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Ciências

Toxicological effects of TiO_2 nanoparticles in two freshwater species: *Carassius auratus* and *Corbicula fluminea*

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To all my family, friends and colleagues.

A special thanks to Célia, Adérito and Ana Carolina.

I dedicate this work to my dearest friend Liliana, always alive in my memories.

"Why cannot we write the entire 24 volumes of the Encyclopeadia Brittanica on the head of a pin?"

(Richard Feynman, 1960)

“No matter how sophisticated knowledge is, it will always be subject to some degree of ignorance. To be alert to — and humble about — the potential gaps in those bodies of knowledge that are included in our decision-making is fundamental. Surprise is inevitable. Just as one basis for scientific research is the anticipation of positive surprises — ‘discoveries’ — so it will always yield the corresponding prospect of negative surprises. By their nature, complex, cumulative, synergistic or indirect effects in particular have traditionally been inadequately addressed in regulatory appraisal.”

(The European Environment Agency)

Resumo

As nanopartículas (NPs) de dióxido de titânio (TiO_2) têm uma vasta utilização, desde aplicações industriais a produtos para os consumidores. O uso crescente de nanomateriais pode levar à entrada de quantidades significativas de NPs no meio ambiente, sendo o meio aquático muitas vezes o seu destino final. Porém, o impacto e possíveis efeitos nocivos das NPs para o biota aquático ainda não estão totalmente clarificados. Neste contexto, este trabalho pretende avaliar a toxicidade de NPs de TiO_2 em duas espécies de organismos de água doce (*Carassius auratus* e *Corbicula fluminae*).

Os organismos foram expostos a suspensões de NPs de TiO_2 (±21 nm) com concentrações desde 0.01 a 800 mg TiO_2/L e processados para a realização de análises enzimáticas e histológicas após períodos de 7, 14 e 21 dias. Foi determinada a actividade enzimática da superóxido dismutase, catalase e glutationa-s-tranferase e o grau de peroxidação lipídica, a fim de avaliar a resposta ao *stress* oxidativo. Os tecidos de órgãos alvo previamente seleccionados de acordo com a sua importância fisiológica foram observados através de microscopia óptica e electrónica e a presença de TiO_2 foi determinada através de análise elementar de raio x.

A exposição dos organismos a suspensões de NPs de TiO_2 não foi letal para os peixes da espécie *C. auratus*, mas níveis de mortalidade significativos ocorreram nos bivalves da espécie *C. fluminae*. Os resultados demonstram que as NPs de TiO_2 podem causar toxicidade sub-letal envolvendo *stress* oxidativo, aumentando a peroxidação lipídica e induzindo variações significativas da actividade antioxidante comparativamente aos controlos e ao longo dos períodos de exposição. Foram observadas alterações histológicas nas branquias, fígado e intestino dos peixes e na glândula digestiva dos bivalves. Verificou-se que as NPs presentes em suspensão foram ingeridas pelos organismos, o que resulta na acumulação de aglomerados de NPs dentro do lúmen intestinal dos peixes. Confirmou-se ainda a ocorrência de internalização celular de NPs de TiO_2, especificamente nas células do tecido branquial dos peixes.

Os resultados obtidos sugerem que existe um potencial risco para o biota aquático, relacionado com a entrada das NPs de TiO_2 no ambiente aquático. O impacto ambiental das NPs é uma questão de enorme relevância e apesar de se observar um aumento dos estudos sobre os efeitos das NPs de TiO_2, os mecanismos de toxicidade destas e de outras NPs metálicas permanecem por esclarecer.

Palavras-chave

Nanoparticulas de TiO_2; *Carassius auratus*; *Corbicula fluminea*; *stress* oxidativo; actividade antioxidante; nanotoxicologia; ecotoxicologia

Abstract

Titanium dioxide (TiO_2) nanoparticles (NPs) have a widespread use, from industrial applications to consumer products. The increasing use of nanomaterials can lead to significant releases of NPs into environment and the aquatic system is commonly the ultimate recipient for NPs. However, the impact and potential detrimental effects of NPs to aquatic biota remains unclear. In this context, the aim of the present work is to evaluate the toxicity of the TiO_2 NPs exposure in two freshwater species (*Carassius auratus* and *Corbicula fluminae*).

Organisms were exposed to suspensions of TiO_2 NPs (±21 nm) within a range of concentrations from 0.01 to 800 mg TiO_2/L and sampled for enzymatic and histological analysis after periods of 7, 14 and 21 days. Lipid peroxidation, superoxide dismutase, catalase and glutathione-s-transferase activity were determined in order to evaluate the response to oxidative stress. Tissues from target organs were analyzed by optical and electron microscopy and x-ray elemental analyses allowed detecting the presence of TiO_2.

The exposure to TiO_2 NPs in aquatic suspensions was not lethal for *C. auratus*, but significant mortality rates were found for *C. fluminea*. Results show that TiO_2 NPs causes toxicity involving oxidative stress, increasing lipid peroxidation and inducing significant variations of the antioxidant activity in the exposed organisms compared to controls and over exposure time. Histological pathologies were observed in *C. auratus* gills, liver and intestine and in *C. fluminea* digestive gland. NPs in suspension are ingested by organisms, resulting in the accumulation of TiO_2 NPs agglomerates inside *C. auratus* intestinal lumen. Cellular internalization of TiO_2 NPs was confirmed in cells from fish gills.

The results suggest that, a potential risk to the aquatic biota exist related to the TiO_2 NPs release to the aquatic environment. The environmental impact of the NPs is a matter of concern and despite an increase of studies of nanosized-TiO_2 effects, the precise mechanisms of toxicity of this and other metal NPs remain unclear.

Keywords

TiO_2 nanoparticles; *Carassius auratus*; *Corbicula fluminea*; oxidative stress; antioxidante activity; nanotoxicology; ecotoxicology

Table of Contents

Acknowledgements iii
Resumo + Palavras-chave vii
Abstract + Keywords ix
Table of Contents xi
Index of Figures xiii
Index of Tables xv
Abbreviations xii

1 Introduction 1
1.1 Background 1
1.3.1 Characterization of Nanoparticles 2
1.4.2.1 Nanoparticles in aquatic environments 6
1.4.2.2 Effects in aquatic organisms 7
1.5 Titanium dioxide nanoparticles 7
1.5.1 Effects and toxicity of TiO_2 NPs 8
1.5.2 Toxicity of TiO_2 NPs to aquatic organisms 9
1.6 Biological models 12
1.7 Oxidative stress enzymes 13
1.7.1 Superoxide Dismutase 14
1.7.2 Catalase 14
1.7.3 Glutathione S-Transferase 15
1.8 Lipid Peroxidation 18
1.9 Microscopy Techniques and histopathology 19
1.9.1 Light and electron microscopy 19
1.9.2 Histological examination 19
2 Objectives 21
3 Materials and methods 22
3.1 Nanoparticles characterization 22
3.2 Experimental procedure 23
3.2.1 Test organisms 23
3.2.2 Acclimation 24
3.2.3 Preparation of Test Nanoparticles 24
3.2.4 Exposure Assays 24
3.3 Sampling the tested organisms 26
3.4 Histology 26

3.4.1 Optical Microscopy 26
3.4.2 Transmission Electron Microscopy (TEM) 27
3.4.3 Scanning Electron Microscopy (SEM) 27
3.5 Determination of Total Protein 28
3.6 Determination of Enzymatic Activity 28
3.6.1 Catalase (CAT) 28
3.6.2 Glutathione-S-Transferase (GST) 29
3.6.3 Superoxide Dismutase (SOD) 29
3.7 Lipid Peroxidation 30
3.8 Statistical analysis 31
4 Results 32
4.1 Nanoparticles characterization in aquatic suspensions 32
4.2 Mortality and macroscopic observations 34
4.2.1 *C. auratus* characterization and macroscopic observations 34
4.2.2 *C. fluminea* characterization and macroscopic observations 35
4.3 Histological observations 37
4.3.1 *C. auratus* histopathological analysis 37
4.3.1.1 Liver 37
4.3.1.2 Gills 39
4.3.1.3 Intestine 41
4.3.2 *C. fluminea* histopathological analysis 43
4.4 Antioxidant activity and Lipid Peroxidation analysis 45
4.4.1 SOD activity in *C. auratus* 45
4.4.2 CAT activity in *C. auratus* 46
4.4.3 GST activity in *C. auratus* 48
4.4.4 Lipid peroxidation in *C. auratus* 50
4.4.5 *C. fluminea* antioxidant activity and lipo peroxidation analysis 52
5 Discussion 54
6 Conclusions 61
7 References 63
Annex I 75
AnnexII 76

Index of Figures

Figure 1 Potential cellular interactions of nanoparticles. 4

Figure 2 Possible routes of environmental exposure to engineered nps after release to the aquatic environment and trough food chains. 6

Figure 3 Catalase heme group. 15

Figure 4 Enzymatic antioxidant defense lines against ROS oxidative damage. 17

Figure 5 Lipid peroxidation chain reactions. 18

Figure 6 Typical image produced by NTA showing particle tracks. 23

Figure 7 Sampling of *C. fluminea* at tagus river´s margin. 23

Figure 8 Organisms in acclimation tanks. 24

Figure 9 *C. auratus* in test tanks. 25

Figure 10 Tanks for *C. fluminea* exposure to TiO_2 NPs suspensions. 25

Figure 11 Tanks for *C. fluminea* exposure to TiO_2 NPs suspensions. 32

Figure 12 Scanning electron microscopy of TiO_2 NPs. 32

Figure 13 Nanoparticle tracking analysis. 33

Figure 14 *C. auratus* macroscopic observations. 34

Figure 15 Macroscopic observations of NPs presence in *C. auratus*. 34

Figure 16 Representation of *C. fluminea* mortality. 35

Figure 17 Macroscopic observation of *C. fluminea* shell. 36

Figure 18 Observation of *C. auratus* liver sections by optical microscopy. 37

Figure 19 Electron microscopy observation of *C. auratus* liver tissue after exposure TiO_2 NPs 38

Figure 20 Observation of *C. auratus* gill sections by optical microscopy. 39

Figure 21 Electron microscopy observation and elemental analysis of *C. auratus* gill tissue after exposure to TiO_2 NPs. 40

Figure 22 Observation of *C. auratus* intestine sections by optical microscopy. 41

Figure 23 Electron microscopy observation and elemental analysis of *C. auratus* intestinal tissues after exposure to TiO_2 NPs. 42

Figure 24 Observation of *C. fluminea* digestive gland sections trough optical microscopy. 43

Figure 25 Electron microscopy observation and elemental analysis of *C. fluminea* after exposure to TiO_2 NPs. 44

Figure 26 SOD activity in *C. auratus*. 45

Figure 27 CAT activity in *C. auratus*. 46

Figure 28 GST activity in *C. auratus*. 48

Figure 29 LPO in *C. auratus*. 50

Figure 30 Activity of antioxidant enzymes and lipid peroxidation level in *C. fluminea*. 52

Index of Tables

Table 1 Modeled concentrations of TiO_2 nanoparticles released into environmental compartments in Europe and United States. 5
Table 2 TiO_2 nanoparticles physico-chemical proprieties, according to the manufacturer. 22
Table 3 Mortality rate, length and weight (mean±sd), after 7, 14 and 21 days of TiO_2 NPs exposure for *C. auratus*. 75
Table 4 Mortality rate, length and weight (mean±sd), after 7, 14 and 21 days of TiO_2 NPs exposure for *C. fluminea*. 76

Abbreviations

BSA	Bovine Serum Albumine
CAT	Catalase
CDNB	1-chloro-2,4-dinitrobenzene
DLS	Dynamic Light Scattering
DNA	Deoxyribonucleic Acid
EDS	Energy Dispersive X-ray Spectrometry
EDTA	Ethylenediamine Tetraacetic Acid
EGF	Epidermal Growth Factor
LC_{50}	Lethal Concentration, 50%
LPO	Lipid Peroxidation
GPX	Glutathione Peroxidase
GR	Glutathione Reductase
GSH	Glutathione
GST	Glutathione-S-Transferase
H&E	Hematoxyline & Eosine
NADPH	Nicotinamide Adenine Dinucleotide Phosphate-oxidase
NBT	Nitroblue Tetrazolium
NF-kB	Nuclear Factor kappa B
MDA	Malondialdehyde
nm	Nanometer
NPs	Nanoparticles
NTA	Nanoparticle Tracking Analysis
ROS	Reactive Oxygen Species
SD	Standard Deviation
SDS	Sodium Dodecyl Dulfate
SEM	Scanning Electron Microscopy
SOD	Superoxide dismutase
TBA	Thiobarbituric Acid
TBARS	Thiobarbituric Acid Reactive Substances
TBT	Tributylin
TCA	Trichloroacetic Acid
TEM	Transmission Electron Microscopy
TMP	Trimethylolpropane
UV	Ultraviolet
XOD	Xanthine-Oxidase
XRD	X-Ray Diffraction

1 Introduction

1.1 Background

The concept of nanotechnology was first introduced about 50 years ago, when the Nobel Prize-winner Richard Feynman presented a talk called “There’s Plenty of Room at the Bottom”, at the annual meeting of the American Physical Society at the California Institute of Technology. The audience was puzzled and intrigued with Feynman’s futuristic vision of how it could be possible to put a huge amount of information written in an exceedingly small space, while he was exploring the possibility of manipulating materials at the scale of individual atoms and molecules (Feynman, 1960).

Over the last 20 years, nanotechnology has emerged and is already a multidisciplinary reality, present in a wide range of fields including chemistry, physics, biology, medicine, engineering and electronics. Nowadays, nanotechnology can be defined as the research and development of structures, devices and systems by controlling shape and size at nanometric scale[1] (RS, 2004), to create materials with new behaviors and chemical properties.

1.2 Nanomaterials

Engineered nanomaterials such as nanoparticles (NPs) are increasingly being used for commercial purposes in products within medicine, electronics, sporting goods, tires, textiles and cosmetics. In the past decade, the Project of Emerging Nanotechnology launched a Nanotechnology Consumer Product Inventory, available at http://www.nanotechproject.org/. From an initial number of 212 products identified in the year of 2006, over 1300 manufacturer-identified nanotechnology-enabled consumer products have entered the marketplace to date, according to this inventory. The nanomaterial potential applications seem endless, promising great benefits for society and bringing high economic expectations. This is considered to be one of the major technology sectors of the 21st century (Delgado, 2010), which is reflected on an increasing global investment of multi-billion euros/dollars.

However, the same special properties that make nanomaterials so distinctive and useful also may represent and be the cause of potential risks and unpredictable effects to living beings and environment. Unfortunately, there is a great knowledge lack between nanotechnology

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[1] In a dimensional scaling, a nanometer (nm) corresponds to one billionth of a meter (10^{-9} m).

and its potential toxicity. Although the nanotoxicology is a very young research field, there is an increasing of studies that demonstrate hazards associated with nanoparticles, bringing the awareness of their potential adverse effects.

1.3 Nanoparticles

Nanoparticles (NPs) comprise diverse types of materials from metals, polymers, ceramic to biomaterials and have been defined as particles with at least one dimension in the order up to 100 nm (RS, 2004). However, without reliable methods for characterization and determination of the physicochemical properties of NPs it is also difficult to assess human or animal exposure to NPs (FSAI, 2008).

1.3.1 Characterization of Nanoparticles

Although NPs characterization is usually performed by using diverse techniques for estimating their physicochemical properties (e.g SEM, TEM, DLS, XRD) there is an urgent need to establish calibration standards and procedures for the characterization of NPs since the reliability, precision and accuracy of these techniques on the nanoscale are however often questioned. In this sense, the ISO TC229 Technical Committee on Nanotechnologies was established in 2005 to address these issues (FSAI, 2008). Accordingly, NPs characterization is a major challenge since it requires considerable care and there are many difficulties and uncertainties, especially regarding NPs aggregation, size, purity, and batch variations. Additionally, characterization is further complicated by the incorporation of NPs into biological matrices which may change their properties and requires further characterization beyond that of the pristine nanoparticle (FSAI, 2008).

Regarding NPs properties despite having the same chemical composition as bulk materials, they may exhibit new or enhanced size-dependent properties compared with larger particles of the same material (Hodes, 2007). According to Nel at al. (2006) the properties of nanomaterials are related to their size, structure and a large surface area-to-volume ratio relative to larger-sized chemicals and materials. In addition, in terms of size they are included in a transitional zone between individual atoms or molecules and the corresponding bulk material. Moreover, as the size of a particle decreases, more molecules are present at the surface giving rise to a larger surface area for chemical interaction. Thus, the higher surface area to the volume ratio plays a major role in the increasing of chemical reactivity and the change of the magnetic, conductive, optical and diffusion properties (Nel et al., 2006). Hence, materials that are inert in larger size can become reactive at the nanoscale. The size at which materials start changing their properties can vary from less than one nanometer to the micrometer range (Hodes, 2007).

Another important feature of NPs is their tendency to aggregate, often as a result of the drying stage during the synthesis process, and with considerable implications for the determination of the size and surface area of NPs but also for assessment of exposure to living organisms. The aggregation is often overlooked or even ignored when characterizing NPs size and surface area because they can exist both in very large and small particle size with a heterogeneous distribution. This is an important fact because sizes recorded are often only a small fraction of the sample, rather than a true representation of the sample composition. In this way, many studies use several techniques to disperse nanoparticles (e.g. ultrasound sonication, dispersing and milling). However, even following these procedures, the dispersions are often very polydisperse, and the surface may have changed, due to exposure of "fresh" surface due to the breaking up of clusters. Therefore, an important issue is to distinguish between the primary particle size, which is typically on the nanoscale, and the cluster size due to aggregation, which may be either nanoscale or micron scale (FSAI, 2008).

In a toxicological point of view size and surface area are extremely important since the small size and a large surface area allows a great proportion of its atoms or molecules to be displayed on its surface rather than within the material´s interior. As a consequence, these nanomaterial´s atoms or molecules may be chemically and biological reactive and have potential negative effects on living organisms. Other factors such as shape, surface coating, aggregation potential and solubility also affects physicochemical and transport properties of nanomaterials but also its toxicity potential. As an example, some types of NPs are associated to DNA damage, production of reactive oxygen species oxidative stress and neurologic problems, among others effects. Moreover, the ever-increasing use of these materials, soon can lead to the release and accumulation of heightened levels of these materials into environment. Still, there's a considerable gap in the regulation of the commercialization of products containing nanotechnologies (Falkner and Jaspers, 2012).

1.4 Environmental and Human Risks

1.4.1 Human and animal exposure to Nanoparticles

Human beings always have been exposed to airborne nanosized particles. However, such exposure has increased dramatically over the last decades due to the development of NPs from anthropogenic sources (Oberdörster et al., 2005).

The same properties that make NPs unique and so wide useful may also become a trap to our health. The higher toxicological potential of NPs is mostly due to their small size, wide surface, increase of their chemical reactivity and biological activity and the capacity to generate free radicals (Nel et al., 2006). NPs also can have the ability to penetrate trough the

biological barriers and to move easily through the biological systems (Oberdörster et al., 2005; Nel et al., 2006).

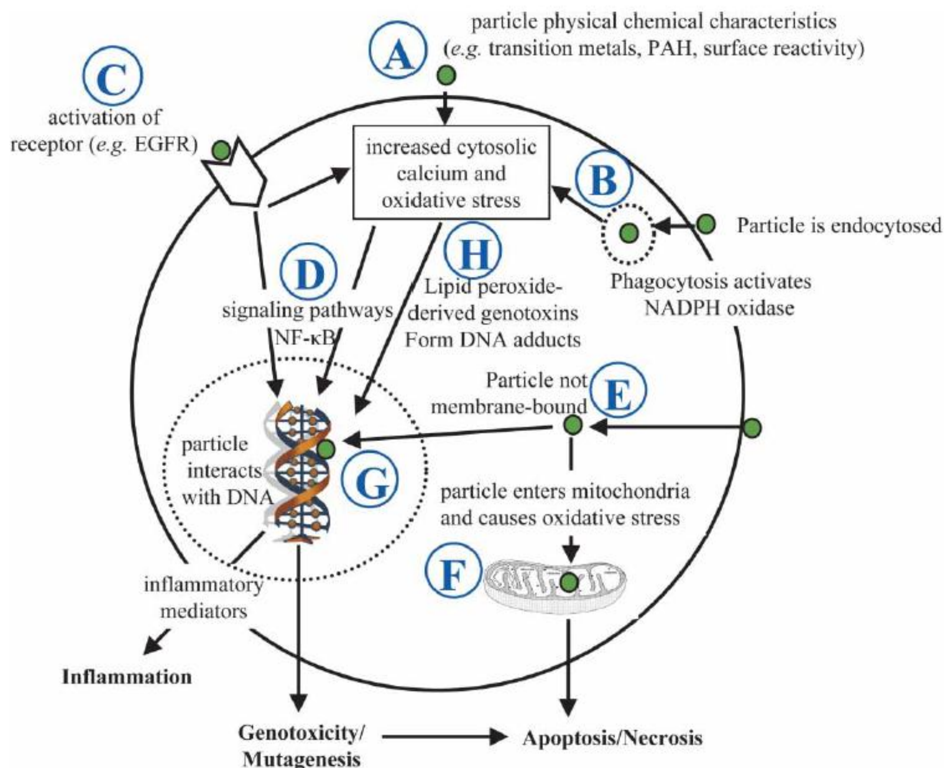


Figure 1 Potential cellular interactions of Nanoparticles.

The diagram shows the potential effects of NP with emphasis on potential oxidative stress induced effects and their consequences. (A) Particle-associated characteristics induce lipid peroxidation, intracellular oxidative stress and increased cytosolic calcium ion concentration; (B) NP may be actively endocytosed. In phagocytic cells phagocytosis triggers activation of NADPH oxidase and generation of ROS; (C) Particles and their associated metals, as well as oxidative stress, can activate the EGF receptor; (D) Oxidative stress, receptor activation and increased calcium ions activate transcription of pro-inflammatory genes via transcription factors such as NF-kB; (E) NP may enter the cell by passive diffusion and remain non-membrane bound from where they may enter mitochondria; (F) and disrupt normal electron transport leading to oxidative stress. (G) Free particles may also enter the nucleus via the nuclear pore complex and interact with the genetic material. (H) Lipid peroxide-derived products such as 4-hydroxynonenal form DNA adducts that may lead to genotoxicity and mutagenesis (in Oberdörster et al., 2005).

