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Maria Inês Alves Ribeiro

Effects of cerium dioxide nanoparticles in *Oncorhynchus mykiss* gills after an acute exposure: assessment of oxidative stress, genotoxicity and histological alterations

University Fernando Pessoa, Master in Pharmaceutical Sciences

Faculty of Health Sciences

Porto, 2015

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I hereby declare that the work carried out in this thesis is entirely original. It was carried out by me and all the resource materials were properly referenced.

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Maria Inês Alves Ribeiro

(Signature)

Post-graduate work/dissertation presented to the University Fernando Pessoa as part of the requirements in order to get the Master degree in Pharmaceutical Sciences, under the supervision of Prof. Alberto Teodorico Correia (PhD) and co-supervision of Prof. Sara Antunes (PhD)

University Fernando Pessoa, Master in Pharmaceutical Sciences

Faculty of Health Sciences

Porto, 2015

This work has been already presented as a poster communication in an international congress: Ribeiro MIA, Antunes SC, Nunes AC, Correia AT. 2015. Effects of cerium dioxide nanoparticles in *Oncorhynchus mykiss* gills after an acute exposure: assessment of oxidative stress, genotoxicity and histological alterations. XV European Congress of Ichthyology. 7th-11st September. Porto, Portugal.

Resumo

Atualmente, o estudo de nanopartículas (NPs) metálicas é cada vez mais comum dentro da comunidade científica. Contudo, os dados sobre ecotoxicologia relativos as NPs de dióxido de cério (CeO_2) é escassa. Este trabalho é um estudo experimental sobre os possíveis efeitos toxicológicos das NPs de CeO_2 nas brânquias de *Oncorhynchus mykiss* (truta arco-íris). Uma exposição aguda semi-contínua foi realizada e os espécimes da truta arco-íris foram expostas a três concentrações de NPs de CeO_2 (0.25, 2.50, ou 25.00 mg/L), incluindo um grupo controlo (não exposto) durante 96 horas. A exposição a NPs de CeO_2 causou determinadas patologias nas brânquias, incluindo aneurismas, elevação epitelial e hiperplasia. A percentagem de lamelas secundárias disponíveis para a troca gasosa (PAGE), não evidenciou diferenças significativas. A exposição a NPs de CeO_2 causou diferenças estatísticas na catalase (CAT) que mostram um aumento da atividade enzimática nas concentrações 2.50 e 25.00 mg/L. No que diz respeito ao teste as substâncias reactivas ao ácido tiobarbitúrico (TBARS), estas apresentaram uma diminuição do valor nas duas concentrações mais elevadas e um aumento na concentração mais baixa. Atividade da Glutationa *S*-transferase (GST) exibiu um aumento nas duas concentrações mais elevadas e uma diminuição no menor. Nenhuma diferença significativa foi observada na atividade da Na^+/K^+-ATPase. Por sua vez, os resultados do ensaio dos cometas revelou uma tendência crescente com diferenças significativas. Declaro que os resultados sugerem que as NPs de CeO_2 causaram danos genéticos, alterações nos tecidos e aumento da atividade da CAT nas brânquias de uma forma dose-efeito.

Abstract

At present the study of metallic nanoparticles (NPs) is increasingly common within the scientific community. However ecotoxicology data about cerium dioxide (CeO_2) NPs is scarce. This work is an experimental study about the possible toxicological effects of the CeO_2 NPs in the gills of *Oncorhynchus mykiss* (rainbow trout). A semi-continuous acute test was conducted and rainbow trout individuals were exposed to three CeO_2 NPs concentrations (0.25, 2.50, or 25.00 mg/L), including a control (unexposed) group for 96 hours. Exposure to CeO_2 NPs caused some gill pathologies including aneurisms, epithelial lifting and hyperplasia. The percentage of secondary lamellae available for gas exchange (PAGE) revealed however no significant differences. Exposure to CeO_2 NPs caused statistical differences in the catalase (CAT) showing an increase of the enzymatic activity in the 2.50 and 25.00 mg/L concentrations. With regard to thiobarbituric acid reactive substances (TBARS) test, showed a decrease value at the two higher concentrations, and an increase at the lowest concentration. Glutathione S-transferase activity (GST) exhibited an increase at the two higher concentrations and a decrease at the lowest. No significant differences were observed in Na^+/K^+-ATPase activity. In turn, comets assay results revealed an increasing trend with significant differences. The hereby results suggest that CeO_2 NP caused genetic damage, tissue alterations and enhances CAT activity on gills in a dose-effect manner.

Keywords: Rainbow trout; nanoparticles; gills; exposure; biomarkers.

Acknowledgements

O espaço limitado desta secção de agradecimentos, seguramente, não me permite agradecer, como devia, a todas as pessoas que, ao longo do meu Mestrado integrado em Ciências Farmacêuticas me ajudaram, direta ou indiretamente, a cumprir todos os meus objetivos e a finalizar mais esta etapa da minha formação académica.

Desta forma, deixo algumas palavras sentidas e profundas, agradecendo e reconhecendo toda a ajuda recebida.

Ao meu Orientador Prof. Doutor Alberto Teodorico Correia, agradeço desde já pelo privilégio que foi ter efetuado a minha tese no CIIMAR (Centro Interdisciplinar de Investigação Marinha e Ambiental). Pela orientação e apoio incondicionais que muito elevaram os meus conhecimentos científicos e, sem dúvida me ajudaram a ultrapassar todas as dificuldades. Agradeço por esta oportunidade, por ter acreditado na minha palavra que iria cumprir os prazos e tudo o que me propôs assim como pela responsabilidade incutida em mim em todas as fases da tese. Agradeço também pela amabilidade, amizade e boa disposição em todos os momentos. Agradeço pela oportunidade de ter trabalhado lado a lado com os alunos que o Professor auxiliava, Diogo Martins e Cláudia Moreira, obrigada a todos.

À minha Co-Orientadora Prof. Doutora Sara Antunes o meu sincero agradecimento pela co-orientação nesta tese. Muito obrigada pela disponibilidade, e profissionalismo demonstrado. Agradeço também pela amabilidade, amizade e boa disposição em todos os momentos. Um agradecimento e reconhecimento sincero da sua ajuda e pela disponibilidade da Faculdade de Ciências do Porto, que contribuiu para realização desta tese

Como não posso deixar de referir, um obrigada muito especial e sincero para a aluna de doutoramento Sara Rodrigues, que foi incansável em todas as minhas dúvidas, com uma total disponibilidade para comigo. Obrigada Sara, o teu apoio foi determinante para a conclusão desta tese.

À minha Família, em especial aos meus pais, obrigada pelo apoio e amor incondicional, obrigada pelos ensinamentos de vida que fizeram de mim uma melhor pessoa e profissional. À minha irmã, Ana Pedro, que sempre me disse que eu era capaz, que sempre foi o meu braço direito e que acima de tudo está comigo em todas as fases

da minha vida. Um obrigado especial à minha avó e ao meu padrinho porque sempre me ajudaram quando necessitei. Não posso deixar de agradecer à Márcia, a minha segunda mãe, que sempre me disse o que precisava ouvir, e não o que queria ouvir. Obrigada a todos por todas as vezes que estava cansada e foram a minha almofada, obrigada por me ajudarem a completar esta fase. Obrigada por estarem presentes quando estive doente mas acima de tudo por se manterem na saúde. Espero que esta etapa, que agora termino, possa, de alguma forma, retribuir e compensar todo o carinho, apoio e dedicação que me ofereceram constantemente. A vocês, dedico todo este trabalho.

Segundo Albert Einstein “A ciência sem a religião é manca, a religião sem a ciência é cega” por isso agradeço a Deus porque a Ele devo tudo o que sou, tudo o que tenho e tudo o que um dia terei.

"Até aqui o Senhor nos ajudou" - 1Sm 7:12.

CONTENTS

Resumo ... VI
Abstract ... VII
Acknowledgements ... VIII
LIST OF TABLES ... XII
LIST OF FIGURES ... XII
LIST OF ABBREVIATIONS AND ACRONYMS ... XIII
1. INTRODUCTION ... 1
2. MATERIALS AND METHODS ... 8
2.1. Fish acquisition and quarantine ... 8
2.1.1. Study organism: *Oncorhynchus mykiss* ... 8
2.1.2. Nanoparticle characterization ... 8
2.1.3. Experimental design: *Acute exposure* ... 8
2.1.4. Tissues homogenization ... 9
2.2. Biochemical assessments: ... 10
2.2.1. Na^+/K^+- ATPase activity ... 10
2.2.2. Glutathione *S*-transferase activity ... 11
2.2.3. Catalase activity ... 12
2.2.4. Thiobarbituric Acid Reaction Substance quantifications ... 12
2.3. Genotoxicity evaluation: Comet assay ... 13
2.4. Histological assessment ... 15
2.4.1. Gills Semi-Qualitative Assessment ... 16
2.4.2. Gills Quantitative Assessment ... 17
2.5. Statistical analysis ... 17
3. RESULTS ... 18
3.1. Biomarkers evaluation ... 18
3.1.1. $Na^+ K^+$- ATPase activity ... 18
3.1.2. Glutathione *S*-transferase activity ... 18
3.1.3. Catalase activity ... 19
3.1.4. TBARS quantifications ... 20
4. Genotoxicity ... 20
5. Histological alterations ... 21
5.1. Gills Semi-Qualitative Assessment ... 21

5.2. Gills Quantitative Assessment 22
6. DISCUSSION 26
6.1. Biomarkers evaluation 26
6.1.1. Na^+/K^+-ATPase activity 26
6.1.2. Glutathione *S*-transferase 26
6.1.3. Catalase activity 27
6.1.4. TBARS quantification 27
6.2. Genotoxicity evaluation 27
6.3. Histological alterations 28
7. CONCLUSIONS 30
8. REFERENCES 31

LIST OF TABLES

Table 1: Importance factor to the histological evaluation 5

LIST OF FIGURES

Figure 1: Blades with the minigels 14
Figure 2: Classes of the comets obtained by the SSGE. They represent classes 0-4 as used for visual scoring 15
Figure 3: Rainbow Trout Gill from fish 1 CC 1; 20.02.2015; HE x40; 50µm) SLL (secondary lamellar length); SLW (secondary lamellar width); ID (interlamellar distance) and BET (basal epithelial thickness) 17
Figure 4: Na+/K+-ATPase activity (µmol/mg protein-1/h-1). CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC)).. 18
Figure 6: CAT activity (µmol.min-1.mg-1 protein). CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC)). 19
Figure 7: TBARS quantification (nmol mg protein^{-1}). CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC)). 20
Figure 9: Gills Semi-Qualitative Assessment. CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC))... 22
Figure 10: Gills quantative assessment (BET, ID, SLL and SLW). CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC))... 23
Figure 13: Aneurysm (black arrow) and hyperplasia of the epithelium (white arrow) from High Concentration 1; Hematoxylin-Gil; x40 magnification;.............................. 24
Figure 12: Aneurysm (white arrow) from Low Concentration 2; Hematoxylin-Gill; x40 magnification 24
Figure 15: Capillary dilatation and Lamellar fusion (circle), Hyperplasia of the mucosal cells (black arrow) and Hyperplasia of the Epithelium (white arrow from High Concentration 1; Hematoxylin-Gill x40 magnification... 24
Figure 14: Intercellular Edema (Circle) from High Concentration 2; Hematoxylin-Gill; x40 magnification 24
Figure 16: Hyperplasia of the chloride cells (circle) from Medium Concentration 1; Hematoxylin-Gill; x40 magnification 25
Figure 17: Lifting of the Epithelium (circle) from Low Concentration 3; Hematoxylin-Gill; x40 magnification.. 25

