Universidade de Aveiro Departamento de Biologia
Ano 2014

Bruno Ricardo da Silva Correia

Evaluation of the genotoxicity effect and antioxidant response of two nanoparticles in *Eisenia andrei*

Avaliação dos efeitos genotóxicos e resposta antioxidante de duas nanoparticulas em *Eisenia andrei*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Ruth Maria de Oliveira Pereira, Professora Auxiliar Convidada do Departamento de Biologia da Universidade do Porto, e coorientação da Doutora Sónia Alexandra Leite Velho Mendo Barroso, Professora Auxiliar c/Agregação do Departamento de Biologia da Universidade de Aveiro.

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Dedico este trabalho aos meus pais, amigos, peixes, namorada e todas a minhocas que matei para o realizar.

o júri

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palavras-chave

minhocas, *Eisenia andrei*, nanopartículas, nanomateriais,genotoxicidade, comet assay, stress oxidativo, enzimas antioxidantes,$TiSiO_4$, SDS/DDAB

resumo

Nos últimos anos tem-se verificado um enorme crescimento da indústria da nanotecnologia. O aumento da produção e descoberta de novos nanomateriais, onde as nanopartículas estão incluídas, leva a um acréscimo do risco da introdução destes no ambiente. Apesar de recentemente se ter verificado um aumento da publicação de estudos relativos aos potenciais efeitos tóxicos destes materiais, estes são manifestamente insuficientes devido à enorme diversidade de nanomateriais. Apesar da elevada importância dos solos, existe uma falta de estudos sobre este compartimento. Como tal, mais estudos sobre os potenciais efeitos nefastos dos nanomateriais no solo são necessários. Para estudos de toxicidade de partículas no solo, as minhocas são um organismo indicado. Estas têm sido usadas durante mais de 30 anos em exposições a contaminantes no solo e são consideradas um organismo essencial para a manutenção deste compartimento.

O nosso trabalho teve como objetivo determinar se diferentes concentrações de dois tipos distintos de nanopartículas, uma inorgânica (titanium silicon oxide -$TiSiO_4$) e outra orgânica (sodiumdodecylsulphate/didodecyldimethylammoniumbromide - SDS/DDAB), são genotóxicas e também se desencadeiam uma resposta antioxidante em organismos terrestres. Para tal, minhocas da espécie *Eisenia andrei* foram expostas durante 30 dias a solos artificiais "*Organisation for Economic Co-operation and Development*" (OECD) contaminados com diferentes concentrações das nanopartículas teste. Após a exposição, coelomócitos foram extraídos das minhocas e os danos no DNA foram quantificados usando o "comet assay". A atividade das enzimas antioxidantes (glutationa S-Transferase, glutationa peroxidase e glutationareductase), bem como produtos da peroxidação lipídica, foram determinados. Os resultados mostraram que ambas as nanopartículas são genotóxicas, em especial o $TiSiO_4$. Tendo em conta a literatura disponível seria esperado que esta genotoxicidade estivesse relacionada com um aumento na produção de espécies reativas de oxigénio, levando a alterações significativas na atividade de enzimas antioxidantes e na peroxidação lipídica, mas tal não se verificou. Foi possível verificar alterações na actividade de algumas enzimas e na peroxidação lipídica nos tratamentos com as NPs, mas estas alterações não foram estatisticamente significativas. Os nossos resultados sugerem que ambas as nanopartículas são capazes de levar a danos no DNA aparentemente não relacionado com o stress oxidativo.

keywords

earthworms, *Eisenia andrei*, nanoparticles, nanomaterials, genotoxicity, comet assay, oxidative stress, antioxidant enzymes, $TiSiO_4$, SDS/DDAB

abstract

In the last few years there has been a growth in the nanotechnology industry. The increase in the discovery and production of new nanomaterials, were the nanoparticles are included, makes their release in the environment more likely. Although in the recent years there has been an increase of published studies related to the toxic effects of these materials, the information available is not enough since a large number of nanomaterials exist. Even though the soils are extremely important for life, there is a lack of toxicity studies available. Taking this in consideration, more studies using the terrestrial compartment are needed. For these studies, earthworms are a recommend species since standard guidelines for toxicity tests in soil using earthworms have been used with success for more than 30 years and this species is essential for the maintenance of properties of this compartment.

The aim of our work was to determine if different concentrations of two distinct types of nanoparticles, one inorganic (titanium silicon oxide- $TiSiO_4$) and other organic (sodium dodecyl sulphate/didodecyldimethylammonium bromide-SDS/DDAB), are genotoxic and also if there is an antioxidant response in terrestrial organisms. For this, earthworms from the species *Eisenia andrei* (weight: from 300 to 600mg) were exposed for 30 days to the"*Organisation for Economic Co-operation and Development*"(OECD) artificial soil contaminated with different concentrations of the tested nanoparticles. After the exposure, coelomocytes were extracted from earthworms and DNA damage was assessed by comet assay. In addition the activity of antioxidant enzymes (e.g. glutathione peroxidase, glutathione reductase and glutathione-S-Transferase) was assessed, as well as lipid peroxidation. The results have shown that both particles were genotoxic, specially the $TiSiO_4$-NPs. Taking in consideration available information about the mechanism by which the nanoparticles can exert their toxicity, it was expected that the genotoxicity would be related with an increase with the production of reactive oxygen species,leading to alterations in the activity of the antioxidant enzymes and the products of lipid peroxidation. Although some alterations could be found in the activity of antioxidant enzymes and in lipid peroxidation, these results are not statistically significant, suggesting that both nanoparticles are capable of causing damage to the DNA, but the mechanism used by these particles might not related with oxidative stress.