Nanotoxicological research has already associated some NPs to several toxicological effects as damage to DNA (Donaldson et al., 1996; Dunford et al., 1997), disruption of cellular function (Sayes et al., 2006), production of reactive oxygen species (Long et al., 2006), asbestos-like pathogencity (Poland et al., 2008), neurologic problems (NIH and NCCAM, 2010), organ

damage including significant lesions on the liver and kidneys (Wang et al., 2007), gill damage, respiratory problems and oxidative stress in fish (Federici et al., 2007).

1.4.2 Environmental Impacts of Nanoparticles

Nanoparticles (NPs) are not only artificial, but they also always existed in environment from natural sources. Carbon NPs have been found in 10 000 years ice cores (Murr et al., 2004). Other natural NPs nanoparticles can also be found in soil, water sources, atmospheric dust or volcanic ash (Handy et al., 2008).

Engineered nanomaterials can enter the environment through deliberated releases, which includes their use to remediate contaminated soils and groundwater, unintentional releases such as atmospheric emissions and also from the use of consumer's products with NPs, as sunscreens and cosmetics (Klaine et al., 2008). NPs have the potential to contaminate soil, migrate into surface and groundwater and interact with biota (Klaine et al., 2008). Also, NPs in solid wastes, wastewater effluents, direct discharges, or accidental spillages can be transported to aquatic systems by wind or rainwater (Klaine et al., 2008).

Table 1 Modeled concentrations of TiO_2 nanoparticles released into environmental compartments in Europe and United States (adapted from Menard et al., 2011).

Environmental Compartment	Predicted environmental concentration	
	Europe	United States
Water	0.012 – 0.057 µg/L	0.002 – 0.010 µg/L
Soil	1.01 – 4.45 µg/kg	0.43 – 2.3 µg/kg
Sludge treated soil/ Sediment	70.6 – 310 µg/kg	34.5 – 170 µg/kg
	273 – 1409 µg/kg	44 – 251 µg/kg
Air	0.0005 µg/m^3	0.0005 µg/m^3
Sewage treatment plant effluent	2.50 - 10.8 µg/L	1.37 – 6.70 µg/L
Sewage treatment plant sludge	100 – 433 mg/kg	107 – 523 mg/kg

1.4.2.1 Nanoparticles in aquatic environments

The aquatic environment receives daily substantial amounts of environmental pollutants that can be up taken by aquatic organisms from sediments, suspended particulate matter with toxic properties and food sources, depending on the particular dietary and ecological lifestyles of the organisms (Valavanidis et al., 2006).

Aquatic systems contain natural complex colloid[2] materials. These include inorganic minerals, typically hydrous iron and manganese oxides, and as well organic matter, such as humic substances, proteins and peptides (Klaine et al., 2008; Lead and Wilkinson, 2006). Their small size and large surface area per unit mass make them important binding phases for both organic and inorganic contaminants. This way, NPs can be accumulated and transported by the colloid fraction (Lead and Wilkinson, 2006). Once within an aquatic environment, NPs can also enter a process of aggregation which is closely related to the deposition and sedimentation of particles (Wiesner et al., 2009). This process is determined by the NPs surface properties, which are mainly dependent on parameters such as temperature, ionic strength, pH, particle concentration and size, among others (Navarro et al., 2008).

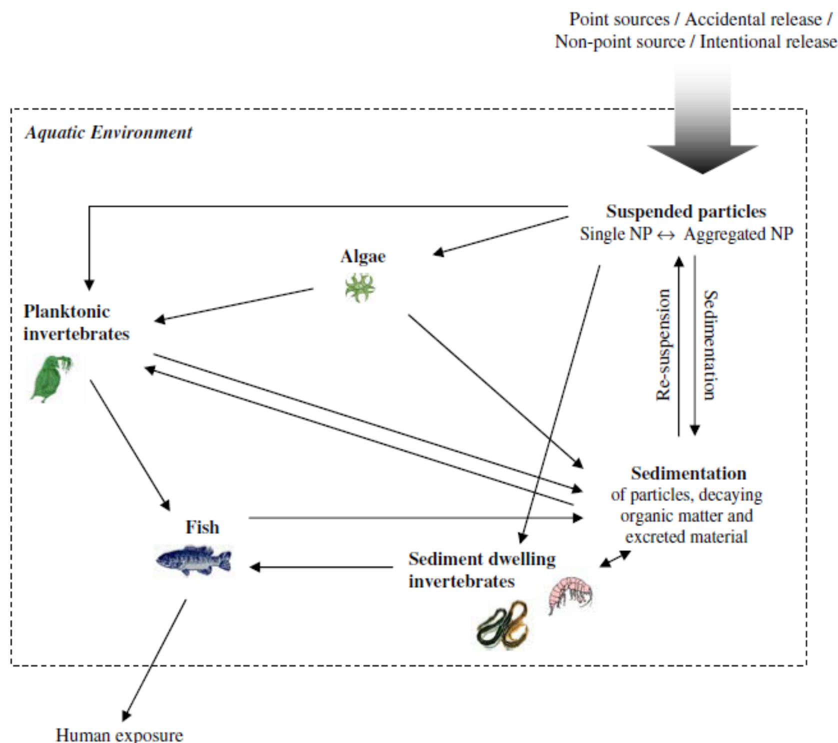


Figure 2 Possible routes of environmental exposure to engineered NPs after release to the aquatic environment and trough food chains. (in Baun et al., 2008)

[2] In aquatic systems, colloid is the generic term applied to particles in a size range between 1 nm to 1 µm.

1.4.2.2 Effects in aquatic organisms

Although several studies and reports have been recently published there is still a lack of knowledge on the ecotoxicity of NPs in aquatic biota. To date most of the data available are on freshwater species, particularly those used as standard species in regulatory toxicology such as *Daphnia magna* or *Danio rerio* and using several types of NPs (e.g. TiO_2, nanodiamonds, C60 fullereens) (Handy et al., 2008). It is also evident that more studies are needed on marine and terrestrial organisms and other vertebrates such as amphibians, reptiles and birds.

In aquatic invertebrates, the exposition to CeO_2 and SiO_2 NPs caused an increase of the mortality of *D. magna* and CeO_2 NPs also induced DNA damage and potential reproduction reduction for these organisms (Lee et al., 2009). Moreover, nanosized copper and silver had a 48 h LC_{50} of less than 1 mg/L for daphnids and algae (Griffitt et al., 2008) and also ZnO NPs showed toxicity to these organisms (Adams et al., 2006). For fish organisms, studies showed that Ag NPs provoked a 48 h LC_{50} value of 1.03 mg/L in Japanese medaka (*Oryzias latipes*), with additional developmental, morphological and histopathological changes, including edema production, abnormalities in the spine, fins, heart, brain, and eyes (Wu et al., 2010). In zebrafish, Ag and Cu NPs had a 48 h LC_{50} of less than 10 mg/L (Griffitt et al., 2008). Aggregation of single walled carbon nanotubes has been visible on the gill mucus of trout (Smith et al., 2007) and also TiO_2 NPs induced histological changes and oxidative stress in the rainbow trout (Federici et al., 2007).

1.5 Titanium dioxide nanoparticles

Conventional titanium dioxide (TiO_2) is a naturally harmless occurring mineral, which has been used since the beginning of the 20th century for numerous industrial applications and consumer products, particularly for coatings and pigments (Chen et al., 2007).

In addition, as the size of TiO_2 particles decreases into the nanoscale, higher is the potential for photocatalytic properties and UV absorption (Shao and Schlossman, 1999). These properties led TiO_2 nanoparticles (NPs) to a wide range of industrial applications and consumer products such as water treatment agents, self-cleaning surface coatings, light-emitting diodes, solar cells, disinfectant sprays, sporting equipment, sunscreens and other cosmetics (Chen et al., 2007).

Consequently, the commercial production of nanosized TiO_2 is increasing at a very high rate every year. For example, the estimated production between 2006 and 2010 was about 5000 tons/year, while the predicted production for between 2011 and 2014 is about 10 000 tons/year (UNEP, 2007). The high production of TiO_2 NPs due to its widespread use, soon can

lead to significant release of these NPs into the environment (Hall et al., 2009). However, little is known about the NPs fate, behavior and toxicity once released into the environment and surface waters.

1.5.1 Effects and toxicity of TiO_2 NPs

Conventional TiO_2 has been considered to be biologically inert and harmless for living beings, and fine TiO_2 particles were used as controls in toxicological studies of various particles, for example in numerous pulmonary toxicity studies (Sager et al., 2008).

TiO_2 reflects and scatters UVB and UVA in sunlight, thus has been applied for e.g. as a safe physical sunscreen. However, TiO_2 absorbs about 70% of incident UV and especially in aqueous environments this leads to generation of hydroxyl radicals which can initiate oxidations (Dunford et al., 1997). Also TiO_2 NPs are photo-inducible, redox active and thus generators of potential reactive oxygen species (ROS) at its surface (Menard et al., 2011). Dunford et al. (1997) showed that TiO_2 present in sunscreen samples was able to catalyze oxidative damage to DNA both *in vitro* and in human cells. Moreover TiO_2 in nanoparticle size showed to be able to produce ROS not only in the presence of UV irradiation (Armelao et al., 2007), but also in the absence of photoactivation (Gurr et al., 2005; Reeves et al., 2008). ROS and free radicals are oxidative stress inductors and may play a major role in the NPs potential toxicity to organisms.

Concerning mammalians, some pro-inflammatory effects resulting from TiO_2 NPs exposure were observed both *in vitro* and *in vivo* in pulmonary cells. Studies reported that TiO_2 NPs can induce respiratory toxicity, epithelial inflammation and cytotoxicity within the lung of rodents (Ferin et al., 1991; Oberdörster et al., 1992; Bermudez et al., 2004; Warheit et al., 2006) but also in human lung cells (e.g. Gurr et al., 2005; Lai et al., 2008).

Exposure of rats by intracheal instillation to a suspension of TiO_2 NPs caused dose-dependent pulmonary damage and inflammation, which persisted 42 days post-exposure (Sager et al., 2008).

The administration of TiO_2 NPs into an air pouch in mice, provoked an acute inflammatory response, inducing a rapidly leukocyte infiltration with predominance of neutrophils and an increase expression of pro-inflammatory mediators as chemokines (Gonçalves and Girard, 2011).

Rabbit erythrocytes treated *in vitro* with TiO_2 NPs underwent hemagglutination and dose dependent hemolysis (Li et al., 2008). Moreover intragastric administration of TiO_2 NPs in mice provoked the damage of blood system haemostasis, reduction of the immunity in association with a seriously damaged liver function (Duan et al., 2010).

After an intraperitoneal injection in mice, TiO_2 NPs were retained in multiple organs and tissues, mainly in spleen but also in liver, kidney and lung, inducing significant pathological changes and various degrees of organ lesions, severely in spleen (Chen et al., 2009). An accumulation of NPs in mice spleen due to a TiO_2 NPs exposition by intraperitoneal injection for consecutive 45 days, was also reported by Li et al. (2010), leading to congestion and lymph nodule proliferation of spleen tissue, spleenocytes apoptosis, ROS accumulation, resulting in a decrease of immune capacity.

TiO_2 NPs induced formation of micronuclei and apoptosis in hamster embryo fibroblasts (Rahman et al., 2002). In addition, several studies showed that TiO_2 NPs caused hepatocyte necrosis in mice livers and changes in some enzymes levels (Wang et al., 2007; Liu et al., 2009) and hepatocytes apoptosis and inflammation related to alterations of both mRNA and protein expression levels of diverse inflammatory cytokines (Ma et al., 2009). Moreover, according to the same author the increase of lipid peroxides in brain and liver mouse tissues caused by TiO_2 NPs was associated to an oxidative attack activated by a decrease of the antioxidative defense mechanisms.

TiO_2 NPs were also associated with changes in gene expression, including alterations concerning brain development in mouse (Shimizu et al., 2009) and expression of apoptosis-related genes (Carinci et al., 2003).

With respect to skin, several studies report that TiO_2 NPs are able to penetrate animal and human skin (Wu et al., 2009, Monteiro-Riviere et al., 2011). Dermal exposure to TiO_2 *in vivo*, using mice as biological model, revealed that these NPs are able to penetrate skin, reach different tissues and induce lesions in different organs mostly at skin and liver (Wu et al., 2009). Results of this study also showed that prolonged exposure to TiO_2 NPs can cause oxidative stress by increasing lipid peroxidation products and cause collagen depletion leading to skin aging, concluding that TiO_2 NPs may pose a risk to human health after dermal exposure over a relative long time period (Wu et al., 2009).

1.5.2 Toxicity of TiO_2 NPs to aquatic organisms

Regarding the ecotoxicity of TiO_2 NPs to aquatic biota, the most studied group of aquatic organisms are freshwater invertebrates, followed by algae and for last freshwater fish (Menard et al., 2011).

Algae play an important role in the equilibrium of aquatic ecosystems, being the first level of the trophic chain to produce organics and oxygen (Sadiq et al., 2011).

The exposition of TiO_2 NPs to freshwater green micro algae produced a growth inhibition (Hartmann et al., 2010; Metzlera et al., 2011; Sadiq et al., 2011) and also a concentration dependent decrease in chlorophyll content (Sadiq et al., 2011).

Hartmann et al (2010) observed that in addition to the generation of reactive oxygen species, possible mechanisms of toxicity to algae included the adhesion of TiO_2 to algal cells and physical disruption of the cell membranes. Microscopy techniques confirmed that TiO_2 NPs have a strong affinity toward the cell surface, demonstrating probable interactions between the particles and the surface active sites of the cell membrane (Metzlera et al., 2011; Sadiq et al., 2011). The adhesion/adsorption of NPs to the cell surface may interrupt the nutrient transfer; enhance the ROS reaction rates and membrane lipid peroxidation (Metzelera et al., 2011).

The observation of TiO_2 NPs aggregates entrapping algal cells, suggested that it may play the major role in the toxicity of TiO_2 NPs to algae species (Aruoja et al., 2009; Sharma, 2009; Sadiq et al., 2011).

Cherchi et al. (2011) showed that the internalization of TiO_2 NPs through multilayered membranes in algal cells can occur, generating observable alteration in various intracellular structures and inducing a series of recognized stress responses. Therefore, NPs may be transported along the ecological food web and ultimately impact important biogeochemical processes, such as the carbon and nitrogen cycle (Cherchi et al., 2011).

Daphnia magna (a cladoceran freshwater water flea) is widely used as a biological model for testing ecotoxicity. *D. magna* is a vital connection in the aquatic food chain between the algae that they consume and the ecologically and economically important fish that consume those freshwaters crustaceans (Lovern and Klaper, 2006).

The exposure of *D. magna* to TiO_2 NPs induced significantly the activity of several antioxidant enzymes as CAT and GST, with a concentration-dependent increase (Kim et al., 2010). This suggested that the toxicity was mediated by ROS, generated by TiO_2 NPs, via oxidative stress in *D. magna* (Kim et al., 2010).

Filtered TiO_2 NPs were reported to cause an increase of the mortality of *D. magna* with the increase of TiO_2 concentration (Lovern and Klaper, 2006). In a chronic bioassay, Kim et al. (2010) observed an increase in mortality, probably due to the accumulation of TiO_2 NPs in the intestine of *D. magna*, which might induce effects such as oxidative stress relating to the induction of antioxidant enzymes.

Other studies also showed that *D. magna* ingest TiO_2 NPs from aqueous suspension and their deposition is visible inside the gastrointestinal tract (Baun et al., 2008; Kim et al., 2010; Zhu et al., 2010a). *D. magna* displayed difficulty in eliminating TiO_2 NPs from their body, resulting in a high level of bioaccumulation, which may interfere with food intake, growth and reproduction (Zhu et al., 2010a and 2010b).

The chronic exposure of *D. magna* to TiO_2 NPs resulted in severe growth retardation, reproductive defects and increasingly mortality (Zhu et al., 2010a). The toxicity of NPs was shown to increase with the exposure duration, demonstrating that it may also be an important factor in the toxicity mediated by NPs (Zhu et al., 2010a).

Zhu et al. (2010b) showed evidence for TiO_2 transfer from *D. magna* to zebrafish (*Danio rerio*) through a simplified freshwater food chain.

In larval zebrafish, TiO_2 NPs affected significantly swimming parameters, as average and maximum velocity and activity level (Chen et al., 2011).

In vitro studies reported intrinsic genotoxic and cytotoxic potential of TiO_2 NPs on fish cell lines derived from rainbow trout gonadal tissue (Vevers and Jha, 2008) and from gold fish (*Carassius auratus*) skin cells (Reeves et al., 2008). Also Reeves et al. (2008) indicated that ·OH radicals are the predominant radical species generated both in aqueous solution as in the fish cells, thus playing the major role in producing the genotoxic effects in terms of oxidative DNA damage.

Griffitt et al. (2009) reported that TiO_2 NPs exposure to zebrafish altered the expression of genes involved in ribosomal function, which may be related to inhibition of protein synthesis by cellular stress. Moreover the microinjection of TiO_2 NPs in zebrafish embryos caused significant changes in the expression of genes related to circadian rhythm, cell kinase activity, intracellular trafficking and immune response, detected by microarray analysis (Jovanovic et al., 2011).

In vivo studies using as a biological model different species of fish, also showed changes in the activity of antioxidant enzymes, in lipid peroxidation levels and histopathological changes, as a result to the exposition to TiO_2 NPs.

The exposure of rainbow trout (*Oncorhynchus mykiss*) to TiO_2 NPs caused respiratory distress and sub-lethal toxicity involving oxidative stress, induction of antioxidant defense system, increase in lipid peroxidation and organ pathologies in gills, liver, intestine and brain (Federici et al., 2007). Also Frederici et al. (2007) suggested that the observation of a severe erosion of the trout gut epithelium can be a consequence of drinking contaminated water with NPs.

Xiong et al. (2011) exposed zebrafish to TiO_2 NPs, concluding that these NPs were able to cause toxicity effects without entering the cells, despite the formation of aggregates in suspensions. They observed that extracellular hydroxyl radical (·OH) generated by TiO_2 NPs could induce oxidative damage directly on the cell membranes of gill tissue.

In juvenile carp (*Cyprinus carpio*), TiO_2 NPs modified the antioxidant enzymatic activity (SOD, CAT, POD) and elevated the lipid peroxidation levels most evidently in liver, inducing liver

disorders (as necrotic and apoptosis hepatocytes) and also gills pathologies (as edema and thickening of gill lamellae and filaments) (Hao et al., 2009).

The effects of dietary exposure to TiO_2 NPs in rainbow trout were studied by Ramsden et al. (2009), showing the occurrence of Ti accumulation in gills, gut, liver, brain and spleen, with Ti not clearing in some organs following recovery, especially the brain. They also observed disturbances of Cu and Zn levels, a 50% inhibition of Na^+/K^+-ATPase activity in the brain and a 50% reduction of thiobarbituric acid reactive substances in the gill and intestine during exposure. Comparing their results for TiO_2 NPs against the know hazard from other metals, Ramsden et al. (2009) concluded that the dietary hazard from TiO_2 NPs might be considered more toxic than dietary Cu and Zn, and similar to Hg at equivalent oral doses.

Recent studies are revealing that the presence of TiO_2 NPs can exacerbate the toxicity of other contaminants, having an indirect impact on aquatic organisms by varying the toxicity of coexisting pollutants. The presence of TiO_2 NPs greatly enhanced the accumulation of cadmium (Cd) and arsenic (As) in carp (*Cyprinus carpio*), especially in viscera and gills (Zhang et al., 2007; Sun et al., 2009), acting as a carrier of these metals into fish. Hartmann et al. (2010) observed that the algal toxicity of Cd was enhanced in the presence of TiO_2 NPs, indicating either a combined effect of Cd and TiO_2 NPs or an increase of the bioavailability of Cd for the algae caused by TiO_2 NPs. Also Hu et al. (2011) showed that TiO_2 NPs in humic acid solutions can act as a carrier to facilitate the Cd bioaccumulation in zebrafish and potentially other heavy metals. The toxicity of tributyltin (TBT, a highly toxic marine antifouling compound) to abalone (*Haliotis diversicolor supertexta*) embryos increased with the presence of TiO_2 NPs, as a result of the combined effects of TBT adsorption onto TiO_2 NPs aggregates and the internalization of these aggregates by abalone embryos (Zhu et al., 20111). Thus, not only the direct impacts of NPs should be a matter of concern but also their interactions with other environmental pollutants.

1.6 Biological models

Aquatic organisms are widely chosen as test species, as they are more sensitive to exposure and toxicity compared to terrestrial organisms including mammals. Besides being easily to cage and having a filtration capacity, they can provide model systems for evaluation of oxidative damage concerning to chronic exposure or sublethal concentrations, mutagenicity and other adverse effects of pollutants (Valavanidis et al., 2006).

1.7 Oxidative stress enzymes

Oxidative stress is an important subject for terrestrial and aquatic toxicology, where its molecular biomarkers found widespread applications in mechanisms of environmental toxicity in aquatic organisms exposed to different chemical pollutants (Livingstone, 2001).

Reactive oxygen species (ROS), such as the superoxide anion radical (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical (·OH) are continually produced in biological systems as toxic byproducts of normal oxidative metabolism, but can be increased by interactions with pollutants by various mechanisms (Livingstone, 2003).

In living aerobic organisms, the neutralization, detoxication and removal of ROS is effected by intracellular antioxidant defense systems, whose roles are to intercept and inactivate reactive radicals (Valavanidis et al., 2006). The antioxidant defense includes specific antioxidant enzymes, such as superoxide dismutase (SOD; converts $O2^-$ to H_2O_2; EC 1.15.1.1), catalase (CAT; converts H_2O_2 to H_2O and O_2; EC 1.1.1.6), glutathione-s-transferase (GST; conjugates and detoxifies products of lipid peroxidation; EC 2.5.1.18), but also non enzymatic cellular defenses, such as reduced glutathione (GSH), vitamins A and E, ascorbate and urate (de Zwart et al., 1999; Livingstone, 2003). The between the generation and the neutralization of ROS by antioxidant mechanisms within an organism is called oxidative stress (Davies, 1995).