LIST OF ABBREVIATIONS AND ACRONYMS

ADP	Adenosine diphosphate
ATP	Adenosine triphosphate
BET	Basal Epithelial Thickness
CAT	Catalase
CDNB	1-Chloro-2,4-dinitrobenzene
Ce O_2	Cerium dioxide
CeO_2 NPs	Cerium dioxide Nanoparticles
CuO	Copper oxide
$CuSO_4$	Copper sulphate
DNA	Deoxyribonucleic Acid
EC50	Effective Concentrations
EtBr	Ethidium bromide
GDI	Genetic Damage Index
GPI	Gills Pathology Index
GST	Glutathione *S*-Transferase
H_2O_2	Hydrogen Peroxide
HC	Higher Concentration
HLA	Human Leukocyte Antigen
IB	Imidazole Buffer
ID	Interlamellar Distance
LC	Low Concentration
LC50	Lethal Concentrations
LDH	Lactic Dehydrogenase
LDH	Lactate Dehydrogenase
LMPA	Loop-mediated isothermal amplification
MC	Medium Concentration
NADH	Nicotinamide Adenine Dinucleotide
NAT_2	Arylamine *N*-acetyltransferase
NPs	Nanoparticles
PAGE	Secondary lamellae available for Gas Exchange
PEP	Phosphoenolpyruvate
PK	Pyruvate Kinase
SiO_2	Silicon dioxide
SLL	Secondary Lamellar Length
SLW	Secondary Lamellar Width
SSGE	Single cell alkaline gel electrophoresis
TBARS	Thiobarbituric Acid Reactive Substances
ZnO	Zinc oxide

1. INTRODUCTION

Nanotechnology is defined as the production and application of structures, devices and systems by controlling shape and size at the nanometer scale (Park *et al.,* 2007). Due to their small size, less than 100 nm, nanoparticles (NPs) display greater specific surface areas and energies; quantum related effects and generally increased surface reactivity comparatively to the corresponding conventional forms, leading to immeasurably different properties and applications (Park *et al.,* 2007; Gaiser *et al.,* 2009). Is one of the fastest-growing fields today and it will represent a projected 20-30 billion dollar market by 2015 (Arnold *et al.,* 2013). The nanotechnology industry has experienced a rapid growth, and its applications include products such as paints, cosmetics, medicines, foods, sunscreens, catalysts. The general public is already exposed to a variety of consumer products containing NPs like sunscreens and cosmetic products containing TiO_2 (titanium dioxide), electronic devices, medical diagnosis devices (Park *et al.,* 2007; Tiede *et al.,* 2009) and even food (Tiede *et al.,* 2009; Sekhon, 2010).

Metal oxide nanoparticles, in particular, have multiple applications in different fields such as industrial, electrical, pharmaceutical, and biomedical ones because of their exceptional physicochemical properties paralleled to bulk chemicals (Nel *et al.,* 2009). The cerium dioxide metal nanoparticles (CeO_2 NPs) or nanoceria, are one of the rarest and most important oxides nanoparticles used at present (average concentration of 50 ppm), found in a variety of mineral classes, primarily including carbonates, phosphates, silicates, oxides and hydroxides. Main sources of industrial cerium include the carbonate mineral bastnasite and the phosphate mineral monazite (Dahle *et al.* 2015). Currently they are one of the most viable nanoparticles produced and economically valuable (Hedrick, 2004; Wang *et al.,* 2008; Jun *et al.,* 2013). They are used mostly in the automotive industry, where they are present in the form of solid oxide electrolyte fuel cells, as diesel fuel additive, in solar panels, as catalysts, and in ceramic and glass applications (Murray *et al.,* 1999; Zheng *et al.,* 2005; Eom, 2009; Park *et al.,* 2008b). Cerium is a lanthanide series rare earth metal element that exists in the states of Cerium (III), Cerium (IV) (Kilbourn, 2003; Dahle *et al.* 2015); between the two, they contain oxygen vacancies that allow the nanoparticles to act as a regenerative catalyst (Heckert, 2008). The cerium can be separated out of the other rare-earth

elements through oxidation, forming CeO_2, because of its distinctive stability in tetravalent state (Winkler, 2002).

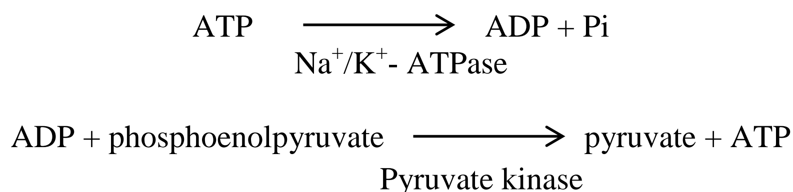
It is important to address the issues regarding the NPs potential impacts on human and environmental health, as the nanotechnology industry grows. The increasing world-wide use of CeO_2 NPs, leads to the necessity of conducting ecotoxicological studies to assess the potential harmful of these nanoparticles in the environment, namely in the aquatic compartiment (Van Hoecke *et al.,* 2009). CeO_2 NPs can be released into the waste waters and to the atmosphere being distributed broadly in the aquatic environment and causing damages to the living organisms (Gaiser *et al.,* 2009). Currently the CeO_2 NPs are on the list of the substances that the Organization for Economic Co-operation and Development (OECD) consider to be priority to test (OECD, 2010).

At present there is limited information about the toxicity of CeO_2 NPs in vivo systems. A few organisms, namely crustaceans and fish, have been experimentally exposed to sub-lethal concentrations of CeO_2 NPs. *Pseudokirchneriella subcapitata,* a unicellular alga, revealed chronic toxicity and with 10% of the population showed algal growth inhibition effects at the concentrations of 2.6-5.4 mg/L CeO_2. However no toxicity was observed in *Danio rerio* embryos following an acute exposure to CeO_2 NPs up to test concentrations of 1000, 5000, and 200 mg/L (Van Hoecke *et al.,* 2009). Exposed *D. rerio* adults to CeO_2 NPs via water and diet resulted in significant CeO_2 NPs uptake in the liver of the *D. rerio* exposed to the contaminant and it is likely that this resulted in limited bioavailability of the unmodified metal oxide NPs in fish (Johnston *et al.,* 2010). The toxic potential of CeO_2 NPs were investigated in two in vivo systems, *D. rerio* and *C. elegans*, employing both wild type and genetically deficient *C. elegans* strains to explore the role of oxidative stress in CeO_2 NP toxicity in vivo (Das et al. 2007). Exposed *Caenorhabditis elegans* to CeO_2 NPs at the concentrations of 2.5, 5.0, 12.5, 25.0, 62.5 and 93.8 mg/L for three days exhibited a decreased growth in wild-types nematodes at all dosing levels (Arnold *et al.,* 2013). Jun *et al.* (2013) tested multiple biomarkers in *Carassius auratus,* freshwater fish, at the concentrations of 20, 40, 80, 160, and 320 mg/L of copper oxide (CuO), zinc oxide (ZnO) and CeO_2 NPs in 4 days of exposure. In this study it was possible to observe an increase of inhibition of Na^+/K^+-ATPase in gill and Catalase (CAT) activity in liver.

The chemical water pollution plays a primary role in destroying ecosystems (Ayas *et al.,* 2007). The environment is continuously overloaded with foreign organic chemicals (xenobiotics). The presence of a xenobiotic compound on the aquatic ecosystem alone does not indicate distressing effects. However, conjoint external levels of exposure, internal levels of tissue contamination and early adverse effects dictates the end result (Oost *et al.,* 2003). Water pollution can cause different changes in fish, ranging from biochemical alterations in single cells up to changes in whole populations. Thus, the presence of those changes is a suitable indicator of the environmental pollution (Bernet *et al.,* 1999; Ayas *et al.,* 2007). It is possible to monitor pollutant concentrations in the tissues of the exposed organisms and to record the biological responses induced by xenobiotics using biomarkers (Oost *et al.,* 2003; Ayas *et al.,* 2007). The concept of biomarkers or ´bioindicator` and ´ecological indicator` was established in the 1990s (Bernet *et al.,* 1999; Oost *et al.,* 2003). They are defined as the measurements in body fluids, cells or tissues indicating biochemical or cellular modifications due to the incidence and magnitude of toxicants, or of host response (NRC, 1987). Biomarkers raise the possibility of determining where an organism is affected and offer a warning sign, making early detection of diseases possible.

According to Oost *et al.* (2003) and NRC (1987), the term biomarker may be divided into: biomarker of exposure; response or toxic effect; and susceptibility. The biomarkers assessment only includes the biomarkers of exposure defined was the detection and measurement of an exogenous substance, its metabolite or the product of an interaction between a xenobiotic agent and some target molecule or cell that is measured in a compartment within an organism [examples: Glutathione S-transferase (GST), CAT, deoxyribonucleic acid (DNA)]. They should be quantitative, sensitive, non-invasive, specific, and easily measurable, relate to the biochemical mechanism and work at realistic doses (Depledge, 1994; Timbrell, 1998).

Na^+/K^+- ATPase activity is a biomarker that can be evaluate the effects on the osmoregulation. The basic reaction of the assay is as follows:



$$\mathrm{ATP} \xrightarrow[\mathrm{Na^+/K^+\text{- }ATPase}]{} \mathrm{ADP + Pi}$$

$$\mathrm{ADP + phosphoenolpyruvate} \xrightarrow[\mathrm{Pyruvate\ kinase}]{} \mathrm{pyruvate + ATP}$$

Thus, the production of ADP in the presence of Na^+/K^+- ATPase results in an equimolar disappearance of NADH. To obtain the µmoles ADP/mg protein/hour on each sample, it is necessary to calculate the total protein and subtract.

The glutathione transferases are a group of related enzymes which catalyze the conjugation of glutathione with various electrophilic compounds.Catalase is an intracellular enzyme that has two functions. Firstly, the decomposition of the H_2O_2 (hydrogen peroxide) and the oxidation of the hydrogen donor (methanol, formic acid and phenols) and secondly, the peroxide consumption (peroxidic activity).

The hydrogen peroxide enzymatic decomposition occurs according to a 1st reaction ($2\ H_2O_2 \xrightarrow{CAT} 2H_2O + O_2$), and its speed is proportional to the amount of peroxide present. However, the kinetics of catalase do not follow a normal pattern. On the one hand, it is not possible to saturate the enzyme with the substrate within the possible concentrations (up to 5M H_2O_2) and on the other hand a rapid inactivation of catalase H_2O_2 concentrations up to 0.1 M occurs when the complex I active enzyme H_2O_2 is converted into inactive complexes II or III (Bradford, 1976).