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List of abbreviations

ANOVA – analysis of variance

ASTM – american society for testing and materials

CAT – catalase

CDNB – 1-chloro-2,4-dinitrobenzene

DDAB–didocyldimethylammonium bromide

DNA – deoxyribonucleic acid

EDTA – Ethylenediaminetetraacetic acid

FETAX – the frog embryo teratogenesis assay xenopus

GPx – glutathione peroxidase

GRED – glutathione reductase

GST – glutathione s-transferase

MDA – malondialdehyde

NADPH – nicotinamide adenine dinucleotide phosphate

NM(s) – nanomaterial(s)

NMP – normal melting point

NP(s) – nanoparticle(s)

OECD – organization for economic co-operation and development

PBS – phosphate buffered saline

RNA – ribonucleic acid

ROS – reactive oxygen species

SCGE – single cell gel electrophoresis

SDS –sodium dodecyl sulfate

SDS/DDAB – sodium dodecyl sulfate/didocyldimethylammonium bromide

SOD – superoxide dismutase

TBARS – thiobarbituric acid reactive substances

$TiSiO_4$ – titanium silicon oxide nanoparticles

Tris-HCl – trishydroxymethylaminomethane hydrochloride

WHC – water holding capacity

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Chapter 1

General Introduction

1. General Introduction

1.1. Nanomaterials (NMs): Definition and classes

At the end of 1959, the physicist Richard Feynman suggested that a field not well explored by that time should be taken in consideration. The field he was talking about was the manipulation of things at a smaller scale[1]. Thanks to his ideas, some years later, the term Nanotechnology was created[2].

Although 55 years have passed, there is still a debate going on regarding the nomenclature associated with Nanotechnology and Nanoscience[3]. Using the definition from *The Royal Society & The Royal Academy of Engineering* and *The Nanoscale Science, Engineering, and Technology Organization* we can say that Nanoscience is usually associated with the study of materials at the nanoscale (from 0.2 to 100 nm), while the term Nanotechnology is often related to the manipulation and creation of things in that scale[2], [4]. In our work, a distinction between these two terms was not made since they both deal with materials at the nanoscale level. The major concern in these two fields is the production and the discovery of different types of NMs with new and/or improved properties[4]. The Directorate-General for the *Environment from the European Commission* defines a NM as "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50 % or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm - 100 nm.". They also added that in some cases the NMs can have a distribution threshold between 1 – 50 % and a size inferior to 1nm[5].

The United States Environmental Protection Agency groups the NMs in three types according to their source: naturally occurring (e.g. volcanoes and forest fires), produced by human activity unintentionally (e.g. automobile exhaust) or intentionally **(**engineered NMs). This agency has taken a special interest in the engineered NMs, defining four major groups based on their chemical composition and physical structure: Carbon-based, dendrimers, metal-/metal oxide-based, and quantum dots[6].

The carbon-based NMS, as their name suggests, contain mainly or only carbon in their constitution. These NMs have unique conductivity and thermal properties. Dendrimers are branched molecules that repeat themselves around a core internal cavity

were other molecules can be present. NMs based on metal-/metal oxides are composed totally or partially by one or more metals. These NMs have a large range of optical, thermal, magnetic, and conductivity properties. Quantum Dots are semiconductors with special electrical and optical properties[6].

The Royal Society & The Royal Academy of Engineering uses another type of NMs classification according to the dimensions they have at the nanoscale: thin films, layers, and surfaces only have one dimension at the nanoscale and other two dimensions extended; carbon nanotubes and nanowires have two dimensions at the nanoscale and one is extended; nanoparticles (NPs) have three dimensions at the nanoscale[4].

1.2. Nanomaterials: Uses and risks of introduction in the environment

The NM industry has a large potential that keeps growing year after year[7]. In 2012, 18.5 billions of american dollars have been invested in the development of this area and research estimates a revenue of 4.4 trillion of American dollars by 2018[8]. In October 2013, *The Project on emerging Nanotechnologies* listed 1628 consumer products that made use of Nanotechnology (Figure 1). Most of the products listed are related to health and fitness (788 products), home and garden (221 products) and food and beverage (194 products). The most used materials were silver, titanium and carbon[9].

The constant growth on the use of NMs and the applications that they have makes their release into the environment inevitable. Although manufactured NMs are released intentionally in the environment for remediation of soils and water, risk of contamination can also come from the discharge/leakage of the materials during their transport, storage, and production[3], [4]. Since NMs have a large range of applications, it is expected that in the near future these particles can enter the environment by other means (e.g. release by domestic effluent or hospital activities). After their release in the environment, the NMs can possibly interact with the biota (Figure 2)[3]. The environmental matrix (e.g. soil and water) were the NMs are released is extremely important when it comes to the potential toxicity of the materials. Abiotic factors like pH, ionic strength, and water hardness can influence the behavior of the NMs and their access to the organisms (e.g. formation of aggregations or alterations in the NMs properties)[10].

The three main routes that NMs can access the human body include inhalation of the materials by air, direct contact (dermal absorption) and ingestion. The use of sprays with NMs (e.g. sunscreens, hairsprays, and pesticides) can result into their incorporation by inhalation and the exposition of the respiratory tract. The use of textiles, creams, lotions, and other products with NMs can lead to dermal absorption of these materials by the skin. NMs used in food packages and supplements can gain access to the organism during their ingestion[11]. It is important to take in consideration that not all the NMs pose the same risk to the environment and organisms, factors like the toxicity, the time of the exposure and the dose have to be taken into account[4].

