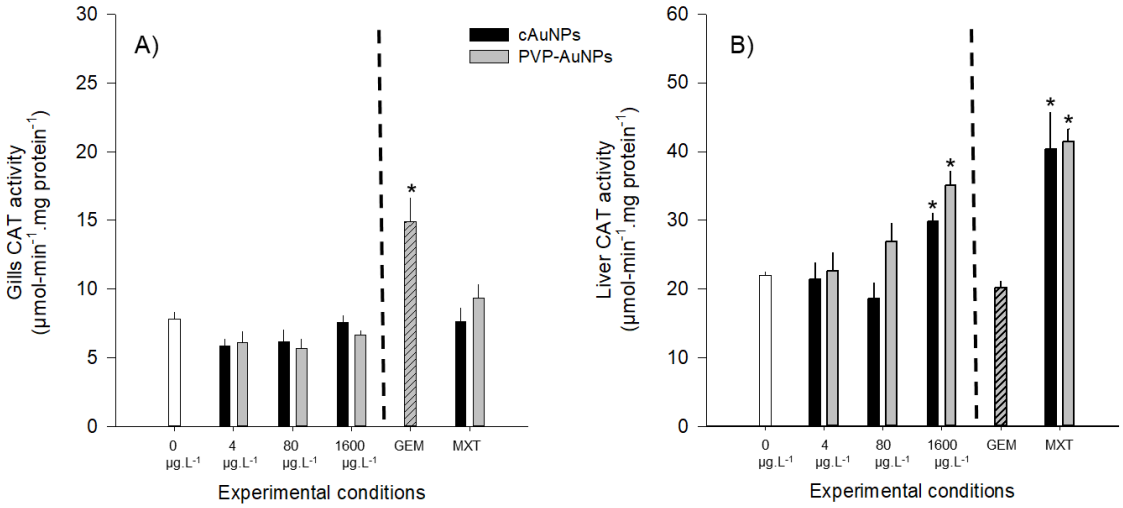

Figure 5. Gills **(A)** and liver **(B)** catalase (CAT) activity of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant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

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

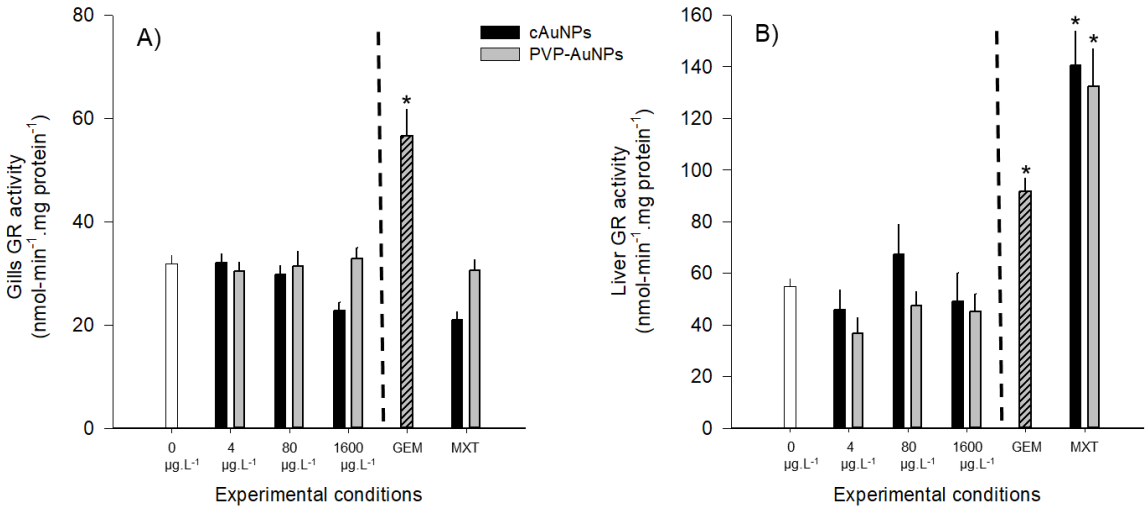

Figure 6. Gills **(A)** and liver **(B)** glutathione reductase (GR) activity of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM.

Regarding GPx, in gills, only 80 $\mu g.L^{-1}$ PVP-AuNPs significantly increased this enzyme activity ($p<0.05$; Dunnett's test; Figure 7A). In the liver, PVP-AuNPs exposure (4 and 1600 $\mu g.L^{-1}$) significantly increased the GPx activity ($p<0.05$; Dunnett's test; Figure 7B).

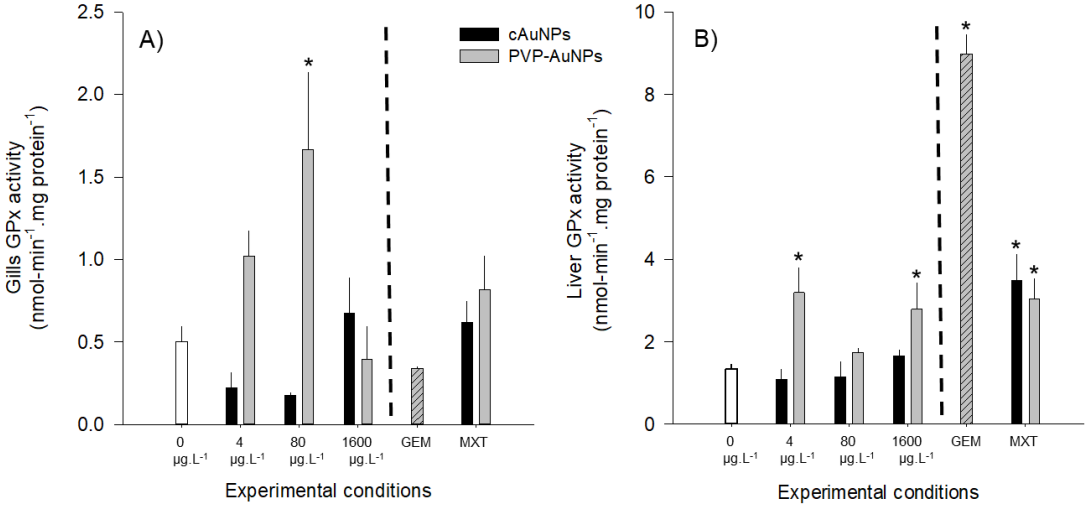

Figure 7. Gills **(A)** and liver **(B)** glutathione peroxidase (GPx) activity of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM.

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

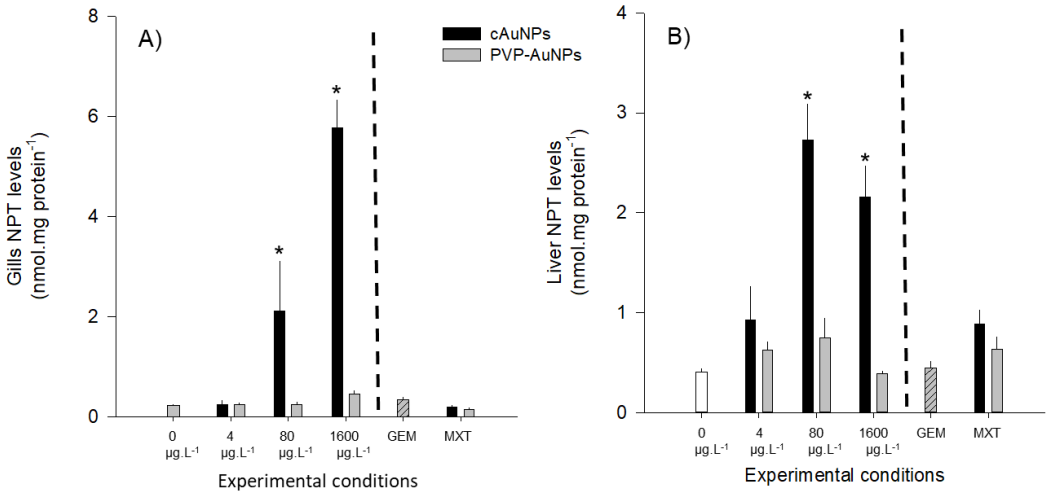

Figure 8. Gills **(A)** and liver **(B)** non-protein thiols (NPT) levels of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM.

In the activity of GST, an enzyme involved in the xenobiotics biotransformation, AuNPs exposures did not have significant effects on gills ($p>0.05$; ANOVA; Figure 9A), In liver, 1600 $\mu g.L^{-1}$ 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).

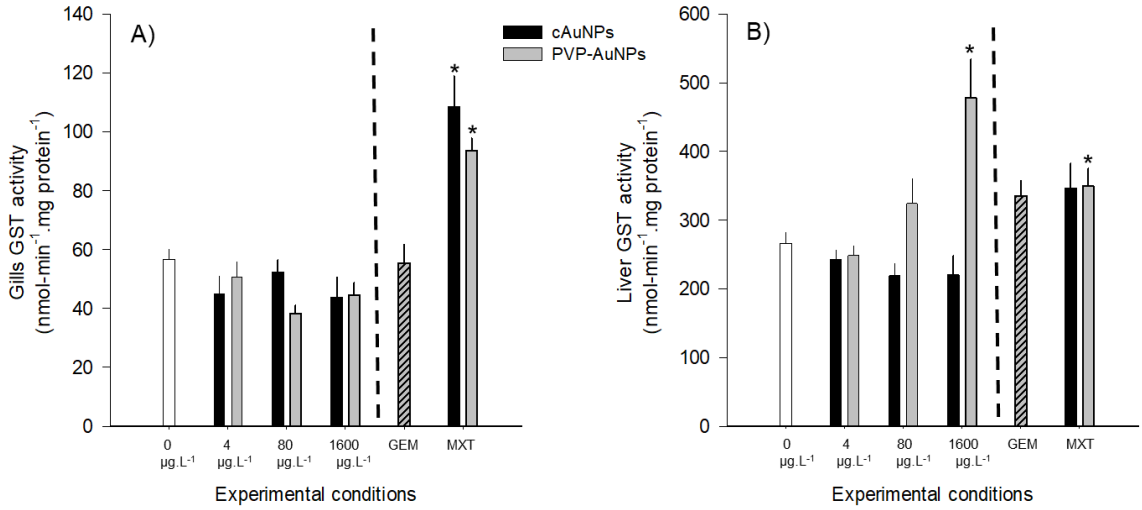

Figure 9. Gills **(A)** and liver **(B)** glutathione *S*-transferases (GST) activity of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM.

As shown in Figure 10A, oxidative damage (assessed as TBARS levels) was found in gills. PVP-AuNPs (4 and 80 µg.L^{-1}) 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) 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.

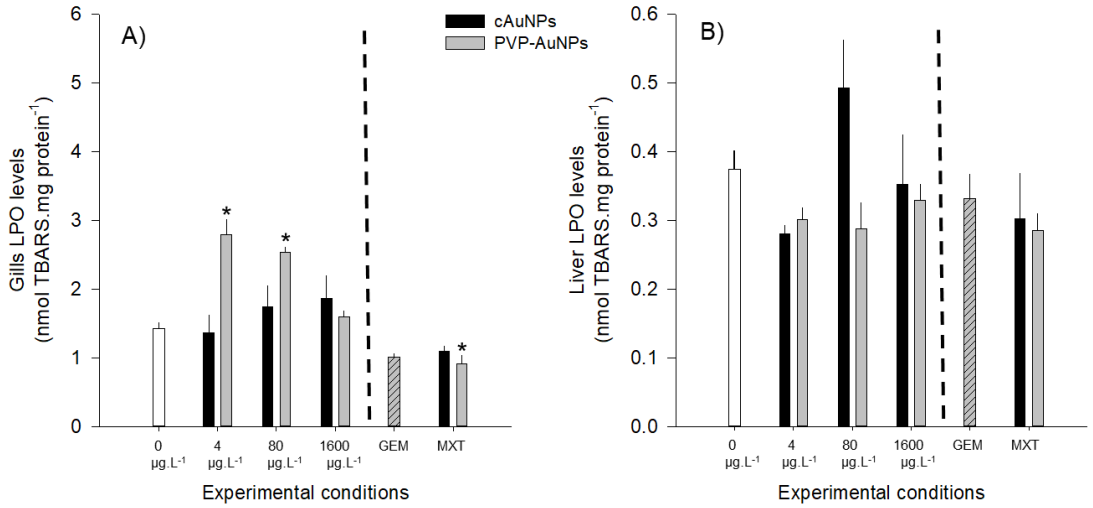

Figure 10. Gills **(A)** and liver **(B)** lipid peroxidation (LPO) levels of *Sparus aurata* after a 96-h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) alone or combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). MXT – AuNPs (cAuNPs or PVP-AuNPs) with GEM.

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 $\mu g.L^{-1}$ of 40 nm cAuNPs. In the present study, the exposure to 80 $\mu g.L^{-1}$ did not induce significant alterations in those enzymes' activity in gills and liver of *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* exposed for 24 h to 750 $\mu g.L^{-1}$ 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 $\mu g.L^{-1}$ for 24 h) increased GR and GPx activity in the digestive gland of the marine bivalve (*Ruditapes philippinarum*). However, in the gills, cAuNPs did not showed 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 reported that AuNPs may cause damage in aquatic organisms in the form of LPO (Tedesco et al. 2010b) as in the present study. 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 $\mu g.L^{-1}$ 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 already shown the ability 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 after the 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/agglomerated 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 µg.L^{-1} AuNPs and 150 µg.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 ($p<0.05$; 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 many 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 eliminate 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, $p<0.05$). ^{x}Significant differences between the combined exposure and the single exposure of GEM. (Tukey´s test, $p<0.05$)

Assessed Endpoints		**% of Effect Related to Control**				
		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

Table 3 (continuation). 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, $p<0.05$). **X**Significant differences between the combined exposure and the single exposure of GEM (Tukey´s test, $p<0.05$).

Assessed Endpoints	**% of Effect Related to Control**					
		cAuNPs	**PVP-AuNPs**	**GEM**	**cAuNPs + GEM**	**PVP-AuNPs + GEM**
Glutathione Reductase Activity	**Gills**	7	2	- 86 *	P: - 79 O: 34	P: - 84 O: 4
	Liver	- 22	17	- 67 *	P: - 89 O: - 156 *#X	P: - 50 O: - 131 *#X
Glutathione Peroxidase Activity	**Gills**	65	- 233 *	32	P: 97 O: - 23	P: - 201 O: - 63 #
	Liver	14	- 30	- 574 *	P: - 560 O: - 162 *#X	P: - 604 O: - 127 *X

Table 3 (continuation). 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, $p<0.05$). **X**Significant differences between the combined exposure and the single exposure of GEM (Tukey´s test, $p<0.05$).

Assessed Endpoints	% of Effect Related to Control					
		cAuNPs	**PVP-AuNPs**	**GEM**	**cAuNPs + GEM**	**PVP-AuNPs + GEM**
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 to *S. aurata*, for many endpoints, 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 effects of NPs and other emerging contaminants of concern. 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 (*Mytilus 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 by humans for the conditions 1600 $\mu g.L^{-1}$ of PVP-AuNPs and 80 $\mu g.L^{-1}$ 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).

Nominal Concentrations ($\mu g.L^{-1}$)	**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 lower and higher concentrations. The present findings support the idea that the bioaccumulation and effects of AuNPs are 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 characterisation 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.

Acknowledgments

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Chapter VII

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Genotoxicity of gold nanoparticles in the gilthead seabream (*Sparus aurata*) after single exposure and combined with the pharmaceutical gemfibrozil

A. Barreto[1*], L.G. Luis[1], E. Pinto[2], A. Almeida[2], P. Paíga[3], L.H.M.L.M. Santos[3,4], C. Delerue-Matos[3], T. Trindade[5], A.M.V.M. Soares[1], K. Hylland[6], S. Loureiro[1], M. Oliveira[1]

[1] Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] LAQV/REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

[3] LAQV/REQUIMTE, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida, 431, 4200-072 Porto, Portugal

[4] Catalan Institute for Water Research (ICRA), Carrer Emili Grahit 101, 17003 Girona, Spain

[5] Departamento de Química & CICECO - Aveiro Instituto de Materiais, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[6] Department of Biosciences, University of Oslo, PO Box 1066, N-0316 Oslo, Norway

*Corresponding author: E-mail: abarreto@ua.pt, Tel +351 234 370 350, Fax +351 234 372 587

Highlights

- Gold nanoparticles (AuNPs) induced DNA damage in *Sparus aurata* at 4 μg.L−1.;
- Erythrocytic nuclear abnormalities (ENAs) increased after exposure to 4 μg.L−1 AuNPs;
- The induction of ENAs decreased with citrate coated AuNPs concentration increase;
- AuNPs combined exposures with gemfibrozil (GEM) induced DNA damage and ENAs;
- AuNPs and GEM combined exposures produced an antagonistic response.

Abstract

Due to their diverse applications, gold nanoparticles (AuNPs) are expected to increase of in the environment, although few studies are available on their mode of action in aquatic organisms. The genotoxicity of AuNPs, alone or combined with the human pharmaceutical gemfibrozil (GEM), an environmental contaminant frequently detected in aquatic systems, including in marine ecosystems, was examined using gilthead seabream erythrocytes as a model system. Fish were exposed for 96 h to 4, 80 and 1600 $\mu g.L^{-1}$ of 40 nm AuNPs with two coatings – citrate or polyvinylpyrrolidone; GEM (150 $\mu g.L^{-1}$); and a combination of AuNPs and GEM (80 $\mu g.L^{-1}$ AuNPs + 150 $\mu g.L^{-1}$ GEM). AuNPs induced DNA damage and increased nuclear abnormalities levels, with coating showing an important role in the toxicity of AuNPs to fish. The combined exposures of AuNPs and GEM produced an antagonistic response, with observed toxic effects in the mixtures being lower than the predicted. The results raise concern about the safety of AuNPs and demonstrate interactions between them and other contaminants.

Keywords: metal nanoparticles; lipid regulator; erythrocytes; mixtures; comet assay; nuclear abnormalities

1. Introduction

The unique properties of nanoparticles (NPs) have led to an increased production and use in different applications (Fouad and Hafez 2017; Schmid 2010) in a wide range of areas, including electronics, medicine, pharmaceutics, industry and agriculture (Khan, Saeed, and Khan 2017). NPs are broadly divided into different categories based on their morphology, size and chemical properties (Khan, Saeed, and Khan 2017). Gold nanoparticles (AuNPs) are metal NPs widely used in biomedical applications, including diagnostics, therapy and prophylaxis (Khan, Vishakante, and Siddaramaiah 2013). The increased use of AuNPs leads to increased environmental inputs and estuarine/marine organisms potential exposure (Barreto et al. 2015). Information available on the current levels of AuNPs in the environment is limited to predicted concentrations arising from use in

consumer products (0.14 µg.L−1 in aquatic environments and 5.99 µg.kg−1 in soil) (García-Negrete et al. 2013; Tiede et al. 2009).

There is also a scarcity of publications on their mode of action in aquatic organisms, specifically the potential genotoxicity of AuNPs in marine organisms. AuNPs have been reported to induce genotoxicity (Balasubramanian et al. 2010; Cardoso et al. 2014; Chueh et al. 2014; de Alteriis et al. 2017; Dedeh et al. 2015; Di Bucchianico et al. 2015; Dominguez et al. 2015; Fraga et al. 2013; Geffroy et al. 2012; Guglielmo et al. 2012; Iswarya et al. 2016; Paino et al. 2012; Renault et al. 2008; Teles et al. 2017; Xia et al. 2017) – Supplementary Information (Table S1), mostly in *in vitro* experiments with cell lines of mammals (Chueh et al. 2014; Di Bucchianico et al. 2015; Fraga et al. 2013; Guglielmo et al. 2012; Li, Lo, et al. 2011; Paino et al. 2012; Schaeublin et al. 2011; Xia et al. 2017), dependent on their surface coating (Dominguez et al. 2015; Fraga et al. 2013; Guglielmo et al. 2012; Iswarya et al. 2016; Paino et al. 2012).

Therefore, one of the aims of the present research was to investigate the genotoxic effects of AuNPs with two different coatings (citrate and polyvinylpyrrolidone – PVP) to the top predator fish Sparus aurata, which is a commercially relevant fish in southern Europe.

Considering the co-occurrence of several contaminants in the environment, such as NPs and pharmaceuticals, organisms were exposed to AuNPs (citrate coated (cAuNPs) or PVP coated (PVP-AuNPs)) also in combination with the pharmaceutical gemfibrozil (GEM). The prescription rates of lipid regulators are continually increasing, and GEM is among the most widely used (Al-Habsi et al. 2016; Prindiville et al. 2011). In Europe, GEM has been found at concentrations up to 1.5 $\mu g.L^{-1}$ in surface waters (Fang et al. 2012). In marine ecosystems, GEM is also among the most frequently detected compounds, with concentrations between 1 and 758 $ng.L^{-1}$ in seawater (Gaw et al. 2014; Vidal-Dorsch et al. 2012). Despite its presence in aquatic ecosystems, there is still limited information concerning mechanisms of toxicity for GEM to aquatic organisms, particularly for marine fish (Teles et al. 2016).

To achieve the goals of the present study, the evaluated endpoints were the erythrocytic DNA strand breaks (molecular endpoint), assessed using the comet

assay, and the presence of erythrocytic nuclear abnormalities (ENAs) (cytogenetic endpoint), using the ENAs assay. Comparisons between the observed percentages of effect in the combined exposures and the predicted percentages of effect which were derived by the sum of single exposure effects were performed to understand if the employed mixture induced similar, lower or greater effects than the sum of both single exposure effects

2. Material and Methods

2.1. Fish maintenance

Juvenile gilthead seabream (*Sparus aurata*), total length 9 ± 0.5 cm, were purchased from a Spanish aquaculture facility and were acclimated in the laboratory for 4 weeks in aquaria containing aerated and filtered artificial seawater (ASW, salinity 35), controlled temperature (20ºC), pH of 7.9 and photoperiod 12:12. During this 4 weeks period, fish were fed daily with commercial fish food (Sorgal, Portugal) and the water renewed daily. All experimental procedures were carried out following the European and Portuguese legislation (authorization N421/2013 of Portuguese competent authority). Animal handling was performed by an accredited researcher. During the experimental assay, photoperiod, temperature and aeration conditions were similar to those used in the acclimation period.

2.2. Gold nanoparticles (AuNPs) – Synthesis and characterisation

AuNPs were prepared by sodium citrate reduction of gold (III) chloride trihydrate (Lekeufack et al. 2010). Part of the resulting cAuNPs were coated with PVP as described by Barreto et al. (2015). The citrate reduction method was chosen due to the non-toxicity of citrate, the use of water as solvent and the fact that cAuNPs have been frequently used in diverse areas (Hanžić et al. 2015; Li et al. 2011; Turkevich, Stevenson, and Hillier 1951). PVP was selected as a second coating agent because it is a water-soluble, nontoxic and biodegradable homopolymer (Min et al. 2009). After synthesis, the AuNPs stock suspensions and the AuNPs suspensions in the exposure media (ASW) and in ultrapure water were characterised at 0, 24 and 96 h. AuNPs combined with GEM were also

characterised in ASW and ultrapure water. AuNPs characterisation was performed by UV-Vis spectra, (Cintra 303, GBC Scientific), dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern), transmission electron microscopy (TEM; Hitachi, H9000 NAR), scanning electron microscopy (SEM; Hitachi, SU70) and zeta potential (ZP; Zetasizer Nano ZS, Malvern).

2.3. Experimental assay

The procedures generally followed the OECD guidelines for fish acute bioassays (OECD 1992). Briefly, 10 fish, per condition, were randomly distributed in the experimental aquaria and exposed for 96 h to the following 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). A negative control (ASW only) and a solvent control (0.003% dimethyl sulfoxide – DMSO) were included. Experimental suspensions of AuNPs were prepared by dilution of cAuNPs (97 $mg.L^{-1}$) and PVP-AuNPs (58 $mg.L^{-1}$) stock suspensions in ASW. A stock solution of GEM (50 $g.L^{-1}$) was prepared in DMSO. GEM (150 $\mu g.L^{-1}$) was prepared by the dilution of the stock solution in ASW. The AuNPs lowest concentration (4 $\mu g.L^{-1}$) was chosen because it is near to the predicted values found in the environment (García-Negrete et al. 2013; Tiede et al. 2009). The concentration range used was based on 20-fold increases. All tested concentrations of AuNPs have earlier been shown to induce behavioural changes, oxidative stress and/or damage (lipid peroxidation increased) in *S. aurata* (Barreto et al. 2019). The chosen concentration of GEM (150 $\mu g.L^{-1}$) is about 100 times higher than relevant environmentally concentrations of GEM (Fang et al. 2012) and has earlier been shown to induce genotoxicity, changes in behaviour, oxidative stress and oxidative damage in *S. aurata* (Barreto et al. 2017, 2018).

Approximately 80% of the experimental media was renewed daily after checking fish mortality and behaviour and measuring water quality (temperature, salinity, conductivity, pH and dissolved oxygen). No food was provided during the experimental period.

After 96 h exposure, animals were anesthetised with 100 $mg.L^{-1}$ tricaine methanesulfonate (MS-222) and a blood sample was collected from the posterior cardinal vein to be used in the comet assay and for the assessment of ENAs.

2.4. Quantification of gold and gemfibrozil (GEM) in the experimental media

Water samples were collected daily at time 0 and 24 h in each aquarium from single exposures (15 ml) and combined exposures (30 ml) for the quantification of gold and/or GEM in the experimental media.

The determination of gold was performed by ICP-MS (inductively coupled plasma mass spectrometry) according to the NIST NCL Method PCC-8 (NIST 2010). Samples were diluted with a 1.5% (v/v) HNO_3 and 4% (v/v) HCl solution and analysed by an iCAPTM Q ICP-MS (Thermo Fisher Scientific, Bremen, Germany). The equipment instrumental conditions were as follow: argon flow rate (14 $L.min^{-1}$); auxiliary argon flow rate (0.8 $L.min^{-1}$); nebulizer flow rate (1.03 $mL.min^{-1}$); RF power (1550 W) and dwell time (100 ms). The elemental isotope ^{197}Au was monitored for analytical determination; ^{159}Tb and ^{209}Bi were used as internal standards. The instrument was tuned daily for maximum signal sensitivity and signal stability.

Quantification of GEM was performed on a Nexera UHPLC (ultra-high performance liquid chromatography) system coupled to a triple-quadrupole mass spectrometer detector LCMS-8030 (Shimadzu Corporation, Kyoto, Japan). Prior to analysis, GEM was extracted from water samples using solid phase extraction (SPE). Standard solution of gemfibrozil-d6 was added to the extract as internal standard before UHPLC-MS/MS (ultra-high performance liquid chromatography tandem-mass spectrometry). Detailed information of the quantification of GEM can be found elsewhere (Barreto et al. 2017). Method detection limit (MDL) for GEM in water was 4.0 $ng.L^{-1}$.