The extent of lipid peroxidation was measured in gills by the quantification of thiobarbituric acid reactive substances (TBARS).

Tissue alterations have/present different consequences to the fish's health. Each reaction pattern can induce several modifications and consequences to the organ's functioning or to its different sections. The classification of the following alterations was performed according to Bernet *et al.* (1999).

Histological evaluation of elements in the fish after exposure to xenobiotics was conducted according the flowing correlation (Table 1) (Bernet, 1999). An importance factor and a score value (1, 2 or 3) were attributed to each alteration observed and the index was calculated using those values.

Table 1: Importance factor to the histological evaluation

Score value	Alteration
1	Minimal pathologic al importance; Easily reversible when the exposure ends.
2	**Moderate pathological importance;** Reversible lesion in most cases if the stressor is defused.
3	**Marked pathological importance;** Lesion is usually irreversible; Leads to partial or total loss of the organ function.

Circulatory disturbances are a consequence of a pathological disorder in blood and tissue flow. The tissue's inflammatory process is related to alterations in the fluid contend. Changes included in the Circulatory disturbances are: Capillary dilatation (1), Hemorrhage (1), Hyperemia (1), Aneurysm (1) and Intracellular edema (1). Hemorrhage and Capillary dilatation are characterized by the blood leaking from blood vessels. When a congestion of blood occurs in an organ, caused by venous and arterial process this change is called Hyperemia. The Aneurysm is a dilation of the arterial blood vessels. Intracellular edema appears on the lamellae's tips and they usually become rounded and filled with red blood cells. Regressive changes are characterized by a reduction in the organ's functioning/function or its complete loss. These alterations include: Architectural and structural alterations (1) (alterations in the tissue structures as a consequence of cell damage) such as epithelium lifting, alterations of the chloride cells, pillar cells, mucosal cells and lamellar fusion and Cytoplasma alterations (1), Deposits (1), Nuclear alterations (2), Atrophy (2) and Necrosis (3). The Cytoplasma alterations are characterized by the presence of changes in cellular plasma caused by hyaline droplets, colloidal droplets, degenerative fatty vacuolization or hydropic glycogen droplets. Deposits are described as intracellular accumulations of substances mainly initiated by degenerative processes. Nuclear alterations are modifications in the nuclear shape and structure of chromatin. Atrophy is a decrease in the cells' number and volume or a reducing quantity of intercellular substances. Necrosis is a morphological state of the cell or tissue which appears after irreversible loss of cell function. Progressive changes are characterized by an amplified activity of cells or tissue

processes. Representative lesions are: Hypertrophy (1) or Hyperplasia (2) of the epithelium, chloride cells, pillar cells or mucosal cells. Hypertrophy is an increase in the organ or tissue's volume due to the enlargement of its component cells, which grow in size not in number. Hyperplasia consists in tissue or organ amplification by a superior number of cells but without change in the cells' volume. The inflammation process is frequently associated with belonging to other reactions (e.g. edema). As a result, it is difficult to characterize inflammatory changes taking only one reaction pattern into account. Inflammatory reactions are divided in Exudate (1) (e.g. proteins) and Infiltration (2). Exudate is a large aggregate of cellular debris inclosing a high protein concentration. Infiltration is characterized by the penetration of leucocytes in the blood vessels walls and eventually infiltrating the adjoining tissue. Neoplastic or tumors are an uncontrolled multiplication of cells and tissue. Tumors are divided in two classes, benign (2) or malignant (3).

The score value used on each sample to evaluate the lesions was: 0 no observed changes; 2 mild occurrences; 4 moderate occurrences and 6 severe occurrences. Each alteration's incidence was calculated in all the samples. Total Pathology Index (TPI) represents a quantification/quantitative analysis of the general health status based on the histological lesions. (Bernet, 1999; Raskovic, 2010).

Rainbow trout (*Oncorhynchus mykiss*) is a native fish from the Pacific drainages of North America and belongs to the family of Salmonidae (Walbaum, 1792). It is a greatly adaptable species distributed worldwide (Fornshell, 2002). *O. mykiss* is a robust fish that is easy to breed; it has a fast development; it is highly tolerant to a varied range of environments and it can be artificially fed (Walbaum, 1792). This species requires a continuous administration of good quality water with high levels of oxygen, and it is sensitive to higher levels of ammonia (NH_3), carbon dioxide (CO_2), pH and to the suspended solids. Controlled temperature is required (Holliman, 2000; Martínez, 2009). The lower oxygen level that is recommended is 5-5.5 mg/L for the growing fish. The reduction of the oxygen levels for a long period of time can cause pathologies or death (Holliman, 2000). The ammonia and the nitrites can appear in the water because of the organic material's degradation (consequence of the excessive feeding) and they present/have toxic relevance when combined with high temperature levels and water pH (Martínez, 2009). The higher ammonia concentration recommended is of 0.025 mg/L (Holliman, 2000).

Concerning temperature, it influences the dissolved oxygen and ammonia concentrations and the decomposition level of the fecal material accumulated on the bottom of the tank. The adequate water temperature for the rainbow trout is around 9-17°C (Holliman, 2000; Martínez, 2009). With relation to the pH, the trout prefers water with a pH level of 7-7.5.

According to previous information presented, this work aimed to assess the ecotoxicological effects of CeO_2 NPs in gills of freshwater fish *Oncorhynchus mykiss*. For this purpose an acute exposure was conducted at a range of CeO2 concentrations and biochemical, genotoxic and histological effects were assessed.

2. MATERIALS AND METHODS

2.1. Fish acquisition and quarantine

2.1.1. Study organism: *Oncorhynchus mykiss*

O. mykiss juvenile diploid individuals were acquired at a mountain range aquaculture facility (Posto Aquícola do Torno—Marão) in northern Portugal, with the initial size of 6-7 cm. The specimens were transported in oxygen filled plastic bags with cold freshwater until arrival at the laboratory facilities. In the laboratory animals kept in quarantine for 2 months in 500 L tanks, with dechlorinated tap water with continuous aeration, controlled temperature (16 ± 2°C) and the photoperiod was of 12h in the light, 12h in the dark. Fish were fed "ad-libitum" every 48h with commercial pellets. During the quarantine period dead or diseased animals were discarded.

2.1.2. Nanoparticle characterization

The nanoparticle used in this study [Cerium (IV) Oxide (CeO_2)] was obtained from Sigma-Aldrich (Schnelldorf, Germany). It is a nanopowder with a cubic crystal structure, particle size of <50 nm, 99.95% trace rare earth metals basis, surface area of $30 m^2/g$ (BET), density of 7.13 g/mL at 25°C, bulk density of 0.53 g/cm^3. Is a water-insoluble nanoparticle (Gehlhaus *et al.*, 2009) and the toxic concentrations (EC50 and LC50, respectively) tested in bacteria, algae, crustaceans and soil enzymes are >100 mg/L according to Velzeboer *et al.* (2008).

2.1.3. Experimental design: *Acute exposure*

A total of 60 individuals of *O. mykiss* were used in the acute assay. The experimental design was five fish per tank, with three replicates per concentration, including the control (non-exposed) group. Tanks were randomly distributed in the exposure room. Test concentrations used in acute exposure were all sub lethal: 0.25; 2.50 and 25.0 mg/L of cerium dioxide nanoparticles. These concentrations of CeO_2 NPs were selected after considering and analyzing the values described in the literature

(Hedrick, 2004; Gaiser, *et al*., 2009) and values already detected in the environment (Tiede, *et al*., 2009; Felix, *et a*l., 2013 and Dahle, and Arai, 2015).

The fish were exposed to the nanoparticle CeO_2 for 96 hours (acute exposure) according to OECD guidelines (OECD, 1992), using a semi-continuous exposure regime (when 80% water change in the third day and a re-dosing after the change). Each tank had a circulating water pump (water-insoluble nanoparticle) to re-suspend the nanopowder in the water (treatments 0.25; 2.50 and 25.0 mg/L) on the days of the contaminant exposure.

For monitoring water quality along of the exposure period, water samples were collected regularly for analysis (total ammonia and nitrites). *In situ*, physical and chemical parameters (dissolved oxygen, pH and temperature) were also measured using the Multi Probe System YSI 556 MPS. Total ammonia and total nitrites were measured using the Palintest Photometer YSI (520 nm for the total nitrites and 640 nm for the total ammonia). Mean values during the exposure values were (means ± S.E.): pH, 6.12±0.03; total ammonia, 0.96±0.06 mg/L; dissolved oxygen, 14.23±0.84 mg/L; total nitrites, 0.20±0.04 mg/L and temperature 14.89±0.35 °C.

After the exposure period (96h) the fish were immediately anesthetized with by immersion in an ice-cold (4°C) water bath and sacrificed (Wilson *et al.,* 2009), collected the gills.

2.1.4. Tissues homogenization

The gills were extracted immediately after sacrifice and half of the second arch was used for the Comet Assay (Collins, 2004; Lorenzo, 2013). The remainder of gills was divided in three aliquots one to be used for biochemical assessments, the second to assess Na+/K+-ATPase activity and the third aliquot for histological analysis. For the Na+/K+-ATPase activity assessment samples were stored in eppendorfs (e.g. 1.5 ml) with SEI Buffer (Sucrose 150mM, Na2EDTA, 10mM, Imidazole, 50mM), initially stored at -80°C (McCormick, 1993). For biochemical assessment an aliquot of liver was stored at -80°C in eppendorfs. For the histological analysis, the gills were placed in plastic cassettes in a Bouin solution for 24 hours (Hughes, Perry, 1979; Smith, 2007).

2.2. Biochemical assessments:

The stored aliquots of gills were analyzed regarding Na^+/K^+-ATPase activity, thiobarbituric acid reactive substances (TBARS), Glutathione *S*-Transferase (GST) and Catalase activity (CAT). For biochemical analysis (GST, CAT and TBARS) gills were homogenized in 2 mL of homogenization buffer (200 mM, pH = 7.0 with Triton X-100 0.1%) and centrifuged at 14000 g for 10 min at 4ºC

2.2.1. Na^+/K^+- ATPase activity

The Na^+/K^+-ATPase activity test was performed according to the microplate method of McCormick (1993) and absorbance was read at 340 nm (Gen5 2.00).

The salt solution (Imidazole 50 mM, NaCl 189 mM, $MgCl_2$ 10.5 mM, KCl 42 mm at pH 7.5 adjusted with HCl) should be made prior to the onset of tissue homogenization and may be stored at 4°C. The assay mixture reagents are: Imidazole Buffer (IB) 50 mM at pH 7.5 adjusted with HCl, Phosphoenolpyruvate (PEP) 2.8 mM, Nicotinamide Adenine Dinucleotide (NADH) 0.22 mM, Adenosine triphosphate (ATP) 0.7 mM, Lactic Dehydrogenase (LDH) 4 U/mL, Pyruvate Kinase (PK) 5 U/mL, Adenosine diphosphate (ADP) 4 mM and Oubain 0.7 mM. The enzymes (PK and LDH) were spun for 8 minutes at 12000 g at 4°C to obtain a distinctive pellet.