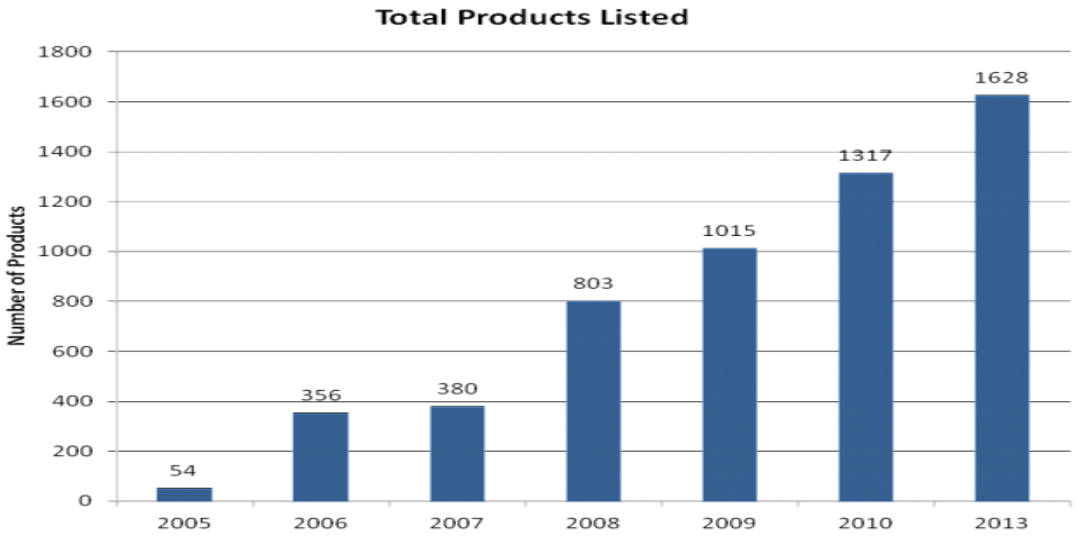

Figure 1: Number of consumer products available that make use of nanotechnology over the last years[9].

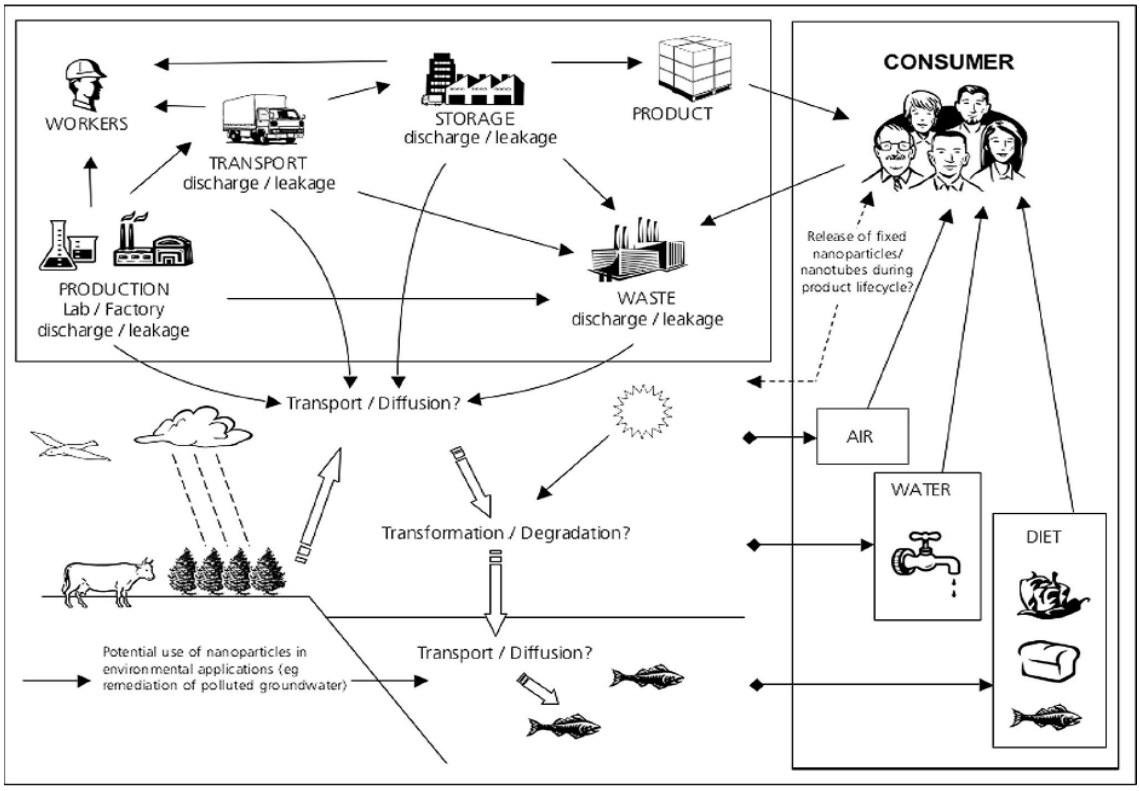

Figure 2: Some of the possible routes by which the nanomaterials might enter the environment[4].

1.3. Nanomaterials: Nanoparticles sources, properties and mechanism of genotoxicity

NPs are a class of NMs that have always existed in the environment and that are mostly produced by natural sources. With the advancement of civilization more particles have been fashioned inadvertently by human activities and with the advent of the Nanotechnology more NPs are intentionally manufactured[12]. Table 1 summarizes the types of NPs arranged in three classes based on their source (natural or anthropogenic). Manufactured NPs show new properties that cannot be found in particles of the same material with larger size[4]. The smaller size not only increases the relative surface area of the particles making them more reactive, but it can also alter the material properties (e.g. electrical conductivity and fluorescence color)[13]. Because of these properties, the NPs have been used in diverse applications like cosmetics, textiles, paints, electronics, and optical devices. In the future, some will be often used to specific drug delivery in the body[4].

Most of the NPs interacting with organisms during their lifetime can be considered benign[14]. One of the biggest problems of the creation and manufacturing of new NPs is that the effects of these particles on human health and other organisms are not well understood. The characteristics that make the NPs so interesting can also be the reasons of their potential toxicity (e.g. the smaller size can make the access to the cells easier and the material more reactive)[15]. As previously stated, not all NPs are toxic, some of them are only toxic when free (e.g. not fixed to an electric circuit) or for a limited period of time (e.g. some NPs can be degraded or transformed in non-toxic forms)[14]. It is also possible to find NPs that are beneficial to the exposed organisms (e.g. cerium and yttrium oxide NPs acted as antioxidants and protected nerve cells from oxidative stress)[16]. To quantify the toxicity and genotoxicity of the NPs, in addition to the size, one should also consider other physical and chemical properties like the shape, crystalline structure, surface area and properties, agglomeration, and solubility[15]. The environment matrix where they are introduced (e.g. air, soil and freshwater) is also important[3].