2.5. Evaluation of molecular damage

For the comet assay, blood samples were diluted with saline phosphate buffer and immediately used. The alkaline comet assay was conducted according to the method of Singh et al. (1988) with some modifications (Barreto et al. 2017). The

different steps included in the comet assay are presented in the Supplementary Information (Figure S1). One slide per fish, anegative (blood from fish maintained in an aquarium with seawater only) and positive (blood from fish treated with 25 µM hydrogen peroxide (H_2O_2) for 10 min) controls were included in the electrophoresis. H_2O_2 was used as a model genotoxic agent since it produces both single-strand breaks and oxidative DNA damage (Termini 2000) and has been used routinely as a positive control in the comet assay (Barreto et al. 2017; Gielazyn et al. 2003; Singh et al. 1988).

Slides were stained with ethidium bromide (20 $\mu L.mL^{-1}$) and, to avoid bias, they were randomly analysed, To avoid bias, slides were randomly analysed, counting 100 randomly selected cells from each sample. Cells were scored visually, according to tail length, into five classes (Collins 2004):

Class 0 – undamaged, without a tail;

Class 1 – with a tail shorter than the diameter of the nucleus;

Class 2 – with a tail length 1-2 times the diameter of the nucleus;

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

Class 4 – comets with no nucleus.

A damage index (DI), in arbitrary units, was assigned to each replicate (for 100 cells) and consequently for each treatment, using the formula:

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

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

2.6. Evaluation of erythrocytic nuclear abnormalities (ENAs)

Blood smears were prepared for ENAs assessment. The ENAs assay was carried out in mature peripheral erythrocytes, as previously described (Barreto et al. 2017; Pacheco and Santos 1996). The ENAs were randomly scored under a light microscope in 1000 intact erythrocytes per fish. Nuclear lesions were scored as previously reported (Barreto et al. 2017): micronuclei, lobed, segmented, kidney-

shaped and vacuolated nuclei. Results were expressed as the frequency mean value (‰) of total ENAs, using the formula:

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

2.7. Data analysis

Data were first tested for normality (Shapiro-Wilk test) and homogeneity of variance (Levene's test), using the Sigma Plot 12.0 software package. Whenever data failed the normality test, it was log 10 transformed to achieve normalization. Differences between controls (negative and solvent) were carried out using a Student t-test. Differences between all the treatments were compared using a two-way analysis of variance (ANOVA), using concentration and coating as factors, followed by a Dunnett´s test, to detect significant differences between the control and treatments. Data from damage classes and types of ENAs were individually compared with control group using a one-way analysis of variance (ANOVA), followed by Dunnett's test, whenever applicable. One-way ANOVA, followed by Tukey´s test, whenever applicable, was used to compare differences between AuNPs single treatments. 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. In addition, differences between GEM, 80 µg.L^{-1} AuNPs (citrate and PVP coating), the mixture and control were tested using a one-way analysis of variance (ANOVA), followed by Dunnett's test.

3. Results

3.1. Gold nanoparticles (AuNPs) – Characterisation and behaviour

The characteristics of AuNPs used in the present study are shown in Table 1. Microscopy analysis confirmed that almost all AuNPs dispersed in ultrapure water

had the same size and presented an approximately spherical shape. In ASW, the highest tested concentration of cAuNPs (1600 µg.L^{-1}) displayed an immediate change the colour from red to light blue, typical from AuNPs agglomeration/aggregation processes. The hydrodynamic size of cAuNPs increased to about 340 ± 42 nm and the characteristic surface plasmon resonance (SPR) peak was not detected (Table 1). Different peaks corresponding to different charges were found in the ZP analysis. In the aquaria containing 1600 µg.L^{-1} of cAuNPs a dark layer became visible within 24 h, corresponding to AuNPs aggregates/agglomerates sedimentation. PVP-AuNPs (1600 µg.L^{-1}) did not alter their colour in ASW, showing the UV-Vis spectra, size and ZP similar to the PVP-AuNPs in ultrapure water after 96 h (Table 1). At 4 and 80 µg.L^{-1}, it was not possible to characterise the AuNPs because of the detection limits of the techniques used.

Table 1. Characteristics of gold nanoparticles (AuNPs) in ultrapure water and artificial seawater after 96 h alone and with gemfibrozil (GEM). cAuNPs – Citrate coated gold nanoparticles; PVP-AuNPs – Polyvinylpyrrolidone coated gold nanoparticles; PdI – Polydispersity Index; SPR – Surface Plasmon Resonance; ZP – Zeta Potential; N. D. – Not detected.

	Size (nm)	**PdI**	**SPR (nm)**	**ZP (mV)**
Ultrapure water				
cAuNPs	35.0 ± 0.2	0.3	534.0	-43.8 ± 0.7
PVP-AuNPs	50.0 ± 0.8	0.3	535.0	-17.0 ± 0.6
cAuNPs + GEM	37.3 ± 0.5	0.3	533.6	-43.2 ± 1.1
PVP-AuNPs + GEM	52.3 ± 1.4	0.2	535.2	-17.3 ± 0.2
Artificial seawater (salinity 35)				
cAuNPs	340.0 ± 42.0	0.9	N. D.	N. D.
PVP-AuNPs	53.5 ± 1.4	0.2	535.4	-17.2 ± 0.2
cAuNPs + GEM	332.1 ± 34.8	0.9	N. D.	N. D.
PVP-AuNPs + GEM	51.6 ± 1.2	0.3	535.1	-17.1 ± 0.4

The study of the interaction of GEM and AuNPs was not possible to be carried out at the concentrations of 80 and 150 $\mu g.L^{-1}$ of AuNPs and GEM, respectively. The UV-Vis spectra analysis of these two compounds' mixture in ultrapure water, within the same ratio but increasing their concentration ten times (800 and 1500 $\mu g.L^{-1}$ of AuNPs and GEM, respectively), revealed that the characteristic SPR peak of AuNPs was maintained (Table 1) and the peak correspondent to GEM was detected in the expected wavelength (around 276 nm). In addition, AuNPs sizes measured by DLS were similar when they were mixed with GEM, as shown in Table 1. In ASW, cAuNPs also aggregated/agglomerated in the presence of GEM, presenting similar behaviour and characteristics as when they were separately in ASW (Table 1). PVP-AuNPs combined with GEM remained stable in ASW, as when they were in single exposure (Table 1).

3.2. Gold and gemfibrozil (GEM) quantification in the experimental media

The results of gold and GEM concentrations obtained by chemical analyses are presented in the Supplementary Information (Table S2). Gold quantified in the experimental media (ASW) was generally lower than the nominal concentrations at 0 h, except for PVP-AuNPs at 4 $\mu g.L^{-1}$. The difference between the nominal and measured concentrations was more evident in the case of cAuNPs, both in single and combined exposures with GEM. For the nominal concentration of 4 $\mu g.L^{-1}$ cAuNPs, the measured concentration of gold was 32% lower than the expected. For the 80 $\mu g.L^{-1}$, the detected concentrations of cAuNPs and PVP-AuNPs in ASW were 62 and 15% lower than the nominal concentrations, respectively. At 1600 $\mu g.L^{-1}$, the concentration of gold was 92 and 9% lower than the expected for cAuNPs and PVP-AuNPs, respectively. The concentration of GEM, at 0 h, was around 60% higher than the nominal concentration (150 $\mu g.L^{-1}$), 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 expected for cAuNPs and PVP-AuNPs, respectively.

After 24 h of exposure, comparing with the gold quantified at 0 h, the concentration of cAuNPs in suspension decreased more than the concentration of PVP-AuNPs. In the nominal concentration 4 $\mu g.L^{-1}$, this decrease was 51 and 19%

for cAuNPs and PVP-AuNPs, respectively. In the nominal concentration 80 $\mu g.L^{-1}$, 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 $\mu g.L^{-1}$, a decrease of gold in suspension 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.

3.3. Evaluation of DNA damage

No significant differences between the negative (ASW only) and solvent (0.003% DMSO) controls were found on the comet and ENAs assays (t-test, $p>0.05$). The negative control displayed a damage index around 27, corresponding to 6.8% DNA damage. The solvent control displayed a damage index around 33, corresponding to 8.3% DNA damage, while the positive control displayed a damage index around 240. AuNPs displayed a significant increase in the genotoxic effects (Dunnett's test, $p<0.05$), assessed by DNA strand breakage, already at 4 $\mu g.L^{-1}$ and at all the concentrations used (Figure 1), independently of the coating. With PVP-AuNPs concentration increase, the DNA strand breakage increased, being the DNA damage index significantly higher at 1600 $\mu g.L^{-1}$, when comparing with exposure to 4 $\mu g.L^{-1}$ (Figure 1).

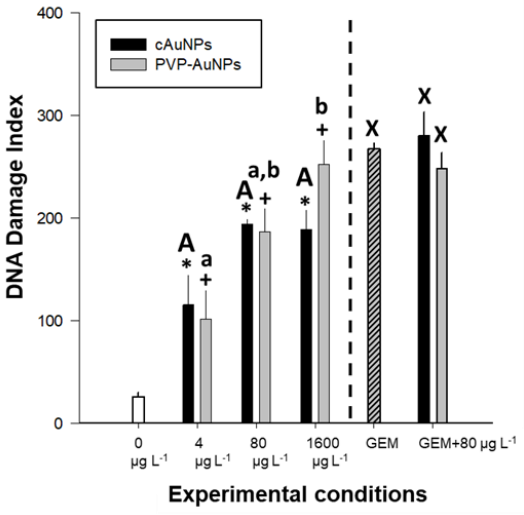

Figure 1. DNA damage index (arbitrary units) of peripheral blood cells from *Sparus aurata* exposed for 96 h to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) single exposure and combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$, citrate coating). $^{+}$Significant differences to control (Dunnett´s test, $p<0.05$, polyvinylpyrrolidone coating). ^{X}Significant differences to control (Dunnett´s test, $p<0.05$, GEM and GEM+80 µg.L^{-1} of cAuNPs or PVP-AuNPs). Different letters correspond to significant differences between the treatments of each type of AuNPs, capital letters to cAuNPs and small letters to PVP-AuNPs (Tukey´s test, $p<0.05$).

A DNA damage index around 267 was detected in organisms exposed to 1600 µg.L^{-1} of PVP-AuNPs, being the maximum DNA damage value detected in the AuNPs single exposures (Table 2).

Table 2. Percentage of DNA damage classes, analysed by the comet assay, of peripheral blood cells from *Sparus aurata* exposed for 96 h to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) single exposure and combined with gemfibrozil (GEM). *Significant differences to control (Dunnett's test, $p<0.05$); data are expressed as mean ± standard error. A. U. – Arbitrary units

Treatment group	DNA damage classes (%)					DNA damage index (A. U.)
	0	1	2	3	4	
Control	77.9±5.8	21.4±5.5	2.6±1.3	0.2±0.2	0	27.2±7.9
4 µg.L^{-1} cAuNPs	24.6±11.4*	44.2±7.0*	25.0±12.6	3.8±2.6	2.4±1.1	115.2±29.0*
80 µg.L^{-1} cAuNPs	9.2±2.7*	12.4±3.5	54.2±7.6*	22.0±5.7	1.8±1.1	194.0±5.3*
1600 µg.L^{-1} cAuNPs	7.0±1.6*	23.0±7.0	48.3±6.3*	18.0±11.0	3.8±2.2	188.5±18.5*
4 µg.L^{-1} PVP-AuNPs	34.0±8.0*	42.3±4.2	15.3±8.4	5.3±2.6	3.3±1.5	101.5±24.7*
80 µg.L^{-1} PVP-AuNPs	9.0±4.4*	23.0±1.3	39.0±7.5	18.5±9.2	6.0±0.6	180.5±37.5*
1600 µg.L^{-1} PVP-AuNPs	7.0±0.9*	11.7±9.0	27.7±0.9	29.7±4.9	24.0±2.8*	267.4±23.0*
150 µg.L^{-1} GEM	0.5±0.3*	3.3±1.5	46.3±6.2*	41.0±3.7*	11.5±2.7	264.8±6.1*
80 µg.L^{-1} cAuNPs + 150 µg.L^{-1} GEM	0*	1.2±0.8	27.6±6.9	48.4±11.7*	11.0±7.1	280.3±22.7*
80 µg.L^{-1} PVP-AuNPs + 150 µg.L^{-1} GEM	0.6±0.6*	5.8±2.6	45.0±7.8*	38.6±8.9*	9.0±4.6	247.6±18.2*

The most abundant class for the negative control group was 0. Class 1 was the most detected in the exposures to 4 µg.L-1 of AuNPs and class 2 in the exposures to 80 and 1600 µg.L-1 of AuNPs. PVP-AuNPs, at 1600 µg.L-1, displayed a DNA damage grouped in class 2, 3 and 4 (Table 2). GEM also displayed a significant increase in DNA damage (Dunnett's test, $p<0.05$; Figure 1), being 2 and 3 the most predominant classes detected (Table 2).

Despite the observed percentage of effect was lower than the predicted – Table 4, DNA damage significantly increased in the combined exposures compared with the control group (Dunnett's test, $p<0.05$; Figure 1). In the combined exposures,

the most detected DNA damage classes were 2 and 3 (Table 2). A DNA damage index around 280 was detected in organisms exposed to the combination of 80 µg.L^{-1} of cAuNPs and GEM, being the maximum DNA damage value detected in both single and combined exposures (Table 2).

3.4. Evaluation of erythrocytic nuclear abnormalities (ENAs) damage

The 96-h exposure of *S. aurata* to all the tested concentrations of AuNPs led to significantly higher ENAs frequency (Dunnett's test, $p<0.05$; Figure 2), independently of the coating. With cAuNPs concentration increase, the ENAs frequency decreased. The effects of cAuNPs were higher in the lowest tested concentration (4 µg.L^{-1}) than in the highest concentration (1600 µg.L^{-1}) (Tukey's test, $p<0.05$; Figure 2). At 1600 µg.L^{-1}, PVP-AuNPs induced significantly more ENAs than cAuNPs (Dunnett's test, $p<0.05$; Figure 2).

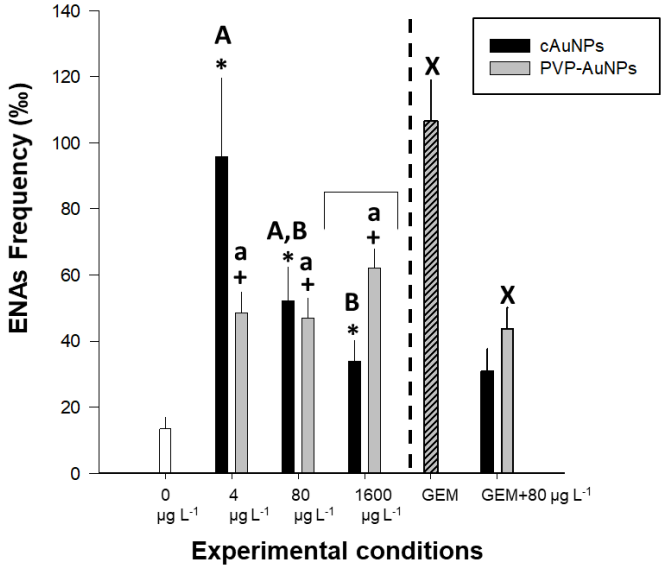

Figure 2. Erythrocytic nuclear abnormalities (ENAs) frequency (‰) in *Sparus aurata* exposed for 96 h to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) single exposure and combined with gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$, citrate coating). $^{+}$Significant differences to control (Dunnett´s test, $p<0.05$, polyvinylpyrrolidone coating). Significant differences between cAuNPs and PVP-AuNPs within the same

concentration (Dunnett´s test, $p<0.05$). ^{X}Significant differences to control (Dunnett´s test, $p<0.05$, GEM and GEM+80 $\mu g.L^{-1}$ of cAuNPs or PVP-AuNPs). Different letters correspond to significant differences between the treatments of each type of AuNPs, capital letters to cAuNPs and small letters to PVP-AuNPs (Tukey´s test, $p<0.05$).

As shown in Table 3, the kidney-shaped nuclei was the abnormality most detected in the single exposures of AuNPs, being statistically significant at 4 $\mu g.L^{-1}$ of cAuNPs and PVP-AuNPs and 80 $\mu g.L^{-1}$ of cAuNPs (Dunnett's test, $p<0.05$). The second most detected abnormality was lobed nuclei, statistically significant at 4 $\mu g.L^{-1}$ for cAuNPs, 80 and 1600 $\mu g.L^{-1}$ PVP-AuNPs (Dunnett's test, $p<0.05$). Segmented, vacuolated and micronuclei were the abnormalities least detected in the exposures to AuNPs (Table 3).

Table 3. Erythrocytic nuclear abnormalities (ENAs) frequency (‰) detected in *Sparus aurata* exposed for 96 h to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) single exposure and combined with gemfibrozil (GEM). *Statistically 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	11.3±3.3	1.1±0.4	0.6±2.2	0.2±0.4	0
4 µg.L^{-1} cAuNPs	45.2±8.8*****	2.5±1.0	33.5±15.8*****	0.8±0.4	0.3±0.2
80 µg.L^{-1} cAuNPs	34.9±9.4*****	1.0±0.2	9.3±2.9	0.1±0.1	0.3±0.2
1600 µg.L^{-1} cAuNPs	23.7±3.6	0.6±0.4	9.4±3.0	0	0.3±0.2
4 µg.L^{-1} PVP-AuNPs	37.6±5.8*****	1.9±0.7	7.4±1.7	1.3±0.8	0.4±0.2
80 µg.L^{-1} PVP-AuNPs	22.7±3.0	0.8±0.3	22.8±4.5*****	0.7±0.4	0
1600 µg.L^{-1} PVP-AuNPs	23.5±3.0	0.2±0.2	37.8±3.1*****	0.7±0.3	0
150 µg.L^{-1} GEM	62.5±4.7*****	24.5±3.3*****	7.5±1.4	9.7±0.6*****	2.5±1.1*****
80 µg.L^{-1} cAuNPs + 150 µg.L^{-1} GEM	23.7±4.5	0.7±0.7	4.7±1.5	1.6±0.4	0.3±0.2
80 µg.L^{-1} PVP-AuNPs + 150 µg.L^{-1} GEM	32.0±4.8	0	5.9±1.0	5.7±3.4	0

As presented in Figure 2, GEM exposure significantly increased ENAs frequency (Dunnett's test, $p<0.05$). The abnormality most detected was kidney-shaped nuclei, followed by segmented and vacuolated nuclei (Dunnett's test, $p<0.05$). The abnormalities less detected were lobed nuclei and micronuclei. The latter was however significantly higher than the control (Dunnett's test, $p<0.05$; Table 3). Some representative images of the different ENAs detected are shown in Figure 3.

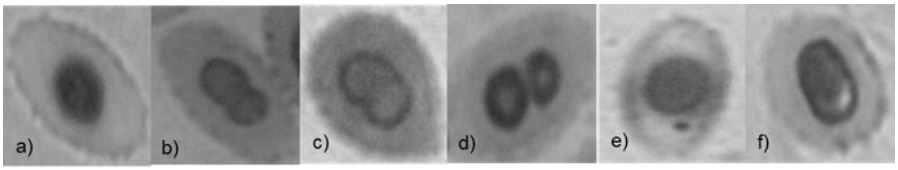

Figure 3. Mature erythrocytes of juvenile Sparus aurata with nuclear normal shape (a) and nuclear abnormalities: lobed nuclei (b), kidney-shaped nuclei (c), segmented nuclei (d), micronuclei (e) and vacuolated nuclei (f). Giemsa stain, 1000x.

In the exposures AuNPs combined with GEM, the ENAs frequency was similar to the control in cAuNPs+GEM (ANOVA, $p>0.05$; Figure 2) whereas after PVP-AuNPs+GEM exposure the frequency of ENAs increased (Dunnett's test, $p<0.05$; Figure 2). The observed percentage of effect in the mixtures was lower than the predicted – Table 4.

Table 4. The relative 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, $p<0.05$).

Assessed endpoints	**% of effect related to control**				
	cAuNPs	**PVP-AuNPs**	**GEM**	**cAuNPs + GEM**	**PVP-AuNPs + GEM**
DNA damage index	653*****	625*****	938*****	**P**: 1591 **O**: 988*****	**P**: 1563 **O**: 862*
Erythrocytic nuclear abnormalities frequency	288*****	250*****	694*****	**P**: 982 **O**: 131	**P**: 944 **O**: 224

4. Discussion

The behaviour of both AuNPs in ASW supported the data obtained in other publications with the same NPs (Barreto et al. 2015). In ASW, a high ionic strength medium, 1600 $\mu g.L^{-1}$ cAuNPs tended to aggregate or agglomerate as a consequence of an imbalance between repulsive and attractive forces (Geffroy et al. 2012; Tedesco et al. 2008; Yoo-Iam, Chaichana, and Satapanajaru 2014). On contrary, for PVP-AuNPs some stability in ASW was attained, as previous reported (Barreto et al. 2015). At 4 and 80 $\mu g.L^{-1}$, the media did not present the typical red colour of AuNPs suspensions because concentrations were low with no detectable alterations in NPs colour typical of agglomeration/aggregation processes (Barreto et al. 2015). Furthermore, the UV-Vis spectrophotometry, DLS and microscopy did not allow the study of AuNPs behaviour at these concentrations because of the weakness of the obtained signal. Some authors have suggested that 20 nm cAuNPs can be considered as resistant to salt-induced aggregation at low $\mu g.L^{-1}$ concentrations (García-Negrete et al. 2013). The lack of alterations in terms of UV-Vis spectra, size and ZP of AuNPs when they were alone or combined with GEM, suggested that GEM and AuNPs did not have a direct chemical association, in terms of exposure. However, a possible interaction between them may occur inside the organisms, which may be related to their modes of action. The chemical analysis revealed differences between the nominal and measured concentrations, mainly evident in the case of the exposure to cAuNPs. This discrepancy may be explained by aggregation/agglomeration and consequent sedimentation of the aggregates/agglomerates of cAuNPs. It may also explain that, comparing with 0 h, the concentration of cAuNPs in suspension, after 24 h, decreased more than the concentration of PVP-AuNPs.

Regardless of the coating, AuNPs significantly induced DNA damage and increased ENAs frequencies in *Sparus aurata* at all tested concentrations. The induction of ENAs decreased with cAuNPs concentration increase. The same response trend was not found for PVP-AuNPs. At 1600 $\mu g.L^{-1}$, PVP-AuNPs induced significantly more ENAs in *S. aurata* than cAuNPs. These differences in toxicity may be associated with the behaviour of AuNPs in the tested media, PVP-AuNPs (1600 $\mu g.L^{-1}$) remained stable in the nano size in ASW, whereas cAuNPs

immediately aggregated/agglomerated, increasing their size. Thus, these dissimilar AuNPs behaviours and characteristics (i.e. size and charge) may alter their bioavailability, uptake and consequently the toxic effects to cells and organisms (Alkilany and Murphy 2010; Barreto et al. 2015; Di Bucchianico et al. 2015; Liu et al. 2014). Rothen-Rutishauser et al. (2006) reported that aggregates larger than 200 nm are able to attach to the human red blood cell membranes, whereas smaller aggregates and single NPs are found inside the cells (Rothen-Rutishauser et al. 2006). Previous genotoxicity studies also reported different effects of AuNPs with different coatings (Fraga et al. 2013; Iswarya et al. 2016; Paino et al. 2012). In a mice model, 96 h exposure to 65 nm PVP-AuNPs induced more effects in the DNA of liver cells (assessed as DNA strand breaks) than 29 nm cAuNPs (Iswarya et al. 2016). Most of the available nanotoxicological studies focus on the toxicity induced by high concentrations of NPs and the effects that arise at lower concentrations have largely been neglected (Fraga et al. 2013). The genotoxic pattern observed for cAuNPs, where low concentrations induced more effects than high concentrations, as previously described (Fraga et al. 2013), may be due to the lower aggregation/agglomeration processes, with higher ability to reach and react with cells inducing DNA damage. As already reported, aggregation/agglomeration is expected to increase with the increase in the number of particles per volume (Barreto et al. 2015). Protective mechanisms activated only in response to high levels of AuNPs may, however, also be responsible for this inverse correlation (Fraga et al. 2013). Nonetheless, the present findings highlight the importance of studying the effects of NPs at low concentrations.

Considering the surface charge, previous studies reported that positively charged AuNPs displayed more effects than negatively charged AuNPs (Broda et al. 2016; Dominguez et al. 2015; Goodman et al. 2004) as a consequence of an electrostatic effect between positively charged AuNPs and the negatively charged cellular membranes and biological molecules such as DNA (Dominguez et al. 2015). Looking at different charges, an earlier gene expression study showed that 1.5 nm AuNPs positively, neutral and negatively charged caused irreparable DNA damage to the human keratinocyte cell line (HaCaT) after 24 h exposure, but affected genes depending on particle charge (Schaeublin et al. 2011). A

microscopic evaluation has furthermore demonstrated the uptake of neutral and negative AuNPs by red blood cells while positively charged AuNPs were attached to the cell surface (Rothen-Rutishauser et al. 2006). cAuNPs used in the present study presented a strong negative ZP (-44 mV) and PVP-AuNPs exhibited a less negative ZP (-17 mV).

Although the available data regarding the genotoxicity of AuNPs is somehow conflicting (Di Bucchianico et al. 2015; Paino et al. 2012), most of the recent studies have reported the potential of AuNPs to be genotoxic (de Alteriis et al. 2017; Dedeh et al. 2015; Di Bucchianico et al. 2015; Dominguez et al. 2015; Xia et al. 2017). The controversy may be explained by a variety of parameters, such as the use of different cells/organisms in the assays, time of exposure, concentrations, surface charge, coating and size of NPs. The genotoxic effects of AuNPs detected in the present study, i.e. erythrocyte DNA strand breaks and nuclear abnormalities, may be caused directly following the entry of NPs into cells or even the nuclei, then binding to DNA; or indirectly, through oxidative stress, which may consequently induce oxidative damage to DNA (Auffan et al. 2009; Cardoso et al. 2014). The production of reactive oxygen species (ROS) following AuNPs exposure has been comprehensively demonstrated in many studies involving aquatic organisms (Farkas et al. 2010; Pan et al. 2012; Tedesco et al. 2008, 2010). ROS has often been described as the major role in terms of oxidative DNA damage by NPs, such as breaks, adducts or mismatches (Catalán et al. 2014; Karlsson 2010). The DNA lesions caused by NPs may still be repaired by cellular DNA repair system (Catalán et al. 2014). Chueh et al. (2014) reported that nanorods AuNPs (72, 180, 360 and 720 $ng.mL^{-1}$ exposure during 24 h in MRC5, human normal lung fibroblast) increased the expression of genes involved in base-excision repair and homologous recombination pathways, indicating that they may cause base damage and double strand breaks (Chueh et al. 2014). A reduction of the 5 nm AuNPs genotoxic effects in yeast *Saccharomyces cerevisiae* cells after 48 and 72 h exposure comparing with 24 h was observed, presumably due to the activation of DNA repair mechanisms (de Alteriis et al. 2017). Therefore, different events may occur at different times of exposure. If the lesions are misrepaired or if unrepaired lesions cause replication errors, gene mutations and chromosomal

mutations may be formed (Catalán et al. 2014) and may display a cascade of biological consequences at the cellular, organ, organism, and finally population and community levels (Lee and Steinert 2003; Theodorakis 2001; Wirgin and Waldman 1998).