Due to the decrease in the activity after the homogenization (even if kept on ice) it is necessary to execute the measurements within half an hour after the beginning of the homogenization and it must be kept on ice throughout. Previously 50 µl of SEID (0.1 g Na Deoxycholic Acid in 20 mL SEI, 0.5 %) were added to eppendorfs and were homogenized in the tube using an Ultrasonic Processor. The tissue was ground for 10 to 15 seconds with the pulser 4 and amplitude of 40. Centrifugation was performed at 16000 g for 5 minutes at 4°C to eliminate the insoluble material present in the sample. The supernatant was extracted and the pellet was re-suspended in IB and it was added to the assay mixture. The assay mixtures Oubain 21 mM and non-oubain must be made immediately before adding the homogenates to the 96 well plate. Each sample was carried out in quadruplicates of 10 µL. The microplate has to be cooled while the homogenate is added on a coolpack with a paper towel. Each well contained 50 µL of the salt solution previously added to the assay mixtures and 150 µL of one of the assay

mixtures, two wells with Oubain and two with the non-Oubain assay mixture per sample. These solution mixtures were brought to the assay at the temperature of 25°C (because of most teolosts' thermal limits) with a water bath just prior to use. The enzymatic activity was determined by measuring the absorbance at 340 nm at 25ºC in thirty seconds intervals for 10 minutes using a microplate reader (BioTek Synergy HT). When first setting up the assay, run and NADH standard curve. The slope should be approximately 20 mOD units/nmole. The Bicinchoninic Acid Assay or BCA Protein Assay is an endpoint assay that determines the total concentration of protein in a solution at a wavelength of 550 nm. The assay was made by pipetting, 10 µL of each standard in quadruplicate (25%, 50%, 75% and 100% of BCA), sample (in triplicate) and blank solution was added to the proper wells. Afterwards, 300 µL of Bradford reagent were added to each well. Mix samples for 10 minutes.

2.2.2. Glutathione *S*-transferase activity

Glutathione *S*-transferase (GST) activity is an enzymatic assay and it was conducted according to the method from Habig *et al.,* (1974) and Ezeji *et al.,* (2012) adapted to microplate with a wavelength of 340 nm. GST catalyzes the conjugation reaction of the glutathione with the 1-Chloro-2,4-dinitrobenzene (CDNB) substrate, forming a thioether (ε= 9,6 $mM^{-1}cm^{-1}$) which can be monitored by the increase in the absorbance at 340 nm using a microplate reader (Thermo Scientific, model Multiskan GO, version 1.00.40, with Skanlt Software 3.2). The reaction solution containing phosphate buffer (0.1 M K_2HPO_4, $K_2HPO_4.12H_2O$, pH 6.5), glutathione solution (10mM) in phosphate buffer (0.1 M, pH 6.5, photodegradable) and CDNB (60 mM) on ethanol (photodegradable) was freshly prepared, just prior to the assay. The wells were filled in with 250 µL of sample plus 250µL of reaction solution. The enzymatic assay was performed on a microplate and the readings were repeated every 10 seconds up until 5 minutes and the GST activity (µmol.min-1.mg-1 protein) was determined using the standard calibration curve.

2.2.3. Catalase activity

Catalase activity is determined using a method that monitors the H_2O_2 decomposition at 240 nm using a microplate reader (Thermo Scientific, model Multiskan GO, version 1.00.40, with Skanlt Software 3.2) according to Góth (1991) (ε 240 = 0.00394 ± 0.0002 liters $mmol^{-1}$ mm^{-1}). The difference in absorbance (Δ 240) per time unit is a measure of catalase activity. First an initial dilution 1:50 of the centrifuged sample was made with 20 µL sample + 980 µL Phosphate Buffer (50 mM KH_2PO_4 and K_2HPO_4 in ultrapure water, pH 7.0). To perform the enzymatic assay, 200 µL of H_2O_2 solution and 400 µL of supernatant (diluted and centrifuged) were added. The blank (e.g. water) contained 200 µL of phosphate buffer and 400 µL of supernatant. The enzymatic assay was assessed/evaluated by the decrease in the absorbance at 240nm for 5 minutes. The CAT activity was expressed in µmol.min-1.mg-1 protein.

2.2.4. Thiobarbituric Acid Reaction Substance quantifications

Thiobarbituric Acid Reactive Substance (TBARS) was determined by lipid peroxidation according to Camejo *et al.,* (1998) and Bouskill *et al.,* (2006). This methodology is based on the reaction of compounds such as malondialdehyde (formed by degradation of membrane lipids by free radical attack) with thiobarbituric acid (TBA) (Buege and Aust 1978).The assay involves adding 200 µL of centrifuged sample/blank (Phosphate Buffer 50 mM, pH 7.0) (5minutes) and 400 µL of trichloroacetic acid solution 10%. Centrifuge at 10000 g for 20 seconds. To 500 µL of supernatant add 500 µL of thiobarbituric acid 1%. Place in boiling bath for 10 minutes. Let it cool down and read the absorbance at 535 nm on a microplate reader (Thermo Scientific, model Multiskan GO, version 1.00.40, with Skanlt Software 3.2). All data from the assay were normalized per nmol/mg protein. The calculations were performed according to the following equation:

$A = \varepsilon * b * C$ (A abs = 535 nm, b = 1 cm and C the concentration in M)

Molar extinction coefficient (ε): 1.56x106 $M^{-1}.cm^{-1}$

2.2.5 Protein content

The final quantification of total proteins was performed by the Bradford method (Bradford, 1976). This method involves the binding of a dye (Bradford reagent – Bradford Biorad) to the total proteins, yielding a colored and stable complex that can be quantified at 595 nm.

2.3. Genotoxicity evaluation: Comet assay

The whole procedure to evaluate the genotoxicity of the rainbow trout gills was adapted from the protocol described in Collins, 2004 and Lorenzo, 2013. This methodology evaluates the index of genotoxic damage - GDI. The GDI is calculated through a visual assessment and classification of the comets (Collins, 2004). The procedure should be made without light influence (preferably in the dark) and at 4°C. The sample preparation and the positive control are made through a cell suspension, using the second gill arch and washing with phosphate buffer solution - PBS (2,7 mM KCl, 1,5 mM KH_2PO_4, 0,14 M NaCl, 8,18 g 2 mM $Na_2HPO_4.12H_2O$). To prepare the samples, place the tissue in a Petri dish with 2 mL of PBS and make 4 or 5 cuts to obtain a cell suspension. Place 1.5 mL in an eppendorf, centrifuge at 200 g during 5 min at 4°C. Discard the supernatant and make up the eppendorf with 1 mL of PBS. Centrifuge again at 200 g during 5 min at 4°C. Discard ~980µL of supernatant and add to the remaining 20µL, 140µL of loop-mediated isothermal amplification (LMPA) at 37°C. The positive control is made with H_2O_2 using a suspension of gill cells. In an eppendorf put 991.3µL of PBS with the suspension of gills cells and add 5.7 µL H_2O_2 8.82 mM. After 4 minutes of exposure the cell suspension were centrifuge at 300 g, 5 min at 4°C. Discard the supernatant and make up the eppendorf with 997 µL of PBS. The incorporation of cells in agarose is performed using 0.8 % of agarose LMPA. Primarily heat the agarose in a microwave for 4-5 seconds and maintain it at 37°C using a hotplate for eppendorf's tubes. Incorporate the suspension in LMPA agarose. In each Eppendorf place 20 µL of cells suspension plus 140 µL of LMPA at 37°C. Fill the blade with minigels according to the image (Fig. 1). In each minigel, place 6µL of the cell suspension with LMPA. Place the slides at 4°C for 15 min to solidify the agarose.

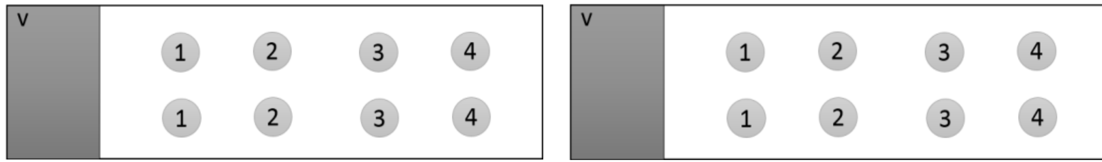


Figure 1: Blades with the minigels

The next procedure is cell lysis. Place the slides in a lysis solution (0.2 M NaOH, 100 mM Na_2.EDTA.$2H_2O$, 100 mM, 10 mM TRIS, 2.5 M NaCl and on the day add 1% Triton X-100 and 10 % DMSO), for 1h-24h at 4°C. Following this step is the alkaline incubation (strand break). Place the slides in an electrophoresis solution (0.3M NAOH, 1 Mm Na_2.EDTA; pH>13) for 20 minutes at 4°C and keep it in the dark. The electrophoresis is carried out at 0.8V/cm, 300MA for 30 minutes at 4°C. After electrophoresis, slides were neutralized and washed 3 times with PBS, to obtain a neutral pH. Then they were washed with distilled water for 15 minutes each. (PBS 15 min and 15 min H_2O). The dehydration of the minigels was made by adding 10 minutes in 70% alcohol and then 10 minutes in 100% ethanol. Keep in the dark during the dehydration. Wash the slides with cold water for 10 minutes. To color the slides, place them in a box with a grid and stained with 0.01 mg/ml ethidium bromide (EtBr) for 20 minutes, using a Pasteur pipette, fully covering the mini-gels. Proceed to washing with cold distilled water for 10 minutes. Leave to dry at room temperature, but protected from light (box).

On the day of the analysis, place 1 drop of distilled water on top of each slide, and cover with a coverslip. The analysis of the slides was made using the LAS Advanced Florescence with a color camera DFC340FX and the LAS-AF software. The fluorescence microscope is equipped with an excitation filter of 510-560 nm and a barrier filter of 590 nm. For each sample 100 comets were analyzed (50 comets by minigel), based on a visual assessment system and on the 5 recognizable comet classes. Class 0 (no damage, no visible tail) to class 4 (almost the entire DNA is in the tail and insignificant in the head) (Illustrated in the Fig. 2). Each comet is given a value according to the class in which it is placed (Collins, 2004).