Depending on the physical and chemical properties of the NPs, they can enter the organism and the cells and be found on different locations (e.g. cytoplasm, mitochondria, lipid vesicles, and near or inside the nucleus)[14]. These locations can probably influence the type of the mechanism by which NPs can exert their genotoxicity[14]. Not much is known about the mechanisms that make the NPs genotoxic. One of the possible tools of the NPs toxicity is the direct interaction with Deoxyribonucleic Acid (DNA) that may cause damage[15]. The NPs can also cause indirect damage to the DNA by interacting with nuclear proteins, interfering on the cell cycle or by directly or indirectly increasing the production of Reactive Oxygen Species (ROS)[15]. The oxidative stress caused by ROS increase, that can lead to damage in lipids, proteins and DNA, is thought to be the main mechanism of NPs genotoxicity[17]. The majority of the ROS are naturally produced in the cell, mostly by the mitochondria and peroxisomes activity[18]. The levels of ROS are maintained at a certain degree by a complex system of antioxidant enzymes like catalase (CAT), superoxide dismutase (SOD) and glutathione peroxidase (GPx), and molecules like glutathione[18]. A certain level of ROS is necessary to maintain the homeostasis but their production can be increased by external sources like ultraviolet light and environmental toxins (e.g. NPs) (Figure 3)[18]. An excess of ROS production, caused by the activity of these external sources may overwhelm the antioxidant system, leading to a condition called

oxidative stress. This stress might result in damage to the genetic material and, in some cases, the death of the cell[19]. This damage can lead to strand breaks, deletions and mutations in the DNA[20].

Table 1: Summary of the natural and anthropogenic sources of nanoparticles[12].

Natural	Anthropogenic	
	Unintentional	Intentional (NPs)
Gas-to-particle conversions	Internal combustion engines	Controlled size and shape, designed for functionality
Forest fires	Power plants	
Volcanoes (hot lava)	Incinerators	Metals, semiconductors, metal oxides, carbon, polymers
Viruses	Jet engines	
Biogenic magnetite: magnetotactic bacteria protoctists, mollusks, arthropods, fish, birds human brain, meteorite (?)	Metal fumes (smelting, welding, etc.)	Nanospheres, -wires, -needles, -tubes, -shells, -rings, -platelets
	Polymer fumes	
	Other fumes	Untreated, coated (nanotechnology applied to many products: cosmetics, medical, fabrics, electronics, optics, displays, etc.)
Ferritin (12.5 nm)	Heated surfaces	
Microparticles (< 100 nm; activated cells)	Frying, broiling, grilling	
	Electric motors	

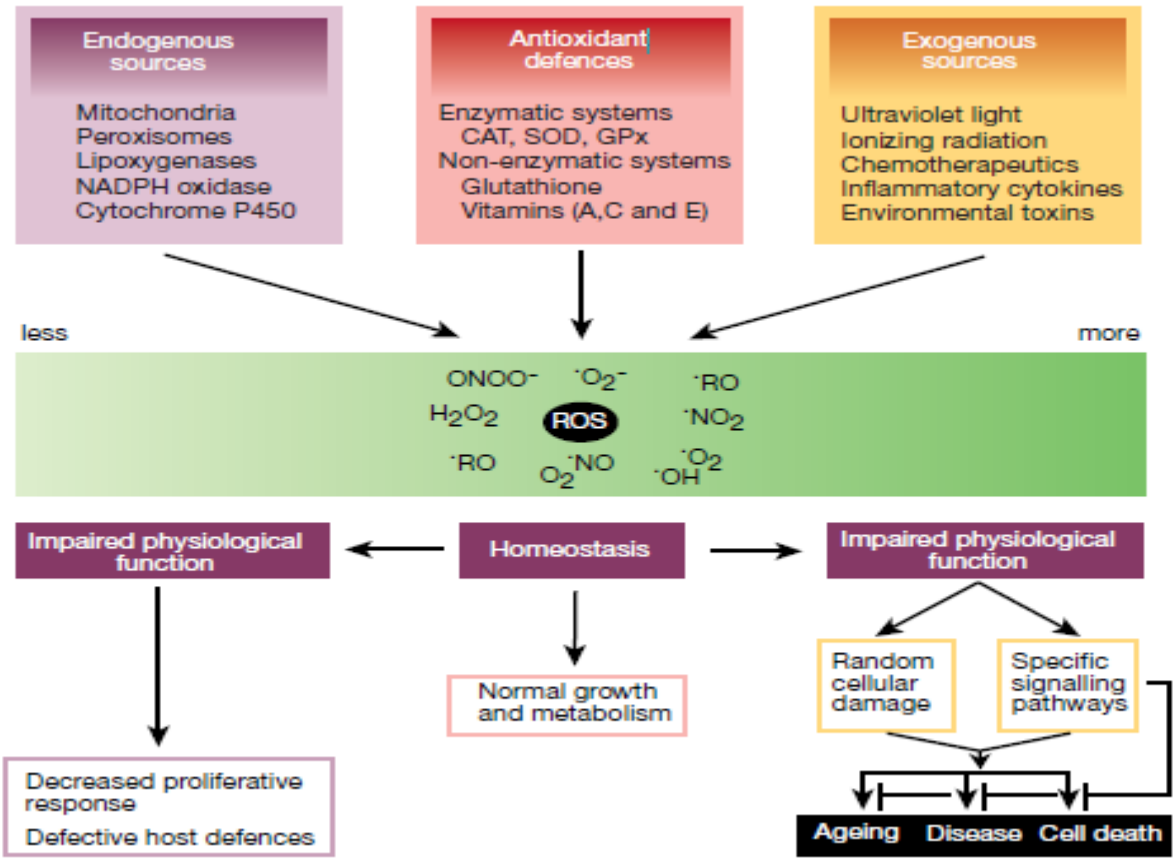

Figure 3: Possible sources of reactive oxygen species and the antioxidant defenses involved in maintaining stable levels of these species. Effects of the alterations on the homeostasis are also shown[18].

1.4. Test organism and genotoxicity and oxidative stress biomarkers

With the growth of the Nanotechnology industry, it is only a matter of time before NPs are introduced in the soil. Not much is known about the potential effects of these particles in the terrestrial ecosystems[3]. Soils are extremely important for life because they support plants growth and habitats for a wide variety of organisms and are also essential for the human economy[21].