Micronuclei, a biomarker of chromosomal damage and/or loss, which represent a permanent damage that can only be fixed through cell removal (Oliveira et al. 2010), were not significantly detected in the present study. Although the mechanisms responsible for the formation of ENAs are not yet completely understood, some nuclear abnormalities (such as lobed and segmented nuclei) may be interpreted as nuclear lesions analogous to micronuclei that may be induced by genotoxicants even if micronuclei are not present (Ayllon and Garcia-Vazquez 2000; Barreto et al. 2017; Guilherme et al. 2008; Harabawy and Mosleh 2014; Stankevičiūtė et al. 2016). The second most nuclear abnormality detected in the present study – lobed nuclei – has earlier been described as a biomarker of reduced repair capacity, misrepair leading to chromosome rearrangement, and a measure of excess DNA that is being extruded from the nucleus (Di Bucchianico et al. 2015). The most detected abnormality, kidney-shaped nuclei, is considered by some authors to have a cytological cause (Bonassi et al. 2006), whereas by other authors it is described as having a genotoxic origin by other authors (Barreto et al. 2017; Carrola et al. 2014; Harabawy and Mosleh 2014). Xia et al. (2017) reported no increase in the frequency of micronuclei in the bone marrow erythrocytes of mice after 4 d exposure of 0.02 to 50 $mg.kg^{-1}$ AuNPs (5, 20 and 50 nm). When the exposure period was extended to 14 d, 5 nm AuNPs did however cause significant clastogenic damage, with a dose-dependent increase of micronuclei frequencies (Xia et al. 2017). Therefore, this suggests that the genotoxic effects of AuNPs may be dependent on the exposure duration. The lack of results in terms of micronuclei induction found in the present study may be associated with the short time of exposure (96 h). On the other hand, the results may also suggest the presence of an effective protection mechanisms and/or increased splenic erythrocyte catabolism and a reduced rate of erythropoiesis (Oliveira et al. 2010; Pacheco et al. 2005).

The combined exposures of AuNPs and GEM produced an antagonistic response, as the observed effects were lower than the predicted based on both single exposure effects. These findings are highly relevant because there is a variety of contaminants in the environment and there is a lack of studies about the combined effects of NPs and other emerging contaminants (Luis et al. 2016). In addition, having responses at different pathway levels can insight on the modes of action regarding chemicals and mixtures.

The results of the present study raise concerns about potential genotoxic effects of AuNPs. Therefore, additional studies are encouraged with AuNPs (alone or combined exposures) at low concentrations, to increase the knowledge about their genotoxic effects to aquatic organisms and the mechanisms involved, assessing the damage at different times of exposure and for longer exposure periods.

5. Conclusions

The present study provides evidence about gold nanoparticles (AuNPs) genotoxicity at molecular and cellular levels, in *Sparus aurata*. Coatings of AuNPs (citrate and polyvinylpyrrolidone) induced different toxicity profiles in *Sparus aurata*. Even at 4 $\mu g.L^{-1}$, AuNPs induced DNA damage (erythrocyte DNA strand breaks) and increased the frequency of erythrocytic nuclear abnormalities. In terms of cytogenetic damage, the effects of citrate coated gold nanoparticles (cAuNPs) were higher in the lowest tested concentration of AuNPs (4 $\mu g.L^{-1}$) than in the highest concentration (1600 $\mu g.L^{-1}$), possibly related to their aggregation/agglomeration behaviour depend on the concentration. The combined exposures of AuNPs and gemfibrozil produced an antagonistic response. Both techniques (comet and erythrocytic nuclear abnormalities (ENAs) assay) were found to be sensitive tools to detect AuNPs genotoxicity in *Sparus aurata*. The obtained data emphasize the importance of study the effects of the contaminants when they are present as mixtures, as in the environment a diversity of pharmaceuticals and nanoparticles may share or not toxicological properties. These results must be considered in aquaculture, biomedical or other areas and environmental risk assessment.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Supplementary Information

Table S1. Summary of studies assessing gold nanoparticles (AuNPs) genotoxicity. N. P. – Information not provided; Ref. – Reference; HepG2 – Human liver HepG2 cells; CHL – Chinese hamster lung fibroblast cells; PBMC – Peripheral blood mononuclear cells; A549 – Human lung adenocarcinoma epithelial A549 cells; MRC-5 – Lung fibroblast cells; 3T3 – BALB/c 3T3 fibroblast cells; HaCaT – Human keratinocyte cell line; MN – Micronuclei; RAPD-PCR – Random amplified polymorphic DNA-PCR analysis; LC-MS/MS – Liquid chromatography tandem-mass spectrometry; PVP – Polyvinylpyrrolidone; CTAB – Cetyltrimethylammonium bromide; PAH – Polyallylamine hydrochloride; MPA – Mercaptopropionic; MUA – 11-mercaptoundecanoic acid; PAMAM – Polyamidoamine dendrimers; TMAT – Trimethylammoniumethanethiol; MES – Mercaptoethanesulfonate; MEEE – Mercaptoethoxyethoxyethanol.

Cells/Organism	Test Type	Period	Endpoints	Methods	Coating	Sizes(nm)/Shapes	Concentration/Dose	Genotoxicity?	Ref.
Sparus aurata	*In vivo*	96 h	Gene expression	Real time PCR	Citrate, PVP	Spherical 40	4, 80 and 1600 $\mu g.L^{-1}$	Yes	Teles et al. (2017)
HepG2, CHL *Mus musculus*	*In vitro* *In vivo*	4h, 24 h, 4d, 14 d	DNA damage MN	Comet assay MN assay	N. P.	Spherical 5, 20 and 50	1.67, 5, and 12.5 $\mu g.mL^{-1}$ 0.02, 0.1, 0.17, 0.5 and 50 $mg.kg^{-1}$ body weight	Yes	Xia et al. (2017)
Saccharomyces cerevisiae	*In vitro*	24, 48and 72 h	DNA damage	Comet assay	N. P.	N. P. 5	N. P.	Yes	de Alteriis et al. (2017)
A549	*In vitro*	48 h	Nuclear abnormalities	Cytokinesis-block MN cytome assay	N. P.	Spherical 2 and 7	0.0046, 0.046, 0.46, 2.3 and 4.6 $\mu g.mL^{-1}$	Yes	Di Bucchianico et al. (2015)
Danio rerio	*In vivo*	20 d	Gene expression	RAPD-PCR	Citrate	Spherical 14	0.25 and 0.8 $\mu g.L^{-1}$	Yes	Dedeh et al. (2015)
Mus musculus	*In vivo*	96 h	DNA damage	Comet assay	Citrate, PVP	Spherical 29, 43 and 65	0.01, 0.05 and 0.1 $mg.kg^{-1}$ body weight	Yes	Iswarya et a. (2015)
Daphnia magna	*In vivo*	24 h	Gene expression	Real time PCR	Citrate, CTAB, PAH and MPA	Spherical 4 Nanorod 50 x 14	1, 10 and 50 $\mu g.mL^{-1}$	Yes	Dominguez et al. (2015)
MRC-5	*In vitro*	24 h	Gene expression	Microarray analysis	N. P.	Nanorod 40 x 10	72, 180, 360 and 720 ng. mL^{-1}	Yes	Chueh et al. (2014)
Rattus norvegicus	*In vivo*	24 h,28 d	DNA damage	Comet assay	Citrate	Spherical 10 and 30	70 $mg.L^{-1}$	Yes	Cardoso et al. (2014)

Table S1 (continuation). Summary of studies assessing gold nanoparticles (AuNPs) genotoxicity. N. P. – Information not provided; Ref. – Reference; HepG2 – Human liver HepG2 cells; CHL – Chinese hamster lung fibroblast cells; PBMC – Peripheral blood mononuclear cells; A549 – Human lung adenocarcinoma epithelial A549 cells; MRC-5 – Lung fibroblast cells; 3T3 – BALB/c 3T3 fibroblast cells; HaCaT – Human keratinocyte cell line; MN – Micronuclei; RAPD-PCR – Random amplified polymorphic DNA-PCR analysis; LC-MS/MS – Liquid chromatography tandem-mass spectrometry; PVP – Polyvinylpyrrolidone; CTAB – Cetyltrimethylammonium bromide; PAH – Polyallylamine hydrochloride; MPA – Mercaptopropionic; MUA – 11-mercaptoundecanoic acid; PAMAM – Polyamidoamine dendrimers; TMAT – Trimethylammoniumethanethiol; MES – Mercaptoethanesulfonate; MEEE – Mercaptoethoxyethoxyethanol.

Cells/ Organism	Test Type	Exposure Period	Endpoints	Methods	Coating	Sizes(nm)/ Shapes	Concentration/ Dose	Genotoxic?	Ref.
HepG2	*In vitro*	24 h	DNA damage	Comet assay	Citrate and MUA	Spherical 20	0.1, 1, 10 and 100 µM	Yes	Fraga et al. (2013)
HepG2	*In vitro*	24 h	DNA damage	LC-MS/MS	Citrate	Spherical 10, 30 and 60	0.0002 and 0.2 $\mu g.mL^{-1}$	No	Nelson et al. (2013)
HepG2 and PBMC	*In vitro*	3 h	DNA damage	Comet assay	Citrate and PAMAM	Spherical 7	1 and 50 µM	Yes	Paino et al. (2012)
Rattus norvegicus	*In vivo*	72 h	DNA damage MN	Comet assay MN assay	N. P.	N. P. 2, 20 and 200	18 µg per rat	No	Schulz et al. (2012)
Danio rerio	*In vivo*	36 and 60 d	Gene expression	RAPD-PCR	Citrate	Spherical 12 and 50	36 and 106 ng/fish/d	Yes	Geffroy et al. (2012)

Table S1 (continuation). Summary of studies assessing gold nanoparticles (AuNPs) genotoxicity. N. P. – Information not provided; Ref. – Reference; HepG2 – Human liver HepG2 cells; CHL – Chinese hamster lung fibroblast cells; PBMC – Peripheral blood mononuclear cells; A549 – Human lung adenocarcinoma epithelial A549 cells; MRC-5 – Lung fibroblast cells; 3T3 – BALB/c 3T3 fibroblast cells; HaCaT – Human keratinocyte cell line; MN – Micronuclei; RAPD-PCR – Random amplified polymorphic DNA-PCR analysis; LC-MS/MS – Liquid chromatography tandem-mass spectrometry; PVP – Polyvinylpyrrolidone; CTAB – Cetyltrimethylammonium bromide; PAH – Polyallylamine hydrochloride; MPA – Mercaptopropionic; MUA – 11-mercaptoundecanoic acid; PAMAM – Polyamidoamine dendrimers; TMAT – Trimethylammoniumethanethiol; MES – Mercaptoethanesulfonate; MEEE – Mercaptoethoxyethoxyethanol.

Cells/ Organism	Test Type	Exposure Period	Endpoints	Methods	Coating	Sizes(nm)/ Shapes	Concentration/ Dose	Genotoxic?	Ref.
3T3	*In vitro*	15, 30 m, 4, 24 and 48 h	DNA damage	Comet assay	Citrate and hyaluronic acid	Spherical 12	500 $\mu g.mL^{-1}$	Yes	Di Guglielmo et al. (2012)
MRC-5	*In vitro*	72 h	DNA damage	Comet assay	Citrate	Spherical 20	1 nM	Yes	Li et al. (2011)
HaCaT	*In vitro*	24 h	Gene expression	Real time PCR	TMAT, MES and MEEE	Spherical 1.5	25 $\mu g.mL^{-1}$	Yes	Schaeublin et al. (2011)
Rattus norvegicus	*In vivo*	24, 96 h, 30 and 60 d	Gene expression	Microarray analysis	Citrate	Spherical 20	0.01 $mg.kg^{-1}$	Yes	Balasubramanian et al. (2010)
Scenedesmus subspicatus and *Corbicula fluminea*	*In vivo*	12 and 24 h	Gene expression	Real time PCR	Citrate	Spherical 10	$1.6x10^2$, $1.6x10^3$, $1.6x10^4$ and $1.6x10^5$ AuNPs/cell	Yes	Renault et al. (2008)

Figure S1. The different steps included in the comet assay.

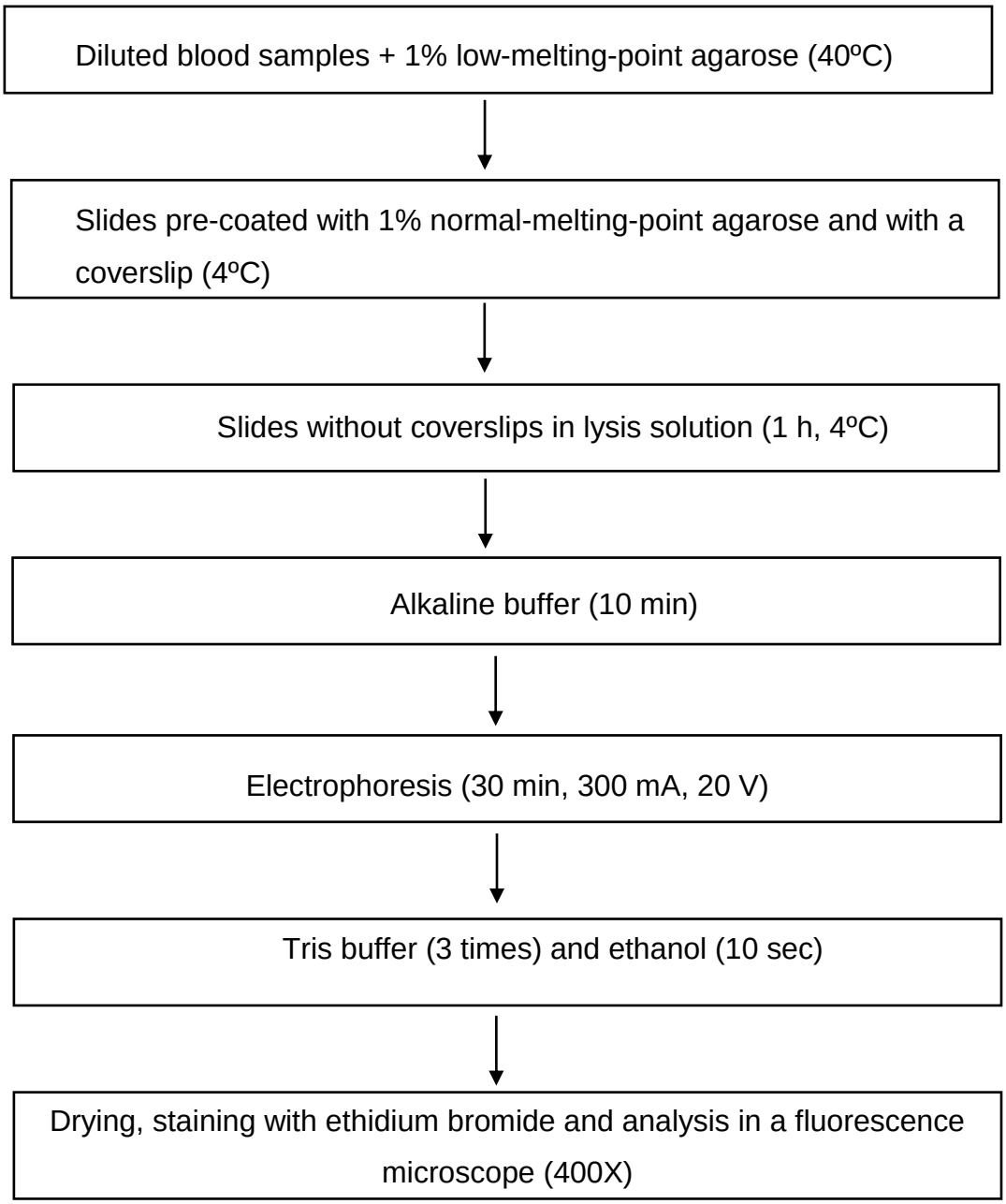

Table S2. Nominal and measured concentrations (µg.L^{-1}) of gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and gemfibrozil (GEM) in the experimental media (artificial seawater) at 0 and 24 h. Results are expressed as mean ± standard error.

Nominal concentration (µg.L^{-1})		**Measured concentration (µg.L^{-1})**		
		cAuNPs	**PVP-AuNPs**	**GEM**
4 AuNPs	**0h**	2.7 ± 0.3	4.2 ± 0.2	-
	24h	1.3 ± 0.3	3.4 ± 0.1	-
80 AuNPs	**0h**	30.5 ± 4.7	67.8 ± 6.1	-
	24h	5.1 ± 0.2	56.9 ± 3.0	-
1600 AuNPs	**0h**	115.1 ± 4.2	1458.7 ± 41.8	-
	24h	61.1 ± 10.1	943.0 ± 11.7	-
150 GEM	**0h**	-	-	240.0 ± 9.3
	24h	-	-	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

Chapter VIII

Effects of gold (ionic or nano form) and gemfibrozil mixtures in gilthead seabream (*Sparus aurata*)

A. Barreto[1*], D. Moreira[1], E. Pinto[2], A. Almeida[2], P. Paíga[3], L. Correia-Sá[3], C. Delerue-Matos[3], T. Trindade[4], A.M.V.M. Soares[1], K. Hylland[5], S. Loureiro[1], M. Oliveira[1]

[1] Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] LAQV-REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

[3] REQUIMTE/LAQV, Instituto Superior de Engenharia do Porto, Instituto Politécnico do Porto, Rua Dr. António Bernardino de Almeida, 431, 4200-072 Porto, Portugal

[4] Departamento de Química & CICECO - Aveiro Instituto de Materiais, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[5] Department of Biosciences, University of Oslo, PO Box 1066, N-0316 Oslo, Norway

*Corresponding author: E-mail: abarreto@ua.pt, Tel +351 234 370 350, Fax +351 234 372 587

Highlights

- The observed effects in the mixtures gold (ionic or nano form) and gemfibrozil were in general different from the predicted;
- For gills and muscle lipid peroxidation levels, erythrocytic nuclear abnormalities and DNA damage index, the observed effects of mixtures were lower than the predicted;
- For gills non-protein thiols content, the observed effect of mixtures was higher than the predicted.

Abstract

Although estuarine/coastal areas are expected to be the final recipient for most contaminants, as well as nanoparticles and pharmaceuticals, our knowledge concerning the effects of combined exposures is still limited. The present study aimed to investigate effects of short-term combined waterborne exposures to gold (nano or ionic form) and gemfibrozil (GEM), a lipid regulator, to the marine fish *Sparus aurata*. The assessed endpoints included different levels of biological organization (behaviour, neurotransmission, biotransformation, oxidative stress/damage and genotoxicity). The observed effects in the mixtures were in general different from the predicted (the sum of single exposure effects). For gills and muscle lipid peroxidation levels, erythrocytic nuclear abnormalities and DNA damage index, the observed effects of mixtures were lower than the predicted, whereas for gills non-protein thiols content was higher. The accumulation of gold in the tissues of *S. aurata* was similar after single and combined exposures. The obtained data emphasize the importance of studying the combined effects of emerging contaminants like gold and pharmaceuticals as antagonistic or synergistic effects may occur.

Keywords: aquatic environments; mixtures; gold nanoparticles; lipid regulators

1. Introduction

While our understanding of how single contaminants affect aquatic ecosystems have increased, there are still large knowledge gaps concerning effects of mixed exposures (Relyea 2009). This is a lack in scientific community once that the study of the effects after combined exposures is more representative of what is found in the aquatic environments that contain mixtures for many emerging contaminants (Luis et al. 2016).

Nanoparticles (NPs) are currently considered as emerging contaminants of concern and the range of different types and total number of manufactured nanomaterials used for technical applications and in consumer products increase continuously (Farkas et al. 2010; Sauve and Desrosiers 2014). NPs are defined as

having at least one dimension between one to one hundred nm (ASTM 2012) and have special physical and chemical properties compared to their bulk materials (Farkas et al. 2010; Niemeyer 2001). Gold nanoparticles (AuNPs) are widely used in different areas, including medical and biological research (Fratoddi et al. 2015), and it is expected that their levels in the aquatic environment will increase. Although the research concerning the effects of AuNPs and ionic gold to aquatic organisms has increased (Botha, James, and Wepener 2015; Dedeh et al. 2015; Farkas et al. 2010; García-Negrete et al. 2013; Tedesco et al. 2008, 2010a, 2010b; Volland et al. 2015; Renault et al. 2008; Teles et al. 2016), there is still a scarcity of studies on their toxicological effects in the presence of other environmental contaminants (Luis et al. 2016).

Pharmaceuticals, as NPs, are considered emerging contaminants of concern and lipid regulators are among the substances most commonly detected in aquatic environments (Bottoni, Caroli, and Caracciolo 2010; Fent, Weston, and Caminada 2006). Gemfibrozil (GEM) is a lipid regulator and has earlier been the focus of studies assessing its effects to aquatic organisms (Barreto et al. 2017, 2018; Gagne, and Blaise 2008; Henriques et al. 2016; Mimeault et al. 2005; Quinn et al. 2011; Schmidt et al. 2011, 2014; Teles et al. 2016; Zurita et al. 2007). There is however limited information on the toxicity of GEM in the presence of other contaminants.

The aim of the present work was to evaluate the biological effects of the combined exposures of gold (nano or ionic form) and GEM to the marine fish *Sparus aurata*. To achieve this goal, 96 h exposure to 80 $\mu g.L^{-1}$ of AuNPs or ionic gold and 150 $\mu g.L^{-1}$ of GEM (single and combined) were performed and different endpoints assessed: swimming performance; activity of enzymes involved in neurotransmission (brain and muscle: cholinesterases – ChE), in biotransformation (gills and liver: glutathione *S*-transferases – GST) and in antioxidant defence (gills and liver: glutathione reductase (GR), catalase (CAT) and glutathione peroxidase (GPx)); non-enzymatic defence (gills and liver: non-protein thiols – NPT); oxidative damage (erythrocytes DNA and cellular membranes of gills, liver, brain and muscle); DNA strand breaks and nuclear abnormalities in erythrocytes. Effect in combined exposures were compared with

the corresponding predicted effect derived from the sum of single exposure effects.

2. Material and Methods

2.1. Synthesis, characterisation and quantification of gold nanoparticles

Citrate coated AuNPs (cAuNPs) with 7 nm diameter were prepared based on the method of Shiba et al. (2013). Part of the resulting cAuNPs were coated with polyvinylpyrrolidone (PVP) as described in detail in Barreto et al. (2015). cAuNPs and PVP-AuNPs were centrifuged to remove most of the citrate molecules, ionic gold and free PVP (in the case of PVP-AuNPs). cAuNPs were chosen due to its extensive use in various applications and the non-toxicity of the reagents used in the citrate reduction method, one of the most widely used in AuNPs synthesis (Hanžić et al. 2015; Li et al. 2011; Turkevich, Stevenson, and Hillier 1951). PVP-AuNPs were selected because PVP is a nontoxic homopolymer, frequently used as AuNPs coating agent to increase its stability (Das et al. 2017; Min et al. 2009). After synthesis, the AuNPs stock suspensions were characterised. AuNPs alone and combined with GEM were also characterised in the experimental media (artificial seawater – ASW) and ultrapure water. The AuNPs were characterised by UV-Vis spectra; size, assessed by dynamic light scattering (DLS), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) and zeta potential (ZP). The determination of gold in the stock suspensions and in the experimental media was performed according to the NIST NCL Method PCC-8 (NIST 2010). An iCAPTM Q ICP-MS (inductively coupled plasma mass spectrometry) instrument (Thermo Fisher Scientific, Bremen, Germany) was used for the analysis. The ICP-MS instrumental conditions were as follow: argon flow rate (14 $L.min^{-1}$); auxiliary argon flow rate (0.8 $L.min^{-1}$); nebulizer flow rate (1.03 $mL.min^{-1}$); RF power (1550 W) and dwell time (100 ms). The elemental isotope ^{197}Au was monitored for analytical determination; ^{159}Tb and ^{209}Bi were used as internal standards. The instrument was tuned daily for maximum signal sensitivity and stability.

2.2. Preparation and quantification of gemfibrozil

A stock solution of GEM (50 $g.L^{-1}$) was prepared daily in dimethyl sulfoxide (DMSO). The extraction of GEM in ASW 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 LCMS-8030 (Shimadzu Corporation, Kyoto, Japan) and operated in the electrospray ionization (ESI) mode was used. Control and data processing was performed using Lab Solutions software (Shimadzu Corporation, Kyoto, Japan). Detailed information about the chromatographic and mass spectrometry experimental conditions as well as the validation parameters can be found elsewhere (Barreto et al. 2017).

2.3. Fish acclimation and exposure design

Juvenile gilthead seabream (*Sparus aurata*), length 6.02 ± 0.04 cm, supplied from an aquaculture facility (Santander, Spain), were acclimated for 4 weeks in aquaria containing aerated and filtered ASW prepared by dissolving the salt in reverse osmosis purified water to obtain a salinity of 30, in a controlled room temperature (18ºC) and natural photoperiod. During this period, animals were fed daily with commercial fish food at a ratio of 1 g per 100 g of fish. During the exposures, the photoperiod, temperature and water parameters (salinity, conductivity, pH and dissolved oxygen) were similar to those used in the acclimation period. No food was provided to the fish during the exposure period.

The bioassay generally followed the OECD guideline (number 203) for fish acute bioassays (OECD 1992). Fish (n=12 per condition) were randomly distributed in the aquaria (3 aquaria per condition) in a ratio of 1 g of fish per 1 L of ASW and exposed during 96 h to the following single exposures: 80 $\mu g.L^{-1}$ of AuNPs (citrate or PVP coating) or ionic gold; 150 $\mu g.L^{-1}$ of GEM and combined exposures of 150 $\mu g.L^{-1}$ GEM with 80 $\mu g.L^{-1}$ AuNPs (citrate or PVP coating) or ionic gold. Test solutions of AuNPs were prepared by dilution of cAuNPs and PVP-AuNPs stock suspensions with 88 $mg.L^{-1}$ and 57 $mg.L^{-1}$ of gold, respectively, in

ASW. Test solutions of ionic gold were also prepared by dilution of gold (III) chloride trihydrate stock solution (2.7 $g.L^{-1}$) in ASW. The test solutions with GEM (150 $\mu g.L^{-1}$) were prepared by appropriate dilutions of the stock solution in ASW. A control (only ASW) and an additional control with DMSO (at 0.003%, the concentration of DMSO used in the GEM treatments) were included in the bioassay. The concentration of 80 $\mu g.L^{-1}$ gold is about 10 times higher than the predicted values of AuNPs for the environment (García-Negrete et al. 2013; Tiede et al. 2009) and already showed to induce significant effects, in terms of genotoxicity and oxidative stress/damage, on *S. aurata* (Chapter III). The GEM concentration used (150 $\mu g.L^{-1}$) is about 100 times higher than the relevant environmentally concentrations of GEM and has previously been shown to induce significant effects, in terms of behavioural changes, genotoxicity and oxidative stress/damage, on *S. aurata* (Barreto et al. 2017, 2018).