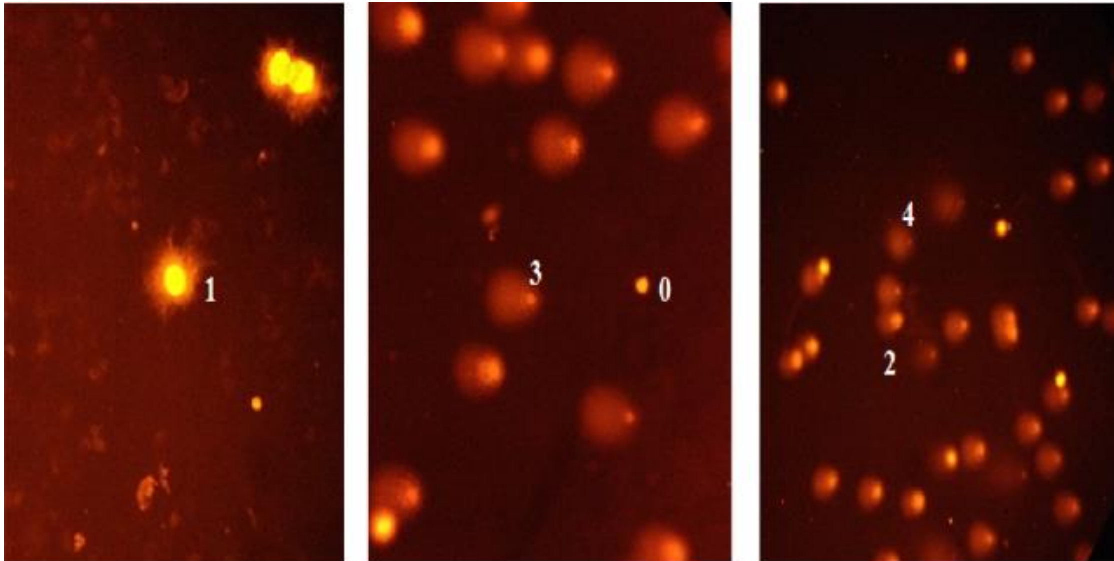


Figure 2: Classes of the comets obtained by the SSGE. They represent classes 0-4 as used for visual scoring

The interpretation of the results is made by calculating the Genetic Damage Index (GDI). The score ranges from 0 to 400 arbitrary units.

Genetic damage index (GDI) = (percentage of cells in class 0 x 0) + (percentage of cells in class 1 x 1) + (percentage of cells in class 2 x 2) + (percentage of cells in class 3 x 3) + (percentage of cells in class 4 x 4)

2.4. Histological assessment

The chemical fixation of gills was performed with a Bouin Solution for 24 hours. In this process the biological tissues are preserved from decomposition or unwanted change, and any biochemical reaction stops. Then the decalcification process in the mineralized structures was executed, using a decalcification solution for 24 hours. The next step was dehydration. Before the inclusion of the material in the paraffin, all the water content should be removed. This process is carried out by immersing the tissue blocks in increasing concentrations of ethanol: 70% ethanol (1h), 80% ethanol (1h), 90% ethanol (1h) and 100% ethanol (1h). This way tissue retraction or irreversible cell structural damage can be avoided. Diaphanization constitutes the infiltration of the tissue by a solvent which is paraffin. Paraffin does not mix with water or ethanol, therefore these should be completely removed, using xylene I (1h) and then xylene II

(1h), so that the paraffin can effectively penetrate into the tissue on the next step. The impregnation in paraffin's purpose is to completely eliminate the xylene contained in the biological material and the complete penetration of the paraffin in the voids left by the water and fat that existed in the tissues. This process is essential to prepare the material for the cuts. First the tissue blocks are placed in Paraffin I (56-58°C for 1h), then Paraffin II (56-58°C for 1h), and Paraffin III (56-58°C left overnight). On the next day the tissue blocks are incorporated in a metal container with Paraffin IV (56-58°C). When the Paraffin solidifies it forms a block, containing the biological tissue ready to cut using a Microtome Reichert-Jung 2030 (5-8µm of thickness). After this process it is possible to obtain slices with the biological material ready to be analyzed. The slice is fixed with distilled water to a histology slide and stays overnight in an oven at 37°C.

Prior to the coloration process (Fig. 8) the deparaffinization with xylene (10 minutes) is carried out to remove the paraffin from the cuts. After that comes the rehydration in 100% ethanol (2 minutes), 90% ethanol (2 minutes), 80% ethanol (2 minutes), 70% ethanol (2 minutes) and distillated water (5 minutes). The coloration process starts with Hematoxylin-Gill (2 minutes), HCL 0.1% (2 seconds), Eosin 1% (2 minutes), Running water (10 minutes) 70% ethanol (2 seconds), 80% ethanol (1 minute), 90% ethanol (1 minute), 100% ethanol (1 minute) and xylene (5 minutes). The assembling was carried out with DPX (room temperature) and the drying took place overnight.

2.4.1. Gills Semi-Qualitative Assessment

Gill injuries were observed in each slide and they were assigned a score value (Bernet, 1999). The pathological changes were classified into five reaction patterns, Circulatory disturbances, Regressive (Epithelium, Supporting tissue) and Progressive changes (Epithelium, Supporting tissue), Inflammation and Neoplastic (Bernet, 1999; Raskovic, 2010). The filaments from each individual gill were measured for: secondary lamellar length (SLL) and width (SLW), interlamellar distance (ID) and basal epithelial thickness (BET). After obtaining the four measures from three different zones (central, distal and proximal), the mean of the values obtained in each treatment was calculated.

2.4.2. Gills Quantitative Assessment

Photomicrographs of the gills tissues were taken arbitrarily under the magnification (40) (Olympus CX41) for the five individual gills per block. Each individual's filaments appearing in the photomicrograph (Fig. 3) were measured for: secondary lamellar length (SLL) and width (SLW), interlamellar distance (ID), and basal epithelial thickness (BET). The secondary lamellar length is essential to the gas exchanges in fish gills. The measurings were made in three zones: central, distal and proximal. The percentage of secondary lamellae available for gas exchange (PAGE) was averaged for each filament of an individual and calculated according to the following equation (Nero, *et al.,* 2006):

$$\text{PAGE}\ (\%) = 100 \times \frac{(\text{mean SLL})}{(\text{mean BET} + \text{mean SLL})}$$

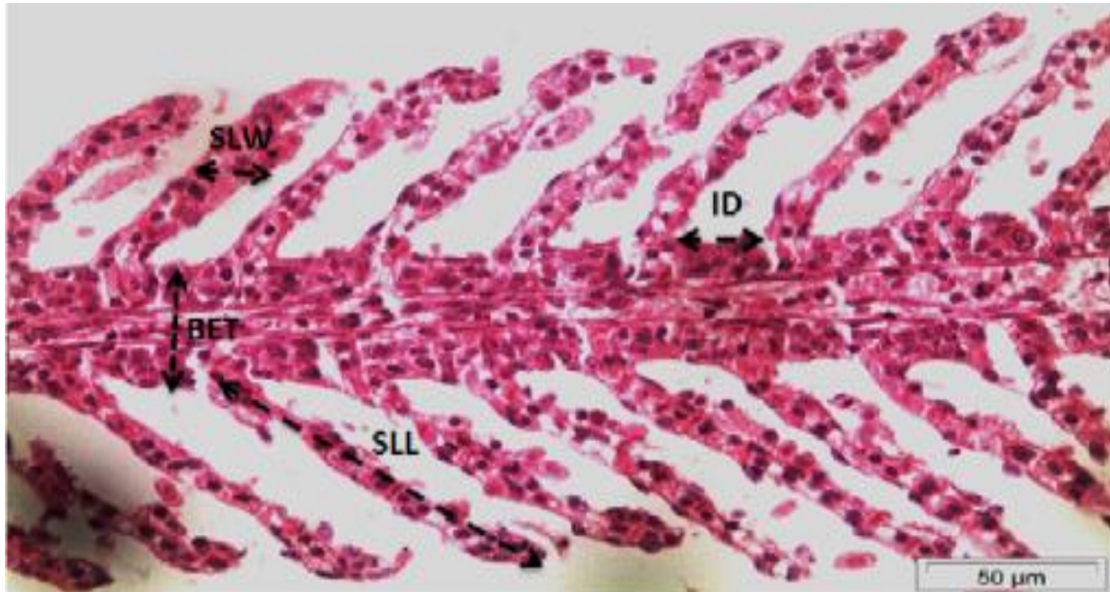


Figure 3: Rainbow Trout Gill from Control concentration 1; Hematoxylin-Gill; x40 magnification) SLL (secondary lamellar length); SLW (secondary lamellar width); ID (interlamellar distance) and BET (basal epithelial thickness)

2.5. Statistical analysis

After testing for normality and homogeneity of variances, data were compared by one-way analysis of variance, followed (if needed) by a Dunnett multi-comparison test to discriminate differences of treatments in relation to the control treatments. The adopted level of significance (α) was 0.05. Data are presented as mean and respective

standard error. Statistical analyses were performed with the software SPSS IBM Statistics v.19.0 (Systal Software).

3. RESULTS

3.1. Biomarkers evaluation

3.1.1. Na^+ K^+- ATPase activity

Na^+/K^+-ATPase activity values are presented at the figure 4. There are no significant differences in the activities between experimental groups ($F_{[3, 45]} = 0.157$; $p<0.925$).

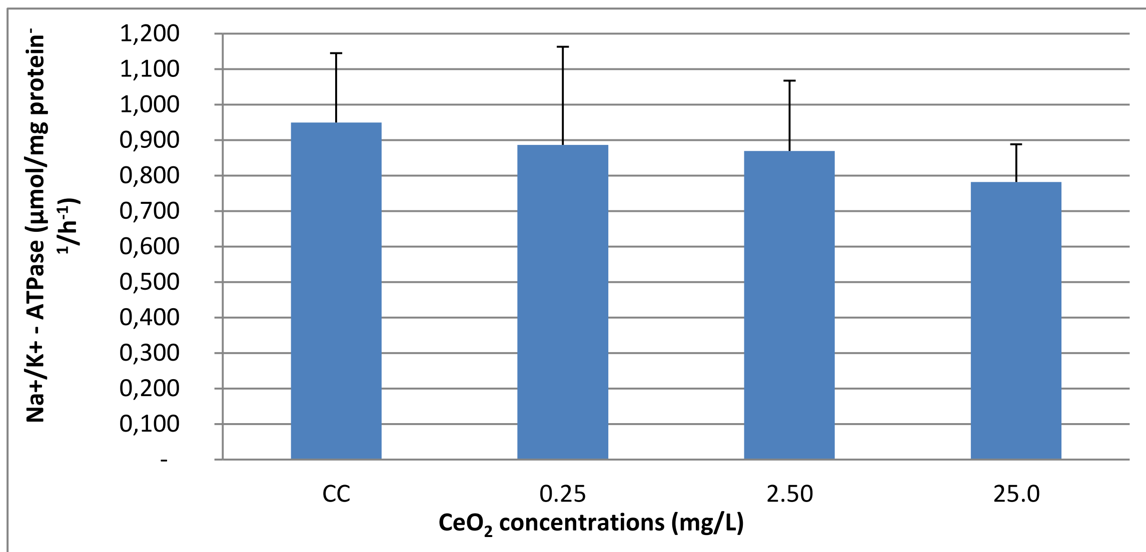


Figure 4: Na+/K+-ATPase activity (µmol/mg protein-1/h-1). CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC)).

3.1.2. Glutathione *S*-transferase activity

The GST activity values are presented at figure 5. There are no significant differences in the GST values between experimental groups and control ($F_{[3, 45]} = 4.064$; $p=0.255$).