To test the ecotoxic effects of NPs in soil organisms, an indicator test species is necessary. Earthworms are extremely important for terrestrial ecosystems, being known as the "engineers of soil". They are essential for the normal operation of this compartment, carrying out crucial functions such as maintaining the soil permeability and aeration, degradation of organic matter, and mixture of soil minerals[22]. Earthworms of the species

Eisenia andrei (Bouché, 1972) are recommended as test species in soil. Standard guidelines have been developed and used for more than 30 years to test possible toxic effects of contaminants in soils[23]. These earthworms are ubiquitous, having a worldwide distribution. They are tolerant to a good range of temperatures and moistures and are easy to handle. They can be fed with animal manure or oatmeal. Their life expectancy can go up to five years, but they usually live two years or less depending on the soil conditions. Their life cycle is short, being around 45 – 51 days under optimal conditions[24].The earthworms *Eisenia andrei/fetida* have also been used before in ecotoxicity tests, using NPs like TiO_2, ZnO, Cu, Ag, Au, Al_2O_3,Ni, SiO_2, ZnO_2, and CeO_2[17], [25]–[29]. Most of these studies were based on standard toxicity tests using mortality and reproduction rates as indicators of toxicity. Even though these indicators are useful, the information given by them is somewhat limited[30]. NPs can have a negative result on these standard toxicity tests, but a closer look at the genetic material may reveal alterations. This may lead, on the long term, to an impairment of the organism fitness and alterations in the ecosystem[17]. Since some NPs are potentially genotoxic, using genotoxicity biomarkers like DNA damage and oxidative stress biomarkers, like alterations in the activity of enzymes involved in the antioxidant response, is recommended[17], [30].

The single cell gel electrophoresis, known as the comet assay, is one of the most used tests for genotoxicity[15]. This method allows the measure of DNA damage and recruited many followers thanks to its "simplicity, sensitivity, versatility, speed, and economy"[31].

Alterations in the production of ROS and the antioxidant system can result in a condition called oxidative stress that can randomly damage molecules in the cell like DNA, proteins and lipids[18]. Since this condition leads to modifications in lipid peroxidation and the antioxidant system, these two markers can be use as biomarkers of oxidative stress[32].

1.5. Test nanoparticles

1.5.1. Titanium silicon oxide nanoparticles ($TiSiO_4$-NPs)

The thermal and electrical properties of these inorganic NPs have aroused the interest of the Nanotechnology industry. These particles are being used in a diverse range of products, therefore there is an increase risk of environment introduction in the future[3].

To our knowledge, only four studies are available about the ecotoxicology of these NPs.

The studies of Pereira et al.[33]and Lopes et al.[34] used the bacterium *Vibrio ficheri* (Microtox assay) to test the toxicity and two strains of *Salmonella typhimurium* (Ames assay) to test the genotoxicity of $TiSiO_4$-NPs. The first study was done with soil spiked with a suspension of $TiSiO_4$-NPs, with a desired concentration of 5 grams of NPs per kilogram of soil. Soil matrix and soil elutriates were sampled and tested with Microtox and Ames assays (only used on soil elutriates) after 2 hours and after 30 days. This study revealed that $TiSiO_4$-NPs are not toxic after 2 hours or 30 days but they can be genotoxic especially after 30 days of exposure[33]. In the second study, $TiSiO_4$-NPs were suspended in two different types of aqueous media with different properties - Milli Q water and American Society for Testing and Materials (ASTM) water (ASTM water was not used in the Ames assay). Five grams of $TiSiO_4$ were added per liter of medium. The assays revealed that the $TiSiO_4$-NPs are only toxic in Milli Q water and that they are not genotoxic[34].

In another work, done by Nogueira et al.[35], soils were spiked and incubated for 30 days with $TiSiO_4$-NPs in an aqueous media (5 g of $TiSiO_4$-NPs per kg of soil). The aim of the study consisted in assessing the alterations of the structure of the soil bacterial community. The study revealed that the bacterial community did not suffer significant changes from the exposition to $TiSiO_4$-NPs[35]. Salvaterra et al.[36] exposed tadpoles of *Pelophylax perezi* to five different concentrations (8.2, 10.2, 12.8, 16, and 20 mg/L) of $TiSiO_4$-NPs in The Frog Embryo Teratogenesis Assay Xenopus (FETAX) medium during 96 hours. In this study, the mortality and other biochemical markers were analyzed and the results showed that the mortality of the tadpoles did not suffer significant changes. However on the biochemical level, some alterations could be found in the catalase and

lactate dehydrogenase activity and lactate and alanine contents, suggesting that $TiSiO_4$-NPs can lead to oxidative stress[36].

In all the works reported, the $TiSiO_4$-NPs showed a tendency to form large aggregates in aqueous suspensions.

1.5.2. Sodium dodecyl sulfate/Didocyldimethylammonium bromide nanoparticles (SDS/DDAB-NPs)

Vesicles of SDS/DDAB are not yet commercialized but, given their potential applications in medicine and cosmetics, it is only a matter of time before they are used and introduced into the environment[34].

To our knowledge, only four studies are available about the ecotoxicology of these NPs.

Pereira et al.[33] and Lopes et al.[34] used the Microtox and the Ames assay to test respectively the toxicity and genotoxicity of SDS/DDAB-NPs. The first assay makes use of the bacteria *Vibrio ficheri* and the second one uses two strains of the bacteria *Salmonella typhimurium* (TA98 and TA100)[33], [34] Pereira et al.[33] spiked soils with aqueous suspensions of SDS/DDAB-NPs and found that soil and soil elutriates were very toxic after 2 hours and after 30 days. Using soil elutriates, they also found that SDS/DDAB-NPs are genotoxic to the strain TA98 of *Salmonella typhimurium* after 30 days[33]. Lopes et al.[34] used aqueous suspensions of SDS/DDAB-NPs. For the Microtox assay they used two different mediums for aqueous suspensions (Milli Q water and ASTM water) and for the Ames assay they only used Milli Q water. Toxicity was found using both waters as mediums and genotoxicity was found on the strain TA98 of *Salmonella typhimurium*[34].