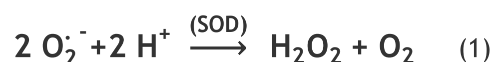
Antioxidant enzyme activities are found widely distributed in tissues of aquatic organisms, with a mostly higher activity in liver of fish and in the digestive gland or equivalent in invertebrate organisms (Livingstone, 2001). Assaying antioxidant enzymes can indicate the antioxidant status of the organisms, working as a potential biomarker for contaminant-mediated oxidative stress (Valavanidis et al., 2006).

Cellular antioxidant defense systems may be increased or inhibited under chemical stress, depending on the intensity, duration of the stress applied and on the susceptibility of the exposed organisms (Cossu et al., 2000). The exposure to organic pollutants and metals may induce significant increases in antioxidant enzymes in response to ameliorate oxidative stress, but these are transient and variable for different aquatic species. Studies with fish observed that, in response to toxicant-induced inflammation by ROS, the concentrations of certain antioxidant enzymes are increased, but under high levels of pollution the antioxidant defenses can be reduced (Valavanidisa et al., 2006). An induction of the antioxidant defense can be considered an adaptation of the organisms to prevent toxicity, while a decrease suggests a precarious state characterized by a higher susceptibility to environmental stress, resulting in adverse effects (Cossu et al., 2000). The knowledge of the regulation of antioxidant systems in aquatic organisms in relation to sources of ROS is limited (Livingstone, 2001). The complexity of pollution in aquatic ecosystems provides a non-specific response to a kind of contaminants, but antioxidants constitute useful biomarkers reflecting not only an

exposure to pollutants but also their toxicity. A multiple marker approach can be more relevant than a single antioxidant parameter to the evaluation of the total antioxidant status.

1.7.1 Superoxide Dismutase

Superoxide dismutase (EC 1.15.1.1) is the antioxidant enzyme that catalyzes the dismutation of the highly reactive superoxide anion (O_2^-) to O_2 and to the less reactive species H_2O_2 (reaction (1)), which can be further destroyed by catalase or GPX reactions.



$$2\,O_2^{\cdot -} + 2\,H^+ \xrightarrow{(SOD)} H_2O_2 + O_2 \qquad (1)$$

Another function of superoxide dismutase is to protect dehydratases (dihydroxy acid dehydratase, aconitase, 6-phosphogluconate dehydratase and fumarases A and B) against inactivation by the free radical superoxide (Matés and Sánchez-Jiménez, 1999). Four classes of SOD have been identified, containing either a di-nuclear Cu, Zn or mononuclear Fe, Mn or Ni cofactors (Matés and Sánchez-Jiménez, 1999).

At physiological pH, the rate of the non-enzymatic dismutation of superoxide is significant, but it is considerably increased in the presence of SOD. The turnover numbers of SODs are indeed very high over a wide range of pH. In animal cells, the fact that intracellular SOD concentrations range from 10^{-6} to 10^{-5} M supports the concept that superoxide is strongly toxic (Chaudière and Ferrari-Iliou, 1999).

1.7.2 Catalase

Catalase (CAT; EC 1.11.1.6) is included in the subclass of oxidorredutases, being present in practically all aerobic organisms and in many anaerobic organisms. It can also be named hydroperoxidase as it catalyzes the conversion of hydrogen peroxide (H_2O_2), a powerful and potentially harmful oxidizing agent, to water and molecular oxygen (reaction (2)).

$$2\,H_2O_2 \xrightarrow{(CAT)} 2\,H_2O + O_2 \qquad (2)$$

Much of the H_2O_2 that is produced during oxidative cellular metabolism comes from the breakdown of one of the most damaging ROS, namely the superoxide anion radical (O_2^-), by superoxide dismutases into hydrogen peroxide and oxygen.

Each catalase molecule can decompose millions of H_2O_2 molecules every second, thus this is one of the most efficient enzymes found in cells. It is so efficient that it cannot be saturated by H_2O_2 at any concentration.

CAT also acts as peroxidase, at low hydrogen peroxide concentration, using reducing co-substrates, as a variety of metabolites and toxins donors of hydrogen such as alcohols, formic acid, formaldehyde or phenols.

Structurally, most catalases exist as tetramers, each subunit containing an active site heme group deep within its structure, but accessible from the surface through hydrophobic channels. This very rigid and stable CAT structure makes it more resistant to unfolding, to pH, thermal denaturation and resistant to proteolysis than most of other enzymes (Chelikani et al., 2004).

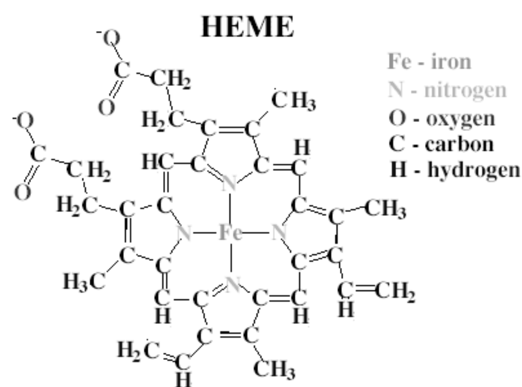


Figure 3 Catalase Heme Group. In the middle of the heme group sits an iron atom. The catalase enzyme uses this iron atom to help it break the bonds in the two molecules of H_2O_2, shifting the atoms around to release two molecules of H_2O and a molecule of O_2.

H_2O_2 is broken down by CAT within a two-stage mechanism in which H_2O_2 alternately oxidises and reduces the heme iron at the active site. In the first step, one H_2O_2 molecule oxidises the heme to an oxyferryl species. In the second step, a second hydrogen peroxide molecule is used as a reductant to regenerate the resting state enzyme, producing water and oxygen (Chelikani et al., 2004).

The ubiquity of the enzyme, its ease of assay, involving a cheap, readily available substrate, H_2O_2, and the outstanding display of oxygen evolution have combined to make it an attractive target for biochemists and molecular biologists alike.

1.7.3 Glutathione S-Transferase

Glutathione-S-transferases (GSTs; EC 2.5.1.18) are large family (cytosolic, mitochondrial, and microsomal) of phase II biotransformation enzymes, with substrates that include products of oxidative stress and electrophilic xenobiotics. Besides their enzymatic activity, GSTs can

protect cells from toxicants through chemical removal of the agents by noncatalytic binding. GSTs and ligandin (a protein binding to physiologic and exogenous ligands) bind toxic xenobiotics and their metabolites and this noncatalytic activity of GSTs can provide protection to the cellular environment by acting as a shield to protect DNA, proteins, and lipids from the deleterious effects of xenobiotics. GSTs are also involved in the biosynthesis of leukotrienes, prostaglandins, testosterone, progesterone, as well as in the degradation of tyrosine. GSTs are among the most abundant proteins in some tissues, including kidneys and especially in the liver, which plays a key role in detoxification.

Oxidative stress usually leads to enhanced generation of endogenous electrophiles and electrophilic toxins generated from lipid peroxidation or that are converted to genotoxic electrophilic intermediates by the catalytic action of cytochromes P450. GSTs catalyze the nucleophilic attack of the sulphur atom of the reduced glutathione (GSH) on electrophilic groups of a range of hydrophobic substrates, thereby neutralizing their electrophilic sites and rendering the products more water-soluble. Glutathione conjugates are metabolized further by cleavage of the glutamate and glycine residues, followed by acetylation of the resultant free amino group of the cysteinyl residue, to produce the final product, a mercapturic acid. The mercapturic acids, i.e. S-alkylated derivatives of N-acetylcysteine, are then excreted. Most substrates are inactivated by conjugation with GSH, however some are converted to more reactive compounds increasing toxicity (bioactivation of a toxin).

Although GSTs do not decompose the ROS per se, these enzymes constitute an all-important second line of defense and provide protection against oxidative stress by attenuating lipid peroxidation and by detoxifying the toxic end-products of lipid peroxidation. As antioxidant enzymes, GSTs complement the role of primary defense enzymes in protecting organisms from the deleterious effects of ROS. While superoxide anion and H_2O_2 are effectively disposed of by the cells through highly efficient enzymes (superoxide dismutase, catalase, and GPx), the ROS escaping this line of defense can initiate the autocatalytic chain of lipid peroxidation through the generation of free radicals capable of abstracting a single hydrogen atom from unsaturated fatty acids. GSTs prevent propagation of lipid peroxidation by reducing lipid hydroperoxides, and GSTs also detoxify the toxic electrophilic end-products of lipid peroxidation. Thus constitute the all-important second and third lines of defense against oxidant toxicity (Sharma et al., 2006).

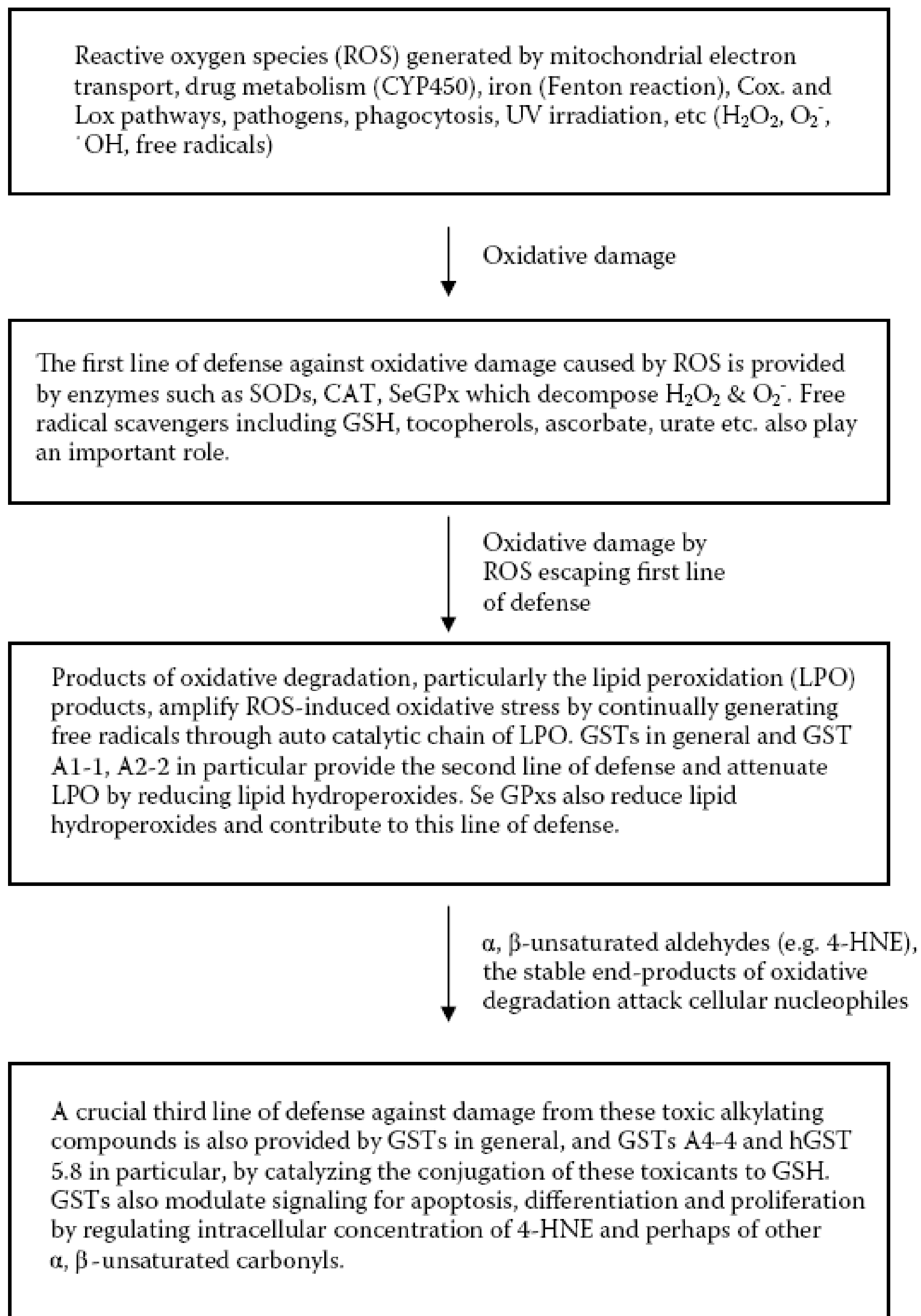


Figure 4 Enzymatic Antioxidant defense lines against ROS oxidative damage. (in Sharma et al., 2006)

1.8 Lipid Peroxidation

Lipid peroxidation is probably the most extensively investigated process induced by free radicals. Membrane phospholipids of aerobic organisms are continually exposed to oxidant challenges, being a target rapidly affected by free radicals. Thus, peroxidized membranes and lipid peroxidation products represent constant threats to aerobic cells. The group of polyunsaturated fatty acids is especially highly susceptible to oxidative reactions by ROS, because of their double bonds.

The process of lipid peroxidation is composed of a set of radical chain reactions, initiated mainly by hydroxyl radicals, especially in transition metal-catalyzed reactions, resulting in the formation of many equivalents of lipid peroxides (LOOH).

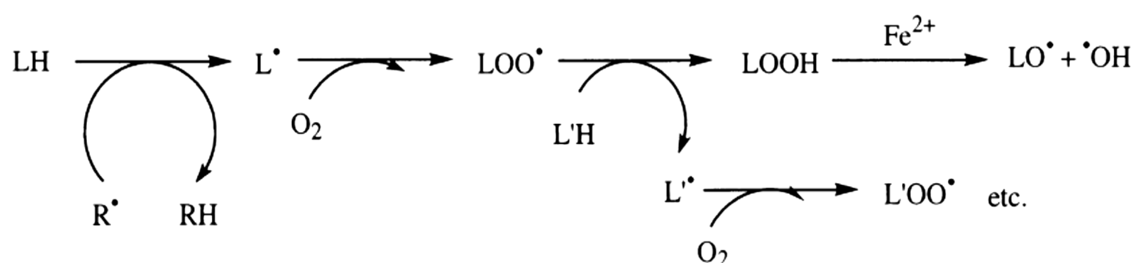


Figure 5 Lipid peroxidation chain reactions. Schematic proceed of lipid peroxidation chain reactions, resulting in the formation of many lipid peroxide radicals.

These degenerative propagation reactions in lipid membranes are usually accompanied by the formation of a wide variety of products, as the resulting LOOH can easily decompose into several reactive species including lipid alkoxyl radicals, aldehydes, alkanes, lipid epoxides, and alcohols. Most of these products are toxic by themselves, especially hydroxyalkenals, and active mutagens, acting as second messengers for radical damage (Valavanidis et al., 2006). These products may form DNA adducts giving rise to mutations and altered patterns of gene expression and the peroxidized membranes become rigid, losing permeability and integrity. Thus, products resulting from lipid peroxidation may be important parameters to monitor radical damage and several of the most important products (de Zwart et al., 1999).

The most widely used assay for lipid peroxidation is malondialdehyde (MDA) formation as a secondary lipid peroxidation product, often assayed with the thiobarbituric acid reactive substances (TBARS) test. MDA levels in hepatic homogenates can be used for metal-induced oxidative stress in fish.

1.9 Microscopy Techniques and histopathology

1.9.1 Light and electron microscopy

The alterations in cell structure resulting from chemical exposure can be evaluated by different types of microscopy, depending on the purpose of the study. Changes which alter the cells and tissues of an organism and do not result in death can be observed under the light microscope or electron microscope. Therefore, light microscopy is commonly used for examination of histopathological alterations caused by exposure to a chemically altered environment while electron microscopy is used for the observation of alterations in the cell ultra-structure and is commonly used to assess tissue and cell changes. Both the scanning electron microscope (SEM) and the transmission electron microscope (TEM) are useful in such evaluations. SEM images provides a tri-dimensional view of tissues and cell surface while TEM provides a bi-dimensional image of cell´s inside and allows for the observation of structural changes which cannot been assessed by light microscopy.

1.9.2 Histological examination

Histology has been widely used for assessment of negative effects on living organism´s tissues and cells. Regarding fish, histopathological biomarkers or cellular changes in tissues such as gill, liver, kidney and spleen have received much attention in assessing the effects of exposure to pollutants and other substances. Therefore, histology can be a powerful tool, especially when used in conjunction with measurements of other biomarkers and other morphological studies. Additionally, the diagnostic power of histopathology may be further enhanced by employment of additional histological techniques such as immunohistochemistry. An added value of histopathology is in its capability to analyse the mechanistic effects of exogenous substances or materials and to characterize effects more specifically. For instance, histopathological analysis of target organs can reflect fish health more realistically than biochemical biomarkers. By comparing the impact of known or unknown samples on indicative histological parameters with that of specific reference compounds, the nature and magnitude of the evoked effects can be then determined (Wester et al., 2002). Thus, it can be used as a useful tool to assess the level of sub-lethal and chronic effects of toxicity, as indicator of the exposure to pollutants and may provide a better extrapolation to community and ecosystem-level (Au, 2004; Bernet et al., 1999).

Histopathological biomarkers are also useful as they can specify the target organs, tissues, cells and organelles of a single or group of toxin(s). Understanding the specificity of a contaminant to damage a particular organ system gives an insight into the mechanism of action of the toxicant which would not be available from doing classical 96h-LC_{50} testing. Chronic exposure to low levels of a toxicant can be studied at a broader scale on a light structure basis rather than conducting LC_{50} test which only study acute exposure. LC_{50} values

may be misleading because they reflect total metal concentration while particular attention should be given to the chemical processes that control chemical speciation. With respect to histopathological indicators they are beneficial since they show the net effect of biochemical and molecular changes in the organism resulting from exposure to a contaminant. Light structure of tissues and organs is altered when levels of the contaminant are still at low levels, therefore histopathological evaluation provide a valuable screening method of an ecosystem before severe ecological damage occurs.

2 Objectives

Within the present context, the main objective of this study is to provide a toxicological assessment of the TiO_2 nanoparticles exposure (which is already present in consumer products) to two different freshwater species, goldfish (*Carassius auratus*) and freshwater clam (*Corbicula fluminea*).

For this propose, the methodology included the evaluation of the activity of some antioxidant enzymes (SOD, CAT, GST), lipid peroxidation and histopathological observations of different potential target organs.

3 Materials and methods

3.1 Nanoparticles characterization

Commercial TiO_2 nanoparticles *Aeroxide TiO_2 P25* (Aerosil, USA), with an average primary particle size of 21 nm in the powder form (Tab. 2), were used for the exposure assays.

Table 2 TiO_2 nanoparticles physico-chemical proprieties, according to the manufacturer.

Test Method	Unit	AEROXIDE TiO_2 P 25
Behavior towards Water		hydrophilic
Appearance		Fluffy white powder
Specific Surface Area (BET)	m^2/g	50 15
Average Primary particle Size	Nm	21
Tapped Density (approximate value)	g/L	130
Loss on Drying (2 hours at 150°C when leaving plant)	wt.%	1,5
Loss on Ignition (2 hours at 1000°C)	wt.%	2,0
pH		3,5 - 4,5
SiO_2	wt.%	0,200
Al_2O_3	wt.%	0,300
Fe_2O_3	wt.%	0,010
TiO_2	wt.%	99,5
Hcl	wt.%	0,300
Sieve Residue	wt.%	0,05
Unit Weight	Kg	10

Nanoparticle Tracking Analysis (NTA) (NanoSight LM10-HS, United Kingdom) was used to characterize the behavior of nanoparticles in liquid suspensions. NTA is a method that uses Brownian motion to locate and follow individual particles (10-1000 nm) in solution (Fig. 6), from which size-distribution profiles can be obtained (Carr, 2009).

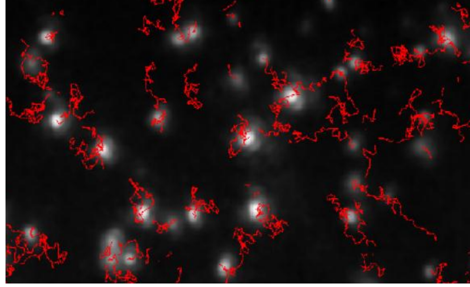


Figure 6 Typical image produced by NTA showing particle tracks. Nanoscale particles can be directly and individually visualized and counted in liquid in real-time (in Carr, 2009).

3.2 Experimental procedure

3.2.1 Test organisms

The biological materials used to carry out the experiments were a freshwater fish (*Carassius auratus*) and a freshwater bivalve (*Corbicula fluminea*). *C. aurautus* has been widely used as a model species in several ecotoxicological studies, since they are commercially available, easy to maintain and handle in laboratory (Ostrander, 2000). *C. fluminea* is also an important tool currently used as a biomarker for monitoring water contamination (Legeay et al., 2005).

The fish were obtained from commercial suppliers (Koi Park, Portugal) and transported to the laboratory facilities for a period of acclimation before the exposure assays.

The bivalves were collected manually on Tagus River, near the locality of Escaroupim and imediatley transported to laboratory for a period of acclimation before being used in exposure tests.

Figure 7 Sampling of *C. fluminea* at Tagus River´s margin.

3.2.2 Acclimation

Previously to the beginning of the assay, both species were acclimated for two weeks in a closed circuit system with filtered de-chlorinated tap water, at a pH of 7.4 ± 0.2, temperature of 19 ± 1ºC, photoperiod: 12hL:12hD and with continuous aeration enough for keeping the dissolved oxygen always higher than 6 mg/L.

Figure 8 Organisms in acclimation tanks. *C. auratus* (A) acclimated in a 400 L polystyrene tank (B). *C. fluminea* in acclimation tank (C).

3.2.3 Preparation of Test Nanoparticles

Stock solutions of TiO_2 nanoparticles were prepared using distilled water and then ultrasonicated (10 min, 35 KHz, 100/400W) using an ultrasound bath (Elma, Germany) for dissolution. Then, solutions were added to 10 L or 2 L of dechlorinated tap water in exposure tanks, in order to obtain nominal concentrations of 0.01, 0.1, 1, 10, 100, 400 and 800 mg TiO_2/L.

3.2.4 Exposure Assays

Carassius auratus

After the acclimation period, *Carassius auratus* (N=105; 5.9 ± 0.4 g; 4.4 ± 0.7 cm standard length), of both sexes, with less than one year of age, were randomly distributed into 15 L capacity polystyrene tests tanks, in groups of 15 fish per tank (Fig. 8). Fish were exposed to different concentrations of TiO_2 nanoparticles, from 0.01 to 800 mg TiO_2/L. An additional tank with clean tap was used for a group of control fish. The fish were tested under a constant temperature of 19 ± 1ºC, pH of 7.4 ± 0.2, photoperiod: 12hL:12hD and continuous aeration. The experimental conditions in each tank were renewed every 48 hours and the assay had duration of 21 days. During the experiments fish were daily fed *ad libitum* with commercial flakes of dry food (Tetra brand). Tanks were monitored constantly for the counting of dead fish.