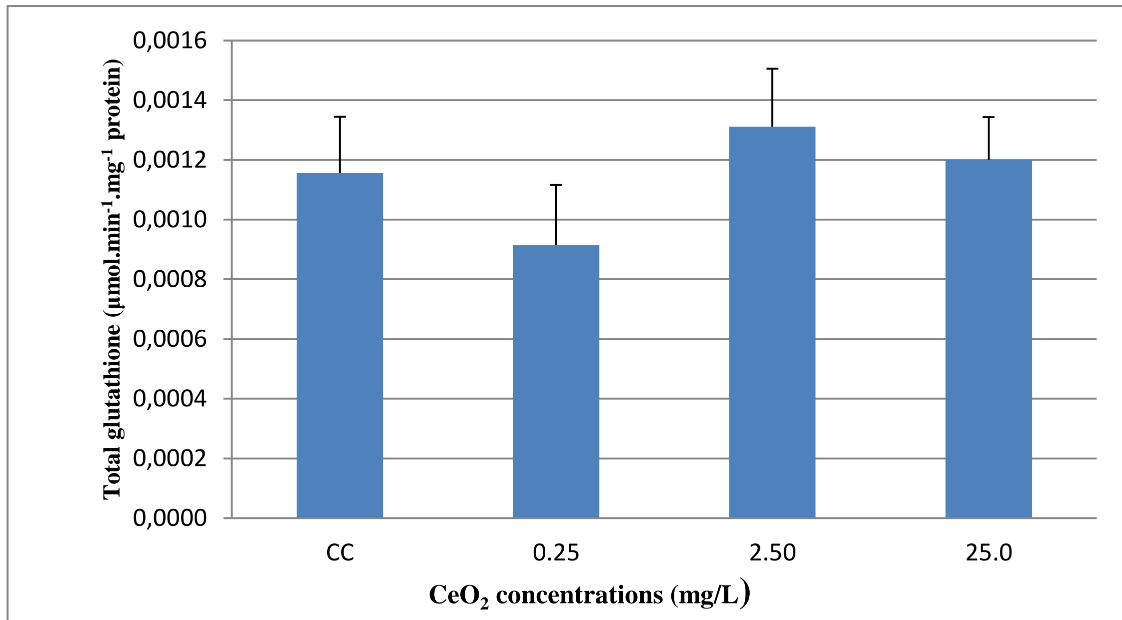


Figure 5: Total glutathione (µmol.min-1.mg-1 protein). CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC)).

3.1.3. Catalase activity

The CAT activity values are shown at figure 6. A significant increase of CAT activity was observed only for the highest concentration tested (25.0 mg/L of CeO_2) ($F_{[3, 45]} = 30.673$; $p<0.001$).

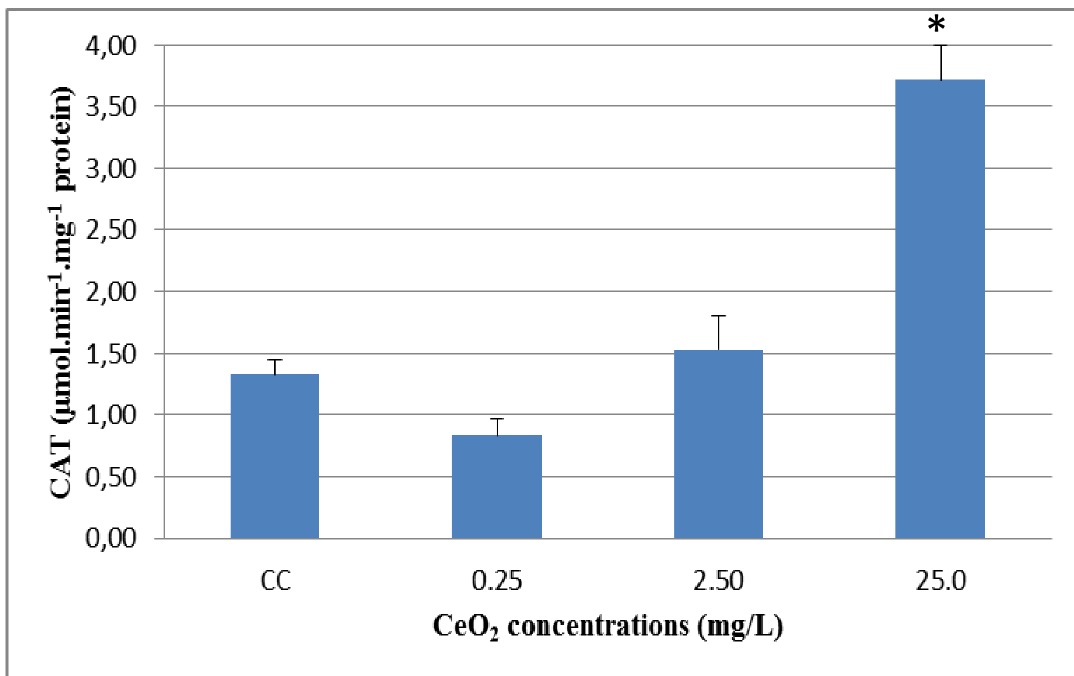


Figure 6: CAT activity (µmol.min-1.mg-1 protein). CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC)).

3.1.4. TBARS quantifications

The TBARS quantification values are presented at figure 7. There are no significant differences in the TBARS values between experimental groups and control ($F_{[3, 56]} = 1.834$; $p<0.151$).

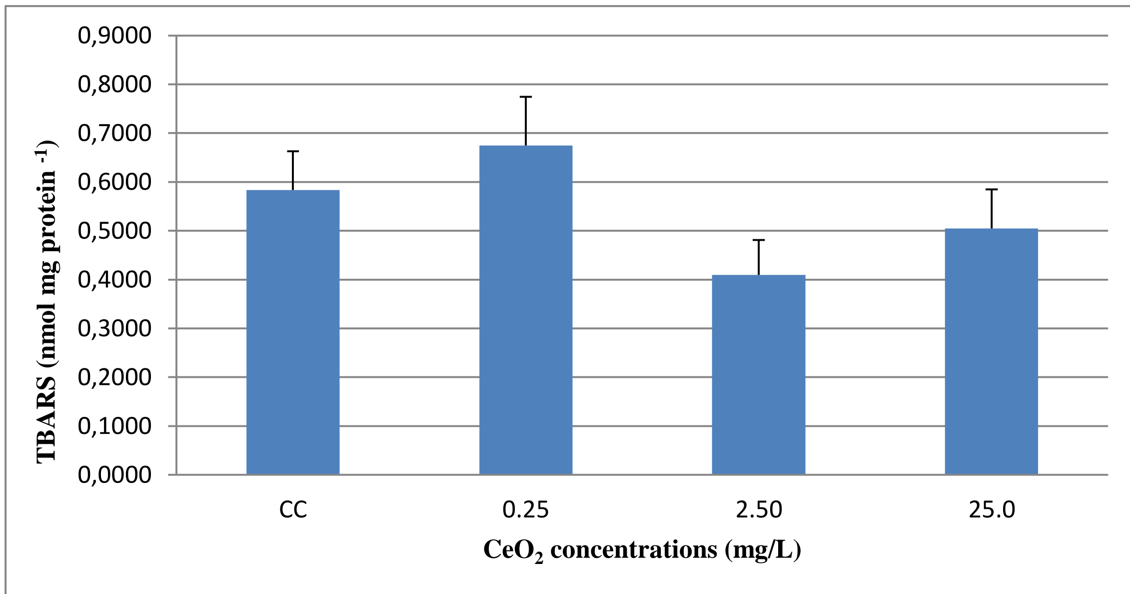


Figure 7: TBARS quantification (nmol mg protein^{-1}). CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC)).

4. Genotoxicity

The GDI values are shown at the figure 8. For all the concentrations tested (0.25; 2.50; 25.00 mg/L of CeO_2 NPs) a significant increase of GDI was observed ($F_{[3, 56]}$ = 29.54; $p<0.001$), making it clear that CeO_2 exposure induce DNA damage.

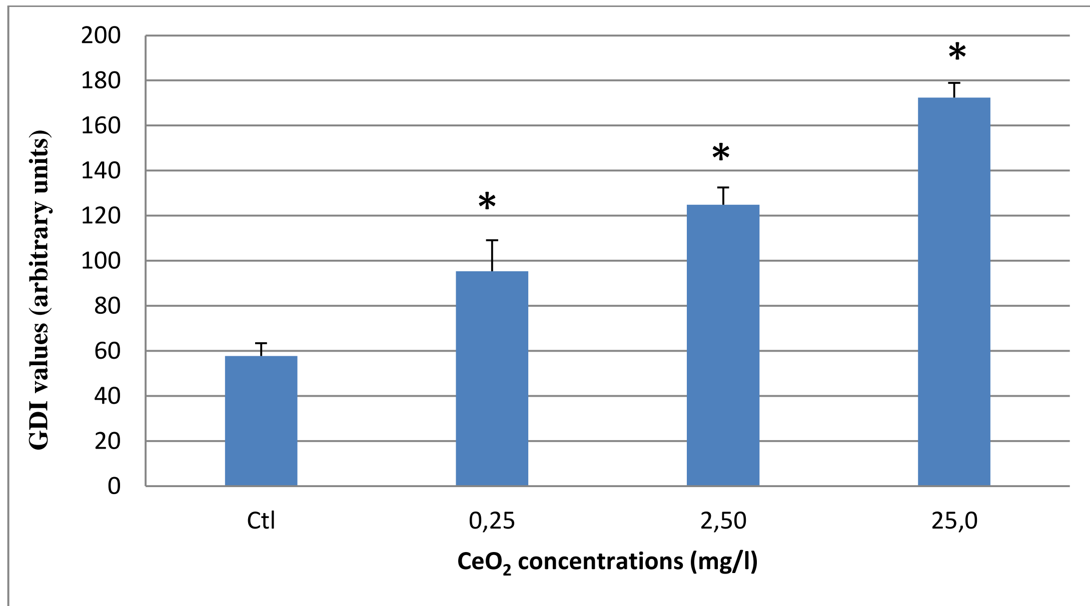


Figure 8: Genetic Damage Index. CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC)).

5. Histological alterations

5.1. Gills Semi-Qualitative Assessment

Histopathological semi-qualitative changes of the *O. mykiss* gills were observed on 0.25; 2.50 and 25.0 mg/L treatment in all studied parameters (Fig. 9). A significant differences of histological alterations were recorded, namely circulatory (for the highest concentration - 25 mg/L CeO_2; $F_{[3, 56]}$ =16.898; $p<0.001$), progressive or proliferative changes (for all concentrations tested; $F_{[3, 56]}$=31.997; $p<0.001$), and Gills Pathological Index (for all concentrations tesed; $F_{[3, 56]}$=35.999; $p<0.001$) (Fig. 9).