Nogueira et al.[35] spiked the soil with SDS/DDAB-NPs suspensions and analyzed it after 30 days to see if alterations on soil bacterial community could be found. Their study revealed that SDS/DDAB-NPs can lead to alterations in the community composition.

Galindo et al.[37] used on their work four types of white-rot fungi - *Trametes versicolor*, *Lentinus sajor caju*, *Pleurotus ostreatus* and *Phanerochaete chrysosporium.* Five concentrations SDS/DDAB-NPs were suspended in an aqueous media and disposed evenly in agar. The growth of the fungi was measured every day to test the toxicity of the NPs

suspension. All the tested concentrations significantly affected the growth of the four types of white-rot fungi[37].

All the previous works, except for Galindo et al.[37] reported a high stability of SDS/DDAB-NPs meaning that large aggregates are not expected and were not found[33]–[35].

2. Purpose of the study

The aim of our work was to find if soils contaminated with $TiSiO_4$ and SDS/DDBA NPs are genotoxic to the earthworms from the species *Eisenia andrei* and also if this toxicity is related to an antioxidant response. For this, earthworms were maintained for 30 days in soil contaminated with different concentrations of these two NPs. After the exposure, genotoxicity biomarkers like DNA damage and oxidative stress biomarkers like alterations in the activity in enzymes related to antioxidant response and products of lipid peroxidation were assessed.

From the information available in the literature, it is expected that possible DNA damage caused by these two NPs will be associated with alterations in the activity of enzymes related to antioxidant response and products of lipid peroxidation.

The present thesis is organized in four chapters:

Chapter 1 – General introduction to nanomaterials, nanoparticles, test species, tested nanoparticles, and purpose of the study.

Chapter 2 – Evaluation of the genotoxicity effect and antioxidant response to titanium silicon oxide in *Eisenia andrei.*

Chapter 3 – Evaluation of the genotoxicity effect and antioxidant response to vesicles composed of sodium dodecyl sulphate/didodecyl dimethylammonium bromide in *Eisenia andrei.*

Chapter 4 – General discussion and conclusion.

3. References

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Chapter 2

Evaluation of the genotoxicity effect and antioxidant response to titanium silicon oxide in *Eisenia andrei*

Evaluation of the genotoxicity effect and antioxidant response to titanium silicon oxide in *Eisenia andrei*

Abstract

In the last few years there has been a fast growth in the nanotechnology related products. Since some of these materials are relatively new, not much information is available about their potential harmful effects to the environment, and when it comes to titanium silicon oxide nanoparticles ($TiSiO_4$-NPs), even less studies are available. With this work we sought to determine whether different concentrations of$TiSiO_4$-NPs are genotoxic and also if there is an antioxidant response in terrestrial organisms. *Eisenia andrei* was the selected species, since they are essential for the maintenance of soil properties and also because they are recognized as a model species in soil toxicity and have been used for more than 30 years in toxicity tests. For this purpose, earthworms (weight: 300 - 600mg) were exposed for 30 days to the OECD artificial soil contaminated with different concentrations of $TiSiO_4$-NPs. After the exposure, coelomocytes were extracted from earthworms and DNA damage was assessed by comet assay. In addition the activity of antioxidant enzymes (e.g. glutathione peroxidase, glutathione reductase and glutathione-S-Transferase) was assessed, as well as lipid peroxidation. Statistical analysis revealed that organisms exposed to the highest concentrations of $TiSiO_4$ ($\geq$ 444.4 mg of NPs per kg of soil) have damage in the DNA when compared to the control. Although some tendencies could be observed, no significant alterations on the antioxidant enzymes or lipid peroxidation were found. Our results suggest that the $TiSiO_4$-NPs are able do damage the DNA. With our tested concentrations and time of exposure no alterations were found on the oxidative stress biomarkers suggesting that the DNA damage found might not be related with oxidative stress.

Keywords: Earthworms, comet assay, DNA damage, $TiSiO_4$, antioxidant enzymes, oxidative stress, nanoparticles

1. Introduction

Titanium silicon oxide ($TiSiO_4$) NPs can be used in different areas of the industry. For example, these NPs can be applied as a catalyst agent in pyrolysis[1], [2], in the production of more efficient thermosyphons[3], antireflective coatings, notch filters, optical devices, electric components (semiconductors and capacitors)[4], [5], composite membranes for fuel cells[6], [7] and also as pharmaceutical and cosmetic excipients[8].

The nanotechnology industry has grown exponentially in the past years[9]. A great number of new NMs have been created and more will probably be discovered and used in the future. This growth will lead to an increase risk of introduction of the NMs in the environment that can interact with the biota[10].

Although in the last few years there has been a great increase in the number of published studies about the toxicity of the NPs[11], only few works reported the potential toxic effects of TiSiO4-NPs. In addition, the results presented by the authors are not consensual[12]–[15]. Table 1 has a brief description of what is known about the toxicity and genotoxicity of these particles.

In the case of $TiSiO_4$-NPs, we have the same number of studies published about the impact in the soil and aquatic compartment, but, when talking about studies of other NMs, there is a higher tendency to find studies about the aquatic compartment when compared with the soil compartment[12]. Having this in consideration, it is important to obtain more information about the effects of NPs and $TiSiO_4$-NPs in special on the soil compartment.

In the present study, earthworms from the species *Eisenia andrei* were exposed to soils contaminated with different concentrations of $TiSiO_4$-NPs for 30 days. There are standard guidelines to test toxicity in earthworms developed by environmental organizations for more than 30 years[16] and earthworms are recognized has one of the most important members of the terrestrial ecosystems[17]. Earthworms from the species *Eisenia andrei* have a ubiquitous worldwide distribution, are tolerant to a large range of temperatures and moistures, they are easy to handle, and have a short life cycle making them a recommended test species for soil ecotoxicity tests[18].