Daily, after checking fish mortality, behavioural alterations and assessing the water parameters, approximately 80% of the experimental media was renewed to prevent significant NPs alteration, GEM degradation and to reduce the build-up of metabolic residues. Experimental media from each aquarium were collected daily (at 0 and 24 h) for the quantification of gold and GEM.

2.4. Assessment of swimming performance

At the end of the exposure period, individual fish were transferred to a long track race flume and induced to swim against a water flow (12 $L.min^{-1}$), generally following the procedure described by Oliveira et al. (2012). The time spent by the fish swimming against the water flow was recorded.

2.5. Collection of biological material

After a 2-h recovery period, animals were anesthetised with tricaine methanesulfonate (MS-222) and weighed. Blood sample was collected from the posterior cardinal vein and fish euthanized by spinal section. For the comet assay, blood samples were diluted with saline phosphate buffer. Blood smears were prepared for the assessment of erythrocytic nuclear abnormalities (ENAs). Liver

were removed and weighed to determine the liver-somatic index (LSI) by the formula (Nunes et al. 2011):

$$LSI = (weight\ of\ liver\ /\ total\ body\ weight)\ x\ 100$$

In addition to liver, other tissues, i.e. gills, muscle and brain were also removed from seven fish and stored at -80ºC until biochemical biomarkers analyses. Liver, gills, spleen and muscle were taken from five individuals and kept at -20ºC for gold quantification.

2.5.1. Biochemical biomarkers determination

Liver and gills were homogenized in potassium phosphate buffer (0.1 mM, pH 7.4) using an ultrasonic homogenizer. The homogenate was then divided into three aliquots: for the quantification of lipid peroxidation (LPO), NPT and for the preparation of post-mitochondrial supernatant (PMS). 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 and aliquots were stored at -80ºC 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 and the obtained supernatant was collected and stored at -80ºC.

Protein concentration was determined according to Bradford (1976), adapted to microplate, using bovine γ – globuline as a 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).

2.5.2. Comet and erythrocytic nuclear abnormalities (ENAs) assays

The alkaline comet assay was conducted according to the method of Singh et al. (1988) with some modifications (Barreto et al. 2017). The sensitivity and specificity of the assay was improved using a lesion-specific endonuclease, formamidopyrimidine DNA glycoslyase (Fpg). Fpg is 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 (Speit et al. 2004; Tudek et al. 1998; Tice et al. 2000; Albertini et al. 2000; Epe et al. 1993; Tchou et al. 1994; Li, Laval, and B. Ludlum 1997). The method for enzyme formamidopyrimidine DNA glycoslyase (Fpg) conjugated with comet assay was performed according to previous procedures (Collins 2014; Collins et al. 1997). A positive control (blood from fish treated with hydrogen peroxide (H_2O_2)), with and without Fpg treatment, was included in the assay. Cells were classified, according to tail length, into five classes (Collins 2004): class 0 – undamaged, without a tail; class 1 – with a tail shorter than the diameter of the nucleus; class 2 – with a tail length 1–2 times the diameter of the nucleus; class 3 – with a tail longer than twice the diameter of the nucleus; class 4 – comets with no nucleus. A damage index (DI), in arbitrary units, was assigned to each slide (for 100 cells) and consequently for each treatment, using the formula:

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

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

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

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

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

2.5.3. Total gold content, bioaccumulation factor and estimated intake for humans

The determination of gold in the fish tissues was performed according to the NIST NCL Method PCC-8 (NIST 2010) using an iCAPTM Q ICP-MS (inductively coupled plasma mass spectrometry) instrument (Thermo Fisher Scientific, Bremen, Germany) and the instrumental conditions the same which were used to the determination of gold in stock suspensions and in the experimental media. Total gold content ($[Au]_{total}$), expressed as $\mu g.g^{-1}$, was calculated as the sum of the gold content in each fish tissue, according the formula:

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

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 $[Au]_{ms}$ the concentration of gold in muscle.

The bioaccumulation factor (BAF), in $L.g^{-1}$, was determined according to Yoo-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 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).

As *Sparus aurata* is a fish for human consumption an extrapolation of gold intake for humans was calculated, using the following 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 ($\mu g.g^{-1}$).

2.6. Data analysis

Using the Sigma Plot 12.0 software package, the assumptions of normality and homogeneity of data were verified. Differences between controls (negative and solvent) were carried out using a Student t-test ($p<0.05$). Differences between treatments and control and between all the treatments were analysed using one-way analysis of variance (ANOVA), followed by Dunnett's or Tukey´s test whenever applicable. Significant differences were assumed for $p<0.05$.

The percentage of effect on *Sparus aurata* after exposure to the tested conditions were calculated relative to control for the assessed endpoints. Observed percentages of effect in the combined exposures were compared with the corresponding predicted percentages of effect, which were calculated as the sum of single exposure effects. These comparisons were performed to clarify whether the combined effect of gold (nano or ionic form) and GEM was similar, smaller or greater than the predicted from single exposures.

3. Results

3.1. Gold Nanoparticles

3.1.1. Characterisation

The cAuNPs presented a well-defined surface plasmon resonance (SPR) peak. DLS analysis showed an average hydrodynamic size of the particles of 7 nm and a strongly negative surface charge – Table 1. TEM analysis confirmed that almost all cAuNPs had the same size and spherical shape. There was a slight shift in the SPR peak to a longer wavelength for PVP-AuNPs when compared with the original cAuNPs. DLS measurements showed a size of around 8 nm and a less negative ZP than cAuNPs – Table 1. SEM analysis allowed the visualization of a PVP layer around some AuNPs metal core.

Table 1. Characteristics of gold nanoparticles (AuNPs) in the stock suspensions. cAuNPs – Citrate coated gold nanoparticles; PVP-AuNPs – Polyvinylpyrrolidone coated gold nanoparticles; PdI – Polydispersity Index; SPR – Surface Plasmon Resonance; ZP – Zeta Potential.

	Size (nm)	PdI	SPR (nm)	ZP (mV)	Concentration ($mg.L^{-1}$)
cAuNPs	6.8	0.5	519.1	-43.2	98
PVP-AuNPs	7.9	0.5	521.2	-12.9	51

3.1.2. Behaviour in the experimental media and combined with gemfibrozil

In the experimental media (ASW), cAuNPs changed the colour from red to light blue, typical of NPs agglomeration/aggregation. PVP-AuNPs did not show colour alteration in ASW. At 80 $\mu g.L^{-1}$, it was not possible to characterise AuNPs due to the detection limits of the used techniques.

A study of the interaction of GEM and AuNPs was also not possible at the tested concentrations of 80 and 150 $\mu g.L^{-1}$ (AuNPs and GEM, respectively). 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 $\mu g.L^{-1}$,

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 alone. PVP-AuNPs combined with GEM remained stable in ASW such as when they were alone.

3.2. Quantification of gold and gemfibrozil in the experimental media

The measured concentrations of gold and GEM in the experimental media (ASW) are present in Table 2.

Table 2. Nominal and measured concentrations (µg.L^{-1}) of gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold 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.

Nominal concentrations (µg.L^{-1})		**Measured concentrations (µg.L^{-1})**			
		cAuNPs	**PVP-AuNPs**	**Ionic gold**	**GEM**
80 gold	**0 h**	5.2 ± 0.5	42.4 ± 4.5	71.8 ± 9.8	N.D.
	24 h	2.9 ± 0.4	27.4 ± 3.4	63.0 ± 27.4	N.D
150 GEM	**0 h**	N.D.	N.D.	N.D.	121.1 ± 20.5
	24 h	N.D.	N.D.	N.D.	93.6 ± 20.6
80 cAuNPs + 150 GEM	**0 h**	5.4 ± 0.5	N.D.	N.D.	85.5 ± 16.9
	24 h	2.9 ± 0.3	N.D.	N.D.	84.2 ± 19.6
80 PVP-AuNPs + 150 GEM	**0 h**	N.D.	40.1 ± 4.3	N.D.	89.9 ± 20.0
	24 h	N.D.	27.4 ± 3.5	N.D.	79.7 ± 18.7
80 Ionic gold + 150 GEM	**0 h**	N.D.	N.D.	70.9 ± 8.9	85.3 ± 27.0
	24 h	N.D.	N.D.	64.7 ± 8.2	79.9 ± 21.0

At 0 h, the gold quantified in the media was lower than the nominal concentrations. The difference between the nominal and measured concentrations was even more evident for cAuNPs. In the single exposures, at 0 h, for cAuNPs, the measured concentration of gold was 94% lower than the expected (80 $\mu g.L^{-1}$). For PVP-AuNPs and ionic gold, were 47 and 10% lower than the nominal concentration (80 $\mu g.L^{-1}$), respectively. In the combined exposures, similar results were observed (Table 2). In the single exposures, the concentration of gold quantified after 24 h of exposure decreased 45, 32 and 12% for cAuNPs, PVP-AuNPs and ionic gold, respectively, when compared to the gold quantification at 0 h. No differences were detected between these results of single exposures and combined exposures (Table 2).

The GEM quantified at 0 h in the experimental media was lower than the nominal concentration (150 $\mu g.L^{-1}$), around 19% less in the single exposures and around 40% less in the combined exposures. At 24 h, in the single exposure, the concentration of GEM decreased 23% and in the combined exposures decreased between 2 and 11% when compared to GEM quantified at 0 h (Table 2).

3.3. Biological responses

Solvent control (with DMSO) did not induce significant effects when compared to the control ($p>0.05$; t-test), for the reported endpoints. Therefore, the treatments were compared to the control.

3.3.1. Swimming performance

As shown in Figure 1A, the ability of *Sparus aurata* to continue swimming against a water flow decreased around 52% ($p<0.05$; Dunnett's test), when fish were exposed to 80 $\mu g.L^{-1}$ of cAuNPs single exposure and combined with GEM.

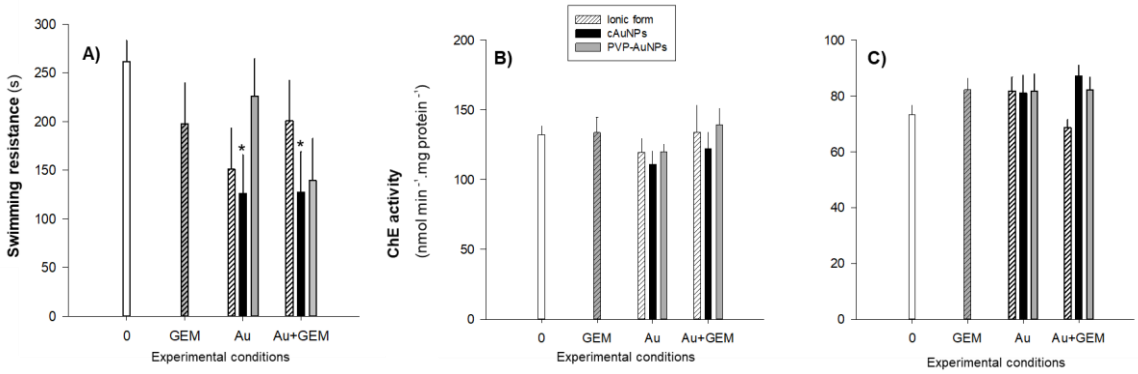

Figure 1. Resistance of *Sparus aurata* to withstand swimming against a water flow **(A),** brain **(B)** and muscle **(C)** cholinesterases (ChE) activity after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$).

3.3.2. Liver-somatic index (LSI)

As shown in Table 3, no differences were found between the LSI of the treatments and the control group ($p>0.05$; ANOVA).

Table 3. Liver-somatic index of *Sparus aurata* after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). Results are expressed as mean ± standard error.

Experimental conditions	Liver-somatic Index
Control	1.7 ± 0.1
GEM	1.9 ± 0.2
Ionic gold	2.0 ± 0.3
cAuNPs	1.5 ± 0.2
PVP-AuNPs	1.7 ± 0.2
Ionic gold + GEM	1.5 ± 0.2
cAuNPs + GEM	1.6 ± 0.1
PVP-AuNPs + GEM	1.7 ± 0.1

3.3.3. Biochemical biomarkers determination

The activity of ChE (both in brain and muscle) was not significantly altered ($p>0.05$; ANOVA) after the exposure to the tested experimental conditions, as shown in Figure 1B and C. The tested experimental conditions did not induce significant alteration in CAT and GR activities of *S. aurata*, both in gills and liver ($p>0.05$; ANOVA; Figures 2 and 3).

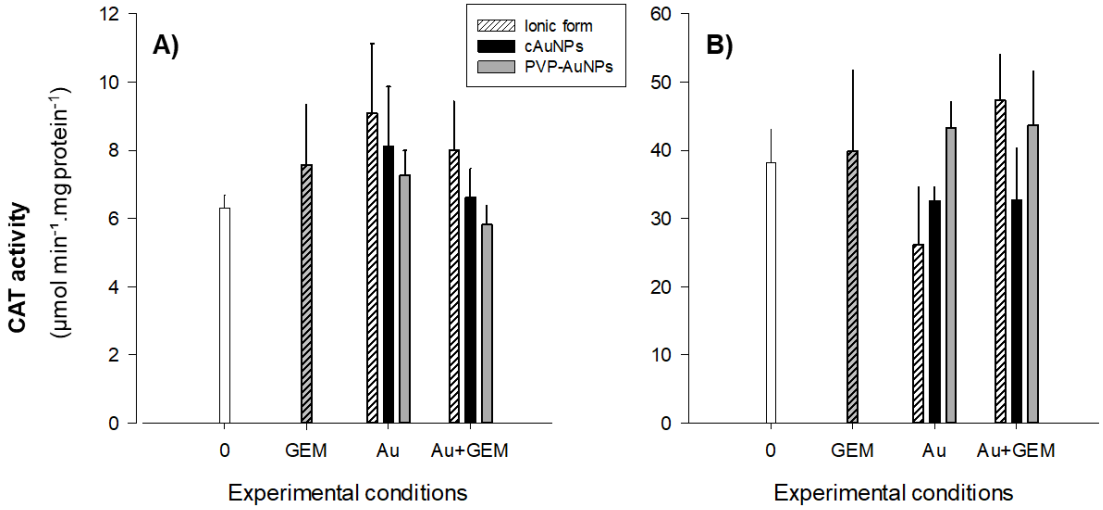

Figure 2. Gills **(A)** and liver **(B)** catalase (CAT) activity of *Sparus aurata* after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). Results are expressed as mean ± standard error.

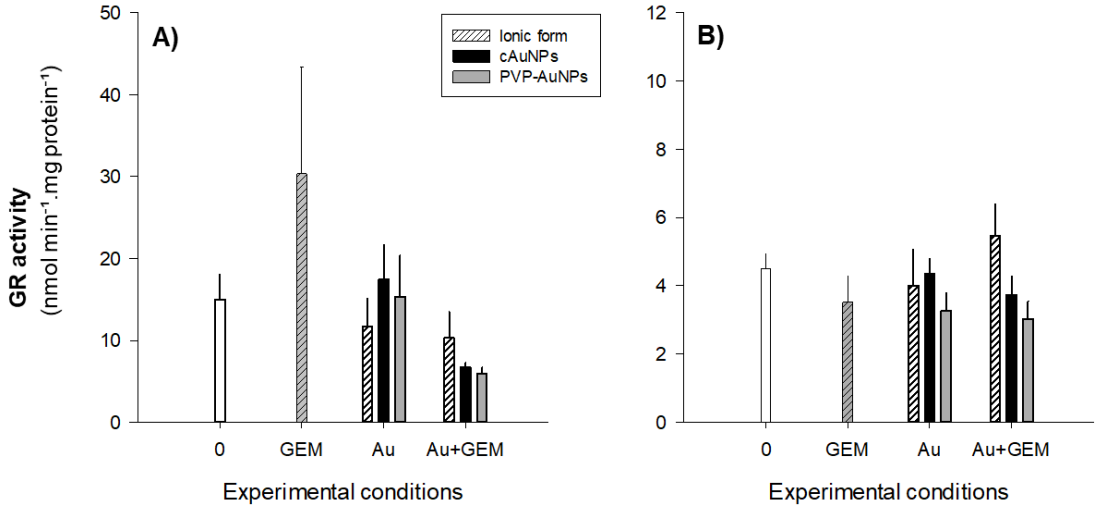

Figure 3. Gills **(A)** and liver **(B)** glutathione reductase (GR) activity of *Sparus aurata* after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). Results are expressed as mean ± standard error.

Concerning GPx activity, in gills, 150 µg.L^{-1} of GEM, GEM plus cAuNPs and GEM plus PVP-AuNPs significantly increased (311, 354 and 247%, respectively) this enzyme activity ($p<0.05$; Dunnett's test; Figure 4A). In liver, only exposure to GEM individually increased (148%) GPx activity ($p<0.05$; Dunnett's test; Figure 4B). In the combination of GEM with gold (both forms) no significant differences were found compared to control ($p>0.05$; ANOVA; Figure 4B).

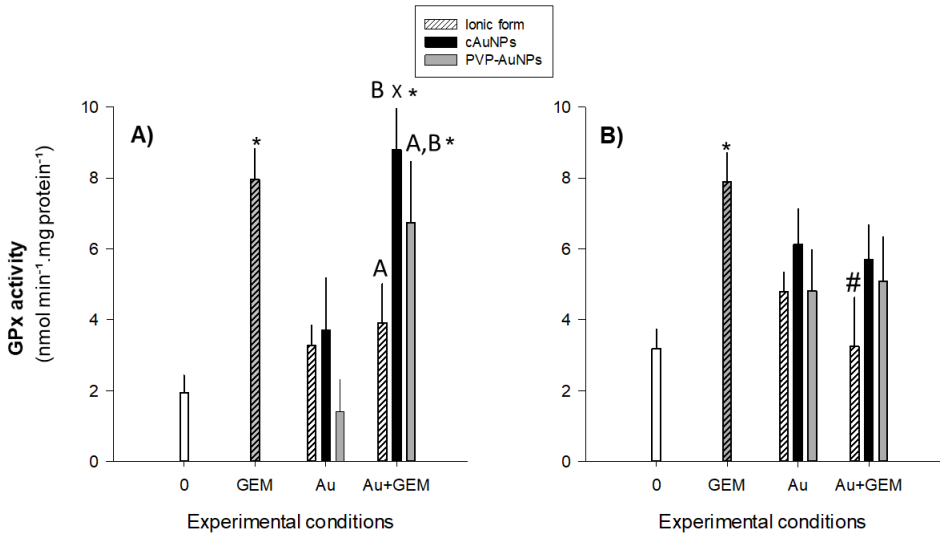

Figure 4. Gills **(A)** and liver **(B)** glutathione peroxidase (GPx) activity of *Sparus aurata* after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05). ^{X}Significant differences between the combination (Au+GEM) and the correspondent single exposure of gold (Tukey´s test, p<0.05). $^{\#}$Significant differences between the combination (Au+GEM) and the single exposure of GEM (Tukey´s test, p<0.05). Different letters correspond to significant differences between ionic gold, cAuNPs and PVP-AuNPs within the combined exposures (Tukey´s test, p<0.05).

Gills NPT levels significantly increased after cAuNPs single and combined exposures, 51 and 93%, respectively (p<0.05; Dunnett's test; Figure 5A). No significant differences were found between hepatic NPT levels of the treatment groups and the control (p>0.05; ANOVA; Figure 5B).

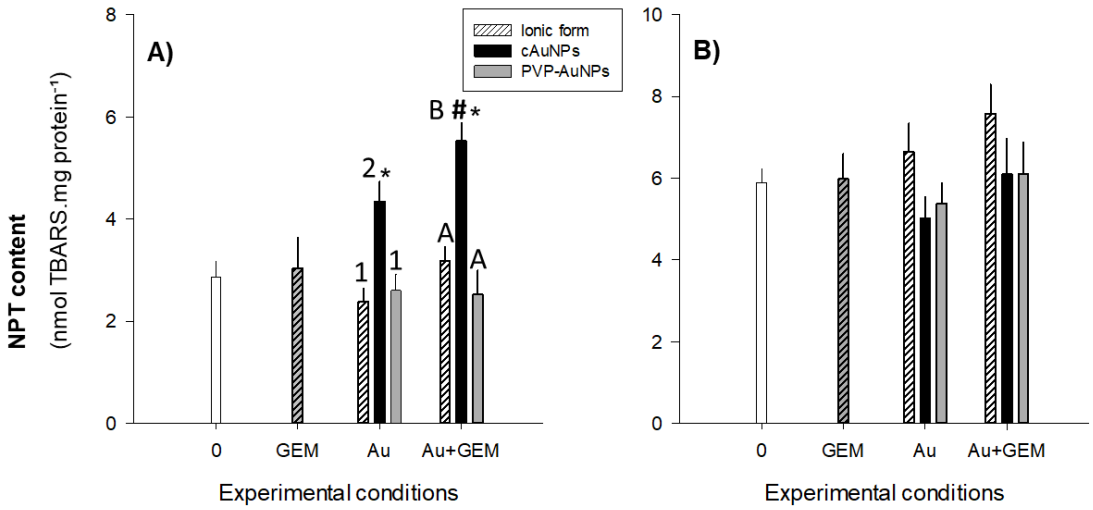

Figure 5. Gills **(A)** and liver **(B)** non-protein thiols (NPT) levels of *Sparus aurata* after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, p<0.05). $^{\#}$Significant differences between the combination (Au+GEM) and the single exposure of GEM (Tukey´s test, p<0.05).

Different numbers correspond to significant differences between ionic gold, cAuNPs and PVP-AuNPs within the single exposures (Tukey´s test, $p<0.05$). Different letters correspond to significant differences between ionic gold, cAuNPs and PVP-AuNPs within the combined exposures (Tukey´s test, $p<0.05$).

Gills GST activity significantly increased (around 81%) following ionic gold single and combined exposures ($p<0.05$; Dunnett's test; Figure 6A). No significant differences were detected for hepatic GST activity between the treatment groups and the control ($p>0.05$; ANOVA; Figure 6B).

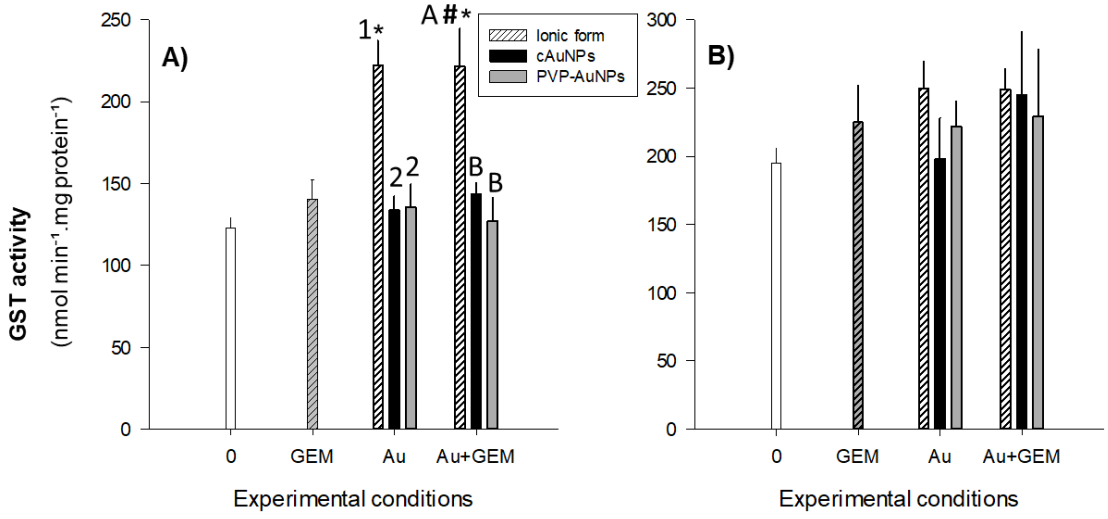

Figure 6. Gills **(A**) and liver **(B)** glutathione *S*-transferases (GST) activity of *Sparus aurata* after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). $^{\#}$Significant differences between the combination (Au+GEM) and single exposure of GEM (Tukey´s test, $p<0.05$). Different numbers correspond to significant differences between ionic gold, cAuNPs and PVP-AuNPs within the single exposures (Tukey´s test, $p<0.05$). Different letters correspond to significant differences between ionic gold, cAuNPs and PVP-AuNPs within the combined exposures (Tukey´s test, $p<0.05$).

As shown in Figure 7A, oxidative damage (assessed as TBARS levels) was found in gills following exposure to cAuNPs single exposure ($p<0.05$; Dunnett's

test). In the combined exposures of cAuNPs with GEM, no significant differences were found compared to control ($p>0.05$; ANOVA; Figure 7A). In muscle, LPO levels significantly increased after the exposure to ionic gold single exposure ($p<0.05$; Dunnett's test; 7D). The combination of ionic gold with GEM did not induce significant differences compared to control ($p>0.05$; ANOVA; Figure 7D). The tested experimental conditions did not induce significant alterations in liver or brain LPO levels ($p>0.05$; ANOVA; Figure 7B and C).

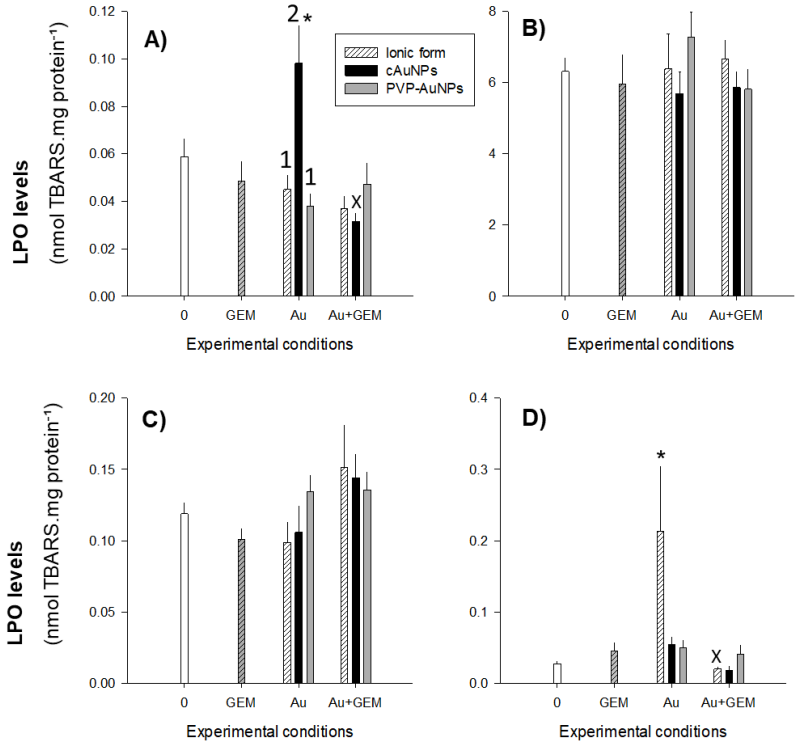

Figure 7. Gills **(A),** liver **(B),** brain **(C)** and muscle **(D)** lipid peroxidation (LPO) levels of *Sparus aurata* after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). ^{X}Significant differences between the combination (Au+GEM) and the correspondent single exposure of gold (Tukey´s test, $p<0.05$). $^{\#}$Significant differences between the combination (Au+GEM) and single exposure of GEM (Tukey´s test, $p<0.05$). Different numbers correspond to significant differences between ionic gold, cAuNPs and PVP-AuNPs within the single exposures (Tukey´s test, $p<0.05$).