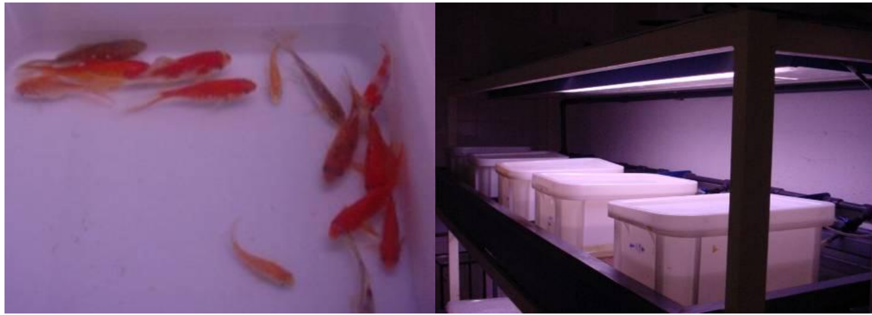


Figure 9 *C. auratus* in test tanks.

Corbicula fluminea

After the acclimation period, *C. fluminea* (N=60; 1.5 ± 0.3 g ; 2.8 ± 0.2 cm standard length), were randomly distributed into 2 L capacity tests tanks, in groups of 10 individual per tank (Fig. 10). The individuals were exposed to the previously prepared solutions of different concentrations of TiO_2 nanoparticles from 0.01 to 100 mg TiO_2/L. An additional tank with clean tap was used for a group of control individuals. The bivalves were tested under a constant temperature of 19 ± 1ºC, pH of 7.4 ± 0.2, photoperiod: 12hL:12hD and continuous aeration. The experimental conditions in each tank were renewed every 48 hours and the assay had duration of 14 days. Tanks were monitored constantly for the counting of dead individuals.

Figure 10 Tanks for *C. fluminea* exposure to TiO_2 NPs suspensions.

3.3 Sampling the tested organisms

Carassius auratus

Fish were collected for sampling after 7, 14, and 21 days of exposure. At the beginning of the experiment, one additional fish group (n=5) was also collected from the acclimation tank for sampling.

Fish were sacrificed by decapitation and dissected to remove the liver, intestine and gills.

Tissue samples from liver, intestine, gills were collected and fixed in a solution of *Bouin-Hollande´s* fixative for histological processing.

For enzymatic and biochemical analyses, tissue samples from the target organs were homogenized on-ice in cold buffer 100 mM potassium phosphate (Sigma-Aldrich, Germany) pH 7.0 containing 2 mM of EDTA (Riedel-Haën, Germany). Tissue homogenates were centrifuged at 10,000x g for 15 minutes at 4 ºC. Supernatant was removed and freeze at -80 ºC for further analysis.

Corbicula fluminea

Organisms were collected for sampling after 7 and 14 days of exposure.

Bivalves were dissected to remove and separate the digestive glandule and gills.. Then, samples of the removed digestive glands were fixed in *Bouin-Hollande´s fixative for 48 hours*.

For enzymatic and biochemical analyses, sub-samples of the tissues were homogenized on-ice in cold buffer 100 mM potassium phosphate (Sigma-Aldrich, Germany) pH 7.0 containing 2 mM of EDTA (Riedel-Haën, Germany). Tissue homogenates were centrifuged at 10,000x g for 15 minutes at 4 ºC. Supernatant was removed and freeze at -80 ºC for further analysis.

3.4 Histology

3.4.1 Optical Microscopy

Histological procedures for light microscopy followed essentially Martoja and Martoja (1967).

Briefly, after a fixation period of 48 h in Bouin's fluid, the samples were washed in tap water and passed through a series of alcohols (70º, 95º and 100º) for dehydration, followed by a bath of xylene (Lab-Scan, Belgium) for intermediate impregnation.

Samples were kept overnight in liquid paraffin (Panreac, Spain) at 56 ºC within an incubator, for impregnation. Afterwards, samples were embedded in paraffin (Panreac, Spain) using Leuckart's bars as mold, in order to obtain solid blocks containing the tissue specimens.

After solidification, tissue samples within the blocks were sliced with a microtome (Leica, Germany) in sections of 5-7 µm thickness. Slices were put in a container with warm water, to avoid a coarse surface and an albumin solution was used for the adhesion of tissues slices into the slides.

Paraffin was removed from slides using xylene as solvent, followed by a treatment in a graduate series of alcohols (100º, 95º and 70º) and a bath with demineralized water.

Slides were stained with hematoxyline, which dyes the cellular nucleous with a violet color, and eosine, which dyes the cellular cytoplasm and intercellular substances with a red-orange color, (H&E), followed by deshydratation steps with alcohols 95º and 100º and xylene.

Alcian blue 8GX (BDH, Poole, UK), at pH=2.5, was carried out according to Carson´s (1990) for differentiating mucous in gills and intestine, using Nuclear Fast Red (SIGMA, St. Louis, MO, USA) as counter staining.

The histological observations were carried out using an optical microscope (Leica-ATC 2000, Wetzal., Germany), with an image system from Leica Microsystems (DMLB model).

3.4.2 Transmission Electron Microscopy (TEM)

Fragments of tissues were fixed sequentially in 3% glutaraldehyde (in cacodylate buffer), osmium tetroxide (in the same buffer) and uranyl acetate (in bi-distilled water). Dehydration was carried out in increasing concentrations of ethanol. After passage through propylene oxide, the samples were embedded in Epon-Araldite.

Thin sections were made with diamond knives and stained with 2% aqueous uranyl acetate and Reynold's lead citrate. The stained sections were studied and photographed in a JEOL 100-SX electron microscope.

3.4.3 Scanning Electron Microscopy (SEM)

Sample preparation for SEM followed essentially Glauert (1975). Sections of 5 to 7 µ thickness of tissues fixed in Bouin-Hollande (48h) were cut from paraffin blocks with a microtome (Leica) and mounted on a carbon slide. Then paraffin was removed from sections with xylene, treated with graded series of ethanol and allowed to dry. Although the carbon tape tended to curl during sample treatment it was possible to analyze samples efficiently. The carbon tape with samples was mounted on aluminum paint, coated with a 3 nm palladium-gold film in a Quorum Q150T ES sputtering system. The surface of the samples was observed in a scanning

electron microscope (SEM) Carl Zeiss AURIGA (Germany) at 1-2 KeV, with an aperture size of 30 microns.

The elemental analysis of the samples was carried out with an Energy Dispersive X-ray spectroscopy (EDS) detector installed in the SEM, with beam energy of 10 keV and aperture size of 120 microns.

3.5 Determination of Total Protein

The determination of total protein concentration was based on the method described by Bradford (1976). This method relies on the binding of the acidic dye Coomassie Blue G-250 to protein, turning the dye into an anionic blue form, which absorbs at 595 nm and thus allows estimating spectrophotometricaly the protein concentration.

Comassie Blue G-250 (50mg) (BioRad, USA) was dissolved in 50 mL methanol and added to 100 mL of 85% H_3PO_4 (Riedel-Haën, Germany). The solution was diluted with distilled H_2O to the final volume of 1 L, then filtered to remove precipitates and stored at 4 ºC.

Bovine Serum Albumine (BSA) was used to prepare the standard curve. A stock BSA (Sigma-Aldrich, Germany) solution was prepared, using distilled water, to a concentration of 1mg/mL. BSA protein standards solutions within a range from 0.0 to 1.0 mg/mL were prepared from the stock solution, by successive dilutions.

The assay was performed using a 96 well microplate (Nunc-Roskilde, Denmark), where 10 µL of BSA standard or sample were added to 190 µL of Bradford reagent in each well. The absorbance at 595 nm was measured using a plate reader (BioRad Benchmark, USA). The protein concentration of samples was calculated using the software program Microplate Manager 4.0, using a BSA standard curve previously prepared, and the results were expressed in mg total protein/mL.

3.6 Determination of Enzymatic Activity

3.6.1 Catalase (CAT)

CAT activity was estimated based on the spectrophotometric method described by Aebi (1984), which follows the decrease in absorbance at 240 nm by H_2O_2 consumption.

A substrate solution of 0.036% (w/w) H_2O_2 was prepared in buffer 50 mM KH_2PO_4 (Sigma-Aldrich, Germany) pH 7.0 containing 1 mM EDTA (Riedel-Haën, Germany), at 25 ºC using 30% (w/w) H_2O_2 (Sigma-Aldrich, Germany).

Bovine liver catalase (Sigma-Aldrich, Germany) was used as standard and positive control.

To perform the assay, 0.1 mL of CAT standard or sample were added to 2.9 mL of the substrate solution in individual quartz cuvettes and absorbance at 240 nm was recorded every 15 seconds for 2 minutes (at 25 ºC, pH 7.0 and path length 10 mm), using a spectrophotometer (Unicam Helios, Portugal).

The change in absorbance per minute (ΔA_{240}) was estimated and the reaction rate at 240 nm was determinate using H_2O_2 extinction coefficient of 40 $M^{-1}cm^{-1}$. The results are expressed in relation to the total protein concentration of the sample (nmol $min^{-1}mg^{-1}$ total protein).

3.6.2 Glutathione-S-Transferase (GST)

GST activity was determinate based on the procedure described by Habig *et al*. (1974), in which the enzyme activity is determined spectrophotometrically at 340 nm by measuring the formation of the conjugate of glutathione (GSH) and 1-chloro-2,4-dinitrobenzene (CDNB).

A substrate solution was prepared using Dulbecco's Phosphate Buffered Saline (Sigma-Aldrich, Germany) with 200 mM L-glutathione reduced (Sigma-Aldrich, Germany) and 100 mM CDNB (Sigma-Aldrich, Germany).

Equine liver GST (Sigma-Aldrich, Germany) was used as standard and positive control.

To perform the assay, 180 µL of substrate solution were added to 20 µL of GST standard or sample in each well of a 96-well microplate (Nunc-Roskilde, Denmark) and the absorbance at 340 nm was recorded every minute for 6 minutes, using a plate reader (BioRad Benchmark, USA).

The change in absorbance per minute (ΔA_{340}) was estimated and the reaction rate at 340 nm was determined using CDNB extinction coefficient of 0.0096 $\mu M^{-1}cm^{-1}$. The results are expressed in relation to total protein concentration of the sample (nmol $min^{-1}mg^{-1}$ total protein).

3.6.3 Superoxide Dismutase (SOD)

The procedure for SOD activity determination followed the nitroblue tetrazolium (NBT) method, adapted from Sun *et al* (1988). In this method, superoxide radical ($\cdot O_2^-$) are generated by the reaction of xanthine with xanthine-oxidase (XOD), and reduce NBT to formazan, which can be assessed spectrophotometrically at 560 nm. SOD competes with NBT

for the dismutation of $\cdot O_2^-$ inhibiting its reduction. The inhibiton level is used as a measure of SOD activity.

SOD from bovine erythrocytes (Sigma-Aldrich, Germany) was used as standard and positive control.

The assay was performed using a 96-well microplate (Nunc-Roskilde, Denmark), adding to each well 200 µL of 50 mM phosphate buffer (pH 8.0) (Sigma-Aldrich, Germany), 10 µL of 3 mM EDTA (Riedel-Haën, Germany), 10 µL of 3 mM xanthine (Sigma-Aldrich, Germany), 10 µL of 0.75 mM NBT (Sigma-Aldrich, Germany) and 10 µL of SOD standard or sample. The reaction was started with the addition of 100 mU XOD (Sigma-Aldrich, Germany) and the absorbance at 560 nm was recorded every minute for 5 minutes, using a plate reader (BioRad Benchmark, USA). Negative control included all components except SOD or sample, producing a maximal increase in absorbance at 560 nm, which allowed determining the inhibition percentage per minute, caused by SOD activity.

The total SOD activity is expressed in units/mg of protein, where one unit is equivalent to the SOD activity that causes 50% inhibition of the reaction rate without SOD.

3.7 Lipid Peroxidation

Lipid peroxidation was determined based on *Thiobarbituric Acid Reactive Species* (TBARS) method, following the procedure described by Ohkawa *et al.* (1979). TBARS method is based on the reaction of malondialdeide (MDA, a compound that results from lipid peroxidation) with thiobarbituric acid (TBA), which produces a compound that absorbs at 532 nm.

Trimethylolpropane (TMP) is an acetal of MDA, which is converted into MDA during an acid incubation, thus TMP (Merck, Germany) was used for the construction of a TBARS standard curve. Standards solutions within a range from 0.0 to 0.3 µM were prepared from a TMP stock solution prepared with distilled water.

The reaction was performed using individual *Eppendorf* tubes for each standard or sample, where was added 5 µL of standard or sample, 45 µL of buffer 100 mM KH_2PO_4 (Sigma-Aldrich, Germany) pH 7.0 containing 2 mM EDTA (Riedel-Haën, Germany), 12.5 µL of 8.1 % Sodium Dodecyl Dulfate (SDS) (Merck, Germany), 93.5 of 20% Trichloroacetic acid (TCA) (Merck, Germany) pH 3.5, 93.5 µL of 1% TBA (Merck, Germany) and 50.5 µL of distilled water. The reaction tubes were put on a boiling water bath for 10 minutes and then were cooled on ice, to stop the reaction. Afterwards, 62.5 µL of distilled water and 312.5 µL of n-butanol:pyridine (15:1 v/v) (Merck, Germany) were added to the tubes and the mixture was then well mixed

and centrifuged. 150 µL of the supernatant were removed and added to the wells of a 96-well plate. The absorbance at 532 nm was measured using a plate reader (BioRad, USA).

The MDA concentration of samples was calculated with the computer program Microplate Manager 4.0, using the TMP standard curve previously prepared. The results were expressed in relation to total protein concentration of the sample (nmol $mg^{-1}mL^{-1}$).

3.8 Statistical analysis

Statistical analysis of the results was carried out by one-way ANOVA, after the data had been checked for assumptions of normality and homogeneity (Leven's test) and, if necessary, appropriately transformed.

The post-hoc Tukey test was used to compare pairs of means and detect significant differences ($P<0.05$). The statistical analysis was performed at the significance level of 5%, using the software Statistica 8.0 (StatSoft Inc., Tulsa, OK, USA, 2007).

4 Results

4.1 Nanoparticles characterization in aquatic suspensions

After the addition of TiO_2 NPs stock solutions to the exposure tanks, a rapid sedimentation of NPs was observed at higher exposure concentrations (≥ 10 mg TiO_2/ L). Despite having a primary size of 21 nm in powder form, once in suspension it was observed the formation of macroscopic aggregates over time. These aggregates deposit at tanks bottoms, covering the organisms (Fig. 11, C). In order to avoid the excessive deposition of NPs, the exposure solutions were renewed every 48 hours. The continuous aeration of the tanks and the fish swimming movements also aided to re-suspend NPs in water.

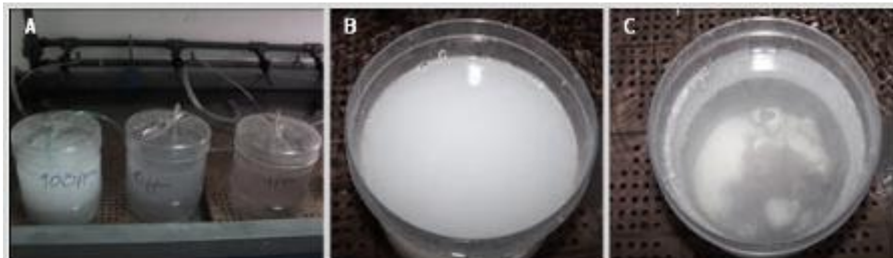


Figure 11 Tanks for the *C. fluminea* exposure to TiO_2 NPs. 100, 10 and 1 mg/L exposure tanks, in the beginning of the experiment (A); 100 mg/L exposure solution, after its preparation (B); 100 mg /L exposure solution, after 48 hours (C). NPs are visible at tanks bottom and covering the organisms (C).

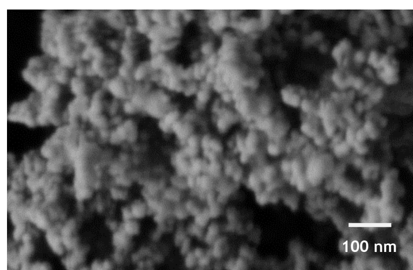
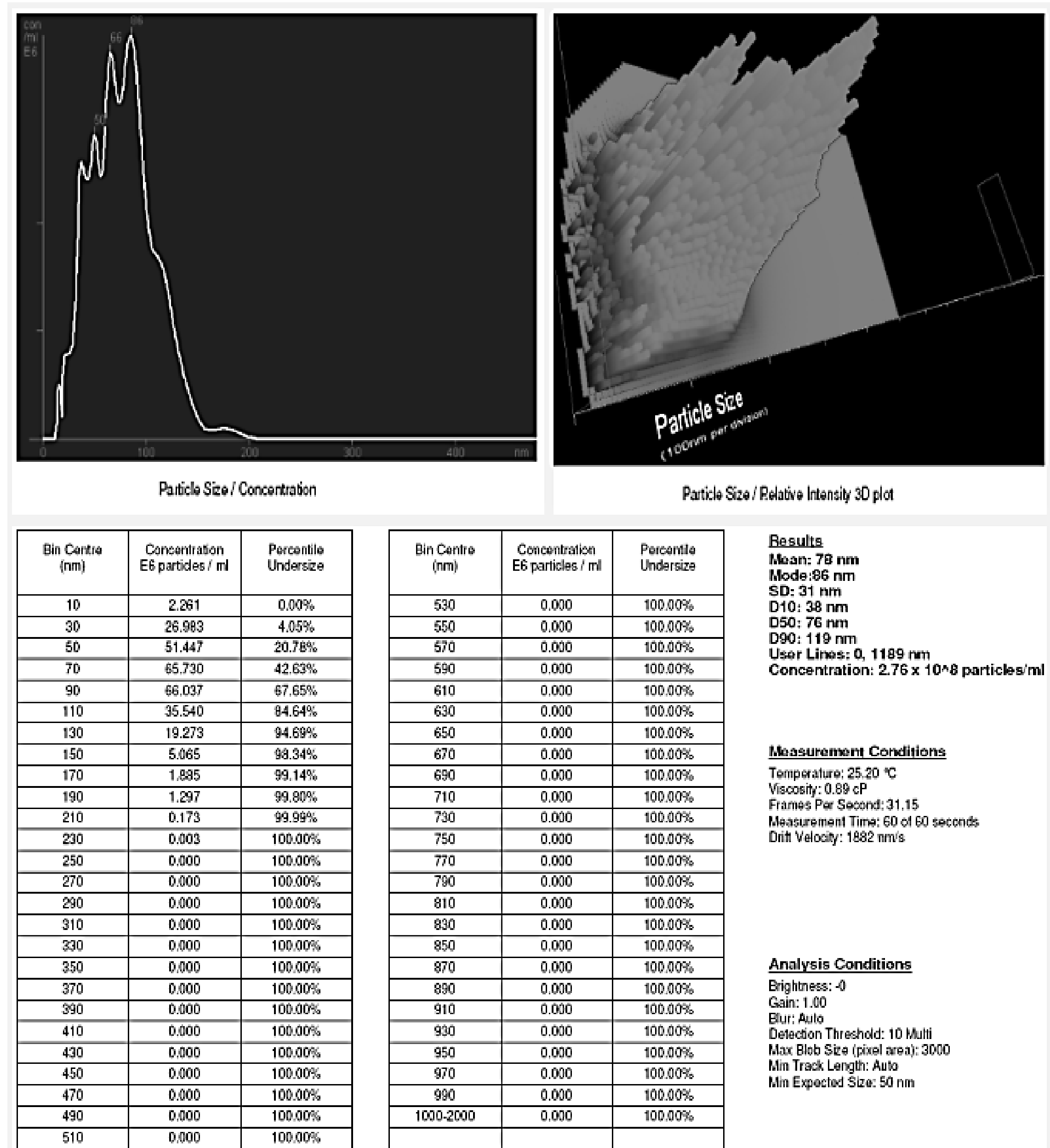


Figure 12 Scanning Electron Microscopy of TiO_2 NPs. SEM picture from an aquatic suspension of TiO_2 NPs, collected from the exposure tanks.

Nanoparticle Tracking Analysis, allowed the visualization of TiO_2 NPs and its movements in real-time and the determination of the particles size/concentration in suspension. For a measurement time of 60 seconds and a concentration of 2.76 x10^8 particles/mL, it was detected the presence of a wide range of different particles sizes, from 10 to 210 nm, with a higher concentration of 86 nm NPs (Fig. 13).



Bin Centre (nm)	Concentration E6 particles / ml	Percentile Undersize
10	2.261	0.00%
30	26.983	4.05%
50	51.447	20.78%
70	65.730	42.63%
90	66.037	67.65%
110	35.540	84.64%
130	19.273	94.69%
150	5.065	98.34%
170	1.885	99.14%
190	1.297	99.80%
210	0.173	99.99%
230	0.003	100.00%
250	0.000	100.00%
270	0.000	100.00%
290	0.000	100.00%
310	0.000	100.00%
330	0.000	100.00%
350	0.000	100.00%
370	0.000	100.00%
390	0.000	100.00%
410	0.000	100.00%
430	0.000	100.00%
450	0.000	100.00%
470	0.000	100.00%
490	0.000	100.00%
510	0.000	100.00%

Bin Centre (nm)	Concentration E6 particles / ml	Percentile Undersize
530	0.000	100.00%
550	0.000	100.00%
570	0.000	100.00%
590	0.000	100.00%
610	0.000	100.00%
630	0.000	100.00%
650	0.000	100.00%
670	0.000	100.00%
690	0.000	100.00%
710	0.000	100.00%
730	0.000	100.00%
750	0.000	100.00%
770	0.000	100.00%
790	0.000	100.00%
810	0.000	100.00%
830	0.000	100.00%
850	0.000	100.00%
870	0.000	100.00%
890	0.000	100.00%
910	0.000	100.00%
930	0.000	100.00%
950	0.000	100.00%
970	0.000	100.00%
990	0.000	100.00%
1000-2000	0.000	100.00%

Results
Mean: 78 nm
Mode:86 nm
SD: 31 nm
D10: 38 nm
D50: 76 nm
D90: 119 nm
User Lines: 0, 1189 nm
Concentration: 2.76 x 10^8 particles/ml

Measurement Conditions
Temperature: 25.20 °C
Viscosity: 0.89 cP
Frames Per Second: 31.15
Measurement Time: 60 of 60 seconds
Drift Velocity: 1882 nm/s

Analysis Conditions
Brightness: -0
Gain: 1.00
Blur: Auto
Detection Threshold: 10 Multi
Max Blob Size (pixel area): 3000
Min Track Length: Auto
Min Expected Size: 50 nm

Figure 13 Nanoparticle Tracking Analysis. Sample report and images acquired from NanoSight's NTA software. Particle size/concentration shows a distribution within 10 – 210 nm, with peaks at 50, 66 and 86 nm.