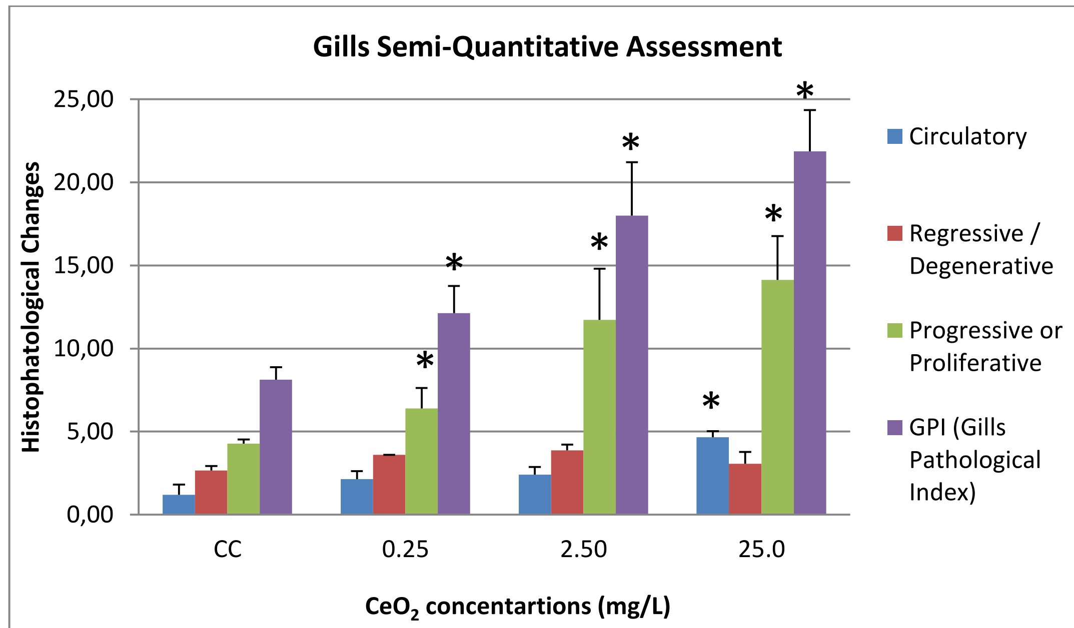


Figure 9: Gills Semi-Qualitative Assessment. CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC)).

5.2. Gills Quantitative Assessment

The values of all the measures acquired are exhibited in the figure 10. There are a significant decrease in BET ($F_{[3, 56]}$ =4.356; p<0.008) values for the concentration of 2.5 mg/L CeO_2. Significant decrease was observed in ID values ($F_{[3, 56]}$ = 8.475; p<0.001), for all concentrations tested. In terms of SLL values a significant decrease was observed in the last two concentrations tested ($F_{[3, 56]}$ =5.369; p<0.003). No significant alterations were recorded for SLW parameter ($F_{[3, 56]}$ =0.499; p<0.684).

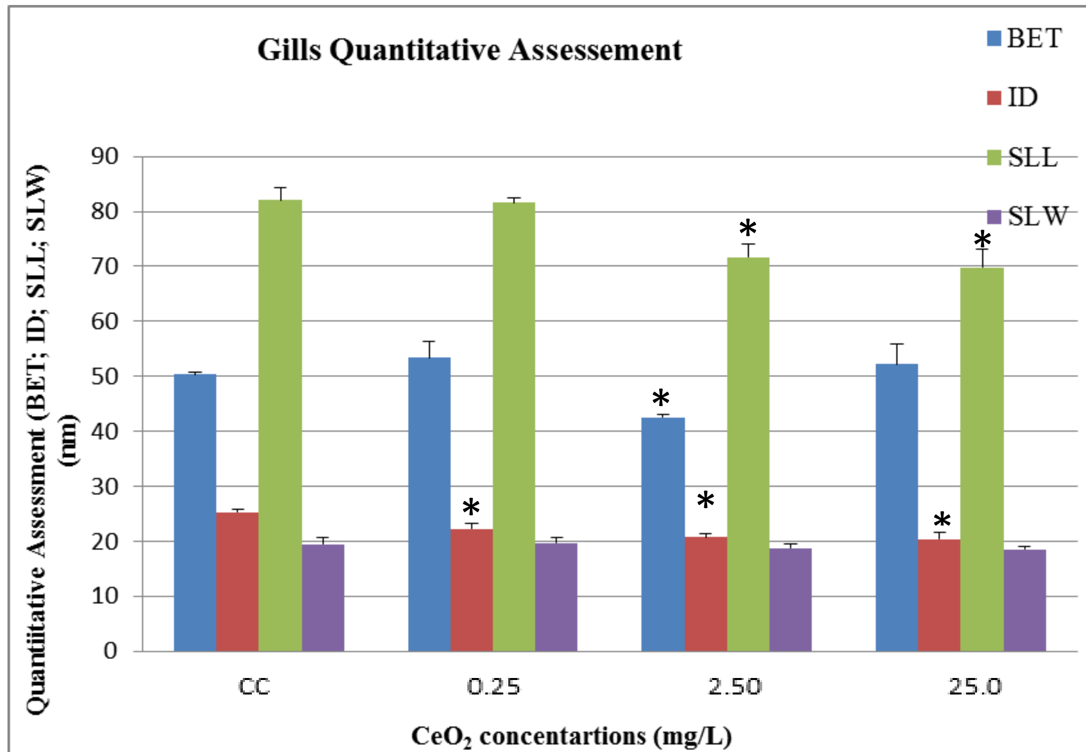


Figure 10: Gills quantative assessment (BET, ID, SLL and SLW). CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC)).

PAGE's measures (%) acquired from the measures of the mean of the BET and SLL. No significant differences in the PAGE values were observed between groups and control treatment ($F_{[3,\ 56]}$ =2.620; p<0.060) (Fig. 11).

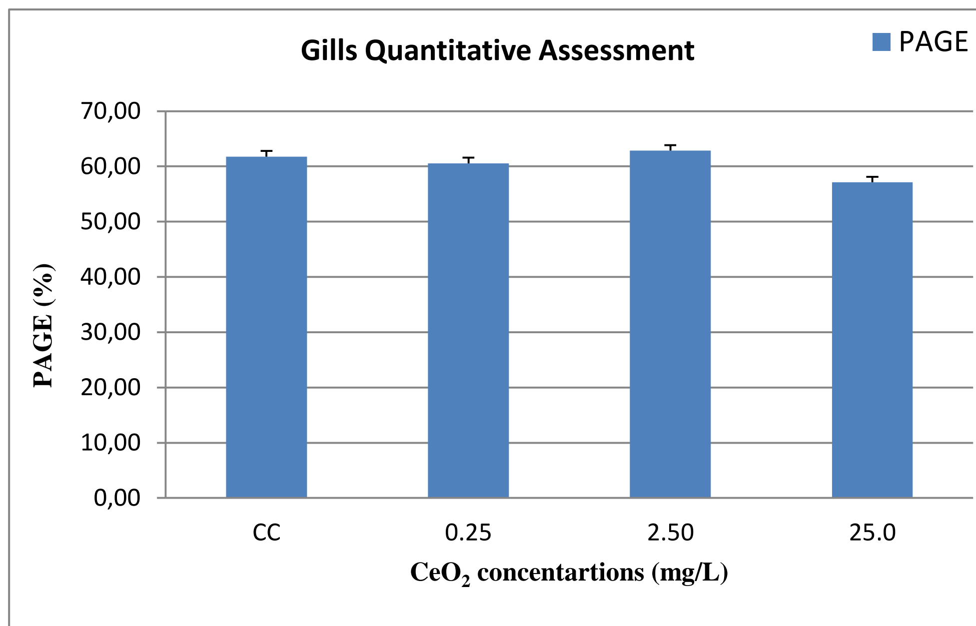


Figure 11: Gills Quantitative Assessment (PAGE %). CC (Control concentration); 0.25 (Low Concentration (LC)), 2.50 (Medium Concentration (MC)), 25.0 (High Concentration (HC)).

At the control group circulatory changes were minimal. The circulatory changes increase at the 25.0 mg/L concentration in every treatment. It was possible to observe capillary dilatation (Fig. 14) and aneurysms (Fig. 12, 13). Intercellular edema (Fig. 15) was found in all treatments but with a lower incidence in the 0.25 and 2.5 mg/L and a higher incidence in the 25 mg/L CeO_2. Regressive changes, as lamellar fusion (Fig. 14) were constant in all treatments however, epithelial lifting (Fig. 17) was observed at the 0.25 and 2.50 mg/L concentrations. Progressive or Proliferative changes were observed in every treatment, but with a higher incidence at the highest concentrations of CeO_2. Epithelial Hyperplasia (Fig. 13, 14) appears in all the fish CeO_2 exposure but in different score values. At the 0.25 mg/L concentration it is possible to observe mucous cells Hyperplasia (Fig. 14); Chloride cells Hyperplasia (Fig. 16) is visible at the concentrations of 2.50 mg/L and 25.0 mg/L.

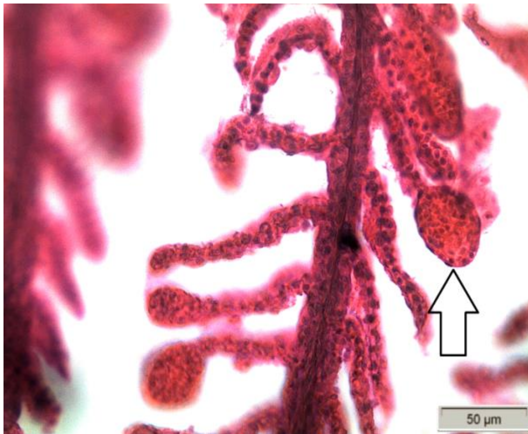


Figure 13: Aneurysm (white arrow) from Low Concentration 2; Hematoxylin-Gill; x40 magnification

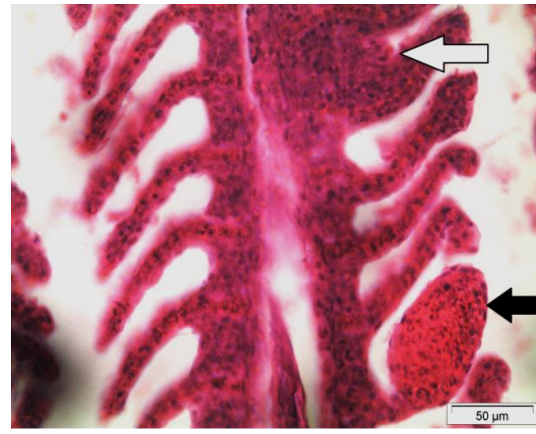


Figure 12: Aneurysm (black arrow) and hyperplasia of the epithelium (white arrow) from High Concentration 1; Hematoxylin-Gil; x40

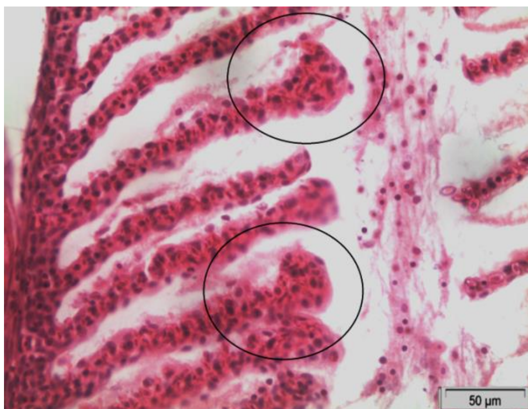


Figure 15: Intercellular Edema (Circle) from High Concentration 2; Hematoxylin-Gill; x40 magnification