3.3.4. Comet and erythrocytic nuclear abnormalities (ENAs) assays

All the treatments resulted in increased DNA strand breakage ($p<0.05$; Dunnett's test), as assessed by comet assay – Table 4.

Table 4. DNA damage classes, measured by the comet assay, of peripheral blood cells from *Sparus aurata* after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). *Significant differences to control (Dunnett's test, $p<0.05$); data are presented as mean ± standard error. $^{+}$Significant differences between the cells treated with Fpg and the correspondent cells without Fpg. 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	47.4±11.4	51.8±10.1	2.6±1.3	0.2±0.2	-	70.1±2.5	63.0±7.1
GEM	0.2±0.2*****	8.0±3.0*****	30.0±2.3*****	50.2±4.8*****	9.6±1.6*****	263.3±4.9*****	325.3±3.2***$^{+}$**
Ionic gold	0.3±0.3*****	12.0±4.0*****	34.7±2.5*****	28.7±1.4*****	27.0±5.7*****	275.3±16.2*****	297.0±13.0*****
cAuNPs	-*****	5.0±2.1*****	44.5±0.9*****	41.8±2.4*****	8.8±0.4*****	254.3±4.9*****	259.5±2.1*****
PVP-AuNPs	- *****	4.3±1.1*****	33.3±1.6*****	56.7±2.9*****	5.7±2.2*****	263.7±3.4*****	268.0±4.9*****
Ionic gold + GEM	0.3±0.3*****	5.3±3.8*****	26.7±2.1*****	34.0±1.6*****	37.0±2.8*****	308.7±4.9*****	320.8±4.2*****
cAuNPs + GEM	- *****	0.5±0.4*****	24.5±7.1*****	59.3±5.2*****	13.3±7.8*****	280.3±22.7*****	292.8±14.8*****
PVP-AuNPs + GEM	0.3±0.2*****	9.5±4.5*****	30.8±3.6*****	32.8±2.9*****	34.3±3.1*****	306.3±6.0*****	311.5±10.9*

A DNA damage index around 308 – the highest DNA damage value detected – was found in organisms exposed to the combination of ionic gold with GEM (Table 4). In terms of damage classes, as shown in Table 4, the most abundant class in the negative control group were class 0 and 1. In all the treatments, when compared to the control, significantly less classes 0 and 1 were observed,

alongside significantly higher levels of classes 2, 3 and 4 ($p<0.05$; Dunnett's test). In general, the DNA damage classes 2 and 3 were the most detected after the exposure to the tested treatments. Comparing the DNA damage index in cells treated with Fpg with the correspondent cells without the enzymatic treatment, no significant differences were found after the single and combined exposures to gold (nano or ionic form) ($p>0.05$; ANOVA). However, in the treatment with GEM, DNA damage index in cells treated with Fpg was significantly higher than those without Fpg ($p<0.05$; Tukey´s test) – Table 4. A DNA damage index around 325 was detected in organisms exposed to GEM, the highest DNA damage value detected, assessed by comet assay plus Fpg (Table 4).

Concerning cytogenetic damage, GEM single exposure led to significantly increased ENAs frequency ($p<0.05$; Dunnett's test), as shown in Figure 8. Gold (nano and ionic forms) did not induce significant effects in this endpoint, at single and combined exposures with GEM ($p>0.05$; ANOVA).

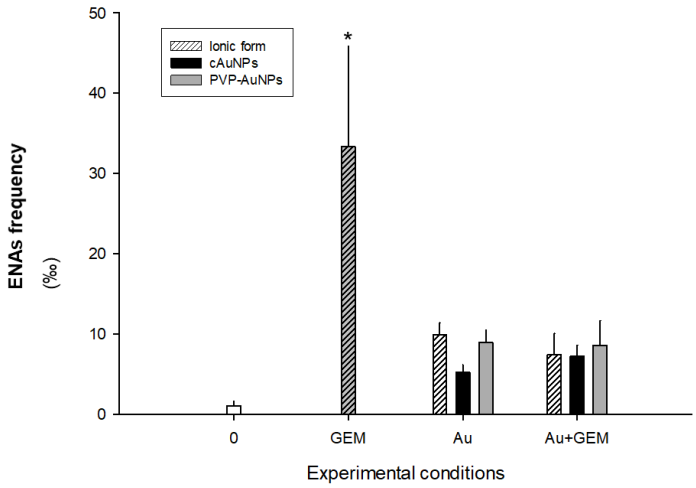

Figure 8. Erythrocytic nuclear abnormalities (ENAs) frequency in *Sparus aurata* after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$).

As shown in Table 5, lobed nuclei abnormality was the most detected in gold single and combined treatments with GEM, being significantly detected after the

single exposures of ionic gold and PVP-AuNPs ($p<0.05$; Dunnett's test). In the case of GEM single exposure, the most detected abnormalities were kidney-shaped and segmented nuclei ($p<0.05$; Dunnett's test) – Table 5.

Table 5. Erythrocytic nuclear abnormalities (ENAs) detected in *Sparus aurata* after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). *Statistically 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	0.1±0.1	0.6±0.4	0.3±0.2	-	-
GEM	17.8±6.8*	11.0±4.5*	2.9±0.9	1.7±1.1	-
Ionic gold	3.4±0.4	0.3±0.2	5.9±1.2*	0.3±0.3	-
cAuNPs	2.3±0.5	0.1±0.1	2.0±0.8	0.3±0.2	-
PVP-AuNPs	3.3±0.7	-	5.6±1.1*	-	-
Ionic gold + GEM	2.8±0.9	0.2±0.2	4.4±1.8	-	-
cAuNPs + GEM	2.4±0.9	0.6±0.6	3.0±0.8	1.2±1.2	-
PVP-AuNPs + GEM	2.0±0.7	-	3.8±1.7	2.8±2.3	-

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

As shown in Table 6, gold accumulated significantly in all assessed tissues after exposure to ionic gold, both individually and in combined exposures ($p<0.05$; Dunnett's test). In the cAuNPs single exposures, gold accumulated significantly in spleen whereas in the combined exposure with GEM it accumulated significantly in both spleen and liver ($p<0.05$; Dunnett's test; Table 6). For single and combined exposures of PVP-AuNPs, gold accumulated significantly in gills and spleen ($p<0.05$; Dunnett's test; Table 6).

Table 6. Gold content in each assessed tissue (gills, liver, spleen and muscle) and total gold content ($[Au]_{total}$) on *Sparus aurata* after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold and gemfibrozil (GEM). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). b.d.l. – Below the detection limit.

Nominal concentrations ($\mu g.L^{-1}$)	Tissues	Gold content ($\mu g.g^{-1}$)		
		cAuNPs	PVP-AuNPs	Ionic gold
0 Au	**Gills**	b.d.l.	b.d.l.	b.d.l.
	Liver	b.d.l.	b.d.l.	b.d.l.
	Spleen	b.d.l.	b.d.l.	b.d.l.
	Muscle	b.d.l.	b.d.l.	b.d.l.
	$[Au]_{total}$	**-**	**-**	**-**
80 Au	**Gills**	b.d.l.	0.6 ± 0.0 *	2.2 ± 0.0 *
	Liver	b.d.l.	b.d.l.	1.0 ± 0.0 *
	Spleen	0.1 ± 0.0 *	0.1 ± 0.0 *	0.3 ± 0.0 *
	Muscle	b.d.l.	b.d.l.	0.2 ± 0.0 *
	$[Au]_{total}$	**0.1 ± 0.0**	**0.7 ± 0.0**	**3.6 ± 0.0**
80 Au + 150 GEM	**Gills**	b.d.l.	0.7 ± 0.0 *	2.0 ± 0.0 *
	Liver	0.1 ± 0.0 *	b.d.l.	0.6 ± 0.0 *
	Spleen	0.1 ± 0.0 *	0.1 ± 0.0 *	0.3 ± 0.0 *
	Muscle	b.d.l.	b.d.l.	0.2 ± 0.0 *
	$[Au]_{total}$	**0.2 ± 0.0**	**0.8 ± 0.0**	**3.0 ± 0.0**

Overall, taking into account all the exposures, the accumulation of gold may be ranked as gills>liver>spleen>muscle. The calculation of BAF showed that values were zero or close to zero after the exposure to the tested conditions. The highest $[Au]_{total}$ values on *S. aurata* were detected after exposure to ionic gold single and combined exposures, 3.6 and 3.0 $\mu g.g^{-1}$, respectively – Table 6. The highest estimated values for gold intake by a Portuguese person were calculated to the conditions: 80 $\mu g.L^{-1}$ of ionic gold and ionic gold plus GEM (Table 7). In the case of AuNPs exposure, no accumulation of gold was found in the muscle, therefore estimated values for gold intake by a Portuguese person were not calculated.

Table 7. Estimated gold intake (µg per kg body weight) per year, by each Portuguese person, after the ingestion of *Sparus aurata*, taking into account the total content of gold detected in muscle of fish after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) and ionic gold alone or combined with gemfibrozil (GEM).

Nominal concentrations ($\mu g.L^{-1}$)	**Estimated gold intake (µg.kg body weight per year)**		
	cAuNPs	**PVP-AuNPs**	**Ionic gold**
80 Au	-	-	0.2
80 Au + 150 GEM	-	-	0.2

3.5. Observed versus predicted percentage of effect

The percentages of effect on *Sparus aurata,* in the different assessed endpoints, after the exposure to single and combined exposures of AuNPs and GEM are shown in the Table 8.

Table 8. The relative percentages of effect on *Sparus aurata,* in the different assessed endpoints, after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold 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, $p<0.05$). ^{X}Significant differences between the combination (Au+GEM) and the correspondent single exposure of gold (Tukey´s test, $p<0.05$). $^{\#}$Significant differences between the combination (Au+GEM) and single exposure of GEM (Tukey´s test, $p<0.05$).

Assessed Endpoints		% of effect related to control						
		Ionic gold	cAuNPs	PVP-AuNPs	GEM	Ionic gold + GEM	cAuNPs + GEM	PVP-AuNPs + GEM
Swimming Resistance		42	52 *	14	25	**P:** 67 **O:** 23	**P**: 76 **O**: 51 *****	**P**: 38 **O**: 47
Cholinesterases Activity	**Brain**	10	16	9	- 1	**P:** 8 **O:** - 1	**P**: 15 **O**: - 7	**P**: 8 **O**: - 5
	Muscle	- 12	- 11	- 12	10	**P**: - 24 **O**: 6	**P**: - 23 **O**: - 19	**P**: - 24 **O**: - 12
Catalase Activity	**Gills**	- 44	- 28	- 15	- 20	**P**: - 64 **O**: - 27	**P**: - 48 **O**: - 5	**P**: - 35 **O**: 8
	Liver	31	15	- 13	- 4	**P**: 27 **O**: - 24	**P**: 10 **O**: 14	**P**: - 18 **O**: - 14
Glutathione Reductase Activity	**Gills**	22	- 16	- 2	- 102	**P**: - 80 **O**: 31	**P**: - 118 **O**: 55	**P**: - 104 **O**: 60
	Liver	11	3	27	22	**P**: 32 **O**: - 22	**P**: 24 **O**: 17	**P**: 49 **O**: 32
Glutathione Peroxidase Activity	**Gills**	- 69	- 91	28	- 311 *	**P**: - 380 **O**: - 102	**P**: - 402 **O**: - 354 *****^{x}	**P**: - 283 **O**: - 247 *****
	Liver	- 51	-93	- 51	- 148 *	**P**: - 199 **O**: - 2 $^{\#}$	**P**: - 241 **O**: - 79	**P**: - 200 **O**: - 60
Non-Protein Thiols Levels	**Gills**	17	- 51 *	9	- 6	**P**: 11 **O**: - 11	**P**: - 57 **O**: - 93 *****$^{\#}$	**P**: 3 **O**: 12
	Liver	- 13	15	9	- 2	**P**: - 14 **O**: - 29	**P**: 13 **O**: - 3	**P**: 7 **O**: - 4

Table 8 (continuation). The relative percentages of effect on *Sparus aurata,* in the different assessed endpoints, after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold 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). ^{X}Significant differences between the combination (Au+GEM) and the correspondent single exposure of gold (Tukey´s test, p<0.05). $^{\#}$Significant differences between the combination (Au+GEM) and single exposure of GEM (Tukey´s test, p<0.05).

Assessed Endpoints		% of effect related to control						
		Ionic gold	cAuNPs	PVP-AuNPs	GEM	Ionic gold + GEM	cAuNPs + GEM	PVP-AuNPs + GEM
Glutathione *S*-Transferases Activity	Gills	- 81 *	- 9	- 10	- 15	**P**: - 96 **O**: - 81 ***** $^{\#}$	**P**: - 24 **O**: - 17	**P**: - 25 **O**: - 4
	Liver	- 28	- 2	- 14	- 16	**P**: - 44 **O**: - 28	**P**: - 17 **O**: - 26	**P**: - 29 **O**: - 18
Lipid Peroxidation Levels	Gills	23	- 67 *	36	17	**P**: 41 **O**: 37	**P**: - 49 **O**: 46 ^{x}	**P**: 53 **O**: 20
	Liver	- 1	10	- 15	6	**P**: 4 **O**: - 5	**P**: 15 **O**: 7	**P**: - 10 **O**: 8
	Brain	17	11	- 13	15	**P**: 32 **O**: - 27	**P**: 26 **O**: - 21	**P**: 2 **O**: - 14
	Muscle	- 676 *	- 100	- 84	- 65	**P**: -741 **O**: 19 ^{x}	**P**: - 164 **O**: 34	**P**: - 149 **O**: -52

Table 8 (continuation). The relative percentages of effect on *Sparus aurata,* in the different assessed endpoints, after 96 h of single and combined exposures to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), ionic gold 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, $p<0.05$). ^{X}Significant differences between the combination (Au+GEM) and the correspondent single exposure of gold (Tukey´s test, $p<0.05$). $^{\#}$Significant differences between the combination (Au+GEM) and single exposure of GEM (Tukey´s test, $p<0.05$).

Assessed Endpoints		% of effect related to control						
		Ionic gold	cAuNPs	PVP-AuNPs	GEM	Ionic gold + GEM	cAuNPs + GEM	PVP-AuNPs + GEM
DNA Damage Index	**Erythrocytes**	-381 *	-344 *	-361 *	-360 *	**P**: -742 **O**: -440 *****	**P**: -705 **O**: -390 *****	**P**: -721 **O**: -435 *****
Nuclear Abnormalities Frequency	**Erythrocytes**	-890	-420	-790	-3233 *	**P**: -4123 **O**: -640	**P**: -3653 **O**: -620	**P**: -4023 **O**: -760

For some endpoints, the predicted percentages of effect were similar to the observed percentages of effect, e.g. for swimming resistance, ChE, CAT and GST activities (Table 8). For other endpoints, such as gills and muscle LPO levels, ENAs frequency and DNA damage index, the observed percentages of effect in the mixtures were, however, lower than the predicted, with values similar to the control group (with the exception of the DNA damage index) – Table 8. For gills NPT content, the observed percentage of effect in the mixture cAuNPs+GEM was higher than the predicted (Table 8).

4. Discussion

The absence of changes in UV-Vis spectra, size and ZP of AuNPs when they were in a mixture with GEM suggests there was no physical association between GEM and AuNPs in the exposure media, as previously reported for 40 nm AuNPs plus GEM (Barreto et al. 2019).

The accumulation of gold in the assessed tissues of *S. aurata* was similar comparing single and combined exposures, with more gold accumulated after exposure to ionic than nano form, as previously reported (Chapter III).

For some endpoints, such as swimming resistance, ChE, CAT and GST activities, the observed percentages of effect in the mixtures were similar to the predicted. For other assessed endpoints, however, the mixtures had no significant effects compared to control when it is expected significant effects. For other parameters, the observed percentage of effect in the mixture was higher than the predicted. Gills and muscle LPO levels were induced by cAuNPs and ionic gold single exposures, respectively, whereas these effects were not detected when they were combined with GEM. Also, ENAs frequency increased after exposure to GEM single exposure, whereas in GEM and gold (nano and ionic form) combined exposures, the ENAs frequency was similar to those of the control group. The results suggest that these compounds eliminate each other effects in combined exposures, suggesting an antagonistic effect [70]. There is, however, also the possibility that the combined exposures activate defences that prevent damage. This hypothesis seems supported by the gills NPT content, which the effects of the combined exposure cAuNPs and GEM were higher than the sum of the effects of

each contaminant alone. In addition, the possible occurrence of synergistic effects between gold and GEM must also be taken into consideration. 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 effects of NPs and other emerging contaminants (Luis et al. 2016). Barreto et al. 2019 also showed differences between the observed and the predicted percentages of effect after the exposure to the mixture of 40 nm PVP-AuNPs or cAuNPs and GEM in *Sparus aurata*. In gills, in general, in the combined exposures to AuNPs and GEM no significant differences were found when compared to control whereas in the single exposures they had significant effects. In liver, in some enzymatic activities (CAT and GR), the observed percentages of effect were higher than the predicted (Barreto et al. 2019). In an *in vitro* study with marine mussels (*Mytilus galloprovincialis*), GST activity increased with the exposure to the pharmaceutical carbamazepine whereas at in a simultaneous exposure with AuNPs (citrate and PVP coated) the enzyme activity was not different from control (Luis et al. 2016). A similar effect with cAuNPs was also reported when combined fluoxetine (Luis et al. 2016). In a study with marine clam (*Ruditapes philippinarum*), using pharmaceuticals – carbamazepine and cetirizine – and the metal cadmium, the combined treatments caused smaller effects in LPO and activity of GST than exposures to single contaminants (Almeida et al. 2018).

Overall, enzymatic and non-enzymatic responses involved in the defence of *S. aurata* against oxidative damage were more active in the gills than in liver. Oxidative damage, assessed as TBARS levels, was only detected in gills after the single exposure to cAuNPs, not being found after the combined exposure with GEM. This may be explained by the higher accumulation of gold detected in gills than the other assessed tissues. Gills are the first organ to be exposed to the contaminants and are considered a good candidate to an early assessment of the effects of waterborne contaminants (Oliveira, Pacheco, and Santos 2008). Liver was not very responsive in the assessed biological responses. The detected similarity between LSI of the treatments and the control suggests no significant change in the fish total energy reserves (Nunes et al. 2011; Sandström et al. 2005).

The present study showed that gold (nano and ionic form), both after single and combined exposures, induced DNA breaks but DNA oxidative damage was not apparent. This may be due to a low potential of the tested conditions to induce oxidative damage in the erythrocytes DNA of *S. aurata*. In addition, the oxidative DNA lesions caused by exposure to gold may have been already repaired by cellular DNA repair systems. Oxidative damage was however detected in cellular membranes, assessed as LPO, following exposure to gold (nano and ionic form). In addition, GEM single exposure induced oxidative damage in DNA, whereas in combination with gold (nano and ionic form) this did not occur. Thus, the hypothesis that NPs may directly interact with the enzyme used to detect oxidative damage to DNA (Fpg), as previously described by others (Kain, Karlsson, and Möller 2012) and in the Chapter III, needs to be considered. This interaction may occur by through different mechanisms: 1) the presence of particles in the comet nucleoid as already observed (Karlsson, Nygren, and Möller 2004; Stone, Johnston, and Schins 2009), may prevent the binding of Fpg with the damaged DNA (Grin et al. 2009; Kain, Karlsson, and Möller 2012); 2) Fpg may attach to the surface of particles and form a corona around the NPs (Lynch and Dawson 2008); 3) Ionic forms may change the conformation or interact with the structure of Fpg, which is crucial for the substrate specificity of the enzyme (Kain, Karlsson, and Möller 2012). Based on the above, the assessment of DNA oxidative damage after exposure to NPs and even ionic Au should be done using other methods and further studies are recommended to clarify this issue.

A maximum amount of gold that each person may be exposed to daily over their lifetimes without considerable health risk – “tolerable daily intake” (TDI) – was previously estimated as 322 $\mu g.kg^{-1}$ (Chapter III). Based on the tested conditions and present results, the estimated maximum gold intake by humans per day was around 0.0005 $\mu g.kg^{-1}$ body weight, not exceeding the previously estimated TDI value for gold.

The obtained data emphasize the importance of the study of the combined effects of contaminants, particularly when NPs are involved as they may adsorb contaminants on their surface and be a “Trojan horse” for increased entry of adsorbed contaminants into organisms and cells.

5. Conclusions

Exposure to a mixture of gold (ionic and nano form) and gemfibrozil caused different effects than the predicted. For instance, in the gills and muscle lipid peroxidation levels, erythrocytic nuclear abnormalities and DNA damage index, the observed percentages of effect in the mixtures were lower than the predicted. In the gills non-protein thiols content, the observed percentage of effect in the mixture was higher than the predicted. Comparing the responses between gills and liver, enzymatic/non-enzymatic defence of *Sparus aurata* against oxidative damage were more active in the gills than in liver. Oxidative damage, determined as increased lipid peroxidation, was only detected in gills. The accumulation of gold in the tissues of *S. aurata* was similar comparing single and combined exposures. The present results are highly relevant since organisms are exposed to mixtures of contaminants in nature.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Chapter IX

Effects of gold nanoparticles in gilthead seabream – a proteomic approach

A. Barreto[1*], A. Carvalho[1], A. Campos[2], H. Osório[3,4,5], E. Pinto[6], A. Almeida[6], T. Trindade[7], A.M.V.M. Soares[1], K. Hylland[8], S. Loureiro[1], M. Oliveira[1]

[1] Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[2] CIIMAR, Centro Interdisciplinar de Investigação Marinha e Ambiental, Universidade do Porto, 4450-208 Matosinhos, Portugal

[3] i3S – Instituto de Investigação e Inovação em Saúde, Universidade do Porto, Porto, Portugal

[4] Instituto de Patologia e Imunologia Molecular da Universidade do Porto, IPATIMUP, Porto, Portugal

[5] Faculdade de Medicina, Universidade do Porto, Portugal

[6] LAQV-REQUIMTE, Departamento de Ciências Químicas, Faculdade de Farmácia, Universidade do Porto, Rua Jorge Viterbo Ferreira, 228, 4050-313 Porto, Portugal

[7] Departamento de Química & CICECO - Aveiro Instituto de Materiais, Universidade de Aveiro, 3810-193 Aveiro, Portugal

[8] Department of Biosciences, University of Oslo, PO Box 1066, N-0316 Oslo, Norway

*Corresponding author: E-mail: abarreto@ua.pt, Tel +351 234 370 350, Fax +351 234 372 587

Highlights

- The proteomic changes of *Sparus aurata* liver after the exposure to different gold nanoparticles (AuNPs) were dependent on the nanoparticles characteristics;
- After the exposure to AuNPs, 26 proteins exhibited differences in abundance compared with control group;
- AuNPs triggered several pathways related to: cell morphology and differentiation; protein synthesis, folding and transport; oxidative stress and response to metals.

Abstract

Despite the widespread use of nanoparticles (NPs), there are still major gaps of knowledge regarding the impact of nanomaterials on human health and the environment. The present work aimed to study the effects of 7 and 40 nm AuNPs (citrate and polyvinylpyrrolidone (PVP) coated) on the liver proteome of the estuarine/marine fish gilthead seabream (*Sparus aurata*), after 96 h exposure. A total of 632 spots were detected in the gels analysis, with 26 proteins exhibiting differences in abundance after the exposure to AuNPs compared with control group. Most of these proteins was structural (actins and tubulins) although other proteins playing different functions in the cells were also identified (e.g. calreticulin, 94 kDa glucose-regulated protein, pyruvate carboxylase b, phosphoenolpyruvate carboxykinase 2, cytohesin-1 and hippocalcin). Data suggest that AuNPs triggered several pathways dependent on the NPs characteristics, related to: cell morphology and differentiation; protein synthesis, folding and transport; oxidative stress and response to metals. Although higher gold accumulation was found in the liver of *S. aurata* after the exposure to 7 nm PVP-AuNPs, 7 nm cAuNPs were the one inducing more effects in liver proteome.

Keywords: nanoparticles, *Sparus aurata*, liver, 2-DE, proteomic

1. Introduction

Despite the benefits that nanotechnology may bring to society, there are still major gaps on the knowledge regarding the impact of nanomaterials on human health and the environment (Matysiak et al. 2016). Gold nanoparticles (AuNPs) are widely used in biomedical applications (Khan, Vishakante, and Siddaramaiah 2013), being one of the fundamental requirements for their wide use, their presumed non-toxic and biocompatible nature. Although, recent studies have highlighted their possible toxicity to human health and ecosystems (Farkas et al. 2010; García-Cambero et al. 2013; García-Negrete et al. 2013; Iswarya et al. 2016; Paino et al. 2012; Teles et al. 2016).

Thus, it seems necessary to conduct further interdisciplinary research to fill the knowledge gaps in nanoparticles (NPs) toxicity, using more holistic approaches

than offered by conventional biological techniques. “OMICS” techniques could give rise to a better insight of the mechanisms involved in the nanotoxicity (Matysiak et al. 2016). Some studies have already shown that proteomic is a good tool to evaluate NPs toxicity, showing potential to reveal metabolic pathways and processes that may be not immediately evident with more conventional biomarkers (Gioria et al. 2014, 2016; Kim et al. 2010; Mirzajani et al. 2014a, 2014b; Otelea and Rascu 2015; Planchon et al. 2017).

The present study aimed to understand the effects on the gilthead seabream (*Sparus aurata*) liver proteome of 96 h exposure to different types of AuNPs, using a gel-based approach. This marine top predator fish was selected as a model organism, due to its importance on European aquaculture (Cordero et al. 2016). AuNPs of two sizes (7 and 40 nm), with citrate or polyvinylpyrrolidone (PVP) coating were tested. The physicochemical properties of NPs, especially size and surface coating, are considered important factors that influence directly and significantly the toxicity of NPs (Khanna et al. 2015).

A proteomic approach has already been performed to assess the effects of AuNPs to human adenocarcinoma Caco-2 cells (Gioria et al. 2016), Balb/3T3 mouse fibroblast cell line (Gioria et al. 2014) and mussel *Mytilus edulis* (Tedesco et al. 2010). The available studies with proteomic in *Sparus aurata* (liver), have been conducted to investigate the effects of different commercial feeds (Ghisaura et al. 2014), maslinic acid (Rufino-Palomares et al. 2011) and ivermectin (Varo et al. 2010). To the best of our knowledge, only two studies have used a fish liver proteomic approach to study the effects of NPs (copper and selenium) (Gupta et al. 2016; Naderi et al. 2017). Exposure to copper NPs induced differences in the abundance of proteins associated with oxidative stress and steroid biosynthesis in *Cyprinus carpio* (Gupta et al. 2016). Selenium NPs altered the abundance of proteins associated with glycolysis, gluconeogenesis and amino acid metabolism in *Oncorhynchus mykiss* (Naderi et al. 2017).