4.2 Mortality and macroscopic observations

4.2.1 *C. auratus* characterization and macroscopic observations

For the different assay periods no significant mortality (<10 %) was observed in all treatments, even at higher concentrations of TiO_2 NPs (Tab. 3, annex I).

During the assay, fish exposed to higher concentrations of TiO_2 NPs ($\geq$ 10 mg/L) seemed to have lower food intake, with a visible reduction or even absence of excretion products. At these concentrations it was also observed a progressive loss of scales. A few fish presented a progressive development of fungal infections, at low exposure (Fig. 14).

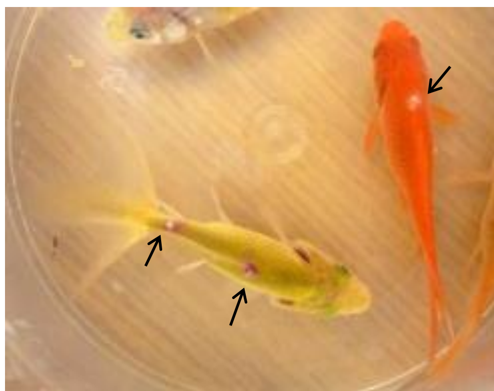


Figure 14 *C. auratus* macroscopic observations. Fish exposed to 0.1 mg/L after 14 days, presenting fungal infection (indicated by arrows).

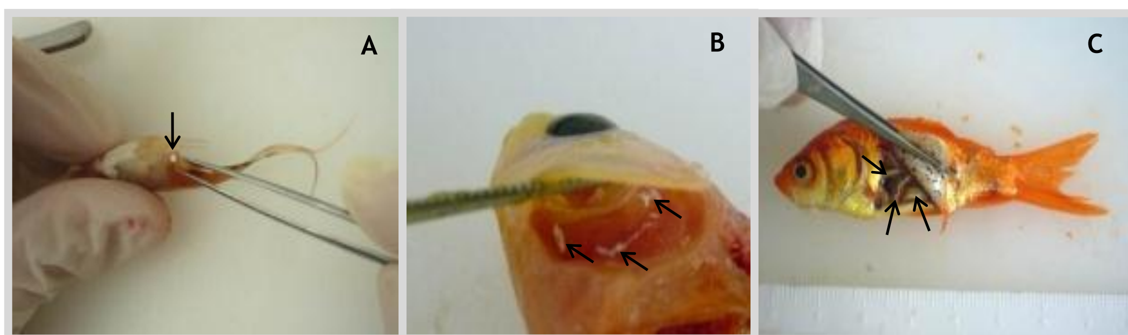


Figure 15 Macroscopic observations of NPs presence in *C. auratus*. Presence of visible aggregates from TiO_2 NPs (indicated by arrows) in exposed fish. NPs visible at urogenital orifice of an 800 mg TiO_2/L exposed fish, after 7 days (A); NPs in the gills surface after 14 days, 400 mg TiO_2/L exposure (B); NPs filling all intestine after 21 days, 10 mg TiO_2/L exposure (C).

After a 7 days exposure, some individual fish exposed to concentrations above 100 mg/L presented visible NPs aggregates within digestive system (Fig. 15 A). After 14 days NPs were also visible on gills surface (≥ 400 mg/L, Fig. 15 B) and inside the intestine of 10 mg/L exposed fish. After 21 days NPs filled almost all intestines of organisms exposed to concentrations of TiO_2 NPs above 10 mg/L (Fig. 15 C) and were also visible on its gills surface.

4.2.2 *C. fluminea* characterization and macroscopic observations

It was observed an increase of the mortality in organisms exposed to TiO_2 NPs, with a significant mortality rate (≥ 10 %) for 0.1, 10 and 100 mg/L concentrations, during the assay periods (Tab. 4, annex II; Fig. 16). After 7 days of 100 mg/L exposure to TiO_2, only one individual has survived to this concentration. Thus, new organisms were exposed to 100 mg/L for another 7 days period in order to obtain a significant number of organisms to sample.

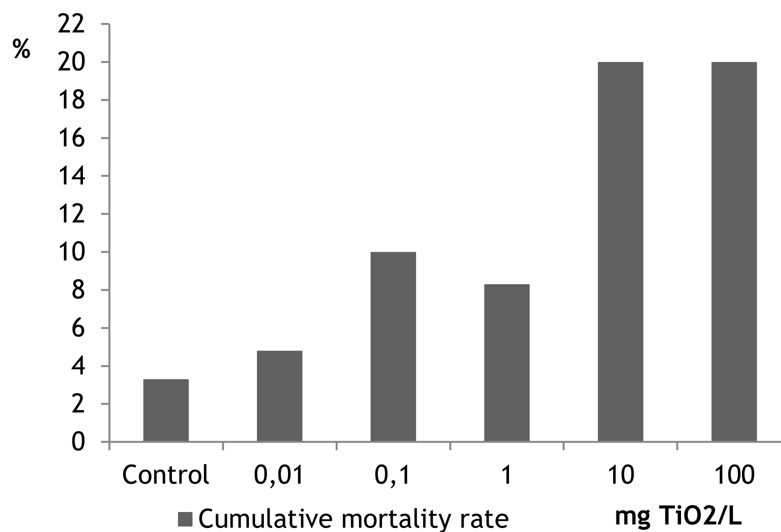


Figure 16 *C. fluminea* cumulative mortality rate.

At concentrations above 1 mg/L, TiO_2 NPs formed visible macromolecular aggregates that deposit at the tanks bottom and cover the organisms' shell (Fig. 17). The NPs affinity to adsorb to the shell surface seems to provoke its degradation over time.

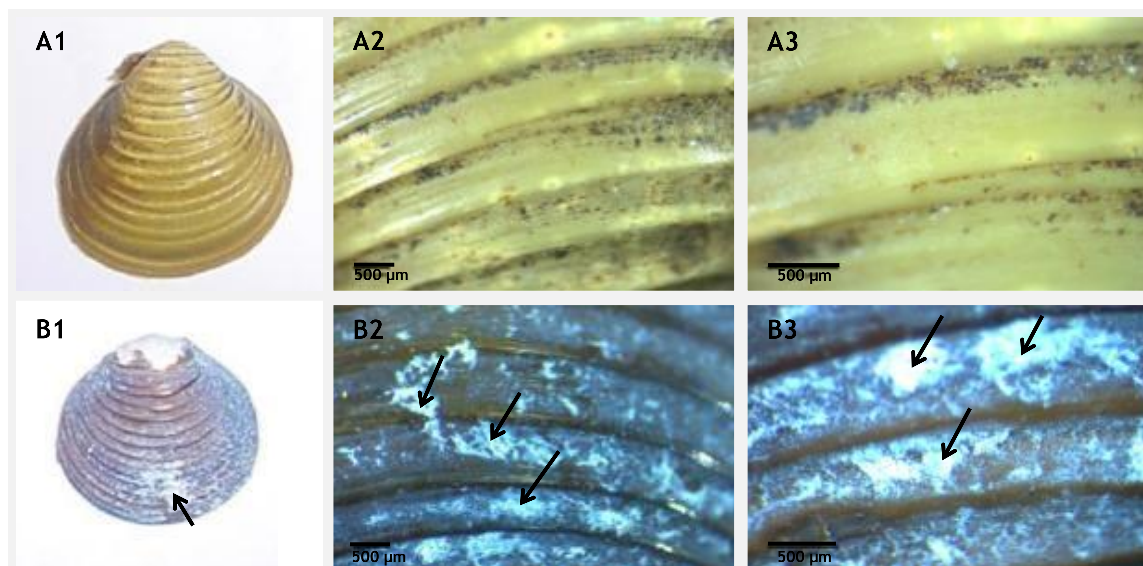


Figure 17 Macroscopic observation of *C. fluminea* shell. Control organism (A1, A2 and A3); organism exposed to 10 mg TiO_2/L, after 14 days (B1, B2 and B3) with visible adsorption of NPs to the shell surface (arrowhead).

4.3 Histological observations

4.3.1 *C. auratus* histopathological analysis

4.3.1.1 Liver

Histological observation of livers from control fish show a normal tissue structure, constituted by a homogeneous hepatic parenchyma. The system of hepatocytes presents a polygonal shape well defined and sustained by a sinusoidal net. The hepatocytes have a large cytoplasm volume with central and spherical nucleus (Fig. 18 A).

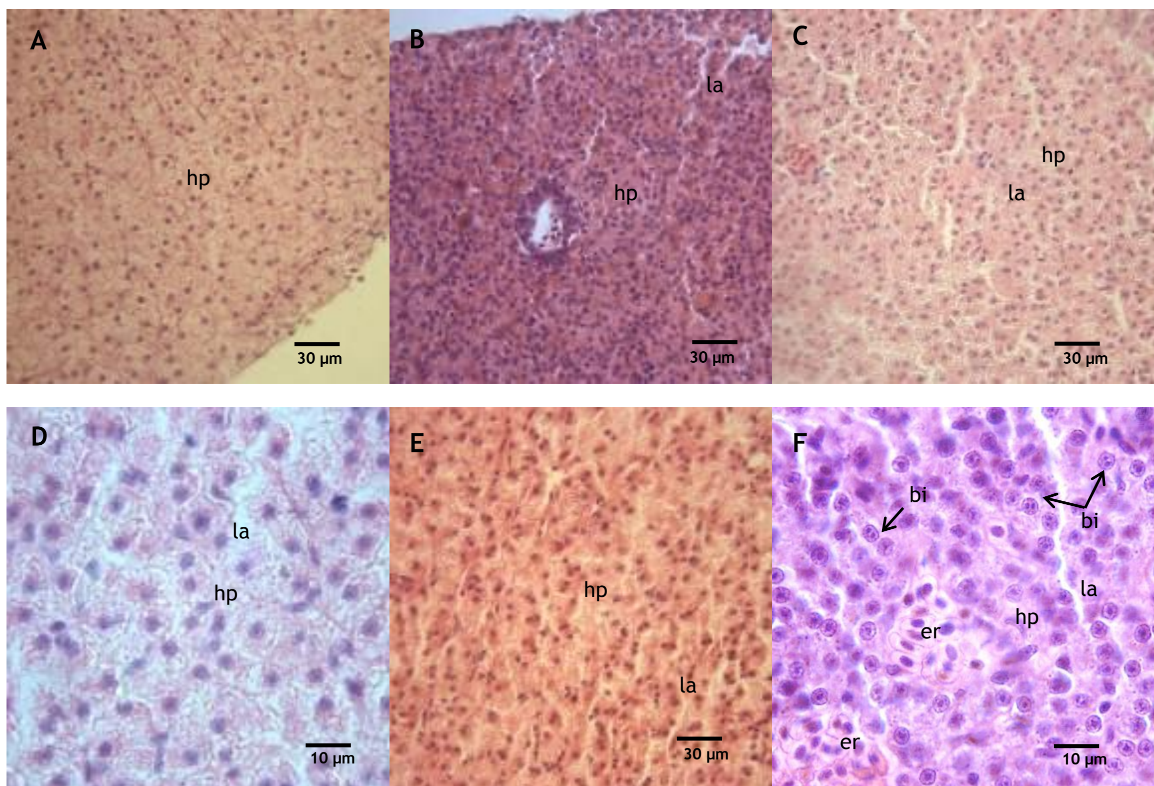


Figure 18 Observation of *C. auratus* liver sections by optical microscopy. Control fish (A); Fish exposed to different concentrations of TiO_2 nanoparticles (B, 0.1 mg/L for 21d; C, 10 mg/L for 21d; D, 100 mg/L for 21d; E, 400 mg/L for 7d; F, 800 mg/L for 14d). Legend: hp, hepatocytes; la, loss of cellular adhesion; bi, binucleated nuclei (arrowhead); er, erythrocytes. Staining: H&E.

Histopathological analysis revealed severe structural changes in the hepatic tissue from fish exposed to the different concentrations of TiO_2 NPs (Fig. 18 B - F), comparatively with control organisms. Exposed organisms suffer a progressive degeneration and loss of integrity of the hepatic tissue, with severity levels increasing with concentration and over the time. Hepatocytes present a reduction of cytoplasmic volume, with loss of their typical cellular polygonal shape. It is visible a disorganization of the cellular tissue, with cytoplasmic membrane disintegration and loss of adhesion between hepatic cells (Fig. 18 B – F). Some cells also present binucleated nuclei (Fig. 18 F).

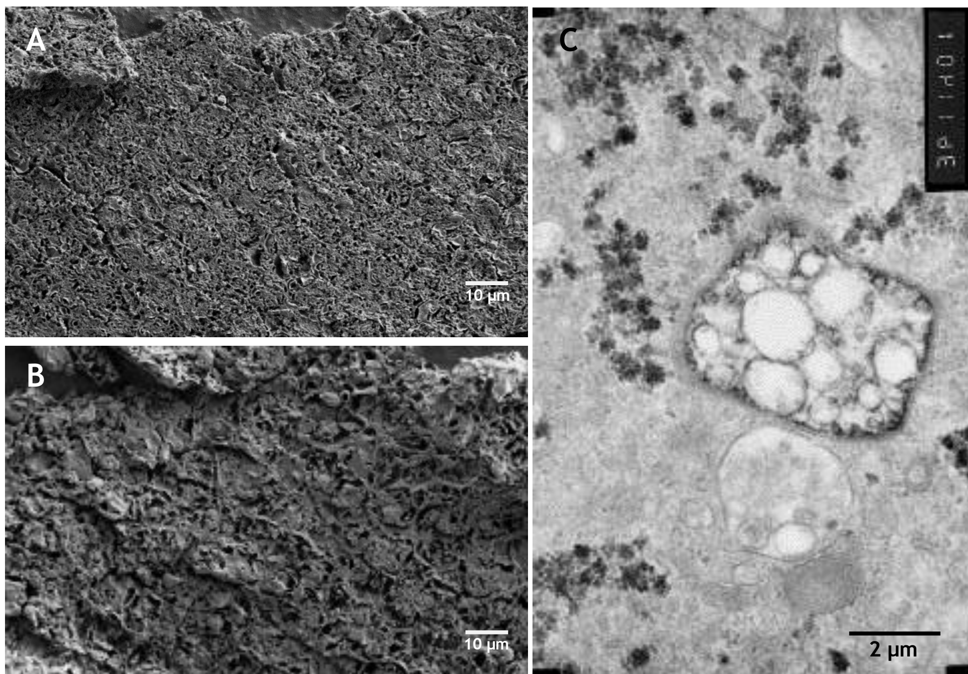


Figure 19 Electron microscopy observations of *C. auratus* liver tissue after exposure to TiO_2 NPs. SEM image of hepatic tissue from control fish (A), and from fish exposed to TiO_2 NPs (B); TEM image of a hepatocyte from fish exposed to TiO_2 NPs (C), showing no visible TiO_2 NPs inside the cell.

The observation of liver sections from individuals exposed to TiO_2 NPs trough scanning electron microscopy showed severe alterations in the hepatic tissue structure (Fig. 19 B), comparatively to control organisms (Fig. 19 A). EDS elemental analysis did not detected TiO_2 in hepatic tissue of tested fish. Transmission electron microscopy showed no presence of TiO_2 NPs inside hepatocytes of exposed livers (Fig. 19 C).

4.3.1.2 Gills

Gills from control fish show normal histology (Fig. 20 A). Gill filaments present a regular structure with evident lamellae spacing. The lamellae are well differentiated and arranged in both sides of branchial filament.

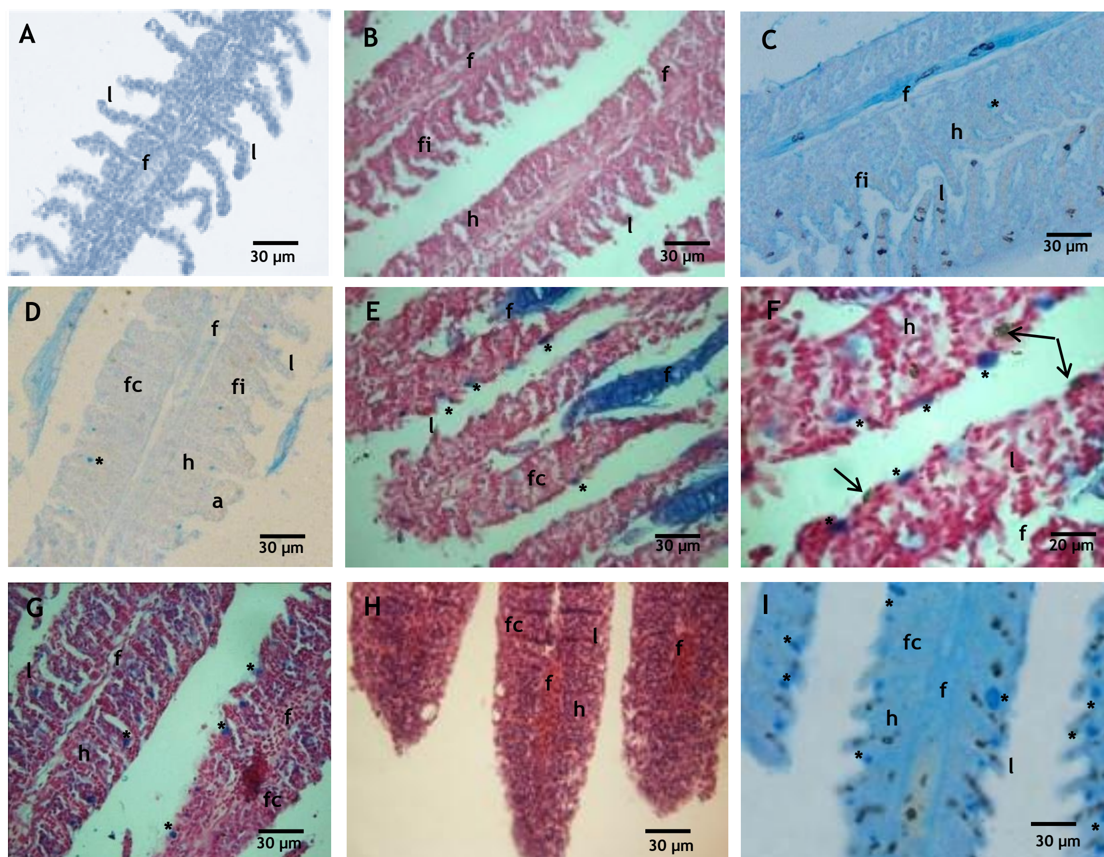


Figure 20 Observation of *C. auratus* gill sections by optical microscopy. Control fish (A); Fish exposed to different concentrations of TiO_2 NPs (B, 0.01 mg/L for 7 days; C, 0.1 mg/L for 7 days; D, 10 mg/L for 14 days; E, 100 mg/L for 14 days; F, 100 mg/L for 14 days; G, 400 mg/L for 14 days; H, 800 mg/L for 21 days; I, 800 mg/L for 21 days). Legend: f, filament; l, lamellae; h, hyperplasia; a, lamellar aneurysms; fc, complete fusion of lamellae; fi, incomplete fusion of lamellae; (*) presence of mucous; (arrowhead) possible presence of TiO_2 NPs agglomerates in the tissue. Staining: H&E and Alcian Blue.

Organisms exposed to the different concentrations of TiO_2 NPs present histopathological alterations in gill tissue (Fig. 20 B – I), visible soon after 7 days of exposure. The most common alteration detected is hyperplasia, with a progressive proliferation of epithelial cells occurring between the inter-lamellar spaces. This disorder is visible in organisms from every

exposure concentration, with different degrees of severity. The generality of the individuals exposed from 0.01 to 10 mg/L show an incomplete fusion of several lamellae (Fig. 20 B – D). A higher degree of hyperplasia is present in organisms exposed to concentrations above 100 mg/L, with individuals suffering a complete fusion of lamellae (Fig. 20 E – I). Tissue alterations also include hypertrophy and displacement of the branchial epithelial cells, cellular and blood vessels alterations. Alcian Blue staining revealed a proliferation of mucous cells in the gill inter-lamellar space of exposed organisms (Fig. 20 B – I), increasing the mucous secretion. Control organisms show an apparent low intensity or absence of mucous production (Fig. 20 A).

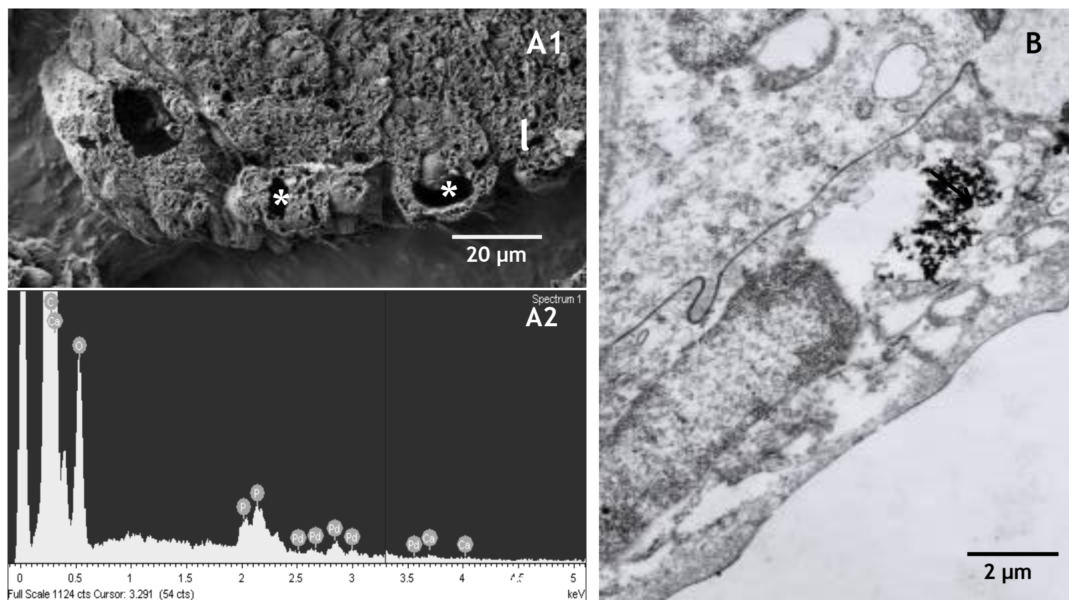


Figure 21 Electron microscopy observation and elemental analysis of *C. auratus* gill tissue after exposure to TiO_2 NPs. SEM image (A1) of a gill filament with lamellae fusion and respective EDS spectrum (A2), with no detection of TiO_2 in the selected section. TEM image (B) showing visible TiO_2 NPs (arrowhead) inside gill cell. Legend: l, lamellae; (*) vacuoles in mucous secretory cells.