NPs can cause direct damage to DNA or can directly or indirectly increase the production of ROS, which change proteins, lipids and DNA[19]. Having this in consideration, we used genotoxicity biomarkers like DNA damage. The damage was

measured using an alkaline version of the comet assay. Since this is assay is “simple, sensitive, versatile, fast, and cheap” it has become the most used genotoxicity tests for NPs[19], [20] and was used in our work. Oxidative stress biomarkers like alterations in the activity of enzymes as Glutathione Reductase (GRED), Glutathione S-Transferase (GST) and Glutathione Peroxidase (GPx) implied in the antioxidant response, and the determination of Thiobarbituric Acid Reactive Substance (TBARS) that results from an increase of lipid peroxidation during stress conditions[21] were also used in our work with aid of spectrophotometry. The objective was to see if DNA damage could be found and if it is related with alterations in the oxidative stress biomarkers.

Table 1: Summary of the information available about the toxicity of $TiSiO_4$ nanoparticles indicating the used organism(s), exposure medium, exposure duration, concentration of $TiSiO_4$ in medium, and results.

Reference	Organism(s)	Exposure medium	Exposure duration	Concentration of $TiSiO_4$ in medium	Results
[13]	*Vibrio ficheri* *Salmonella typhimurium*(Strain TA98 and Strain TA100)	Soil contaminated with aqueous suspension of $TiSiO_4$	2 hours and 30 days	5g/kg	Microtox assay (*Vibrio ficheri*) revealed no toxicity after the 2 hours and 30 days exposition. The Ames (*Salmonella typhimurium)* assay revelead genotoxicity after 30 days in both strains.
[14]	*Vibrio ficheri* *Salmonella typhimurium*(Strain TA98 and Strain TA100)	$TiSiO_4$ suspended in two aqueous media	N/A	5g/l	Microtox assay (*Vibrio ficheri*) revealed that suspensions in Milli Q water are toxic. The Ames (*Salmonella typhimurium)* assay revealed no genotoxicity.
[12]	Soil microbial community	Soil contaminated with aqueous suspension of $TiSiO_4$	30 days	5g/kg	No alterations in the structural diversity of the soil microbial community.
[15]	*Pelophylax perezi*	$TiSiO_4$ suspended in aqueous media (FETAX solution)	96 hours	8.2; 10.2; 12.8; 16 and 20 mg/L	Reduction in lactate and alanine concentrations. Increase in activity of catalase and LDH activity for some concentrations.

2. Material and Methods

2.1. Test soil

The standard artificial *Organization for Economic Co-operation and Development (*OECD) soil was used in this work[16]. This soil has approximately the following constitution: 75% industrial sand, 20% kaolin and 5% of sphagnum peat (5 mm sieved). The pH of the soil was adjusted to 6.0±0.5 using calcium carbonate. To assess the Water Holding Capacity (WHC), samples of the soil were inserted in flasks with the bottom removed and replaced with filter paper. These flasks were immersed for two to three hours in water. After the immersion, the weight of the soil was assessed. After that the soil was dried for 24 hours at 105ºC and the weight was measured again. To determine the WHC the difference between the two weights was used[22].

2.2. Test organism

Earthworms of the species *Eisenia andrei* were used on this study. The organisms were obtained from laboratorial cultures under controlled conditions: temperature of 21ºC and photoperiod of 16 hours of light and 8 hours of dark. Once a week, the earthworms in culture were feed horse manure or oatmeal. From the cultures, 240 earthworms with a body mass between 300 and 600 mg were washed with deionized water and let to acclimatize in containers containing OECD soil for 24 hours.

2.3. Tested nanomaterial

In this work, the $TiSiO_4$-NPs were obtained in a powder state. The NPs were supplied by Sigma Aldrich and had the following specifications: particles size less than 50 nm and 99.8% of purity.

2.4. Test procedure

In this study, five concentrations of $TiSiO_4$ were tested: 197.5, 296.3, 444.4, 666.7, and 1000.0 mg/kg. We named the concentrations respectively as 1, 2, 3, 4, and 5. A control with only OCDE soil was also used. For each concentration and control, four replicas were used. For the exposure, 24 buckets with pierced lids with an approximate volume of 0.6 L were used. In each bucket, 500 g of OECD soil were added followed by the addition of $TiSiO_4$ powder to match the desired concentration. After this, the soil was homogeneously mixed. Deionized water matching 40% of the WHC was added and we mixed the soil again. The 240 earthworms previously acclimatized were cleaned with deionized water. Ten earthworms with a mass between 300 and 600 mg were added to each bucket. Five earthworms, randomly collected in each replicate, were used for the DNA damage quantification and the other ones for the biochemical analysis. In Figure 1 we have a quick illustration of this procedure. The 24 buckets with the 240 earthworms were incubated for 30 days in controlled conditions (temperature of 21ºC and a photoperiod of 16 hours of light and 8 hours of dark). Once a week, oatmeal was used to feed the earthworms.

After the 30 days exposure, the earthworms were removed from the soil and washed with deionized water. The earthworms from each exposure bucket were transferred and depurated for 24 hours on smaller recipients (making a total of 24 smaller recipients) with filter paper in the bottom embedded with deionized water (Figure 2). Earthworms used for the DNA damage quantification were used on the same day and the other ones were frozen in nitrogen and stored at -80 ºC.