Thus, this is the first study to assess the effects of AuNPs on the protein abundance of a marine/estuarine fish species. The liver was selected as a target tissue due to its major role in several key metabolic processes (Alves et al. 2010) and the reported accumulation of AuNPs (Chen et al. 2013; Iswarya et al. 2016;

Khan, Vishakante, and Siddaramaiah 2013; Mateo et al 2014; Simpson et al. 2013).

2. Material and Methods

2.1. Fish maintenance

Juvenile gilthead seabream (*Sparus aurata*), length 7.7 ± 0.6 cm, were purchased from a Spanish aquaculture facility and were acclimated in the laboratory for 4 weeks in aquaria containing aerated and filtered artificial seawater (ASW, salinity 30), under controlled temperature (18ºC) and natural photoperiod. During these 4 weeks period, fish were fed daily with commercial fish food (Sorgal, Portugal) and the aquaria water renewed daily. All experimental procedures were carried out following the European and Portuguese legislation (authorization N421/2013 of Portuguese competent authority). Animal handling was performed by an accredited researcher. During the experimental assay, photoperiod, temperature and aeration conditions were similar to those used in the acclimation period.

2.2. Gold nanoparticles (AuNPs) synthesis and characterisation

Citrate-coated AuNPs (cAuNPs) with 7 nm diameter were synthesized by pH-shifting method, with reduction of gold (III) chloride trihydrate by citric acid, followed by neutralization with NaOH (Shiba 2013). cAuNPs with 40 nm diameter were prepared, using 15 nm seeds, by sodium citrate reduction of gold (III) chloride trihydrate (Lekeufack et al. 2010). Part of the cAuNPs were coated with PVP as described by Barreto et al. (2015). The citrate reduction method was chosen due to the non-toxicity of citrate, the use of water as solvent and the fact that cAuNPs have been frequently used in diverse areas (Hanžić et al. 2015; Li et al. 2011; Turkevich, Stevenson, and Hillier 1951). PVP was selected as a second coating and stabilizing agent because it is a water-soluble, nontoxic and biodegradable homopolymer (Min et al. 2009). After synthesis, the AuNPs stock suspensions were characterised by UV-Vis spectra (Cintra 303, GBC Scientific), dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern), transmission electron

microscopy (TEM; Hitachi, H9000 NAR), scanning electron microscopy (SEM; Hitachi, SU70) and zeta potential (ZP; Zetasizer Nano ZS, Malvern).

2.3. Experimental assay and biological material sampling

The procedures generally followed the OECD guidelines for fish acute bioassays (OECD 1992). Briefly, 9 fish 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 5 conditions: control (only ASW); 80 $\mu g.L^{-1}$ of 7 nm of cAuNPs; 80 $\mu g.L^{-1}$ of 7 nm of PVP-AuNPs; 80 $\mu g.L^{-1}$ of 40 nm of cAuNPs and 80 $\mu g.L^{-1}$ of 40 nm of PVP-AuNPs. Experimental suspensions of AuNPs were prepared by dilution of AuNPs stock suspensions in ASW. The concentration, 80 $\mu g.L^{-1}$, was chosen because it has earlier been shown to induce genotoxicity in *S. aurata* after 96 h exposure to all the tested types of AuNPs (Chapter VII).

Approximately 80% of the experimental media was renewed daily after checking fish mortality and behaviour and measuring water quality (temperature, salinity, conductivity, pH and dissolved oxygen). No food was provided during the experimental period. Water samples were collected daily (at 0 and 24 h) from each experimental aquarium for the gold quantification.

After 96 h exposure, animals were anesthetized with tricaine methanesulfonate (MS-222) and euthanized by spinal section. Liver was removed from six fish and stored at -80ºC until proteome analysis. Liver, gills, spleen and muscle were taken from three animals and kept at -20ºC until gold quantification.

2.4. Gold quantification

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

standards. The instrument was tuned daily for maximum signal sensitivity and stability.

2.5. Total gold content and bioaccumulation factor

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

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

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 $[Au]_{ms}$ the concentration of gold in muscle.

The bioaccumulation factor (BAF), in $L.g^{-1}$, was calculated according to Yoo-Iam et al (2014), 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 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).

2.6. Liver proteome analysis

2.6.1. Protein extraction and purification

Proteins were extracted from liver samples following a protocol adapted from Campos et al. (2013). Briefly, each liver tissue (~ 0.1 g) was mixed in 1 mL of extraction buffer (7 M urea, 2 M thiourea, 4% (w/v) CHAPS, 65 Mm DTT (1,4-dithiothreitol), 0.8% (v/v) ampholytes IPG (immobilized pH gradient) Buffer pH 3-10 and 1% (v/v) protease inhibitor), homogenized with a probe sonicator (Vibra-CellTM, Sonics & Materials) and incubated for 1 h, under agitation, at room temperature. After centrifugation (17 000 g, for 10 min, at room temperature) proteins were precipitated with a solution containing 10% (w/v) trichloroacetic acid

(TCA), acetone and 0.07% (v/v) β-mercaptoethanol (β-ME) in a 1:10 (v/v) ratio of sample and precipitation solution. This mixture was left at -20˚C for 1 h and then centrifuged (16 000 g for 20 min at 4˚C). After discarding the supernatant, the protein pellet was washed twice with 0.07% β-ME in acetone. Afterwards, the protein pellet was allowed to dry at room temperature for 1 h, resuspended in rehydration buffer (7 M urea, 2 M thiourea, 4% w/v CHAPS, 65 mM DTT and 0.8 % v/v ampholytes of a pH range of 3-10) with agitation for 20 min and centrifuged (15 000 g for 20 min at room temperature). The supernatant was then recovered and stored at -20˚C. Protein concentration was determined according to the Bradford method, adapted to microplate, using bovine serum albumin (BSA) as standard.

2.6.2. Two-dimensional gel electrophoresis (2-DE)

Protein extracts (400 µg) were diluted in 300 µL rehydration buffer and applied to 17 cm (pH 3-10) ReadyStrip IPG Gel Strips (Bio-Rad). The first dimension (isoelectric focusing) was carried out in a Protean IEF Cell (Bio-Rad). The gel strips were actively rehydrated for 14 h (50 V). After rehydration, voltage was set at constant 250 V for 15 min, followed by a linear increase to 10 000 V for 3 h and then a linear increase until reaching 60 000 V for the complete separation of proteins. A 500 V was applied to the system until the gel strips were stored at -20ºC.

IPG gel strips were incubated, for 15 min, in equilibration solution 1 (50 mM Tris-HCl, 6 M urea, 30% (v/v) glycerol, 2% (w/v) sodium dodecyl sulfate (SDS) and 1% (w/v) DTT under slow agitation. The solution was drained and gel strips incubated again with equilibration solution 2 (50 mM Tris-HCl, 6 M urea, 30% (v/v) glycerol, 2% (w/v) SDS and 2.5% (w/v) iodoacetamide) for 15 min. Completed this step, the gel strips were washed in electrophoresis buffer (24.8 mM Tris-HCl, 192 mM glycine and 0.1% (w/v) SDS) and assembled in SDS-PAGE gels. The second-dimension (SDS-PAGE – Sodium dodecyl sulfate polyacrylamide gel electrophoresis) was performed in 12% (w/v) acrylamide gels, using a Hoefer SE900 multi-gel system (Hoefer, Inc.). The electrophoresis ran overnight, with constant voltage (80 V) at 16˚C. When second dimension finished, gels were

stained with Coomassie Blue Colloidal stain, according to Neuhoff et al. (1988) (Volker et al. 1988).

2.6.3. Quantitative analysis of gel images and statistical analysis

Gels were scanned using a GS-800 Calibrated Densitometer (Bio-Rad). To analyse differences between the five conditions (control and the treatments with AuNPs) in the proteins pattern, the PDQuest 8.0 software (Bio-Rad) was used. An initial automatic detection and matching of the protein spots, applying the same sensitivity parameters for all gels, followed by manual inspection of matched spots were performed. Mismatched protein spots were then corrected, and spot artefacts eliminated from the analysis. Four 2-DE gels per experimental condition were analysed (n = 4). Protein spot densities were normalized by the software, according to their total density. The spot densities were subsequently used as a measure of protein abundance in the sample.

Only spots exhibiting abundance ratios of at least 3.0-fold change with respect to the control were considered. To increase the confidence of this analysis, spots without quantitative information in at least three gel replicates in each condition were ignored. Additionally, one-way analysis of variance (ANOVA) followed by Dunnett's test, whenever applicable, was performed using JMP 12 software (SAS Institute, USA). Significant differences were assumed for $p<0.05$.

2.6.4. Protein identification

Protein spots were excised from 2-DE gels and proteins subjected to in-gel digestion using the protease trypsin (Pandey and Mann 2000). The tryptic digests were desalted, concentrated using reversed phase micro-columns (Gobom et al. 1999) and directly eluted onto the matrix-assisted laser desorption/ionization (MALDI) plate with the matrix α-cyano-4-hydroxycinamic acid (5 $mg.mL^{-1}$) in 70% acetonitrile (v/v) and 0.1% trifluoroacetic acid (v/v). Peptides were analysed by MALDI-TOF (matrix assisted laser desorption/ionisation time of flight; 4800 Proteomics Analyser, SCIEX, FosterCity,CA, USA) in MS (mass spectrometry) and MS/MS (tandem mass spectrometry) mode. The ten S/N (signal-to-noise ratio) best precursors from each MS spectrum were selected for MS/MS analysis. The

generated mass spectra were searched against all sequences from species of the class Actinopterygii available in the Uniprot database (1434448 sequences, January 2018) or the *Danio rerio* sequences, using the algorithm MOWSE, from MASCOT server 2.3 (Matrix-Science). Two trypsin missed cleavages, carbamidomethylation of cysteine as fixed modification as well as four dynamic modifications (methionine and tryptophan oxidation, tryptophan dioxydation and tryptophan tokynurenin) were allowed. Mass accuracy was set to 100 ppm for parent ions and 0.5 Da for MS/MS fragments. Homology identification was retained with probability set at 95%.

3. Results and Discussion

3.1. Gold nanoparticles (AuNPs) – Characterisation

Microscopy analysis confirmed that AuNPs presented an approximately spherical shape (Figure 1), the expected sizes (around 7 and 40 nm) and allowed the visualization of a PVP layer around the metal core of AuNPs (Figure 1B and D).

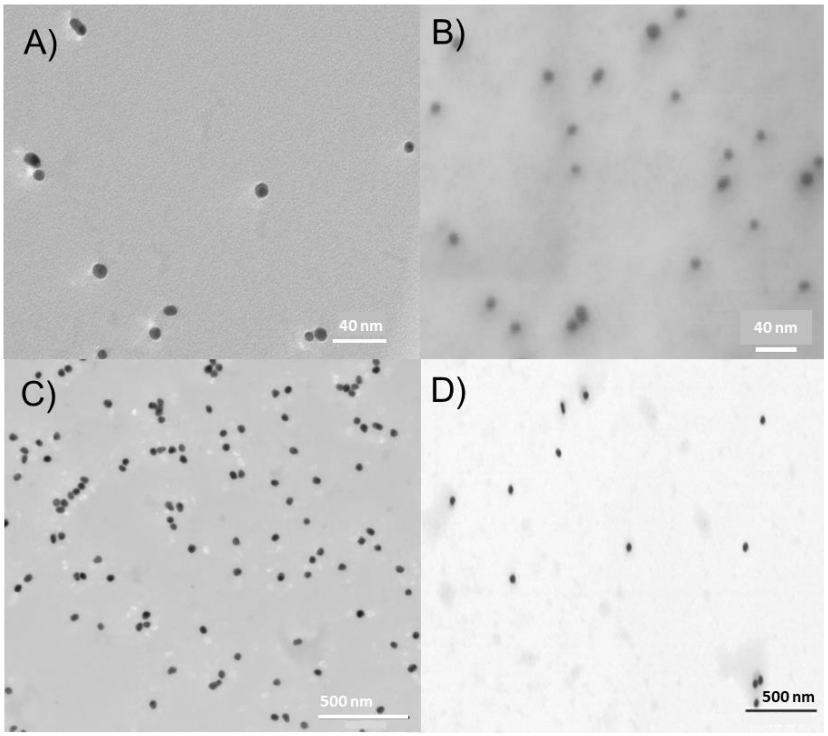

Figure 1 – Electron microscopy images of 7 and 40 nm citrate (cAuNPs) and polyvinylpyrrolidone (PVP-AuNPs) gold nanoparticles: **A)** 7 nm cAuNPs; **B)** 7 nm PVP-AuNPs; **C)** 40 nm cAuNPs; **D)** 40 nm PVP-AuNPs.

The characteristics of AuNPs used in the present study are shown in Table 1.

Table 1. Characteristics of gold nanoparticles (AuNPs) stock suspensions. cAuNPs – Citrate coated gold nanoparticles; PVP-AuNPs – Polyvinylpyrrolidone coated gold nanoparticles; PdI – Polydispersity Index; SPR – Surface Plasmon Resonance; ZP – Zeta Potential.

	Concentration ($mg.L^{-1}$)	Size (nm)	PdI	SPR (nm)	ZP (mV)	pH
7 nm cAuNPs	98	6.7	0.5	519	-43.3	6.4
7 nm PVP-AuNPs	51	7.6	0.5	521	-12.8	6.9
40 nm cAuNPs	97	35.0	0.3	534	-44.1	5.9
40 nm PVP-AuNPs	58	50.3	0.3	535	-17.2	6.4

At the tested concentration, 80 $\mu g.L^{-1}$, it was not possible to characterise the AuNPs due to the detection limits of the techniques used. However, it was possible see that 7 nm cAuNPs when in ASW, immediately changed its typical colour red to light blue, as a result of AuNPs agglomeration/aggregation, whereas 7 nm PVP-AuNPs did not show colour alteration. Concerning 40 nm AuNPs, it was not possible detect if the colour changed or not when they were added to ASW. This may be explained by the fact that, for the same concentration of AuNPs, a higher number of particles are in suspension in lower sizes. The NPs agglomeration/aggregation are expected to increase with the increase in the number of particles per volume (Barreto et al. 2015). Another important aspect is that the surface energy of AuNPs increases as the diameter decreases. Thus, smaller AuNPs may interact more strongly with the compounds present in the medium leading to size-dependent aggregation/agglomeration of AuNPs (Iswarya et al. 2016; Zeng et al. 2012). A previous study (Barreto et al. 2015) demonstrated that PVP-AuNPs were stable in ASW for more than 30 d whereas cAuNPs immediately altered their characteristics and aggregated/agglomerated, increasing their size. These characteristics (size and surface coating) may thus influence their bioavailability, accumulation and effects to the organisms.

3.2. Gold quantification in the test media

The gold concentrations in experimental media (ASW) are presented in Table 2.

Table 2. Measured concentrations (µg.L^{-1}) of 7 and 40 nm gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) in the experimental media (artificial seawater) at 0 and 24 h. Results are expressed as mean ± standard error.

Size AuNPs (nm)	Time (h)	Measured concentrations (µg.L^{-1}) cAuNPs	PVP-AuNPs
7	**0**	20.6 ± 0.1	22.7 ± 0.2
	24	8.4 ± 0.1	18.7 ± 0.1
40	**0**	10.1 ± 0.1	22.5 ± 0.1
	24	6.4 ± 0.1	16.5 ± 0.2

The amount of gold quantified in the experimental media (ASW) was generally lower than the nominal concentration (80 µg.L^{-1}). At 0 h, the measured concentrations of gold were around 20 µg.L^{-1}, with exception to 40 nm cAuNPs which were quantified as 10 µg.L^{-1}. After 24 h of exposure, the concentration of cAuNPs in suspension decreased more than the concentration of PVP-AuNPs, compared with 0 h (Table 2). For 7 nm cAuNPs and PVP-AuNPs, a 59 and 18% decrease was respectively found. For 40 nm cAuNPs and PVP-AuNPs, the concentrations of gold decreased by 37 and 27% respectively, after 24 h exposure.

The higher decrease of gold in suspension in ASW after 24 h observed in the exposures to cAuNPs, may be explained by the aggregation/agglomeration of these particles and subsequent sedimentation. As PVP-AuNPs may remain stable in ASW, the concentration of gold in suspension in ASW after 24 h was closer to the initial concentration than for cAuNPs.

3.3. Total gold content and bioaccumulation factor

As shown in Table 3, gold significantly accumulated in all assessed tissues (gills, liver, spleen and muscle) after exposure to 7 nm PVP-AuNPs ($p<0.05$; Dunnett's test). The exposure to 7 nm cAuNPs and 40 nm PVP-AuNPs also

resulted in significant gold accumulation in all the assessed tissues with the exception to muscle ($p<0.05$; Dunnett's test; Table 3). However, for 40 nm cAuNPs, gold only significantly accumulated in the liver of *S. aurata* ($p<0.05$; Dunnett's test; Table 3).

Table 3. Gold concentration in tissues of *Sparus aurata* (gills, liver, spleen and muscle) exposed to 7 and 40 nm gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs) for 96 h and respective estimated bioaccumulation factor (BAF). Results are expressed as mean ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$). $[Au]_{total}$ – Total gold content. b.d.l. – Bellow the detection limit.

Size AuNPs (nm)	Tissues	Gold Content ($\mu g.g^{-1}$)		BAF ($L.g^{-1}$)	
		cAuNPs	PVP-AuNPs	cAuNPs	PVP-AuNPs
7	**Gills**	1.9 ± 0.1 *	6.3 ± 0.1 *	0.1	0.3
	Liver	7.8 ± 0.1 *	9.8 ± 0.1 *	0.4	0.4
	Spleen	17.4 ± 0.2 *	15.8 ± 0.1 *	0.8	0.7
	Muscle	b.d.l.	2.2 ± 0.1 *	-	0.1
	[Au]total	**27.1 ± 0.1**	**34.1 ± 0.1**	**1.3**	**1.5**
40	**Gills**	0.11 ± 0.0	3.6 ± 0.1 *	0.0	0.2
	Liver	0.7 ± 0.0 *	1.4 ± 0.1 *	0.1	0.1
	Spleen	b.d.l.	17.7 ± 0.1 *	-	0.8
	Muscle	b.d.l.	b.d.l.	-	-
	[Au]total	**0.8 ± 0.1**	**22.7 ± 0.0**	**0.1**	**1.1**

Overall, taking into account all the exposures, the accumulation of gold may be ranked as spleen>liver>gills>muscle. The $[Au]_{total}$ values and the calculated BAF were higher for 7 nm AuNPs than 40 nm AuNPs and may be ranked as follow 7 nm PVP-AuNPs>7 nm cAuNPs>40 nm PVP-AuNPs>40 nm cAuNPs – Table 3. Taking into account the AuNPs tested sizes, the accumulation of gold was higher after the exposure to the smallest tested AuNPs – 7 nm. It has already been described that the NPs size may influence its accumulation on the organisms with smallest AuNPs showing higher levels of accumulation on the cells (Bajak et al. 2015; Huang et al. 2012).

The greater accumulation of gold in tissues when fish were exposed to PVP-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. On the contrary, for cAuNPs, aggregates/agglomerates may deposit on the tanks' bottom, leading to a lower concentration of AuNPs in the water column and, consequently, a lower uptake by fish. As previously reported, when aggregates become too large for direct transport across the cell membrane, uptake may be reduced (Vale et al. 2016).

3.4. Two-dimensional gel electrophoresis (2-DE) gels

Twenty 2-DE gels (n=4 per condition) were performed to analyse the effects of AuNPs in the liver proteome of *S. aurata* after 96 h exposure. An average of 632 protein spots were detected from these gels which may be considered a reasonable number taking in consideration the methodology used. It is recognized that conventional 2-DE gels enable to resolve, with good accuracy, between 800-1000 protein spots (Osório et al. 2017).

Regarding gel quality, all gels showed similar staining intensities meaning that gels were correctly normalized in terms of protein amount (equal amount of protein loaded in each gel). The detected spots were mostly located in gel areas corresponding to proteins with high isoelectric point (pI) between 7 and 10. In terms of molecular mass, most of the detected proteins have between 20 to 70 kDa. This protein profile is consistent with a previous one in which 2-DE enabled to resolve 564 proteins along a pI gradient of 3 to 10 and molecular masses of 19 to 115 kDa, also from the liver of *S. aurata* (Rufino-Palomares et al. 2011).

3.5. Differential protein analysis

Of the 632 spots detected, 26 exhibited differences in abundance (either 3.0-fold changes in regard to control or statistical differences at $p<0.05$). The 26 proteins displaying differences in abundance are marked in a reference 2-DE gel from the control group – Figure 2, being 16 proteins over-abundant and 9 under-abundant (Table 4).

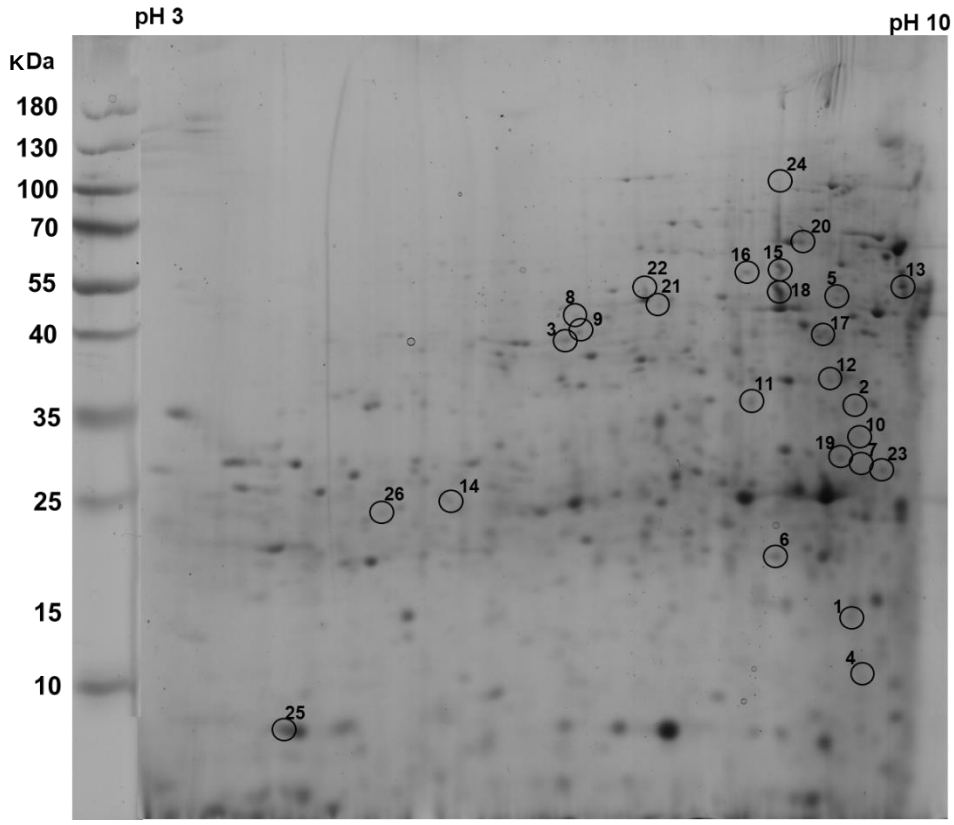

Figure 2. Proteomic map of *Sparus aurata* liver. Protein identities corresponding to the numbers indicated in the figure are reported in Table 4. Proteins were separated in the first dimension with pH 3–10 (IPG) immobilized pH gradient strips, followed by SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) on 12% w/v gels. Gels were stained with Coomassie Blue Colloidal. The 26 spots excised for MALDI-TOF MS (matrix assisted laser desorption/ionisation time of flight mass spectrometry) analysis are encircled.

One protein increased in abundance by 7 nm PVP-AuNPs but decreased by 40 nm cAuNPs. The abundance of seven proteins was altered at least for two of the tested conditions with, for instance, one protein being more abundant after the exposure to all the conditions comparing with the control (Table 4).

Table 4. Proteins displaying differences in abundance in *Sparus aurata* liver after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), assessed by 2-DE. Values of protein expression are represented as mean spot density in gel ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$) $^{\#}$3.0-fold changes in regard to control. Proteins identified with *Danio rerio* and Actinopterygii databases. (1) Uniprot database accession numbers. ID – Identification; MS – Mass Spectrometry; MS/MS – Tandem-Mass Spectrometry.

		Protein Expression									Matched Peptides	
Protein Number	Gene ID	Control	7 nm cAuNPs	7 nm PVP-AuNPs	40 nm cAuNPs	40 nm PVP-AuNPs	Protein Name	Accession Number (1)	Species	Protein Score	MS	MS/MS
1	*EEF1G*	1129.3 ± 287.4	2935.7 ± 1462.8*****	726.2 ± 350.8	1250.3 ± 299.3	459.6 ± 279.2	Elongation factor 1-gamma	A0A0F8C4B3	*Larimichthys crocea*	80	58	0
2	GRP-94	694.8 ± 201.3	1129.1 ± 835.6*****	518.8 ± 303.7	254.4 ± 207.7	295.0 ± 119.4	94 kDa glucose-regulated protein	M9NZ74	*Sparus aurata*	200	34	4
3	pck2	805.0 ± 206.8	1858.1 ± 559.2*****	1110.1 ± 654.5	1851.8 ± 882.2*	707.7 ± 181.1	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	F1R9Y5	*Danio rerio*	73	22	1
4	gkup	695.2 ± 349.5	525.2 ± 177.3	10.2 ± 10.2*****	955.1 ± 417.9	594.0 ± 442.2	Glucuronokinase with putative uridyl pyrophosphorylase	A0A0R4IGN7	*Danio rerio*	86	34	0
5	atp5f1b	451.4 ± 168.5	247.6 ± 148.4	1291.2 ± 798.7	2056.9 ± 1088.7*****	302.2 ± 175.5	ATP synthase subunit beta	A8WGC6	*Danio rerio*	396	30	6
6	shmt2	926.5 ± 321.9	277.1 ± 277.1	1640.8 ± 1057.9	1245.3 ± 426.4	174.9 ± 76.9*****	Mitochondrial serine hydroxymethyltransferase	A9LDD9	*Danio rerio*	95	17	3
7	CPA	0.0 ± 0.0	226.4 ± 226.4	923.7 ± 319.5***#**	135.5 ± 135.5	0.0 ± 0.0	Carboxypeptidase	G3NFY9	*Gasterosteus aculeatus*	144	7	2
8	pcxb	0.0 ± 0.0	0.0 ± 0.0	50.6 ± 50.6	1787.4 ± 117.3***#**	100.4 ± 100.4	Pyruvate carboxylase b	B0S5R6	*Danio rerio*	71	19	1
9	selenbp1	277.7 ± 109.4	88.1 ± 88.1	644.7 ± 406.0*	621.2 ± 441.2*****	549.7 ± 120.6	Selenium-binding protein 1	Q6PHD9	*Danio rerio*	138	11	3

Table 4 (continuation). Proteins displaying differences in abundance in *Sparus aurata* liver after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), assessed by 2-DE. Values of protein expression are represented as mean spot density in gel ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$) #3.0-fold changes in regard to control. Proteins identified with *Danio rerio* and Actinopterygii databases. (1) Uniprot database accession numbers. ID – Identification; MS – Mass Spectrometry; MS/MS – Tandem-Mass Spectrometry.