The observation of sections from gill filaments through scanning electron microscopy combined with elemental analysis by an EDS analyzer, showed no apparent detection of TiO_2 in cellular tissues of individuals exposed to TiO_2 NPs (Fig. 21 A1, A2). However, transmission electron microscopy was able to provide a clear image of the presence of TiO_2 NPs inside gill cells from organisms exposed to TiO_2 NPs (Fig. 21 B).

4.3.1.3 Intestine

Histological analysis of the intestine from control fish showed the usual tissue structure for this organ, with presence of normal food residues within intestine lumen (Fig 22 A, B). The intestinal epithelium has numerous villi, well separated, lined by a simple epithelium of columnar cells, and an apical striated border (microvilli) interspersed with goblet (mucus-secreting) cells.

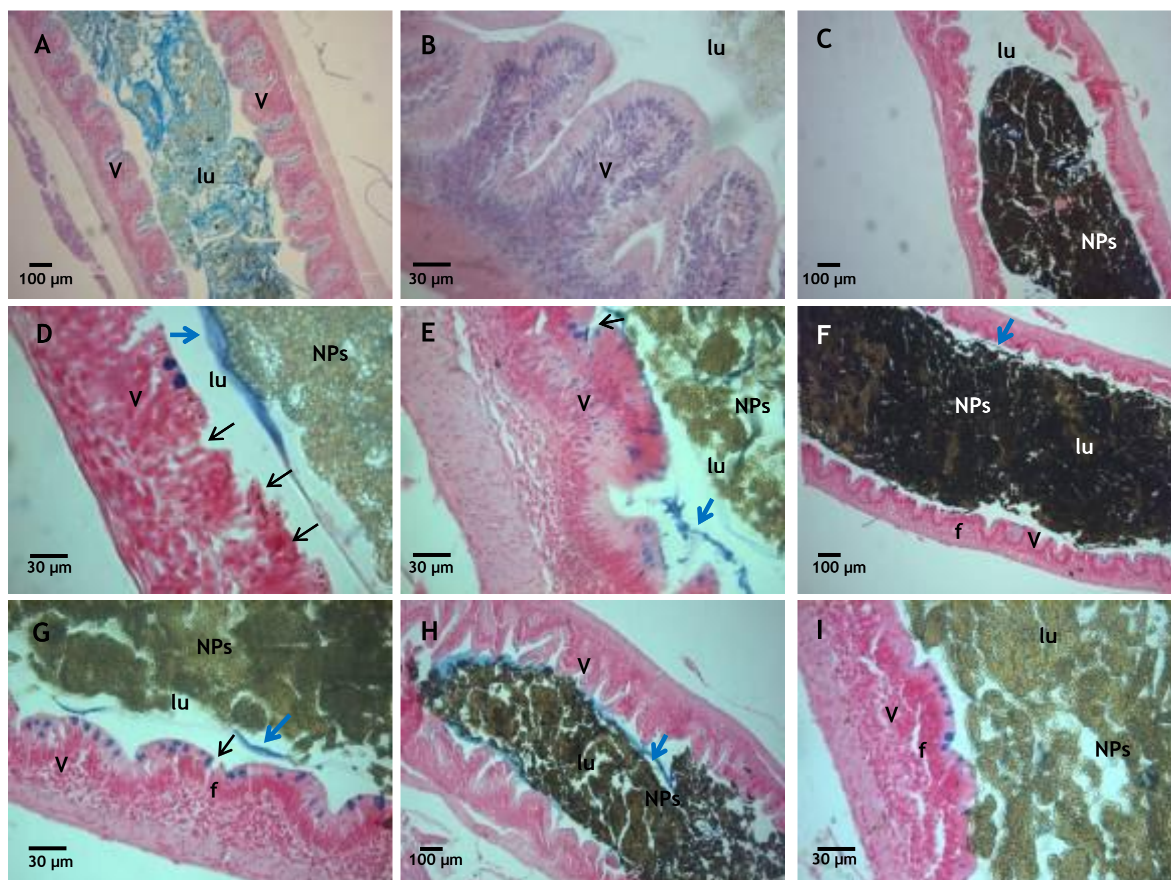


Figure 22 Observation of *C. auratus* intestine sections by optical microscopy. Control fish (A and B); Accumulation of nanoparticles inside intestine lumen of fish exposed to different concentrations of TiO_2 NPs (C and D, 10 mg/L for 21d; E, 100 mg/L for 21d; F, 400 mg/L for 21d; G, 400 mg/L for 21d; H and I, 800 mg/L for 21d). Legend: f, tip fusion; In, Intestine epithelium; NPs, nanoparticles aggregates; lu, intestine lumen; black arrowheads, epithelium erosion; blue arrowheads, mucous barrier; V, villi. Staining: H&E and Alcian Blue.

Concerning to exposed fish (Fig. 22 C - I), it was observed the presence of huge compact clusters of nanoparticles accumulated inside intestine lumen, for exposures above 10 mg TiO_2/L. Histological examination indicates that intestine aggregates of NPs increase in accordance with exposure time and tested treatments. Trough light microscopy, NPs were observed not only in the intestinal contents, but also in contact and apparently within the intestine epithelium (Fig. 22 D). The microscopic analysis showed significant changes in intestinal tissues in fish exposed above to 10 mg TiO_2/L. Comparatively to control organisms, exposed organisms showed tissue alterations with loss of the regular cellular structure, including the erosion of the intestinal epithelium, fusion of some villi and presence of a mucus “barrier” between NPs accumulated inside intestine lumen and epithelium cells.

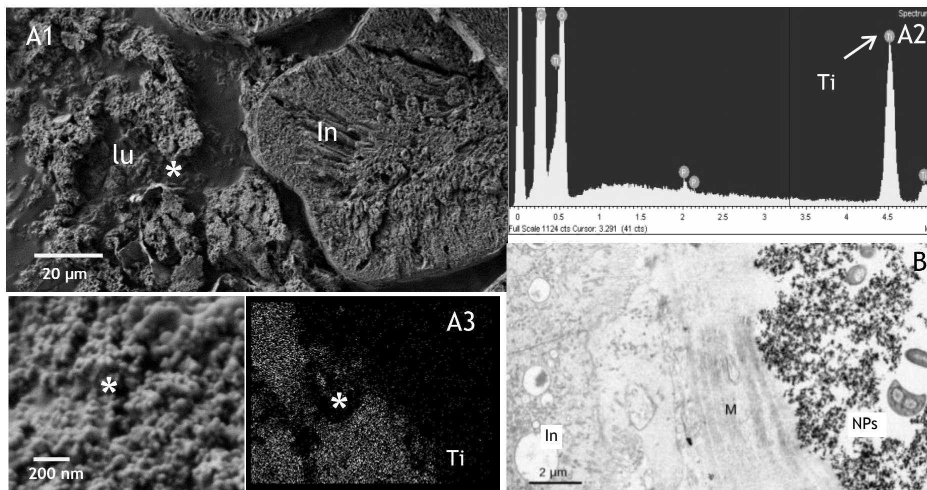


Figure 23 Electron microscopy observation and elemental analysis of *C. auratus* intestinal tissues after exposure to TiO_2 NPs. SEM from nanoparticles aggregates (*) inside intestine lumen (A1), respective EDS spectrum (A2) revealing the presence of Ti (arrowhead) among other elements, and Ti distribution within intestine. TEM image of intestine from fish exposed to 100 mg TiO_2/L (21days). Legend: In (intestine epithelium cells); lu (intestine lumen); M (mucous layer); NPs (nanoparticles aggregates).

The EDS analysis performed on tissue sections (Fig. 23 A1, A2 and A3) confirmed that the aggregates observed by light microscopy in the intestine lumen are composed by titanium as shown by the respective EDS spectrum (Fig. 23 A2). With respect to the intestine epithelium no evidence of NPs internalization by cells was found by EDS neither by SEM observation.

4.3.2 *C. fluminea* histopathological analysis

Control organisms (Fig. 24 A) present the typical cellular structure for this species. Cells from the digestive gland have the normal membrane thickness, there is no cellular atrophy and the digestive lumen is nearly occluded.

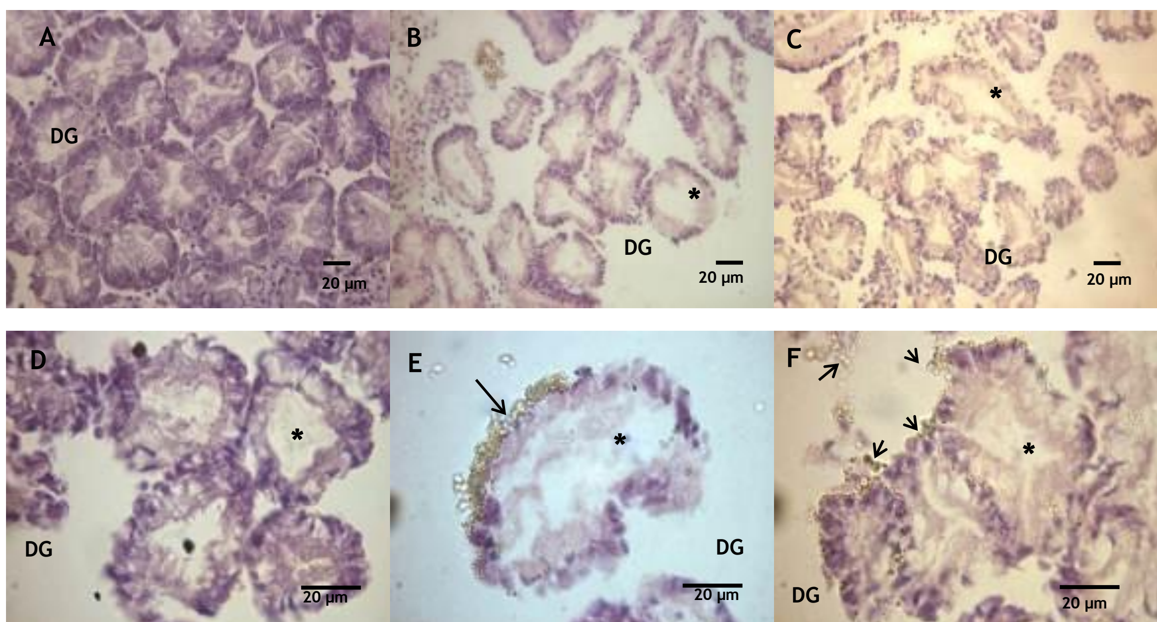


Figure 24 Observation of *C. fluminea* digestive gland sections trough optical microscopy. Digestive gland cells of a control organism (A) and of organisms exposed 14 days to different concentrations of TiO_2 NPs (B, 0.1 mg/L; C and D, 10 mg/L; E and F, 100 mg/L). Legend: DG, digestive gland; arrowheads, nanoparticle; *, cell atrophy. Staining: H&E.

The histological observations showed that exposed organisms suffer a progressive alteration and degeneration of the tissue structure, mainly on the digestive gland cells (Fig 24 B – F), even at the lowest concentration of TiO_2 NPs. The cellular membrane loss its normal thickness and the great majority of cells are atrophied. It seems that there is a bioaccumulation of TiO_2 NPs in the tissue, with adhesion of NPs to the digestive cells wall (Fig. 24 F), especially to those exposed to 100 mg/L.

The presence of TiO_2 NPs in exposed organism was confirmed by x-ray elemental analyze and it showed that these NPs have a major affinity to adsorb to the shell (Fig. 25).

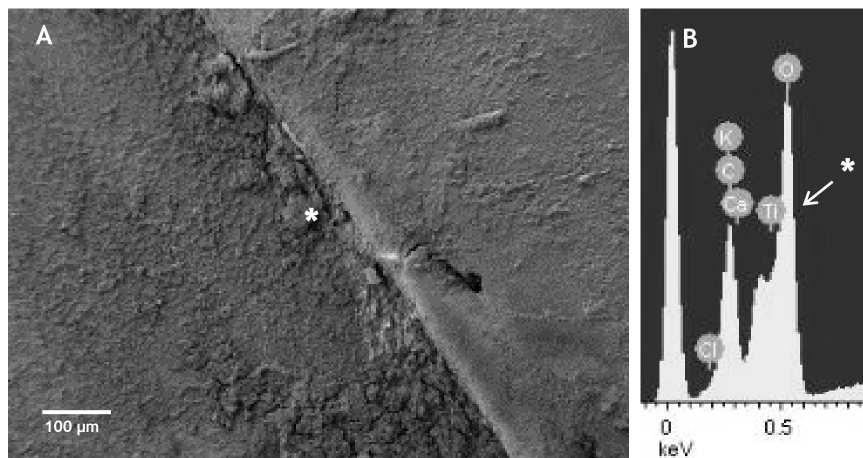


Figure 25 Electron microscopy observation and elemental analysis of *C. fluminea* after exposure to TiO_2 NPs. SEM of the *C. fluminea* shell surface (A) and respective EDS spectrum (B), showing the presence of TiO_2 (arrowhead).

4.4 Antioxidant activity and Lipid Peroxidation analysis

4.4.1 SOD activity in *C. auratus*

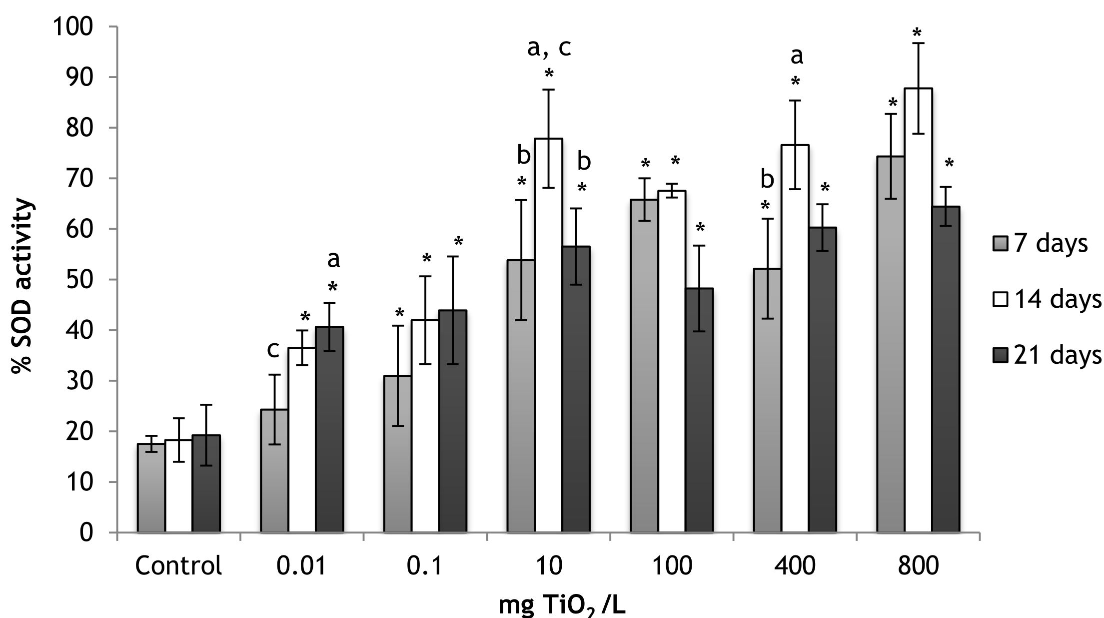


Figure 26 SOD activity in *C. auratus*. SOD activity (mean±SD) in livers of *C. auratus* exposed to different concentrations of TiO_2 NPs, for 7, 14 and 21 days. Statistically significant differences comparatively to controls if * (p<0.05). Letters indicate significant differences between exposure periods (p<0.05), comparatively to 7 days (a), 14 days (b) and 21 days (c).

The activity of SOD was measured in livers from *C. auratus*, and presented in terms of percentage in Figure 26. Statistical analysis showed no significant difference (p>0.05) in SOD activity before the beginning of test (16.95±1.09 %) and activity measured in controls at 7, 14 and 21 days. Results show an increase of enzymatic activity in exposed organisms, proportional to the tested concentrations. After 7 days a significant increase of SOD activity (p<0.05) was found for fish exposed from 0.1 to 800 mg/L, after 14 and 21 days the differences were significant (p<0.05) for every exposure concentration, in comparison to controls. The highest SOD activity (87.78 %) was measured in the livers of fish exposed to 800 mg TiO2/L during 14 days. Significant increases (p<0.05) were found in concentrations of 0.01 mg/L from 7 to 21 days, 10 mg/L and of 400 mg/L from 7 to 14 days. There was a general decrease of SOD activity from 14 to 21 days, at concentrations above 10 mg/L, with a significant difference (p<0.05) for the concentration of 10 mg TiO_2/L.

4.4.2 CAT activity in *C. auratus*

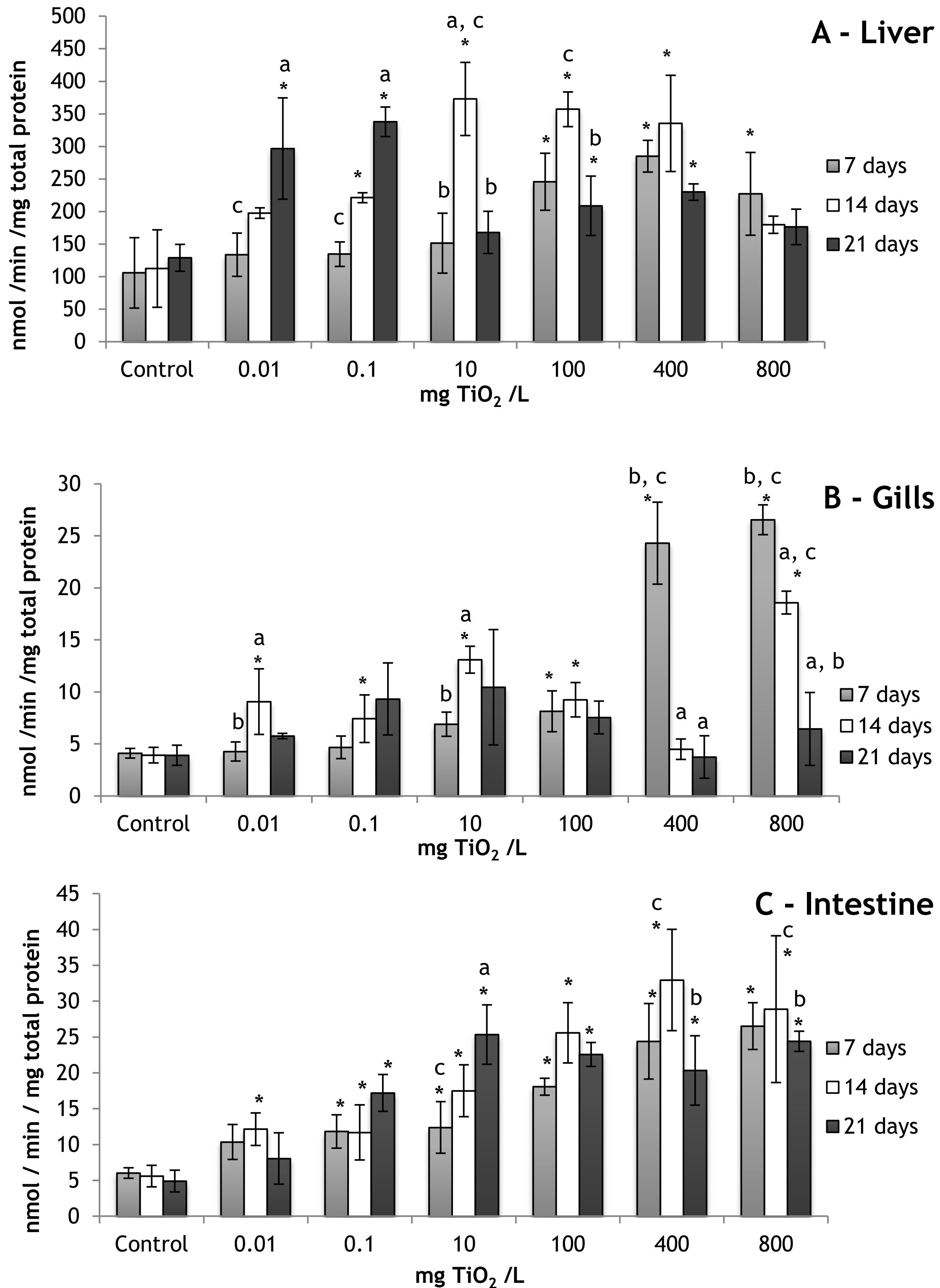


Figure 27 CAT activity in *C. auratus*. CAT total activity (mean±SD), measured in liver (A), gills (B) and intestine (C) of *C. auratus*, after 7, 14 and 21 days of exposure to TiO2 NPs. Significant differences (p<0.05) comparatively to controls if *. Significant differences between exposure periods (p<0.05), comparatively to 7 days (a), 14 days (b) and 21 days (c).

CAT activity was measured in livers, gills and intestines from *C. auratus* and the activity concentrations are presented in Figure 27. For each organ there was no statistically significant difference ($p>0.05$) in measured activity before the beginning of test (135.65±5.63 nmol/min/mg total protein in livers; 4.09±0.82 nmol/min/mg total protein in gills; 6.03±0.72 nmol/min/mg total protein in intestines) and activity in controls at 7, 14 and 21 days. The measured CAT activity was higher in livers than in gills and intestines.