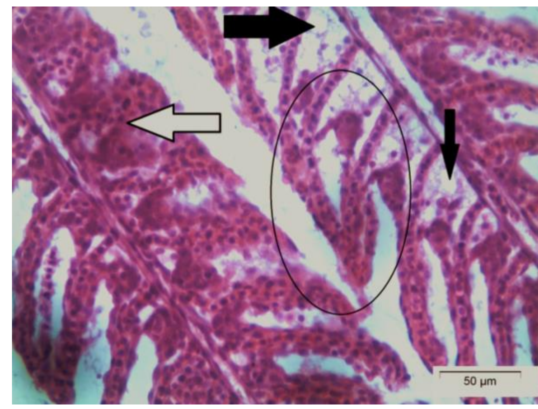


Figure 14: Capillary dilatation and Lamellar fusion (circle), Hyperplasia of the mucosal cells (black arrow) and Hyperplasia of the Epithelium (white arrow from High Concentration 1; Hematoxylin-Gill x40 magnification

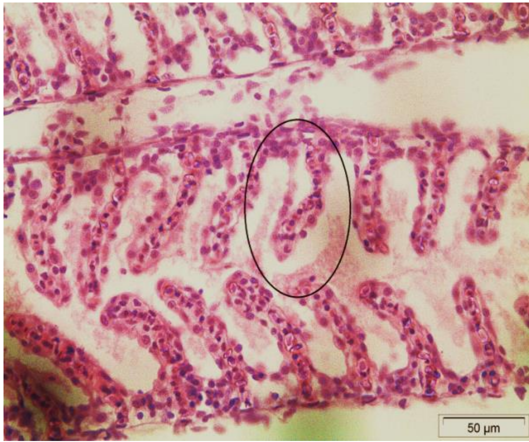


Figure 17: Lifting of the Epithelium (circle) from Low Concentration 3; Hematoxylin-Gill; x40 magnification

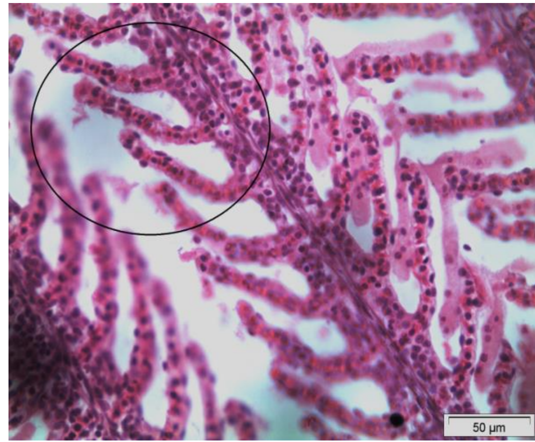


Figure 16: Hyperplasia of the chloride cells (circle) from Medium Concentration 1; Hematoxylin-Gill; x40 magnification

6. DISCUSSION

6.1. Biomarkers evaluation

6.1.1. Na^+/K^+-ATPase activity

In terms of Na^+/K^+-ATPase activity there were no significant differences between experimental groups. Gill Na^+/K^+-ATPase is a membrane-bound enzyme that catalyzes the active Na^+ and K^+ transport in animals, providing a dynamic force in the gill epithelium (Torre *et al.,* 2007). Despite some inhibition of brachial Na^+/K^+-ATPase activity, there were no major disturbances in osmotic and ionic balance. Jun, *et al*., 2013 tested multiple biomarkers in *Carassius auratus* using concentrations of 20, 40, 80, 160, and 320 mg/L of CuO, ZnO and CeO_2 NPs, after 4 days of exposure. In this study it was possible to observe a significant increase of Na^+/K^+-ATPase activity in gills of *C. auratus* at concentration of copper oxide, zinc oxide and cerium dioxide NPs at 80 mg/L. Ramsden, *et al.,* 2009 tested Na^+/K^+-ATPase activities in the gill and intestine and conclude that were unaffected by TiO_2 exposure. Shrimpton and McCormick (1999) tested in vitro gill of juvenile *O. mykiss* and conclude that Na^+/K^+-ATPase activity was significantly affected by cortisol concentration in the incubation medium ($p<0.001$).

6.1.2. Glutathione *S*-transferase

The conjugation of electrophilic compounds (or phase I metabolites) with GSH is catalyzed by the glutathione S-transferase (GST), a multigene superfamily of dimeric, multifunctional, primarily soluble enzymes. Separately from their essential functions in intracellular transport (heme, bilirubin and bile acids) and the biosynthesis of leukotriene's and prostaglandins, an important role for GSTs is obviously defense against oxidative damage and peroxidative products of DNA and lipids. Based on the results obtained it was not possible to conclude that *O. mykiss* gills were significantly affected at the GST levels. Ramsden, *et al.,* 2009 used 10, or 100 mg kg^{-1} TiO_2 NPs diets for 8 weeks and tested total glutathione (GSH) levels in the gill, intestine, liver and brain homogenates with only the gill showing statistically significant changes in GSH.

6.1.3. Catalase activity

Catalase is an intracellular enzyme that decomposition of the H_2O_2 (hydrogen peroxide) and is responsible for the oxidation of the hydrogen donor (methanol, formic acid and phenols) or the peroxide consumption (peroxidic activity) (Habig, *et al.*1974, Jun, *et al*. 2013). CAT is primarily located in the peroxisomes. Sideways with glutathione peroxidase, is accountable for the reduction of H_2O_2 produced from the metabolism of long chain fatty acids in peroximes (Jun, *et al*., 2013). CAT activity exhibits a significant increase after acute exposure at 25 mg/L CeO_2. These results demonstrate that CeO_2 possibly induce an activation of the anti-oxidant response in the gills by altering the levels of CAT. In the work of Jun, *et al*., 2013 using *Carassius auratus* at the concentrations of 20, 40, 80, 160, and 320 mg/L of CuO, ZnO and CeO_2 NPs, after 4 days of exposure, metal oxide NPs did not significantly alter liver CAT activity at the lowest concentrations compared to the controls. Exposures of CuO, CuO and ZnO NPs (≥40 mg/L), and CeO_2 (≥160 mg/L) significantly inhibited CAT activity, and the inhibition rates increased in a concentration-dependent manner.

6.1.4. TBARS quantification

The measurement of lipid peroxidation offers another value to compare the potential oxidative stress damage in the gills caused by CeO_2 (Oost *et al.* 2003). Ramsden, et al., 2009 used 10, or 100 mg kg^{-1} TiO_2 NPs diets for 8 weeks and the *O. mykiss* exposed to TiO_2 NPs generally showed a decrease in TBARS compared to controls at the end of the experiment. Significant differences were seen in the gills and intestine of TiO_2 NP exposed fish at week 8 with maximum decreases of 49% (gill) and 50% (intestine) in the 100 mg kg^{-1} TiO_2 NP treatment compared to the control.

6.2. Genotoxicity evaluation

The comet assay or single cell alkaline gel electrophoresis is frequently used to measure and evaluate the DNA damage. It can be used in various applications such as genotoxicity testing, human biomonitoring and molecular epidemiology (Collins, 2004; Azqueta, 2011; Lorenzo, 2013). The detection of DNA strand-breaks that are produced, either directly by the toxic chemical (or its metabolite) or by the processing of structural

damage are detected in this assay. DNA base composition, oncogene activation, cytogenetic effects also have the potential to be used as biomarkers (Oost *et al.* 2003). Exposure to CeO_2 caused a significant exponential increase of the Genetic Damage Index (GDI) along concentrations showing a concentration-dependent in brachial GDI. It means that CeO_2 caused genotoxic changes i.e., DNA damage in the exposed rainbow trout's. Genetic damage can lead to mutations and diseases that can change the future one species ultimately affecting humans because of the phenomenon of bioaccumulation and biomagnification exposed to this nanoparticle. Lopez-Moreno *et al.* 2010 tested ZnO and CeO_2 NPs on *Glycine max*, they amplified polymorphic DNA assay and applied to detect DNA damage and mutations caused by NPs. Results obtained from the exposure of *Glycine max* to CeO_2 NPs show the appearance of four new bands at 2000 mg/L^{-1} and three new bands at 4000 mg/L^{-1} treatment. In this study was possible to demonstrate genotoxic effects from the exposure of *Glycine max* to CeO_2 NPs. Lee *et al*. 2009 assess genotoxic and ecotoxic effects of CeO_2, silicon dioxide (SiO_2) and TiO_2 NPs test solutions in freshwater crustacean, *D. magna* and the larva, *Chironomus riparius*. CeO_2 may have genotoxic effects on *D. magna* and *C. riparius*, given that the DNA strand breaks increased in both species.

6.3. Histological alterations

Histological changes are likely to appear even in sub-lethal doses, in various tissues and organs (Johnson, et al., 1992). The here-obtained results show that exposure at CeO_2 caused aneurysms, intercellular edema and capillary dilatation, suggesting some degree of vascular wall injury in the braquial capillary bed or interruptions of capillary flow. Regressive changes as lamellar fusion and, epithelial lifting were observed the 0.25 and 2.5 mg/L concentrations. Progressive or proliferative changes were observed in every treatment, but with a higher incidence as the CeO_2 NP concentration increased. Epithelial hyperplasia, mucous cells hyperplasia and chloride cells hyperplasia were visible at all concentrations. The decrease in surface area reduces xenobiotic entrance, nonetheless can compromise organ function.

The evaluation of lesions on the rainbow trout gills are biomarkers of environmental contamination affected by the CeO_2 NPs (Depledge, 1994). Bairuty *et al.* 2013 studied the Histopathological effects of waterborne Cu NP and copper sulphate

($CuSO_4$) on the O. mykiss organs with the concentration of 20 or 100 µg/L^{-1}. This study showed that CuSO4 and Cu-NPs caused gill injuries (e.g., hyperplasia, edema, lamellar fusion, clubbed tips, etc) after 4 days of exposure. Smith et al. 2007 tested the toxicity of carbon nanotubes in O. mykiss for 10 days with the concentration of 0.1, 0.25 or 0.5 mg/L-1. The gills displayed normal anatomy in the freshwater controls; however the exposure gills reveled some increase in the incidence of edema in the secondary lamellae, changes in monocytes morphology, and hyperplasia in the primary lamellae.

7. CONCLUSIONS

CeO_2 NPs is by far one of the most used cerium compounds, with applications in various areas as electronics, automotive industry, solar panels for energy, etc. Moreover, this study provides one of the first overviews of organ integrity and physiological effects of CeO2 NPs in rainbow trout. However, little is known about the possible ecotoxicological effects of CeO_2 NPs, its bioaccumulation in the environment and the potential effects on human health.

The low acute toxicity of dispersed CeO_2 NPs does not mean that there are no toxicological concerns. In fact, this study reports an assortment of important sub-lethal effects in rainbow trout subjected to acute exposition inducing lesions in the gills, biochemical disturbances (CAT increase activity) and genotoxicity. The gills are organs responsible for vital functions such as breathing, excretion and accumulation/biotransformation of xenobiotics, therefore, lesions in this organ can dramatically affect the organism survival.

The evaluation of potential impacts of the nanoparticles on human and environmental health has high priority because of its increasing use. In this study, it was possible to demonstrate that the CeO_2 NP´s is a nanoparticle that leads to histological, biochemical and genotoxic alterations, at ecological relevant concentrations and acute exposure time.

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