		Protein Expression									Matched Peptides	
Protein Number	**Gene ID**	**Control**	**7 nm cAuNPs**	**7 nm PVP-AuNPs**	**40 nm cAuNPs**	**40 nm PVP-AuNPs**	**Protein Name**	**Accession Number (1)**	**Species**	**Protein Score**	**MS**	**MS/MS**
13	calr	3174.4 ± 1340.4	1099.5 ± 476.3	142.3 ± 142.3*	1696.5 ± 660.1	733.5 ± 468.1	Calreticulin	F1Q8W8	*Danio rerio*	110	12	1
14	bhmt	101.0 ± 101.0	1100.4 ± 382.7*	223.8 ± 223.8	666.1 ± 262.3	250.9 ± 150.2	Betaine--homocysteine S-methyltransferase 1	F1QU55	*Danio rerio*	156	16	2
15	actba	2490.9 ± 371.4	902.7 ± 662.3	3935.9 ± 2108.4*	1933.9 ± 1378.2*	774.8 ± 366.2	Actin, cytoplasmic 1	Q7ZVI7	*Danio rerio*	528	22	7
16	actba	2794.4 ± 2523.6	285.5 ± 285.5*	0.0 ± 0.0*	0.0 ± 0.0*	58.1 ± 58.1*	Actin, cytoplasmic 1	Q7ZVI7	*Danio rerio*	222	19	4
17	actba	938.7 ± 592.3	979.8 ± 575.1	3794.2 ± 1889.1*	4371.8 ± 2139.1*	1559.7 ± 1258.0	Actin, cytoplasmic 1	Q7ZVI7	*Danio rerio*	309	15	5
18	actba	2490.9 ± 1259.5	5749.4 ± 2359.8	6084.7 ± 2182.6*	4040.1 ± 1586.1	2275.9 ± 385.7	Actin, cytoplasmic 1	Q7ZVI7	*Danio rerio*	282	24	4
19	actbb	622.8 ± 68.7	486.6 ± 301.4	195.8 ± 195.8	682.6 ± 682.6*	130.0 ± 130.0	Actin, cytoplasmic 2	Q7ZVF9	*Danio rerio*	74	16	1
20	actbb	589.1 ± 239.2	1228.5 ± 1020.7*	292.7 ± 292.7	298.3 ± 298.3	102.1 ± 102.1	Actin, cytoplasmic 2	Q7ZVF9	*Danio rerio*	84	22	1
21	actbb	552.8 ± 494.1	235.4 ± 146.7*	0.0 ± 0*	0.0 ± 0.0*	78.2 ± 78.2*	Actin, cytoplasmic 2	Q7ZVF9	*Danio rerio*	84	18	2

Table 4 (continuation). Proteins displaying differences in abundance in *Sparus aurata* liver after 96 h exposure to gold nanoparticles (citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs), assessed by 2-DE. Values of protein expression are represented as mean spot density in gel ± standard error. *Significant differences to control (Dunnett´s test, $p<0.05$) $^{\#}$3.0-fold changes in regard to control. Proteins identified with *Danio rerio* and Actinopterygii databases. (1) Uniprot database accession numbers. ID – Identification; MS – Mass Spectrometry; MS/MS – Tandem-Mass Spectrometry.

		Protein Expression									Matched Peptides	
Protein Number	**Gene ID**	**Control**	**7 nm cAuNPs**	**7 nm PVP-AuNPs**	**40 nm cAuNPs**	**40 nm PVP-AuNPs**	**Protein Name**	**Accession Number (1)**	**Species**	**Protein Score**	**MS**	**MS/MS**
22	fah	922.8 ± 536.0	409.7 ± 541.5*****	333.9 ± 270.8	109.5 ± 109.5	174.9 ± 112.8*****	Fumarylacetoacetate hydrolase (Fumarylacetoacetase)	Q803S0	*Danio rerio*	165	14	3
23	*HPCA*	995.5 ± 995.5	722.4 ± 487.6	641.4 ± 641.4	905.5 ± 644.0	986.8 ± 437.0*****	Hippocalcin	I3JLG1	*Oreochromis niloticus*	64	16	1
24	fgf1b	100.1 ± 58.5	943.4 ± 361.4*****	158.0 ± 158.0	231.1 ± 231.1	61.8 ± 61.8	Fibroblast growth factor	A7YT71	*Danio rerio*	64	16	0
25	*PPIA*	2107.6 ± 899.0	15.8 ± 15.8*****	973.2 ± 973.2	1957.8 ± 1131.0	939.2 ± 670.5	Peptidyl-prolyl cis-trans isomerase	Q4S1X7	*Tetraodon nigroviridis*	230	12	2
26		558.7 ± 234.1	377.6 ± 222.6	0.0 ± 0.0***#**	0.0 ± 0.0***#**	0.0 ± 0.0*#	Uncharacterised protein	A0A0E9WUZ5	*Anguilla anguilla*	62	10	0

Considering each tested condition: eight proteins were more abundant and three were less abundant after exposure to 7 nm cAuNPs; four proteins were more abundant and five were less abundant after exposure to 7 nm cAuNPs; six proteins increased in abundance and four decreased in abundance after exposure to 7 nm cAuNPs; two proteins were more abundant and four were less abundant after exposure to 7 nm cAuNPs. cAuNPs led to a higher number of proteins with increased abundance whereas PVP-AuNPs led to a higher number of proteins with decreased abundance.

In terms of effects produced in liver proteome of *S. aurata* compared with the control group, the tested AuNPs may be ranked as 7 nm cAuNPs>40 nm cAuNPs>7 nm PVP-AuNPs>40 nm PVP-AuNPs, with eleven proteins displaying differences in abundance after the exposure to 7 nm cAuNPs; ten by 40 nm cAuNPs; nine by 7 nm PVP-AuNPs and six by 40 nm PVP-AuNPs. Although 7 nm PVP-AuNPs accumulated more in the assessed tissues of *S. aurata,* including liver, 7 nm cAuNPs induced more effects in the fish liver proteome. It seems that the coating was the characteristic that had more influence on the effects of AuNPs comparing with size and behaviour of AuNPs in ASW. Overall, cAuNPs induced more effects than PVP-AuNPs. A previous study in the Caco-2 cells' proteome showed that effects of AuNPs exposure were size specific, with 5 nm AuNPs inducing more proteins displaying differences in abundance compared with the 30 nm AuNPs (Gioria et al. 2016). It was also described that 88 and 83 proteins displayed differences in abundance in Balb/3T3 mouse fibroblast cell line after exposure to 5 and 15 nm AuNPs, respectively (Gioria et al. 2014). No study was found analysing the fish liver proteome after the exposure to AuNPs. However, previous studies with others NPs showed that abundance of several proteins of the liver of fish *Cyprinus carpio* and *Oncorhynchus mykiss* was altered after the exposure to copper and selenium NPs, respectively (Gupta et al., 2016; Naderi et al., 2017).

3.6. Protein identification

MALDI-TOF analysis retrieved MS and MS/MS information that were combined and used to search in the protein database UNIPROT the identity of the proteins

affected by AuNPs. Most of proteins of interest were identified by this approach, except one that could not be identified even after performing a homology search with all protein sequences from the taxonomic class Actinopterygii. The results of this analysis are shown in Table 4. Most of the identified proteins were structural (actins and tubulins) which is expected as structural proteins are among the major constituents of the biological tissues and organs including liver. Nevertheless, other proteins playing different functions in liver cells were identified such as calreticulin, 94 kDa glucose-regulated protein, pyruvate carboxylase b, phosphoenolpyruvate carboxykinase 2, cytohesin-1 and hippocalcin (Table 4). More information regarding the identification of the proteins is shown in supplementary information (Table S1). As in the present study, abundance of actins was one of the most altered after 72 h exposure to 5 and 30 nm AuNPs in Caco-2 cells (Gioria et al. 2016).

Concerning biological functions of the proteins displaying abundance differences, some are putatively involved in protein biosynthesis, folding or transport (elongation factor 1-gamma, selenium-binding protein 1, calreticulin, peptidyl-prolyl cis-trans isomerase), others in microtubule-based process (tubulin), cell structure, motility and membrane organization (actins) – Table 5. Proteins involved in cellular response to calcium ion (hippocalcin) and involved in gluconeogenesis, glycine and ATP synthesis (mitochondrial phosphoenolpyruvate carboxykinase 2, mitochondrial serine hydroxymethyltransferase and ATP synthase subunit beta) were also identified. Moreover, a putative 94 kDa glucose-regulated protein (GRP-94), involved in the response of the organisms to stress, and pyruvate carboxylase b, involved in the response to metals, were also identified.

Table 5. Molecular function and biological process of proteins displaying differences in abundance in *Sparus aurata* liver after 96 h exposure to gold nanoparticles. Proteins identified with *Danio rerio* and Actinopterygii databases. cAuNPs – Citrate coated gold nanoparticles; PVP-AuNPs – Polyvinylpyrrolidone coated gold nanoparticles.

Protein Number	Protein Name	Accession Number	Molecular Function	Biological Process	Type of AuNPs which induced effects
1	Elongation factor 1-gamma	A0A0F8C4B3	- Elongation factor	- Protein biosynthesis	7 nm cAuNPs
9	Selenium-binding protein 1	Q6PHD9	- Methanethiol oxidase activity - Selenium binding	- Protein transport	40 nm cAuNPs
2	94 kDa glucose-regulated protein	M9NZ74	- ATP binding - Unfolded protein binding	- Protein folding - Response to stress	7 nm cAuNPs
13	Calreticulin	F1Q8W8	- Calcium ion binding - Unfolded protein binding	- Protein folding	7 nm PVP-AuNPs
25	Peptidyl-prolyl cis-trans isomerase	Q4S1X7	- Peptidyl-prolyl cis-trans isomerase activity	- Protein folding	7 nm cAuNPs
7	Carboxypeptidase	G3NFY9	- Serine-type carboxypeptidase activity	- Protein catabolism	7 nm PVP-AuNPs
12	Cytohesin-1	A0A146RY54	- ARF guanyl-nucleotide exchange factor activity - Phospholipid binding	- Regulation of ARF protein signal transduction	7 nm cAuNPs
3	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	F1R9Y5	- GTP binding - Phosphoenolpyruvate carboxykinase (GTP) activity	- Gluconeogenesis	7 and 40 nm cAuNPs
4	Glucuronokinase with putative uridyl pyrophosphorylase	A0A0R4IGN7	- Glucuronokinase activity - Nucleotidyltransferase activity	- Biosynthetic process - Ascorbate metabolism	7 nm PVP-AuNPs
6	Mitochondrial serine hydroxymethyltransferase	A9LDD9	- Lycine hydroxymethyltransferase activity - Ethyltransferase activity - Pyridoxal phosphate binding	- Glycine biosynthetic process from serine - Tetrahydrofolate interconversion	40 nm PVP-AuNPs
5	ATP synthase subunit beta	A8WGC6	- ATP binding - Proton-transporting ATP synthase activity, rotational mechanism	- ATP synthesis coupled proton transport	40 nm cAuNPs
14	Betaine-homocysteine S-methyltransferase 1	F1QU55	- Betaine-homocysteine S-methyltransferase activity - Zinc ion binding	- Methionine biosynthetic process	7 nm cAuNPs
22	Fumarylacetoacetate hydrolase (Fumarylacetoacetase)	Q803S0	- Fumarylacetoacetase activity	- Aromatic amino acid family metabolic process	7 nm cAuNPs; 40 nm PVP-AuNPs
8	Pyruvate carboxylase b	B0S5R6	- ATP binding - Metal ion binding	- Response to cadmium ion	40 nm cAuNPs
23	Hippocalcin	I3JLG1	- Calcium ion binding	- Cellular response to calcium ion	40 nm PVP-AuNPs

Table 5 (continuation). Molecular function and biological process of proteins displaying differences in abundance in *Sparus aurata* liver after 96 h exposure to gold nanoparticles. Proteins identified with *Danio rerio* and Actinopterygii databases. cAuNPs – Citrate coated gold nanoparticles; PVP-AuNPs – Polyvinylpyrrolidone coated gold nanoparticles.

Protein Number	Protein Name	Accession Number	Molecular Function	Biological Process	Type of AuNPs which induced effects
10	Tubulin beta chain	Q32PU7	- GTPase activity - GTP binding - Structural constituent of cytoskeleton	- Microtubule-based process	7 nm cAuNPs
11	Tubulin beta chain	Q32PU7	- GTPase activity - GTP binding - Structural constituent of cytoskeleton	- Microtubule-based process	40 nm PVP-AuNPs
15	Actin, cytoplasmic 1	Q7ZVI7	- ATP binding	- Cell structure, cell junction assembly, cell motility, membrane organization	7 nm PVP-AuNPs; 40 nm cAuNPs
16	Actin, cytoplasmic 1	Q7ZVI7	- ATP binding	- Cell structure, cell junction assembly, cell motility, membrane organization	All tested types.
17	Actin, cytoplasmic 1	Q7ZVI7	- ATP binding	- Cell structure, cell junction assembly, cell motility, membrane organization	7 nm PVP-AuNPs; 40 nm cAuNPs
18	Actin, cytoplasmic 1	Q7ZVI7	- ATP binding	- Cell structure, cell junction assembly, cell motility, membrane organization	7 nm PVP-AuNPs
19	Actin, cytoplasmic 2	Q7ZVF9	- ATP binding	- Cell structure, cell junction assembly, cell motility, membrane organization	40 nm cAuNPs
20	Actin, cytoplasmic 2	Q7ZVF9	- ATP binding	- Cell structure, cell junction assembly, cell motility, membrane organization	7 nm cAuNPs
21	Actin, cytoplasmic 2	Q7ZVF9	- ATP binding	- Cell structure, cell junction assembly, cell motility, membrane organization	All tested types

Table 5 (continuation). Molecular function and biological process of proteins displaying differences in abundance in *Sparus aurata* liver after 96 h exposure to gold nanoparticles. Proteins identified with *Danio rerio* and Actinopterygii databases. cAuNPs – Citrate coated gold nanoparticles; PVP-AuNPs – Polyvinylpyrrolidone coated gold nanoparticles.

Protein Number	Protein Name	Accession Number	Molecular Function	Biological Process	Type of AuNPs which induced effects
24	Fibroblast growth factor	A7YT71	- Fibroblast growth factor receptor binding - Growth factor activity - Heparin binding	- Angiogenesis - Cell differentiation - Fibroblast growth factor receptor signaling pathway - Positive regulation of cell division	7 nm cAuNPs
26	Uncharacterised protein	A0A0E9WUZ5			7 nm PVP-AuNPs; 40 nm cAuNPs; 40 nm PVP-AuNPs

Different proteins were affected in this study which means that the AuNPs are most likely affecting different metabolic pathways in liver cells, being the effects dependent on the NPs characteristics. The only common target of all tested AuNPs was proteins involved in cell morphology – actins – being over or under-abundant after the exposure to 7 nm AuNPs and 40 nm cAuNPs and under-abundant by 40 nm PVP-AuNPs.

Analysing each tested condition, 7 nm cAuNPs led to an increase in abundance of proteins involved in protein biosynthesis (elongation factor 1-gamma), response to stress (94 kDa glucose-regulated protein), gluconeogenesis (phosphoenolpyruvate carboxykinase 2), microtubule-based process (tubulin beta chain), regulation of ARF protein signal transduction (cytohesin-1), methionine biosynthetic process (betaine-homocysteine S-methyltransferase 1), cell differentiation (fibroblast growth factor) and to a decrease in abundance of proteins involved in protein folding (peptidyl-prolyl cis-trans isomerase) and aromatic amino acid metabolism (fumarylacetoacetate hydrolase). Several actin isoforms were affected (increased or decreased in abundance) with the exposure to 7 cAuNPs – Table 5. The exposure to 40 nm cAuNPs led to the increase in abundance of

proteins involved in protein transport (selenium-binding protein 1), gluconeogenesis (phosphoenolpyruvate carboxykinase 2), ATP synthesis (ATP synthase subunit beta), response to metals (pyruvate carboxylase b) and also affected the abundance (increased or decreased in abundance) of actins (Table 5). The 7 nm PVP-AuNPs decreased the abundance of proteins involved in protein folding (calreticulin), ascorbate metabolism (glucuronokinase with putative uridyl pyrophosphorylase) and in cell morphology (actins). However, increased abundance of actins was also detected after the exposure to 7 nm PVP-AuNPs (Table 5). The 40 nm PVP-AuNPs decreased the abundance of proteins involved in glycine biosynthetic process (mitochondrial serine hydroxymethyltransferase), aromatic amino acid metabolism (fumarylacetoacetate hydrolase), cellular response to calcium ion (hippocalcin), cell morphology (actins) and increased the abundance of proteins involved in microtubule-based process (tubulin beta chain) – Table 5.

A previous study with 5 and 30 nm AuNPs 72 h exposure in Caco-2 cells reported a decrease in abundance of proteins associated with cellular growth and proliferation, whereas proteins found mostly over-abundant were involved in antioxidant activity and apoptosis (Gioria et al. 2016). In the same study, proteins involved in protein synthesis and amino acid transport were under or over-abundant (Gioria et al. 2016), as in present study, being dependent on the characteristics of AuNPs. Also, 5 and 15 nm AuNPs triggered changes in several pathways related to cellular growth and proliferation, cell morphology, cell cycle regulation, cellular function and maintenance, oxidative stress and inflammatory response in Balb/3T3 cell line (Gioria et al. 2014). Proteins such as elongation factor 1-gamma (protein biosynthesis), tubulin (microtubule-based process), peptidyl-prolyl cis-trans isomerase (protein folding), ATP synthase (ATP synthesis) displayed differences in abundance in Balb/3T3 cell line after the exposure to AuNPs (Gioria et al. 2014), as in the present study.

Overall, the proteomic approach used in the present study proved important to reveal the metabolic processes affected in the liver of *S. aurata* by NPs with different characteristics. To complement the present findings, further studies to

analyse posttranslational modifications of proteins, metabolome or transcriptome of *S. aurata* liver after the exposure to different AuNPs are encouraged.

4. Conclusions

The present work reveals the proteomic changes of *Sparus aurata* liver after the exposure to different gold nanoparticles (AuNPs). From the 2-DE gels analysis, 26 proteins, mainly involved in cell morphology, diverse metabolic pathways such as protein synthesis, folding and transport, oxidative stress and response to metals, presented differences in abundance (under or over-abundance) after the exposure to AuNPs. The effects were dependent on the characteristics of AuNPs, with citrate-coated AuNPs (cAuNPs) inducing more effects than polyvinylpyrrolidone-coated AuNPs (PVP-AuNPs). Despite 7 nm PVP-AuNPs accumulating more in the tissues of *S. aurata,* including liver, 7 nm cAuNPs induced more effects in the fish liver proteome than the other tested AuNPs. Proteomic approach was a sensible tool to identify subtle effects of AuNPs at a molecular level, infer the mechanisms of action of AuNPs and their putative toxicity, allowing to differentiate responses to AuNPs with different characteristics.

Conflict of interest statement

The authors declare that there are no conflicts of interest.

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Supplementary Information

Table S1. Matrix assisted laser desorption/ionisation time of flight (MALDI-TOF) identification of the proteins displaying differences in abundance in *Sparus aurata* liver after 96 h exposure to gold nanoparticles. Proteins identified using the UNIPROT database. Mr – Molecular Weight; MS – Mass Spectrometry; MS/MS – Tandem-Mass Spectrometry.

							Matched Peptides			
Protein Number	**Gene ID**	**Mr (KDa)**	**Protein Name**	**Accession Number**	**Species**	**Protein Score**	**MS**	**MS/MS**	**Ion Score**	**Peptide Sequence**
1	*EEF1G*	168,763	Elongation factor 1-gamma	A0A0F8C4B3	*Larimichthys crocea*	80	58	0	-	-
2	GRP-94	92,772	94 kDa glucose-regulated protein	M9NZ74	*Sparus aurata*	200	34	4	58 76 32 22	R.GLFDEYGSK.K K.SILFVPTSAPR.G K.GVVDSDDLPLNVSR.E K.EVEEDEYTAFYK.T
3	pck2	69,778	Phosphoenolpyruvate carboxykinase 2 (mitochondrial)	F1R9Y5	*Danio rerio*	73	22	1	51	K.IFHVNWFR.K
4	gkup	56,095	Glucuronokinase with putative uridyl pyrophosphorylase	A0A0R4IGN7	*Danio rerio*	86	34	0	-	-
5	atp5f1b	55,130	ATP synthase subunit beta	A8WGC6	*Danio rerio*	396	30	6	31 70 27 56 93 49	R.IPVGPETLGR.I K.AHGGYSVFAGVGER.T R.VALTGLTVAEYFR.D R.LVLEVAQHLGENTVR.T R.DQEGQDVLLFIDNIFR.F R.AIAELGIYPAVDPLDSTSR.I

Table S1 (continuation). MALDI-TOF identification of the proteins displaying differences in abundance in *Sparus aurata* liver after 96 h exposure to gold nanoparticles. Proteins identified using the UNIPROT database.

							Matched Peptides			
Protein Number	**Gene ID**	**Mr (KDa)**	**Protein Name**	**Accession Number**	**Species**	**Protein Score**	**MS**	**MS/MS**	**Ion Score**	**Peptide Sequence**
6	shmt2	54,439	Mitochondrial serine hydroxymethyltransferase	A9LDD9	*Danio rerio*	95	17	3	26 17 25	K.YSEGYPGKR.Y K.LIIAGTSAYAR.L R.GLELIASENFCSR.A
7	CPA	53,490	Carboxypeptidase	G3NFY9	*Gasterosteus aculeatus*	144	7	2	133 1	K.NELFLTGESYGGIYIPTLAER.V R.LFPEFSKNELFLTGESYGGIYIP TLAER.V
8	pcxb	52,015	Pyruvate carboxylase b	B0S5R6	*Danio rerio*	71	19	1	41	K.YGNVIHLYER.D
9	selenbp1	50,983	Selenium-binding protein 1	Q6PHD9	*Danio rerio*	138	11	3	28 46 49	R.LILPSLISSR.I R.EEIVYLPCIYR.N R.FLYFSNWLHGDIR.Q
10	tubb2b	49,717	Tubulin beta chain	Q32PU7	*Danio rerio*	124	21	4	21 23 42 6	R.FPGQLNADLR.K R.INVYYNEASGGK.Y K.GHYTEGAELVDSVLDVVR.K R.SGPFGQVFRPDNFVFGQSGAG NNWAK.G
11	tubb2b	49,717	Tubulin beta chain	Q32PU7	*Danio rerio*	169	20	2	69 56	R.FPGQLNADLR.K K.GHYTEGAELVDSVLDVVR.K
12	CYTH1	48,704	Cytohesin-1	A0A146RY54	*Fundulus heteroclitus*	64	28	0	-	-

Table S1 (continuation). MALDI-TOF identification of the proteins displaying differences in abundance in *Sparus aurata* liver after 96 h exposure to gold nanoparticles. Proteins identified using the UNIPROT database.

							Matched Peptides			
Protein Number	**Gene ID**	**Mr (KDa)**	**Protein Name**	**Accession Number**	**Species**	**Protein Score**	**MS**	**MS/MS**	**Ion Score**	**Peptide Sequence**
13	calr	48,640	Calreticulin	F1Q8W8	*Danio rerio*	110	12	1	94	K.YDSIGVIGLDLWQVK.S
14	bhmt	44,082	Betaine--homocysteine S-methyltransferase 1	F1QU55	*Danio rerio*	156	16	2	106 23	R.LNAGEVVIGDGGFVFALEK.R R.AGSNVMQTFTFYASDDKLENR.G
15	actba	41,767	Actin, cytoplasmic 1	Q7ZVI7	*Danio rerio*	528	22	7	36 56 33 58 104 105 61	K.AGFAGDDAPR.A R.GYSFTTTAER.E R.AVFPSIVGRPR.H K.IWHHTFYNELR.V K.SYELPDGQVITIGNER.F R.VAPEEHPVLLTEAPLNPK.A K.DLYANTVLSGGTTMYPGIADR.M
16	actba	41,767	Actin, cytoplasmic 1	Q7ZVI7	*Danio rerio*	222	19	4	30 20 74 59	R.GYSFTTTAER.E K.IWHHTFYNELR.V K.SYELPDGQVITIGNER.F R.VAPEEHPVLLTEAPLNPK.A
17	actba	41,767	Actin, cytoplasmic 1	Q7ZVI7	*Danio rerio*	309	15	5	6 59 112 94 9	R.GYSFTTTAER.E K.IWHHTFYNELR.V K.SYELPDGQVITIGNER.F R.VAPEEHPVLLTEAPLNPK.A K.DLYANTVLSGGTTMYPGIADR.M
18	Actba	41,767	Actin, cytoplasmic 1	Q7ZVI7	*Danio rerio*	282	24	4	36 94 48 18	K.IWHHTFYNELR.V K.SYELPDGQVITIGNER.F R.VAPEEHPVLLTEAPLNPK.A K.DLYANTVLSGGTTMYPGIADR.M

Table S1 (continuation). MALDI-TOF identification of the proteins displaying differences in abundance in *Sparus aurata* liver after 96 h exposure to gold nanoparticles. Proteins identified using the UNIPROT database.

							Matched Peptides			
Protein Number	**Gene ID**	**Mr (KDa)**	**Protein Name**	**Accession Number**	**Species**	**Protein Score**	**MS**	**MS/MS**	**Ion Score**	**Peptide Sequence**
19	actbb	41,753	Actin, cytoplasmic 2	Q7ZVF9	*Danio rerio*	74	16	1	47	K.SYELPDGQVITIGNER.F
20	actbb	41,753	Actin, cytoplasmic 2	Q7ZVF9	*Danio rerio*	84	22	1	52	K.SYELPDGQVITIGNER.F
21	actbb	41,753	Actin, cytoplasmic 2	Q7ZVF9	*Danio rerio*	84	18	2	9 98	K.IWHHTFYNELR.V K.SYELPDGQVITIGNER.F
22	fah	38,754	Fumarylacetoacetate hydrolase (Fumarylacetoacetase)	Q803S0	*Danio rerio*	165	14	3	34 33 87	R.LPVGYHGR.A R.DHATNVGIMFR.G R.DIQAWEYVPLGPFLGK.N
23	*HPCA*	22,317	Hippocalcin	I3JLG1	*Oreochromis niloticus*	64	16	1	5	R.QMDLNNDGKLSLEEFIKGAK.S
24	fgf1b	17,855	Fibroblast growth factor	A7YT71	*Danio rerio*	64	16	0	-	-
25	*PPIA*	17,732	Peptidyl-prolyl cis-trans isomerase	Q4S1X7	*Tetraodon nigroviridis*	230	12	2	69 141	K.FADENFQLK.H K.HVVFGKVVEGIDVVK.A
26		5,067	Uncharacterised protein	A0A0E9WUZ5	*Anguilla anguilla*	62	10	0	-	-

1. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

1.1. Effects of gold nanoparticles

The present thesis has provided information about the possible effects and bioaccumulation of waterborne gold nanoparticles (AuNPs), to an economically important predatory marine fish species, the seabream *Sparus aurata*. Until the present there was limited and conflicting information concerning toxic effects of gold nanoparticles (AuNPs) and a scarcity of studies on the effects of AuNPs to marine organisms, particularly fish species.