In livers, CAT activity increased after 14 days for the majority of test concentrations. Significant increases of CAT activity ($p<0.05$) were found in exposed fish comparatively to controls, after 7 days for fish exposed from 100 to 800 mg/L, after 14 days for exposure concentrations between 0.01 and 400 mg/L and after 21 days for concentrations of 0.01, 0.1, 100 and 400 mg TiO_2 /L, in comparatively to controls. The highest CAT activity (372.96 nmol/min/mg total protein) was measured after 14 days exposure to 10 mg TiO_2/L. From 7 to 21 days it was observed a significant increase ($p<0.05$) inside the concentrations of 0.01 and 0.1 mg/L and from 7 to 14 days for an exposure of 10 mg TiO_2/L. A significant activity decrease ($p<0.05$) occurred from 14 to 21 days in the concentrations of 10 and 100 mg TiO_2/L.

Concerning to gills and comparatively to controls, significant increases of CAT activity ($p<0.05$) were found in concentrations above 100 mg/L for a 7 days exposure and in concentrations of 0.01, 0.1, 10, 100 and 800 mg/L for a 14 days exposure and concentrations. At the end of 21 days no significant differences ($p<0.05$) were found between treatments and controls. The highest CAT activity (26.55 nmol/min/mg total protein) was measured at 7 days exposure to 800 mg TiO_2/L. Significant activity increases ($p<0.05$) from 7 to 14 days were found inside exposure concentrations of 0.01 and 10 mg/L. From 7 to 21 days occurred significant decreases ($p<0.05$) of CAT activity inside concentrations of 400 and 800 mg TiO_2/L.

In respect to intestine, there was a general increase of CAT activity in exposed fish comparatively to controls, statistically significant ($p<0.05$) for exposure concentrations above 0.1 mg TiO_2/L after 7 and 21 days, and for every concentration after 14 days of exposure. The highest CAT activity (32.96 nmol/min/mg total protein) was measured at the concentration of 400 mg TiO_2/L, after 14 days of exposure. There was a significant increase of activity ($p<0.05$) for the concentration of 10 mg/L from 7 to 21 days of exposure. Significant decreases of activity ($p<0.05$) were found between 14 and 21 days for exposure to concentrations of 400 and 800 mg TiO_2/L.

4.4.3 GST activity in *C. auratus*

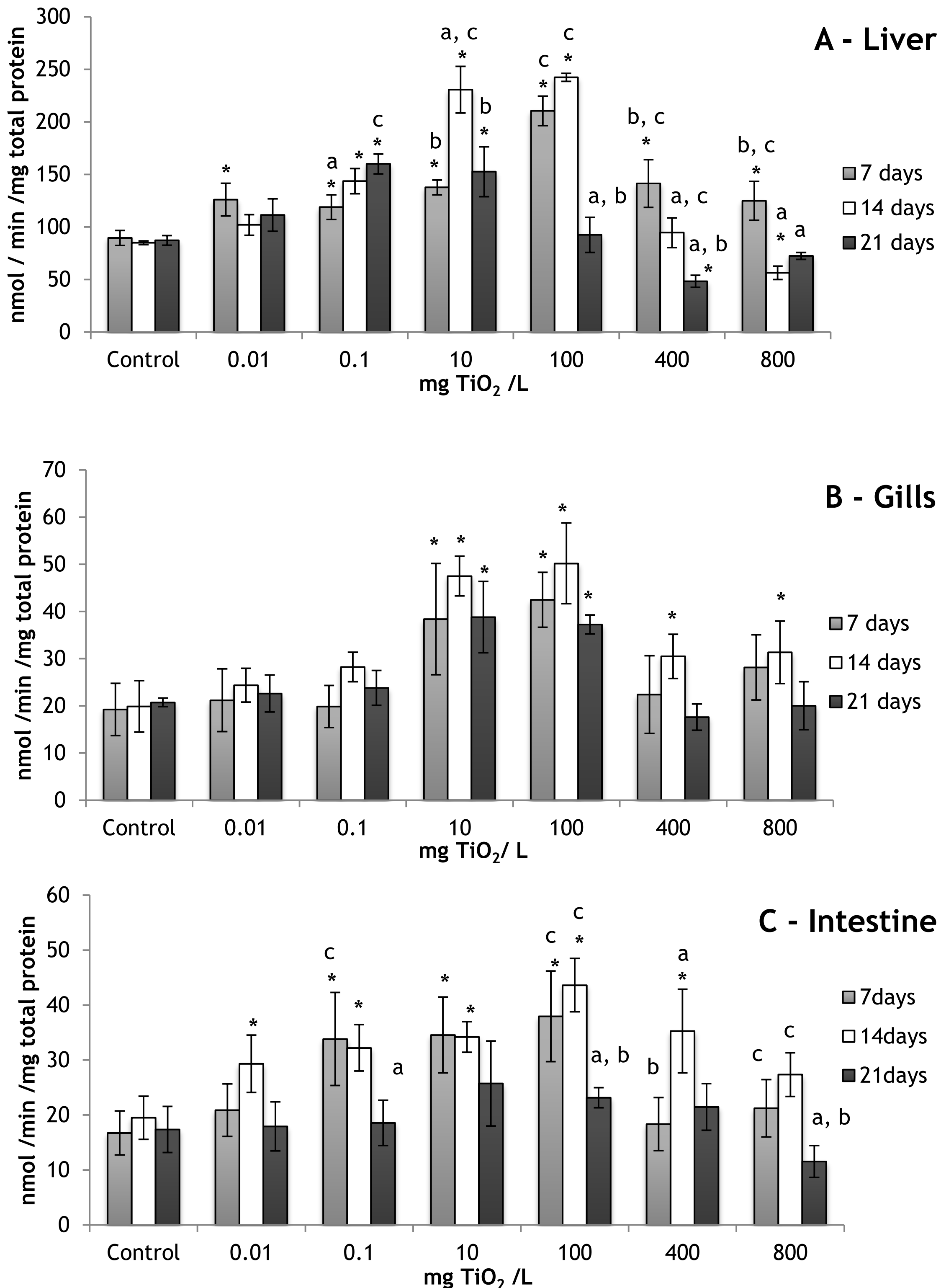


Figure 28 GST activity in *C. auratus*. GST total activity (mean±SD), measured in liver (A), gills (B) and intestine (C) of *C. auratus*, after 7, 14 and 21 days of exposure to TiO_2 NPs. Significant differences ($p < 0.05$) comparatively to controls (*).

Average concentration of the cytosolic GST total activity was measured in livers, gills and intestines of *C. auratus* and results are showed in Figure 28. For every organ, statistical analysis showed no significant difference (p>0.05) between the measured GST activity in the beginning of test (87.18±6.72 nmol/min/mg total protein in livers; 20.43±3.25 nmol/min/mg total protein in gills; 16.73±4.00 nmol/min/mg total protein in intestines) and activity in controls after 7, 14 and 21 days. GST presented a higher total activity in livers than in gills and intestines.

GST activity in livers increased significantly (p<0.05) in fish exposed to every tested concentration after 7 days and in fish exposed from 0.1 to 100 mg TiO_2/L after 14 and 21 days, comparatively to controls. A significant decrease of activity (p<0.05) occurred after 14 days for the exposure to 800 mg TiO_2/L and after 21 days for 400 mg TiO_2/L, comparatively to controls. The highest concentrations of GST activity (242.31 nmol/min/mg total protein) were measured in livers of fish exposed to 100 mg TiO_2/mL after 14 days, whereas the lowest concentrations (48.36 nmol/min/mg total protein) were found in the concentration of 400 mg TiO_2/L after 21 days. It was observed a significant increase of activity (p<0.05) from 7 to 14 days in fish exposed to 10 mg TiO_2/L and from 7 to 21 days in exposure to 0.1 mg TiO_2/L. Significant activity decreases (p<0.05) occurred from 14 to 21 days in the exposure concentrations of 10 and 100 mg TiO_2/L and from 7 to 21 days for concentrations of 400 and 800 mg TiO_2/L.

In gills, significant increases of GST activity (p<0.05) comparatively to controls were observed in concentrations of 10 and 100 mg TiO_2/L after every exposure period, and in concentrations of 400 and 800 mg/L after 14 days of exposure. GST presented a higher activity (50.20 nmol/min/mg total protein) at 7 days exposure to 100 mg TiO_2/L. No significant alterations (p>0.05) of GST activity in gills were found between exposure periods in every treatment.

Concerning to intestine, it was observed a general increase of the GST activity after 7 and 14 days of exposure, between exposed organisms and controls. Comparatively to controls, the activity increase was statistically significant (p<0.05), in concentrations from 0.1 to 100 mg TiO_2/L after 7 days and in concentrations from 0.01 to 400 mg TiO_2/L after 14 days. No statistical differences (p<0.05) were found between controls and treatments after 21 days of exposure. Fish exposed to 100 mg TiO_2/L for 14 days presented the highest GST activity (43.62 nmol/min/mg total protein). From 7 to 14 days a significant activity increase (p<0.05), was found at 400 mg TiO_2/L exposure. At the end of the experiment was observed a general decrease of GST activity in exposed organisms, statistically significant (p<0.05) for the TiO_2 concentrations of 0.1, 100 and 800 mg/L.

4.4.4 Lipid peroxidation in *C. auratus*

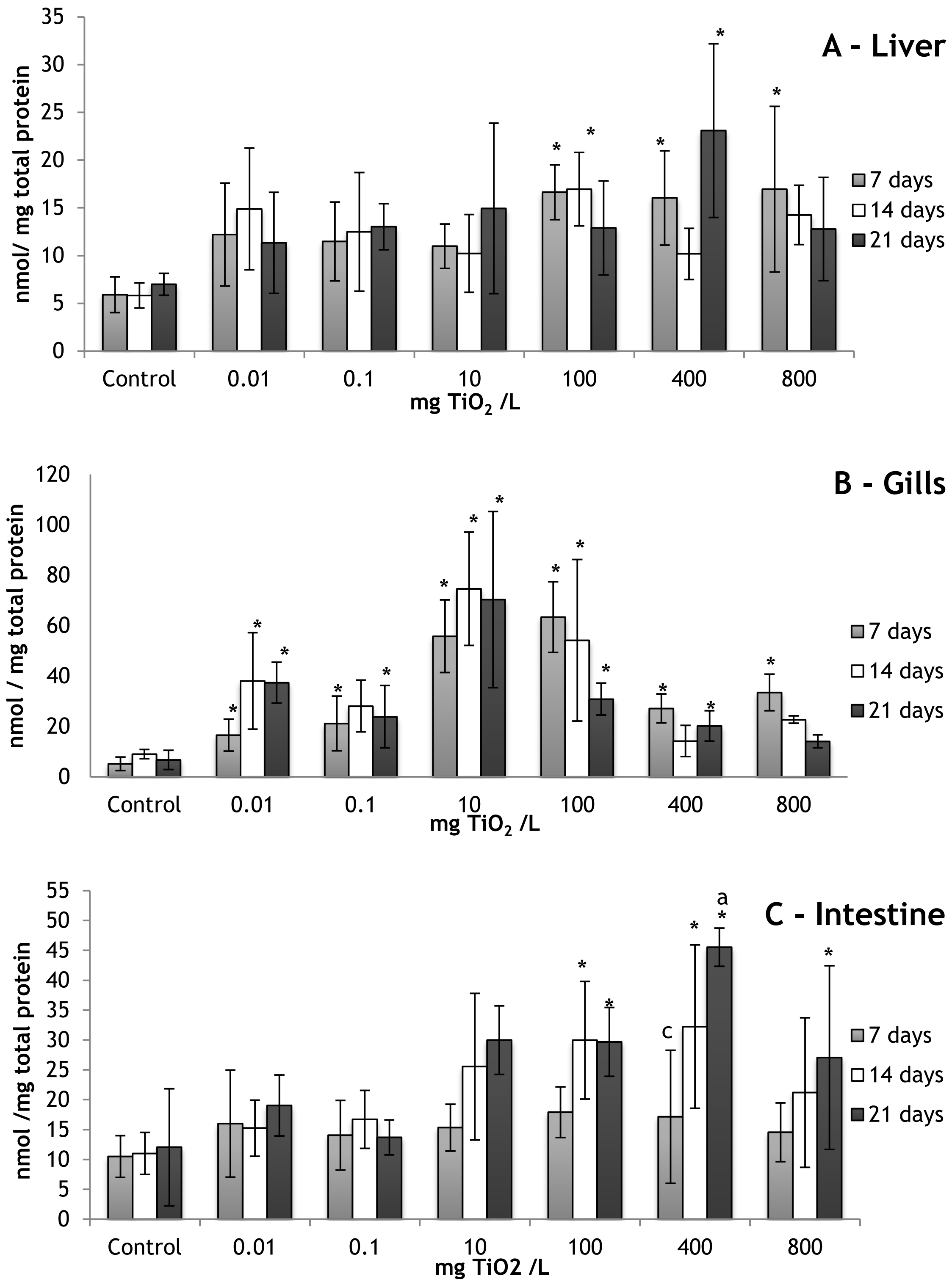


Figure 29 LPO in *C. auratus*. LPO (mean±SD), measured in liver (A), gills (B) and intestine (C) of *C. auratus*, after 7, 14 and 21 days of exposure to TiO_2 NPs. Significant differences ($p<0.05$) comparatively to controls (*).

The level of lipid peroxidation (LPO) was measured in livers, gills and intestines of *C. auratus* and results are represented in Figure 29. All organs showed no significant differences ($p>0.05$) between the measured LPO concentration level in the beginning of experiment (6.65±1.10 nmol/mg total protein in livers; 5.75±2.78 nmol/mg total protein in gills; 9.48±2.93 nmol/mg total protein in intestines) and activity in controls after 7, 14 and 21 days. Higher levels of LPO were found in gills and intestines. No significant alterations ($p>0.05$) of LPO levels in every organ were found between exposure periods.

Livers presented increased LPO values at higher exposure concentrations, significantly different from controls ($p<0.05$) at concentrations of 100 to 800 mg TiO_2/L after 7 days exposure, 100 mg TiO_2/L after 14 days and 100 mg TiO_2/L after 21 days. The highest concentration level of LPO (23.09 nmol/min/mg total protein) was measured in livers of fish exposed to 400 mg TiO_2/mL after 21 days.

LPO levels measured in gills showed significant increases ($p<0.05$) at every exposure concentration, comparatively to controls. Statistical differences from control ($p<0.05$) were observed in every exposure concentration after 7 days, in concentrations of 0.01, 10 and 100 mg/L after 14 days of exposure and from 0.01 to 400 mg/L after 21 days. Higher levels of LPO (74.62 nmol/min/mg total protein) were observed at 14 days exposure to 10 mg TiO_2/L.

Concerning to intestine, it was observed an increase of the LPO levels after 14 and 21 days of exposure, for concentrations above 10 mg TiO_2/L. At 7 days, no significant differences ($p<0.05$) were found between exposed fish and controls. Significant increases ($p<0.05$) were observed after 14 days in fish exposed to 100 and 400 mg/L and after 21 days from 100 to 800 mg/L, comparatively to controls. The higher concentration value of LPO (45.53 nmol/min/mg total protein) was found in fish exposed to 400 mg TiO_2/L at the end of the experiment. It was also observed a significant increase ($p<0.05$) of LPO from 7 to 21 days at the concentration of 400 mg TiO_2/L.

4.4.5 *C. fluminea* antioxidant activity and lipo peroxidation analysis

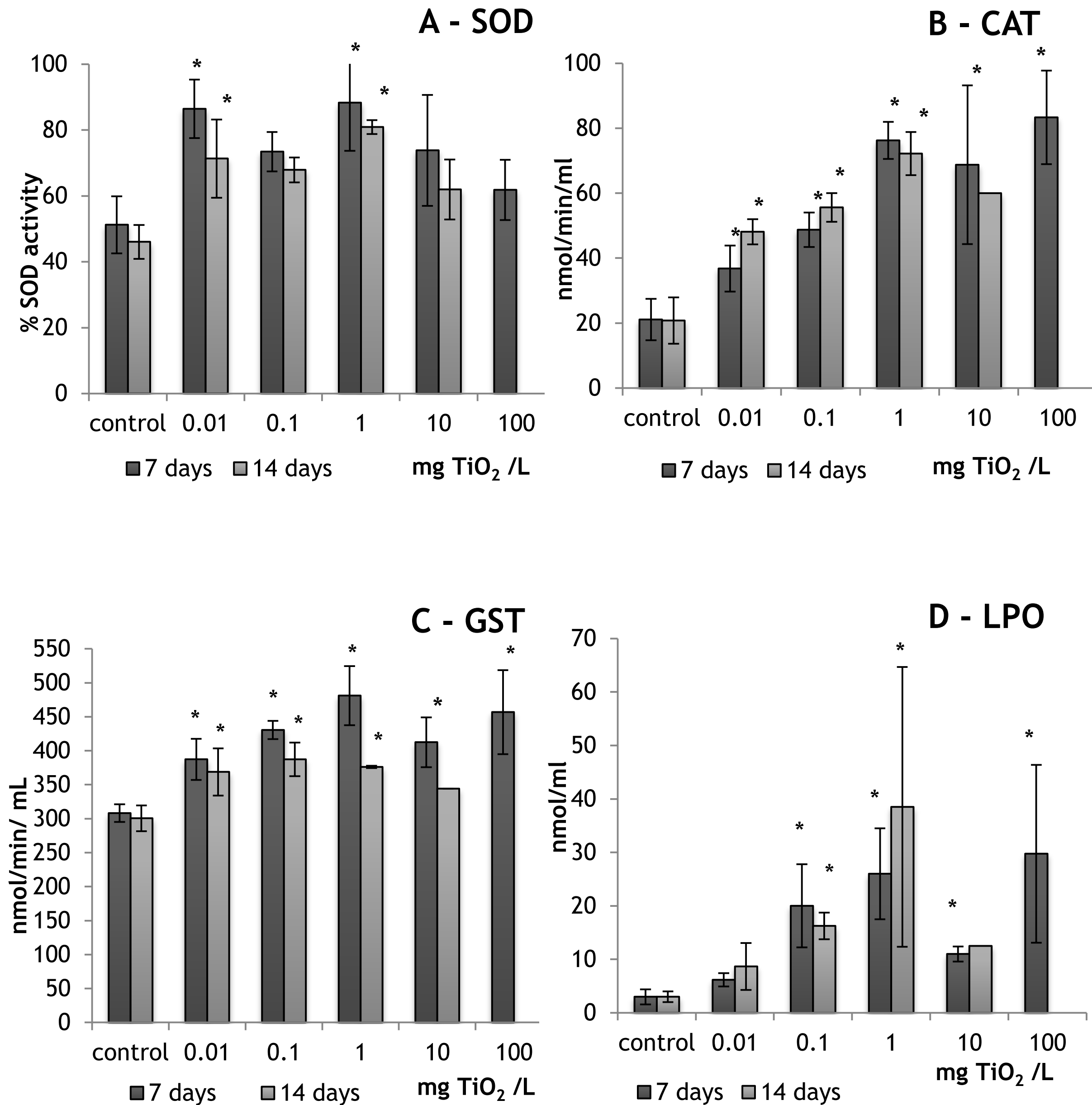


Figure 30 Activity of antioxidant enzymes and Lipid peroxidation level in *C. fluminea*. Specific activity of antioxidant enzymes SOD (A), CAT (B) and GST (C) and LPO level (D), measured in digestive tissue homogenate of *C. fluminea*, after 7 and 14 days of exposure to TiO2 NPs. Data are reported as means ± SD. Significant differences ($p<0.05$) comparatively to controls (*). No significant differences were found between the different exposure periods (7and 14 days) for each exposure concentration.

Enzymatic activity (SOD, CAT and GST) and LPO levels were measured in the digestive system of *C. fluminea*, and results are shown in Figure 30. For each different analysis, no statistically differences ($p>0.05$) were found in controls between the exposure periods (enzymatic activities for controls: 51% for SOD; 21.11 nmol/min/mL for CAT; 308.12 nmol/min/mL for GST). The activity of each antioxidant enzyme analyzed in exposed organisms showed a general increase comparatively to controls.

After 7 and 14 days exposure periods, SOD activity increased significantly ($p<0.05$) at 0.01 and 1 mg TiO_2/L, in contrast with controls. The higher activity of SOD (88.30%) occurred in organisms exposed to 1 mg TiO_2/L, after 7 days. Both CAT and GST activity had a significant increase ($p<0.05$) in every tested concentration. CAT showed a higher value of activity (83.33 nmol/min/mL) in 100 mg TiO_2/L, after a 7 day exposure. High activity of GST (481.13 nmol/min/mL) was found in organisms exposed to 1 mg/L, after 7 days.

Organisms suffered a significant increase ($p<0.05$) of LPO levels, from concentrations above 0.1 after 7 and 14 exposure days, comparatively to controls. The most accentuated LPO value (38.5 mg/min/mL) was observed in the concentration of 1 mg TiO_2/L, after 14 days of exposure.

5 Discussion

There are many studies regarding nanoparticles toxicity in vertebrates, both in vitro and in vivo, which have raised concerns about the deleterious effects in living organisms with particular emphasis in humans (Dunford et al., 1997; Gurr et al., 2005; Lai et al., 2008; Elsaesser and Howard, 2012). However, ecotoxicological studies regarding TiO_2 NPs are much more limited. For instance, most of the toxicity studies using freshwater invertebrates and exposure to TiO_2 NPs focused mainly in freshwater invertebrates (e.g. *Daphnia magna* and *Ceriodaphnia dubia*), followed by a few reports on freshwater fish.

Characterization of nanomaterials is particularly important to a full understanding of toxicity mechanisms and effects in a biological system. Proprieties such as size, chemical composition, surface area, shape, solubility and aggregation contribute for NPs toxicity. Still, nanomaterials characterization is a complex issue, particularly for studies carried out in aquatic environments. For example, particle size can have a dramatic effect in the response of organisms upon exposure: (a) Size can govern where and how the body reacts to the particulates (exposure); (b) Size is a factor in the ability of the body to clear foreign particles; (c) Size can be a factor in the ultimate fate of particles that are not cleared (translocation, accumulation); (d) Particle size can potentially influence direct mechanisms and extent of toxicity (cytotoxicity, necrosis and mutagenicity); and (e) Size directly affects the surface to mass ratio (specific surface area) and can have dramatic effects on surface reactivity and solubility of particulate systems (Powers et al., 2007).