The overarching question for this thesis was "**Will AuNPs affect molecular, biochemical and behavioural responses of seabream** (*Sparus aurata*)**?"**. To answer this question, *in vitro* and *in vivo* assays were performed with AuNPs with different sizes (7 and 40 nm) and coatings (citrate and polyvinylpyrrolidone – PVP), alone and combined with a human pharmaceutical, gemfibrozil (GEM). From the findings of the work developed within this thesis, several highlights may be referred.

- Gold nanoparticles accumulated in Sparus aurata in all analysed tissues, with gills, liver and spleen being the tissues accumulating the highest concentrations of gold (Chapters VI, VIII and IX).

Knowledge concerning accumulation of nanomaterials in the tissues of marine organisms is still limited and contradictory (Krysanov et al. 2010). In fish, nanoparticles (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 and Ranville 2012; Yoo-Iam, Chaichana, and Satapanajaru 2014). 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). In a freshwater fish species, zebrafish (*Danio rerio*), exposed for 36 d to a diet containing 4.5 $\mu g.g^{-1}$ AuNPs (12

nm), gold was found in brain and liver (4.6 and 3.0 $\mu g.g^{-1}$, respectively) (Geffroy et al. 2012), whereas after exposure for 20 d to sediments spiked with 14 nm AuNPs (16 and 55 $\mu g.g^{-1}$), gold was detected in the gills (between 0.01 and 0.03 $\mu g.g^{-1}$), digestive tract (between 0.22 and 1.40 $\mu g.g^{-1}$), but not in brain and muscle (Dedeh et al. 2015). In the marine mussel *Mytilus edulis*, gold bioaccumulation has been observed in the digestive gland (61 $\mu g.g^{-1}$), gills (0.5 $\mu g.g^{-1}$) and mantle (0.02 $\mu g.g^{-1}$) after 24 h exposure to 13 nm AuNPs (750 $\mu g.L^{-1}$) (Tedesco et al. 2008). The data from the present study showed that after 96 h waterborne exposure, *S. aurata* had higher accumulation of gold in gills, liver and spleen than in muscle. The observed gold accumulation was dependent on the characteristics of AuNPs, mostly on the coating, with gold accumulating more in the fish after exposure to polyvinylpyrrolidone coated gold nanoparticles (PVP-AuNPs) than with citrate coated gold nanoparticles (cAuNPs) – Chapters VI, VIII and IX. In the assays aiming to assess the effects of combined exposure to AuNPs and GEM, data suggested that the presence of environmental contaminants can interfere with the accumulation of gold in *S. aurata*. Gold accumulated more in the muscle of *S. aurata* in combined exposure to 40 nm PVP-AuNPs and GEM than in exposure to PVP-AuNPs only (Chapter VI).

- *Induction of enzymatic (e.g. catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx) and glutathione S-transferases (GST)) and non-enzymatic (non-protein thiols - NPT) defences were found after exposure to gold nanoparticles, both in the gills and liver of Sparus aurata (Chapters II, III and VI).*

In previous studies with marine bivalves, the potential of AuNPs to induce defence responses against oxidative stress was already reported (Abdelhafidh et al. 2018; Pan et al. 2012; Tedesco et al. 2008, 2010; Volland et al. 2015). A 16-d exposure of *Scrobicularia plana to* 100 $\mu g.L^{-1}$ of 15 and 40 nm AuNPs resulted in increased CAT and GST activities (Pan et al. 2012). In *Ruditapes decussatus*, 7 d exposure to 5 and 10 $\mu g.L^{-1}$ of triangular AuNPs with a length of 150 nm increased gills and digestive gland CAT and GST activities (Abdelhafidh et al. 2018). In

Ruditapes philippinarum, 7 d exposure to 20 nm cAuNPs (0.75 µg.L^{-1}) increased GR and GPx activities in the digestive gland but did not affect the gills (Volland et al. 2015). In *M. edulis* exposed for 24 h to 13 nm AuNPs (750 µg.L^{-1}), the digestive gland and mantle CAT activity was also stimulated (Tedesco et al. 2008). In fish, no data was available on the effects of AuNPs on gills and liver antioxidant defences prior to this thesis. 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). Two studies with *S. aurata* has reported that 96-h exposure to 40 nm AuNPs modulated the expression of target genes related to oxidative stress in liver (Teles et al. 2016) and head kidney (Teles et al. 2017). In the present study, *S. aurata* liver organ culture exposed for 24 h to 7 and 40 nm AuNPs induced CAT and GR activities (Chapter II). *In vivo* exposure of *S. aurata* for 96 h to 7 and 40 nm AuNPs increased GPx, CAT, GR and GST activities and NPT levels in liver and gills (Chapters III and VI). Overall, the range of the effects depended on concentration, size and coating of AuNPs.

- *Decreased swimming performance of Sparus aurata was observed after gold nanoparticles exposure (Chapters III and VI).*

Nanosized materials, such as fullerene, copper, titanium and silver NPs have been shown to have the ability to affect fish behaviour (Boyle et al. 2013; McNeil et al. 2014; Sovová et al. 2014; Sumi and Chitra 2015). The already reported altered behavioural patterns include: erratic swimming activity and slow opercular movements in the cichlid *Etroplus maculatus* exposed through diet to 100 µg.L^{-1} fullerene NPs for 96 h (Sumi and Chitra 2015); decreased time spent moving at high speed in rainbow trout (*Oncorhynchus mykiss*) exposed to 1 mg.L^{-1} titanium NPs (Boyle et al. 2013); impaired behavioural response of *O. mykiss* to alarm substances after 12 h exposure of 50 µg.L^{-1} copper NPs (Sovová et al. 2014); reduction of the ability of the embryos zebrafish 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). In terms of AuNPs, no study has so far reported alterations on the swimming behaviour of fish, but a decreased feeding performance was reported for marine fish *P. microps* after 96 h exposure to 5 nm AuNPs (Ferreira et al. 2016). In the present study, exposure of *S. aurata* 96 h to 7 and 40 nm AuNPs decreased the ability of fish to continue swimming against a water flow (Chapters III and VI), clearly an ecologically relevant effect of NPs exposure.

- Gold nanoparticles exposure caused oxidative damage (increased lipid peroxidation (LPO) levels) in gills and liver of Sparus aurata (Chapters II, III and VI).

The potential of AuNPs to induce oxidative damage in the form of LPO has earlier been reported for marine organisms (Abdelhafidh et al. 2018; Tedesco et al. 2010), despite no oxidative damage has been reported (Ferreira et al. 2016; Pan et al. 2012; Tedesco et al. 2008). Pan et al. (2012) reported that 16-d exposure of *S. plana* to 15 and 40 nm AuNPs (100 $\mu g.L^{-1}$) did not induce oxidative damage. Tedesco et al. (2008) reported that 24 h exposure to 13 nm AuNPs (750 $\mu g.L^{-1}$) caused a moderate level of oxidative stress in *M. edulis*, without increased LPO levels. Although, 5 nm AuNPs increased LPO in digestive gland, gills and mantle of *M. edulis* (Tedesco et al. 2010). In *R. decussatus*, 7 d exposure to triangular AuNPs (5 and 10 $\mu g.L^{-1}$) also enhanced LPO in gills and digestive gland (Abdelhafidh et al. 2018). 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). In the present study, the induction of LPO depended on the characteristics of AuNPs. Increased LPO was found in *S. aurata* liver organ culture following 24 h exposure to 7 nm AuNPs and in *S. aurata* gills and liver after 96 h exposure to 7 nm cAuNPs and 40 nm PVP-AuNPs (only in gills) – Chapters II, III and VI.

- *Gold nanoparticles induced DNA damage in liver organ culture and erythrocytes and increased frequency of nuclear abnormalities in erythrocytes of Sparus aurata (Chapters II, III and VII).*

There is a scarcity of publications on the potential genotoxicity of AuNPs to aquatic organisms (Dedeh et al. 2015; Geffroy et al. 2012; Dominguez et al. 2015; Teles et al. 2016, 2017), specially to marine species (Teles et al. 2016, 2017). In *D. rerio*, expression of genes involved in DNA repair, detoxification processes, apoptosis, mitochondrial metabolism and oxidative stress were modulated in response to an exposure for 36 d to a diet containing 12 nm AuNPs (4.5 $\mu g.g^{-1}$) (Geffroy et al. 2012) and for 20 d to sediments spiked with 14 nm AuNPs (16 and 55 $\mu g.g^{-1}$) (Dedeh et al. 2015). In *S. aurata*, 96 h exposure to 40 nm AuNPs modulated the expression of genes related to oxidative stress, cell-tissue repair, immune function and apoptosis in liver (Teles et al. 2016) and head (Teles et al. 2017). In the present study, 7 and 40 nm AuNPs induced DNA strand breaks in *S. aurata* liver organ culture (DNA strand breaks), after 24 h – Chapter II – and in the erythrocytes (DNA strand breaks and nuclear abnormalities), after 96 h – Chapters III and VII.

- *Exposure of gold nanoparticles induced alterations in the hepatic protein abundance of Sparus aurata (Chapter IX).*

The use of proteomics is a promising tool to evaluate NPs toxicity, as it has the potential to reveal pathways and processes that may be not immediately evident with conventional biomarkers (Gioria et al. 2014, 2016; Kim et al. 2010; Mirzajani et al. 2014a, 2014b; Otelea and Rascu 2015; Planchon et al. 2017). Few studies have however used a proteomics approach to assess effects of AuNPs (Gioria et al. 2014, 2016; Tedesco et al. 2010). The only study with aquatic organisms, performed with *M. edulis*, revealed a decrease of thiol-containing proteins in the digestive gland after 24 h exposure to 5 nm AuNPs (750 $\mu g.L^{-1}$) (Tedesco et al.

2010). In the present study, a 96-h exposure of *S. aurata* to AuNPs induced alterations in liver protein abundance with 26 proteins displaying differences in abundance. Most of the identified proteins were structural (actins and tubulins) but also other proteins with different functions in the cells were identified, such as calreticulin, 94 kDa glucose-regulated protein, pyruvate carboxylase b, phosphoenolpyruvate carboxykinase 2, cytohesin-1 and hippocalcin. Exposure to AuNPs triggered several hepatic pathways in *S. aurata*, dependent on the characteristics of NPs, involved with e.g. cell morphology and differentiation; different metabolic pathways such as protein synthesis, folding and transport; and oxidative stress (Chapter IX).

1.1.1. The importance of size and coating on the effects of gold nanoparticles

The ionic strength of media where the NPs are present plays a determinant role on its characteristics and behaviour, and thus bioavailability (Barreto et al. 2015). The tested AuNPs displayed different behaviour in the seawater with salinity 30-35. PVP-AuNPs remained stable in the seawater, maintaining their nano-size and therefore presumably more available for uptake by fish. The data from the quantification of gold in gills, liver, spleen and muscle support this concept, with a higher accumulation of gold found in *S. aurata* after the exposure to PVP-AuNPs than cAuNPs (Chapters VI, VIII and IX). However, 7 nm cAuNPs, even almost immediately aggregating/agglomerating and increasing their sizes (160 nm) in seawater, induced more effects on *S. aurata* than PVP-AuNPs (7 and 40 nm), despite PVP-AuNPs observed stability. Also, 7 nm cAuNPs induced more effects than 40 nm cAuNPs which formed agglomerates/aggregates of 340 nm in seawater (Chapters III and VI). In the liver organ culture experiments (24 h), this ranking of effects was not observed, as 7 nm PVP-AuNPs induced more effects in *S. aurata* than cAuNPs. In terms of observed effects in the *in vitro* assays the following rank was established: 7 nm PVP-AuNPs>7 nm cAuNPs>40 nm PVP-AuNPs>40 nm cAuNPs. All tested AuNPs aggregated/agglomerated within 12 h of exposure in cell culture media, increasing their sizes (Chapter II). Based on the *in*

vivo assays (96 h), the overall effects of the tested AuNPs can be ranked as follow: 7 nm cAuNPs>40 nm PVP-AuNPs>40 nm cAuNPs>7 nm PVP-AuNPs (Table 1). For 7 nm AuNPs, cAuNPs induced more effects than PVP coated (Chapter III). An opposite pattern was however observed for 40 nm AuNPs (Chapter VI).

The rank of effects based on the abundance of proteins in the liver of *S. aurata* after 96 h exposure to 80 $\mu g.L^{-1}$ AuNPs can be ordered as: 7 nm cAuNPs>40 nm cAuNPs>7 nm PVP-AuNPs>40 nm PVP-AuNPs (Chapter IX). As observed for the biochemical endpoints (Table 1), 7 nm cAuNPs also induced more effects than the other tested AuNPs. The effects of AuNPs on liver proteome were dependent on the NPs characteristics. The only common target of all tested AuNPs was proteins involved in cell morphology – actins (Chapter IX).

The data of the *in vitro* and *in vivo* assays show that that the smaller AuNPs induce more alterations but, in terms of coating, assay specific responses were found, with PVP and citrate coating AuNPs being more biologically active in *in vitro* and *in vivo* assay, respectively.

Table 1. Overall effects on *Sparus aurata* in the indicated endpoints after 96 h exposure to gold nanoparticles (7 and 40 nm; citrate coated – cAuNPs and polyvinylpyrrolidone coated – PVP-AuNPs). ↓ Decreased of the assessed endpoint at least at one of the tested concentrations (4, 80 and 1600 $\mu g.L^{-1}$) comparing with control group. ↑ Increased of the assessed endpoint at least at one of the tested concentrations (4, 80 and 1600 $\mu g.L^{-1}$) comparing with control group. ━ Similar with control group.

Assessed Endpoints	Tissues	Types of AuNPs			
		7 nm cAuNPs	7 nm PVP-AuNPs	40 nm cAuNPs	40 nm PVP-AuNPs
Swimming Resistance		1600 ↓	-	-	1600 ↓
Cholinesterases Activity	Brain	-	-	-	-
	Muscle				
Catalase Activity	Gills	-	80 ↓	-	-
	Liver		-	1600 ↑	1600 ↑
Glutathione Reductase Activity	Gills	1600 ↑	80 ↓	-	-
	Liver	80; 1600	-		
Glutathione Peroxidase Activity	Gills	4 ↑	-	-	80 ↑
	Liver	-	80 ↓		4; 1600
Non-Protein Thiols Levels	Gills	4; 80; 1600 ↑	-	80; 1600 ↑	-
	Liver	1600		80; 1600	
Glutathione *S*-Transferases Activity	Gills	4 ↑	-	-	-
	Liver	-			1600 ↑
Lipid Peroxidation Levels	Gills	4; 80; 1600 ↑	-	-	4; 80 ↑
	Liver	1600			-
DNA Damage Index	Erythrocytes	80; 1600 ↑	4; 80; 1600 ↑	4; 80; 1600 ↑	4; 80; 1600 ↑
Nuclear Abnormalities Frequency	Erythrocytes	4; 80; 1600 ↑	4; 80; 1600 ↑	4; 80; 1600 ↑	4; 80; 1600 ↑

In the available literature, different results have been reported depending on the tested coatings. In the bacteria *Bacillus aquimaris*, the alga *Chlorella* sp. and the cervical cancer cell line SiHa cells, cAuNPs displayed more effects than PVP-

AuNPs whereas in Swiss Albino Mice, PVP-AuNPs were more toxic than cAuNPs (Iswarya et al. 2016). In the present study, for the tested exposure periods, the size appeared, in general, to play a more determinant role in the biological activity of the AuNPs than the coating. The smaller particles were more biologically active which is in agreement with Tedesco et al. (2008, 2010) studies where 5 nm AuNPs induce LPO in *M. edilus* unlike 13 nm.

The analysis of the tissues responses showed that the gills of *S. aurata* were more sensitive than the liver (both in the single and combined exposures to AuNPs) – Chapters III, VI and VIII. Gills are the first organ to be exposed to waterborne contaminants and provide a large surface area of contact (Oliveira, Pacheco, and Santos 2008). In this tissue, despite the enzymatic and non-enzymatic antioxidant defences were more activated than in the liver, after exposures of 7 nm AuNPs (single and combined exposures) more oxidative damage (LPO increase) was found. An example is the response to 7 nm cAuNPs, where the NPT and LPO levels in gills were significantly increased following exposure to all tested concentrations, whereas in the liver, only 1600 $\mu g.L^{-1}$ caused increased levels (Chapter III). For 40 nm AuNPs (for single and combined exposures), a different pattern of response was observed. The enzymatic and non-enzymatic antioxidant defences of *S. aurata* were more active in the liver than in gills but oxidative damage was only found in gills. This result may be associated with the generally higher gold accumulation in gills than in liver and the less responsive defence mechanisms in gills (Chapter VI).

1.1.2. Gemfibrozil and effects of gold nanoparticles

It is not really known which role the presence of environmental contaminants may have on the behaviour, bioavailability and effects of NPs. In this study, GEM showed the ability to increase LPO levels, induce DNA damage (both *in vitro* and *in vivo*) and to decrease the swimming resistance of fish at an environmentally relevant concentration, 1.5 $\mu g.L^{-1}$ (Chapters II, IV and V). *In vivo*, the assessment of the combined effects of AuNPs (7 or 40 nm) and GEM showed that for endpoints such as swimming resistance and ChE activity, the predicted

percentage of effect (the sum of the percentage of the single exposures) was similar than the observed percentage of effect (Chapters VI and VIII), but for many of the tested endpoints they were considerably different. For endpoints like erythrocytic nuclear abnormalities and DNA damage index, the observed percentage of effect was lower than the predicted, indicating that when in mixture, AuNPs and GEM have antagonistic effects (Chapters VII and VIII). A successful activation of defences that prevented damage and/or activation of repair mechanisms upon these combined exposures may however also be involved. In other cases, like hepatic CAT and GR activities (for 40 nm AuNPs with GEM) and gills NPT content (7 nm AuNPs with GEM), the effects of the combined exposures were higher than the sum of the effects of each contaminant alone – i.e. apparent synergistic effects (Chapters VI and VIII). The liver organ culture combined exposures to AuNPs and GEM also resulted in synergistic effects in CAT, GR activities and LPO levels (Chapter II). A previous *in vitro* study with marine mussels *Mytilus galloprovincialis* showed that the observed percentages of effect of the combination of the pharmaceuticals fluoxetine or carbamazepine with AuNPs were lower than the predicted (Luis et al. 2016), highlighting the importance to study the effects of mixtures of contaminants.

This approach to test the effects of combined exposure to NPs and other environmental contaminants is highly relevant because, in the environment, organisms are exposed to a variety of contaminants that may alter NPs characteristics and/or influence its bioavailability. In the case of AuNPs and GEM combined exposures, the study of the behaviour of AuNPs showed no effect of GEM presence on the NPs characteristics but the assessment of the effects in *S. aurata* of the combined exposure revealed interaction.

1.1.3. *In vitro* versus *in vivo* effects of gold nanoparticles

In vitro methodologies have gained an increasing importance in toxicology due to the concerns with animal welfare, time and cost constraints. These assays allow reducing the number of experimental animals, control of environmental conditions,

reduction of the genetic heterogeneity and the amounts of tested chemicals and toxic wastes (Oliveira et al. 2003; Soldatow et al., 2013).

In this study, the liver organ culture of *S. aurata* were sensitive to low concentrations of the tested contaminants and could be used to differentiate responses to AuNPs with different characteristics, supporting its use as an alternative to *in vivo* testing. Taking into account the concentrations of AuNPs (4, 80 and 1600 $\mu g.L^{-1}$) and the endpoints (CAT, GR and GST activities, LPO levels and DNA damage) assessed in both *in vitro* (Chapter II) and *in vivo* (Chapters II and VI), increased CAT and GR activities and DNA damage were observed after the exposure to AuNPs, despite differences in exposure length (24 *vs* 96 h). However, some dissimilar results were also detected between *in vitro vs in vivo* experiments. For instance, GST activity was not altered after *in vitro* exposures whereas *in vivo* exposure of 1600 $\mu g.L^{-1}$ 40 nm PVP-AuNPs increased hepatic GST activity. LPO levels were not altered *in vitro* in the range of concentrations 4 to 1600 $\mu g.L^{-1}$ although 1600 $\mu g.L^{-1}$ 7 nm cAuNPs increased *in vivo* liver LPO levels. These dissimilar results may be due to aggregation/agglomeration state of AuNPs. In seawater, PVP-AuNPs maintained their nano-size for the exposure period (96 h) and cAuNPs aggregated/agglomerated almost immediately. In cell culture media, the size of the all tested AuNPs was altered within 12 h of exposure with the formation of aggregates/agglomerates larger than 100 nm. Aggregates/agglomerates of 7 nm PVP-AuNPs had smaller sizes than 7 cAuNPs and 40 nm AuNPs. Additionally, in the *in vivo* tests the whole living organism is used, and a range of mechanisms can occur in different tissues/organs to protect/eliminate a contaminant whereas in the *in vitro* test, only the mechanisms involved in cell, tissue or organ used are evaluated. Therefore, this fact must be take into consideration for some dissimilar results found in this study between *in vitro* versus *in vivo* assays.

1.1.4. Mechanisms underlying the toxicity of gold nanoparticles

The results from this thesis showed that AuNPs may induce oxidative stress and damage, genotoxicity, altered protein abundance and behaviour alterations on

S. aurata. Some authors suggest that NPs do not possess a unique toxicity mechanism (Khalili Fard, Jafari, and Eghbal 2015; Tang et al. 2007), being reactive oxygen species (ROS) generation the most widely accepted nanotoxicity mechanism. This was corroborated by the results from this thesis, which showed the induction of gills and liver enzymatic and non-enzymatic antioxidant defence components (CAT, GR and GPX activities and NPT levels) and oxidative damage (increased LPO levels) in *S. aurata* (Chapters III and VI). AuNPs have already shown the ability to induce ROS production to different aquatic organisms such as *M. edulis, O. mykiss* (hepatocytes) and *D. rerio* (Dominguez et al. 2015; Farkas et al. 2010; Pan et al. 2007; Tedesco et al. 2008, 2010). Another important finding in the present thesis was that NPT levels were only increased after exposure to cAuNPs. 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 in gills and liver of *S. aurata*. On the other hand, the obtained data suggest a lack of interaction between PVP-AuNPs and NPT as levels remained unchanged at different exposure concentrations.

The changes detected in the swimming performance of *S. aurata* revealed the potential effect of AuNPs exposure at an individual level which could suggest a decrease in the activity of cholinesterases (ChE), some of which are critical enzymes for neurological function (Hernández-Moreno et al. 2011; Oliveira et al. 2013). However, *S. aurata* ChE activity was unaltered both in brain and muscle after exposure to the tested AuNPs, suggesting the involvement of other factors in the altered swimming performance (Chapters III and VI). This 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. The behaviour alteration in *S. aurata*, observed in the present study, could be a result of a direct effect of NPs on the brain (Kashiwada 2006; Mattsson et al. 2015). In the brain, a lipid-rich organ, NPs may affect the organization and function of tissue membranes due to their strong affinity to lipids (Mattsson et al. 2015). The decreased swimming performance of *S. aurata* may also be related to a metabolic trade off in

the energy budget of the fish, where AuNPs exposed fish redirect energy from locomotion to other activities involved e.g. in the antioxidant defence system.

This study also reported genotoxic effects of AuNPs in liver organ culture (DNA strand breaks) – Chapter II – and in erythrocytes (DNA strand breaks and nuclear abnormalities) – Chapters III and VII, which may be due to a direct action following the entry of NPs into cells; or indirectly, through oxidative stress, which may consequently induce oxidative damage to DNA (Farkas et al. 2010; Tudek et al. 1998). ROS has often been described as playing a major role in terms of DNA damage caused by NPs (Li, Laval, and Ludlum 1997; Tchou et al 1994). However, in the present study, this was not confirmed using formamidopyrimidine DNA glycoslyase (Fpg), used for the detection of oxidative DNA base damage (Chapter III). This may be due to low potential of the tested conditions to induce oxidative damage in the erythrocytes DNA of *S. aurata* or efficient repair systems. Nonetheless, oxidative damage was detected in cellular membranes, assessed as LPO, after exposure to AuNPs. Although GEM single exposure induced oxidative damage in DNA, in combination with AuNPs this was not observed (Chapter VIII). Thus, a possible interaction of NPs with Fpg, as previously described (Kain, Karlsson, and Möller 2012), needs to be taken into consideration. This interaction may occur through the presence of particles in the comet nucleoid as already observed (Karlsson, Nygren, and Möller 2004; Stone, Johnston, and Schins 2009), preventing the binding of Fpg with the damaged DNA (Grin et al. 2009; Kain, Karlsson, and Möller 2012) or Fpg may attach to the surface of particles and form a corona around NPs (Lynch and Dawson 2008).

In this study, different hepatic proteins were affected by the AuNPs exposure which means that the AuNPs are most likely affecting different metabolic pathways in liver cells involved with e.g. protein biosynthesis, folding or transport; cell structure, motility and membrane organization; oxidative stress and response to metals.

To increase knowledge about the mechanisms underlying the toxicity of AuNPs, toxic effects of AuNPs and ionic gold were assessed. The obtained results showed that the nano form induced more effects than ionic form, after 24 h *in vitro* exposure (Chapter II). The opposite results were, however, observed following *in*

vivo exposure for 96 h (Chapter III). Contrasting results have been reported earlier. A higher cytotoxicity of AuNPs compared to ionic gold was reported for two types of human cell lines (U-937 and HL-60) (Barbasz and Oćwieja 2016), whereas ionic gold was found to have higher toxicity in *O. mykiss* hepatocyte cells (Farkas et al. 2010) and *M. galloprovincialis´* hemolymph (Luis et al. 2016).

The present thesis has given an important contribution to understanding the potential effects of AuNPs to marine fish, as well as, knowledge concerning interactions between AuNPs and the pharmaceutical GEM. The results showed that AuNPs are not inert, raising concern about its safety, for use in aquaculture, biomedical or other applications.

This thesis has emphasized the value of using *in vitro* methodologies. *S. aurata* liver organ culture was sensitive to the tested xenobiotics (AuNPs and GEM) supporting its use in future research as an alternative to *in vivo* testing.

Further studies are encouraged with different AuNPs (single or combined), assessing various endpoints at different times of exposure, to increase the knowledge about AuNPs effects to marine fish species and the mechanisms involved, using longer exposure periods and lower concentrations.

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