With respect to TiO2 NPs, although the physicochemical properties tested as powder form are well described by the manufacturer, once in suspension it behaves in a different mode by aggregating, forming clusters and sedimentating. Samples of TiO_2 in aqueous suspensions were observed by electron microscopy and Nanoparticle Tracking Analysis. Despite a primary size of 21 nm, results showed the presence of a wide range of different TiO_2 particles sizes in suspension, with a heterogeneous size distribution from nanoscale to microscale.

The presence of a variety of nanoscale structures following the behavior of test materials in biological environments makes difficult the attempts to characterize nanoparticles as administered and as they interact with biological systems. Consequently, it is complicated to conduct reproducible and reliable toxicological studies concerning nanomaterials.

Throughout the period of exposure to TiO2 NPs (21 days) it was found that tested concentrations were not lethal for tested fish. This can be partially explained by the known resistance of *C. auratus* to environmental perturbations and to man-made pollutants, such as heavy metals or organochlorine insecticides (Balon, 2004).

Though, the apparently low acute toxicity in found in goldfish does not mean there are no toxicity effects. In fact, as our results report, the organism´s organs show several histological and biochemical changes. Similar results were obtained by Frederici et al (2007) for rainbow trout upon 14 days of exposure to TiO_2 NPs. However, there are evidences that exposure to NPs can have long term effects. For instance, Wiesner et al. (2009) suggested that exposure to NPs may have long-term, evolutionary influences on organisms affecting their physiology and potentially their ability to reproduce. Additionally, TiO_2 NPs have been already associated with changes in gene expression concerning fish species (Griffitt et al., 2009; Jovanic et al., 2011).

On the other hand, significant mortality rates were found in exposed bivalve's species *C. fluminea*. Bivalve mollusks are widely used in biomonitoring programs and environmental perturbations, as they can provide accurate and integrated information about chemicals impact and bioavailability (Bilos et al., 1998). This is due to the great ability to filter large volumes of water, processing microalgae, bacteria, sediments, particulates, and natural nanoparticles, potentially accumulating different chemicals in their tissues and therefore being used as environmental indicators (Canesi et al., 2012).

After entering aquatic ecosystems TiO_2 NPs behavior affects organisms through different toxicity mechanisms. For instance, the deposition and accumulation of TiO_2 NPs aggregates in sediment may present a higher risk of exposure and acute toxicity to filter feeders and sediment-dwelling organisms of aquatic ecosystems. A very interesting finding from the present study is the NPs adhesion to the skin and consequent loss of fish scales, while in bivalves NPs showed a high affinity to adsorb to the shell surface as shown by microscopy analysis. NPs may be able to enter through some fish organs, such as gills or intestine, which are in direct contact with water contaminated with NPs as confirmed by our results. With respect to other organisms, TiO_2 NPs have been already reported as able to penetrate mammalian skin, reaching different tissues and induce lesions in different organs (Wu et al., 2009).

Histological analysis revealed moderate to severe structural alterations in liver, gills and intestinal tissue from *C. auratus* and in the digestive gland tissue from *C. fluminea*, according to the different concentrations of TiO_2 NPs.

Livers from *C. auratus* exposed to TiO2 NPs, showed loss of adhesion between hepatocytes and disorganization of the cellular structure, revealed by cellular membrane disruption. Presence of bi-nucleated nuclei, necrotic cells, some apoptotic bodies, few foci of lipidosis and macrophages presence were also observed in livers from fish exposed to higher concentrations (100 to 800 mg TiO2/L). TEM analysis revealed also severe changes in liver cells compatible with oxidative stress, but no proof of NPs internalization was found inside cells. Hepatocytes of exposed fish showed glycogen depletion, swollen mitochondria and

increased lysosomes, compared to controls. Overall, the results suggest a severe degeneration of hepatic tissues as a result of exposure to sub-lethal concentrations of TiO2 NPs that eventually ends in focal or total loss of tissue integrity. Similar observations have also been reported by Hao et al. (2009) in juvenile carps following exposure to concentrations of TiO2 NPs within a range from 10 to 200 mg/L.

Gills from exposed fish presented several changes, including different degrees of hyperplasia (from low to complete fusion of lamellae) and an increase of mucous secretion. Since gills are in direct contact with the exposure medium, increase of mucous detected in branchial tissues may work as a barrier and defense mechanism against external aggression, also indicating the NPs toxicity to this organ. Indeed mucus secretion by the gills is a common response to aqueous pollutants (Mallat, 1985), in order to prevent direct exposure of the sensitive gill epithelium (Smith et al., 2007). However, histological analysis showed that even at low exposure concentrations this defense was not sufficient to protect the gill from cellular pathologies as also reported by other studies reporting similar findings, in mammals (Warheit et al., 2005, 2006) and in fish (Federici et al., 2007; Hao et al., 2009). In addition, TEM analysis revealed that TiO2 NPs were internalized by gills epithelial cells and accumulating in cell vacuoles.

It was observed that NPs are ingested by fish from the aqueous suspensions and accumulated in fish intestine as clusters or aggregates over the exposure period. The histological observations suggest that NPs aggregates increase according to the different exposure treatments, showing higher NPs concentrations in fish exposed to concentrations above 10 mg TiO_2/mL. Electron microscopy analysis was not able to detect NPs inside the intestine cells, possibly because the mucous produced by intestine cells worked as a barrier. Nevertheless, the presence of TiO_2 NPs in the intestine may interfere with food intake and potentially obstruct the absorption of nutrients. It was also noticed that the great amounts of TiO2 accumulated in intestine lumen were difficult to be excreted normally by fish. Other studies showed similar accumulation of TiO_2 NPs inside gastrointestinal tract of different organisms, such as *D. magna*, and the difficulty in eliminating these NPs from their body *(*Baun et al., 2008; Kim et al., 2010; Zhu et al., 2010). The NPs ability to penetrate trough the biological barriers makes it easily for NPs to move through the biological systems and get into organs that are not directly exposed (Oberdörster et al, 2005; Nel et al, 2006). A study concerning effects of TiO2 NPs in juvenile rainbow trout following dietary exposure, showed the occurrence of Ti accumulation in gills, gut, liver, brain and spleen, with Ti not clearing in some organs following recovery in clean water, especially the brain (Ramsden et al., 2009).

Histological analysis also revealed alterations of the gastric mucosa. NPs were observed in the intestinal lumen but also in direct contact with the intestine epithelium. Histological analysis showed significant changes in intestinal tissues in fish exposed above to 10 mg TiO_2/L, such as the erosion of the intestinal epithelium, fusion of some villi and an apparent presence of a

mucus “barrier” between NPs accumulated inside intestine lumen and epithelium cells as previously referred. Our results are in agreement with studies from Frederici et al. (2007) which suggested that the observation of a severe erosion of the trout gut epithelium can be a consequence of drinking contaminated water with NPs.

Concerning *C. fluminea* digestive gland presented a progressive alteration and degeneration of the tissue structure, including normal thickness loss of cellular membrane and a general atrophy of cells. Apparently, there is accumulation of TiO_2 NPs in the tissue, with adhesion of NPs to the surface of the digestive cells, especially visible to individuals exposed to 100 mg/L. As suspension-feeders, bivalves have highly developed processes for cellular internalization of particles from nano to microscale particles (endo- and phagocytosis), essential to key physiological functions such as intra-cellular digestion and cellular immunity (Canesi et al., 2012). This may represent a higher susceptibility for these organisms to suffer toxicological effects from nanomaterials exposure and a cause for the acute toxicity observed in this study.

Other studies using microscopy techniques also confirmed that TiO2 NPs have a strong affinity toward the cell surface, demonstrating probable interactions between the particles and the surface active sites of the cell membrane (Metzlera et al., 2011; Sadiq et al., 2011). Thus, NPs TiO_2 toxicity to cells may result not only from the cellular internalization of NPs, but also from the adhesion/adsorptions of NPs to cell surface.

Strong evidences to explain TiO2 NPs toxicity comes from the ability to generate free radicals which cause oxidative stress and cell damage in organisms. It is known that TiO_2 NPs are able to produce reactive oxygen species (ROS) in the presence of UV irradiation but also in the absence of photoactivation (Armelao et al., 2007; Gurr et al., 2005; Reeves et al., 2008). The generation of ROS may induce direct oxidative stress in organisms, playing a major role in the TiO_2 NPs potential toxicity. Studies reported that the formation of hydroxyl (OH) radical is a predominant source of biological damage (Reeves et al., 2008). Indeed, OH radical is the primary damaging species produced upon irradiation of TiO_2 NPs (Dodd and Jha, 2009). It was also observed the additional formation of secondary products, as carboxyl radical anions (CO_2^{-}) and superoxide radical anions (O_2^{-}). A proposed pathway for damage, involves primary generation of OH radicals in the cytoplasm, which react to give CO_2^{-} radicals, that can react with cellular oxygen to form O_2^{-} and genotoxic hydrogen peroxide (H_2O_2) (Dodd and Jha, 2009). Free radicals and consequent oxidative stress may cause peroxidation of the lipid membranes and direct damage to proteins, affecting cellular enzymatic defense activities (Reeves et al., 2008; Dodd and Jha, 2009).

In aquatic organisms the efficiency of the antioxidant ability to control ROS production is marked by an array of antioxidant enzymes pool which leads to ROS detoxification (Abele and Pantarulo, 2004). This important and potent group of antioxidant enzymes include superoxide

dismutase (SOD), which converts O_2- in H_2O_2; catalase (CAT) and glutathione peroxidase (GPx), which remove H_2O_2 avoiding its accumulation in cells and tissues; glutathione-S-transferase (GST), and reduced glutathione (GSH), that transform xenobiotics into other conjugates; and glutathione reductase (GR), which delivers GSH to cells (Lesser, 2006).

Therefore, in the present work it was used a multiple biomarker approach to evaluate oxidative stress. Levels of antioxidant enzymes (SOD, CAT and GST) and lipid peroxidation measured in target organs of *C. auratus* and *C. fluminea* showingsignificant differences between exposed organisms and controls overtime. In general results suggest that exposure to TiO2 NPs caused oxidative stress in both organisms.

With respect to SOD, innumerous studies revealed an increase of SOD activity as a first line of defense mechanism against oxidative stress in different aquatic organisms such as mollusks, crustaceans, polychatea and fish (Abele-Oeschger and Oeschger, 1995; Abele et al., 1998; Cooper et al., 2002; Oliveira et al., 2005; Romero et al., 2007; Tremblay et al., 2011). Additionally, since SOD is the first enzyme to act with oxyradicals it can be used as oxidative stress signal for the early warning of environmental pollution. Thus, the depletion of SOD activity is used as an indication of free radical scavenging ability, showing that the antioxidant defense system is overwhelmed by ROS (Vander et al., 2003). Results showed a trend to increase of SOD enzymatic activity in livers of *C. auratus* according to tested concentrations after 7 days of exposure to TiO_2 NPs. After 14 days a significant increase of SOD activity ($p<0.05$) was found for every tested concentrations, in comparison to controls, which might be due to the synthesis of new enzymes or the enhancement of pre-existing enzyme levels under lower concentrations. From 14 to 21 days, SOD activity continued to increase in individuals exposed to lower TiO_2 NPs concentrations (0.01 and 0.1 mg/L), while a trend to decrease was found in organisms exposed to concentrations above 10 mg TiO_2/L. This trend for the depletion of SOD activity indicates that the antioxidant defense systems are under exhaustion and losing efficiency.

GST is an intracellular enzyme of the phase II of xenobiotic metabolism involved in detoxification by catalyzing the conjugation of a wide variety of electrophilic exogenous substances, and plays a role in preventing from oxidative damage by conjugating the breakdown products of lipid peroxides to reduced glutathione (GSH). Thus GST plays an important role by protecting cells and tissues from oxidative stress induced damages (Siddiqui et al., 1993; Slatinska et al., 2008), while CAT is involved in stress oxidative response catalysing the decomposition of H_2O_2. Usually, a reduction in CAT concentrations is associated to accumulation of hydrogen peroxide and other oxyradicals contributing to oxidative damage. In the present study, CAT and GST in *C. auratus* tissues (liver, gill and intestine) followed a similar pattern to SOD with a remarkable enzymatic activity increase at lower concentrations of TiO2 NPs and a significant reduction at higher concentrations. Major decreases were found in CAT and GST activities in fish liver, generally after 14 days of

exposure to TiO_2 NPs concentrations above 10 mg/L. The results suggest that after an initial increase of antioxidant activity, the defense capability decreases overtime due to over-produced ROS and oxidative stress occurred. In *C. fluminea* digestive gland, SOD activity presented significant differences at exposure concentrations of 0.1 and 10 mg TiO_2/L, showing a trend to increase followed by a trend to decrease at 14 days._While for CAT and GST activities a significant increase was found in every treatment, for exposure periods of 7 and 14 days.

Similar fluctuations of SOD and CAT activity over concentration and exposure time were found in Juvenile Carp (*Cyprinus carpio*) upon TiO2 NPs exposure (Hao et al., 2009). The authors suggest that as the antioxidant enzymes are inhibited, ROS scavenging is weakened and they may be accumulated gradually in the major tissues of fish. Moreover, according to some authors, the GSH and enzymes such as GST, catalase and superoxide dismutase may increase caused by a compensatory mechanism to slight oxidative stress through an increase in its synthesis but severe oxidative stress may suppress their activities due to a loss in adaptive response mechanisms to induced stress (Zhang et al., 2005, Yi et al., 2007). A study from Zhang et al. (2005) which exposed goldfish to a chemical (2,4- dichlorophenol) for 40 days showed a similar response pattern reporting a liver GST activity increase until day 10 and then returned to the control level in the final days. Considering the GST activity results and that no significant mortality was observed in the present study this may indicate that for wild fish, potentially exposed to low concentrations, no adverse effects are expected.

Studies reported that in response to toxicity induced by ROS, the concentrations of certain antioxidant enzymes are increased, but under high levels of pollution the antioxidant defenses can be reduced (Valavanidis et al., 2006). Significant increases in antioxidant enzymes can be a response to ameliorate oxidative stress, while the decrease of enzymatic activity may be a consequence of an exhaustion of the detoxification mechanisms (Jacobson & Reimschuessel, 1998), suggesting a precarious state characterized by a higher susceptibility to environmental stress and potential adverse effects (Cossu et al., 2000). This can explain the variations of antioxidant activity, from increase to depletion, over TiO_2 NPs exposure periods in the present work. Thus, these results reflect not only the exposure to ROS, but also its toxicity.

IT is well known that, lipid peroxidation (LPO) is commonly used as an indicator of oxidative stress of cells and tissues (Botsoglou et al., 1994). In fact, ROS can also react with organism's lipids especially membrane-associated ones, a process designated by "peroxidation", which is considered one of the most frequent cellular injury mechanisms (Lesser, 2011). The lipid peroxidation process is usually determined by the quantification of malondialdehyde (MDA) levels, one of the terminal products of the peroxidative breakdown of lipids (Uchiyama and Mihara, 1978).

The LPO results in *C. auratus*, suggest that gills are the most affected organ presenting highest levels of LPO, with significant increases at every TiO_2 NPs exposure concentration, comparatively to controls. Thus, cellular defenses as mucous secretion and antioxidant enzymes were not enough to prevent oxidative damage, even at low exposure concentrations (0.01 and 0.1 mg TiO_2/L) and LPO may be related to the cellular damage observed in gill tissue. In intestine increased LPO levels were observed after 14 days of exposure to TiO_2 NPs concentrations above 10 mg/L, where NPs are visible inside intestine lumen. Both gills and intestine are in direct contact with TiO_2 NPs, the first one through direct contact with aquatic medium and the second one through NPs ingestion. These observations suggest that direct contact with NPs may enhance LPO in tissues. Nevertheless, elevation of LPO was also found in livers, with significant increases comparatively to controls. Frederici et al (2007) also observed elevation of LPO in internal organs, as fish brain, that had no direct access to the aquatic media. This suggests some indirect mediation of oxidative stress to the internal organs, such as diffusion of the highly mobile hydroxyl radical from sites of direct contact and initial injury (as gills or skin), occurring a rapidly distribution of ROS around the body. Clearly, nanoparticles do not have to be internalized to generate systemic oxidative stress.

Results from *C. fluminea* digestive gland showed increased levels of LPO in TiO2 NPs tested concentrations following exposure in comparison to controls. As observed through microscopy, TiO_2 NPs are accumulated inside organisms in close contact with tissues and cells. The LPO results suggest that for this bivalve species TiO_2 cause severe toxicity and damage to cells. However, since no similar studies using freshwater bivalves were found in current scientific literature it is difficult to compare the present results. The bivalve responses to TiO_2 NPs exposure are possibly associated to the sedimentation (increasing bioavailability) of NPs and organism´s biology as described before. As a final remark the present study indicates that further studies with different bivalve species should be performed to increase knowledge on toxicity responses and to allow comparisons with other species.

These results are generally in accordance with GST and CAT results. It was shown that LPO cause various negative effects in terms of cellular integrity namely at the level of membranes of the cells - they may lose permeability and function - among other changes as the production of pro-inflammatory agents and potentially toxic substances (Greenberg et al., 2008).

6 Conclusions

In this study, the exposure to TiO2 NPs in aquatic suspensions was not lethal for C. auratus, but significant mortality rates were found for C. fluminea.

The exposure of *C. auratus* and *C. fluminea* to TiO_2 NPs caused toxicity involving oxidative stress, induction of antioxidant activity, increase in lipid peroxidation and organ pathologies in fish gills, liver and intestine and in bivalves' digestive gland.

It was observed that NPs in suspension are ingested by fish, resulting in the accumulation of TiO2 NPs agglomerates inside intestine lumen. Besides, apparently a bioaccumulation of TiO2 NPs occurs in bivalves, exposing inner tissues (as digestive gland) directly to NPs and making cells easily susceptible to suffer adverse effects.

Using electron microscopy, cellular internalization of TiO_2 NPs was confirmed in cells from fish gills. Though, it is still necessary to clarify the presence/absence of NPs inside cells from other important organs.

The results suggest that releases of TiO_2 NPs into aquatic ecosystems may pose a potential risk to organisms' health and may cause deleterious effects in aquatic organisms. As nanomaterials (NMs) are increasingly entering in the world's daily life, it is evident that effects of NMs, particularly TiO_2 NPs, on environment are a matter of great concern and the precise mechanisms of toxicity of this and other types of NPs must be clarified. Moreover, the release of NPs into freshwater ecosystems may also pose a risk of human exposure via drinking water and food chains.

Despite an increase of studies revealing toxicity of TiO_2 NPs to aquatic organisms, there is an absence of studies focusing on the aquatic sediment-dwelling organisms. This should be a matter of great concern since findings from the present study suggest that these organisms may have a higher risk of exposure to NPs, caused by deposition and accumulation of NPs in the sediment.

Finally, there is an urgent need of regulation concerning NMs. Thus, nanotoxicology data are required to evaluate the exposures and risks associated to NMs in order to protect human health and the environment.

The specific properties and characteristics of nanomaterials need to be considered for any potential health risks, taking into account hazard assessment of nanotechnology products along the entire food chain (food, novel foods, food additives, food contact materials, feed,

pesticides). It is claimed that nanotechnology products could have a substantial impact on the food and feed sector in the future, offering benefits for industry and for the consumer.

Companies and institutes worldwide are currently researching and developing applications in fields such as the treatment of the mechanical and sensorial properties of food – where for example taste or texture can be changed – and improvements in nutritional value.

Recent EU legislation on novel foods will make it obligatory for producers to label products containing ingredients in the form of nanomaterials.

7 References

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Annex I

Table 3 Mortality rate, length and weight (mean ± SD), after 7, 14 and 21 days of TiO_2 NPs exposure for *C. auratus*.

[Nano-TiO_2] mg/L	Control	0.01	0.1	10	100	400	800
Initial (n)	15	15	15	15	15	15	15
7d Mortality %	0.95	0	0.95	0.95	0	0	1.9
Sacrificed (n)	5	5	5	5	5	5	4
Length (cm)	5.5±0.7	5.2±0.6	6±0.3	6.1±0.8	5.1±0.7	5.7±1.1	5.5±0.9
Weight (g)	8.1±1.2	4.4±1.6	5.7±2.3	4.7±1.9	2.9±1.3	4.7±1.2	3.8±1.2
14d Mortality %	0.95	0.95	0.95	0	0	1.9	0.95
Sacrificed (n)	4	4	4	4	5	4	3
Length (cm)	6.4±1.2	6.1±0.6	6.3±0.3	6.0±0.4	5.6±0.7	5.8±0.8	5.6±0.7
Weight (g)	5.6±3.4	3.8±1.1	5.3±1.1	3.9±0.9	3.4±1.3	4.3±1.0	4.8±0.2
21d Mortality %	0	0.95	0	0	0.95	0.95	0
Sacrificed (n)	4	4	4	5	4	3	4
Length (cm)	6.3±1.0	5.4±1.3	6.5±0.7	5.5±0.7	6.4±1.0	6.0±1.3	6.1±0.6
Weight (g)	4.9±0.7	3.3±1.7	5.5±1.6	2.9±1.1	4.5±1.5	4.1±2.1	5.2±2.3
Cumulative Mortality rate %	1.9	1.9	1.9	0.95	0.95	2.85	2.85

Annex II

Table 4 Mortality rate, length and weight (mean ± SD), after 7, 14 and 21 days of TiO_2 NPs exposure for *C. fluminea*.

[Nano-TiO_2] mg/L	**Control**	**0.01**	**0.1**	**1**	**10**	**100**
Initial (n)	10	10	10	10	10	10 ; 5
7d Mortality %	1.67	1.67	10	5	6.67	15 ; 5
Sacrificed (n)	4	4	2	3	3	1 ; 2
Length (cm)	3.1±0.4	2.9±0.3	3.1±0.1	2.7±0.2	2.7±0.1	2.6±0.1
Weight (g)	2.1±0.2	1.7±0.3	1.9±0.5	1.4±0.3	1.5±0.13	1.1±0.3
14d Mortality %	1.67	3.3	0	3.3	3.3	-
Sacrificed (n)	4	3	2	2	1	-
Length (cm)	3.0±0.3	2.8±0.1	2.8±0.4	2.6±0.1	2.7±0.0	-
Weight (g)	1.4±0.3	1.3±0.08	1.3±0.13	1.2±0.2	1.06±0.0	-
Cumulative Mortality rate %	3.3	4.8	10	8.3	10	20