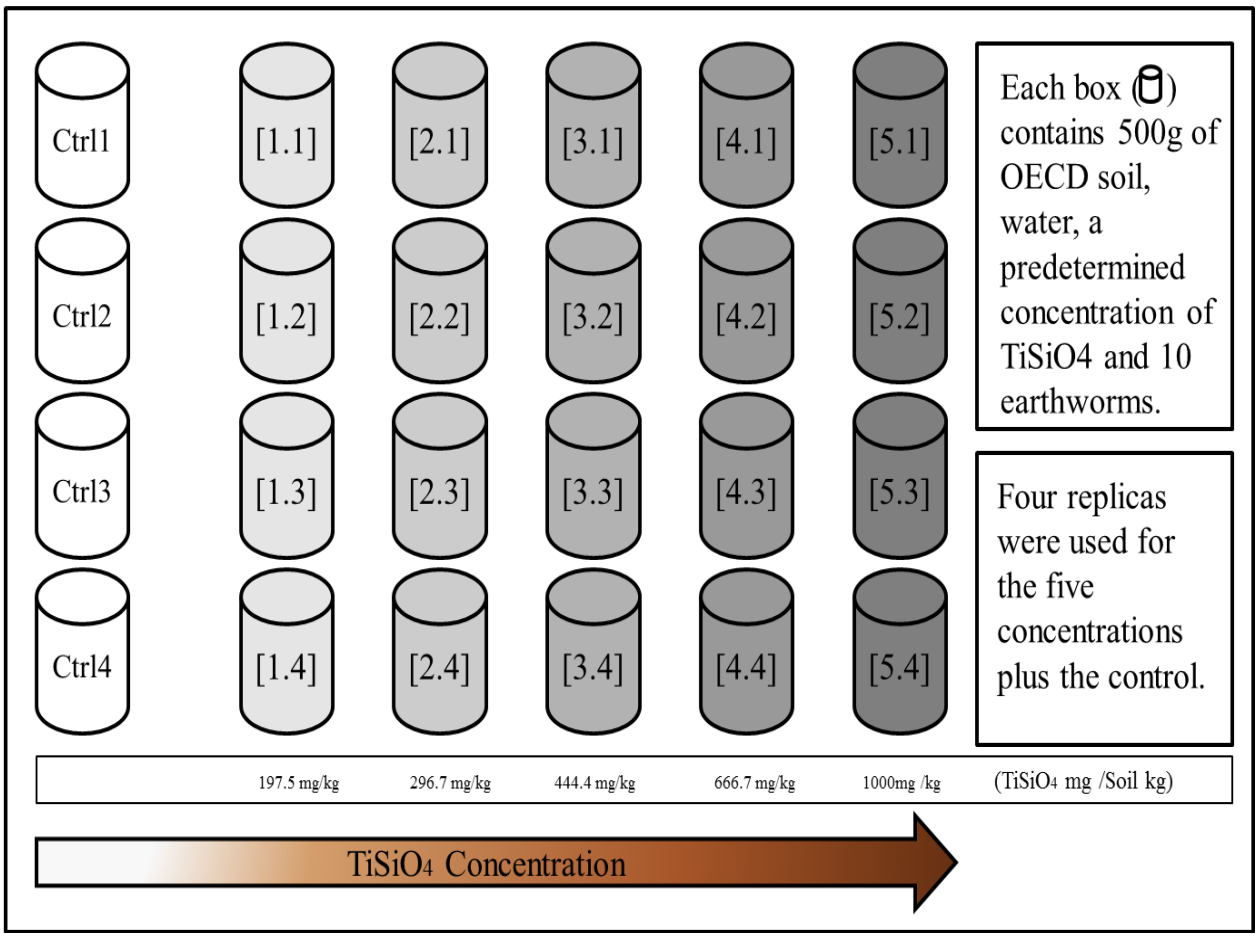

Figure 1: Schematic representation of the test procedure used in the exposition of the earthworms to $TiSiO_4$ nanoparticles.

Figure 2: Flask with earthworms inside being depurated.

2.5. DNA damage quantification

For the DNA damage quantification we used the Single-Cell Gel Electrophoresis (SCGE), also known as comet assay. This method has become a standard when it comes to the assessment of DNA damage[20] and it is the most used technique in genotoxicity studies related to NPs[19]. Coelomocytes from earthworms have been used before with the comet assay for the detection of genotoxic compounds in soils, thus they were also used in this work[23].

2.5.1. Non-invasive coelomocytes extrusion

The coelomocytes were obtained from the earthworms using an adapted protocol from Reinecke et al.[24] and Eyambe et al.[25]. For the extrusion, a solution containing Phosphate Buffered Saline (PBS), ethanol and Ethylenediaminetetraacetic acid **(**EDTA) was distributed between 24 Eppendorf tubes (one for each exposition bucket). In each Eppendorf the earthworms were introduced and removed separately on periods of one minute. Ethanol was used to aid the extrusion of the cells. The Eppendorfs containing the cells and the extrusion fluid were centrifuged and the supernatant was removed to obtain a cell pellet. This pellet was placed in new Eppendorfs and stored in ice.

2.5.2. Comet assay

The alkaline version of the comet assay followed the protocol used by Lourenço et al.[26]. All the steps were performed under yellow light to reduce the possible extra UV-induced damage.

2.5.2.1. Slide preparation

Microscopes slides (one for each sample) were identified and covered with 1% Normal Melting Point agarose (NMP) and let to dry overnight. From each Eppendorf containing the cell suspensions (24 Eppendorfs), 10 μL (containing cells) were mixed with 0.5% low melting point agarose and added to the top of each microscope slide containing NMP

agarose. Ice was used to aid the solidification of the microscope slides. After the solidification the slides were immersed for 24 hours in a lysing solution (2.5M NaCl, 100mM EDTA, 10mM Tris hydroxymethyl aminomethane hydrochloride (Tris-HCl), triton X-100, DMSO, pH set to 10 using NaOH, and deionized water). The microscope slides were removed from the lysing solution and cleaned with PBS. After that they were immersed in an electrophoresis buffer (0.2 M Na_2EDTA, NaOH and deionized water) for 15 minutes. An electrophoresis was run for 10 minutes. After this the slides were washed with Tris-HCl and submerged in absolute ethanol and left to dry. Each slide was stained with 80 μL of ethidium bromide for DNA visualization on fluorescence microscope.

2.5.2.2. Image analysis

The exposition of the cells to the lysing solution leads to the formation of the nucleoid, a structure composed mainly by Ribonucleic Acid (RNA), DNA and proteins. Damage to the DNA can lead to strand breaks that can be detected using the comet assay. When we run the electrophoresis, the DNA extends toward the anode. If the DNA has little to no damage, the extension to the anode is smaller because the DNA is more compacted. More damage leads to more strand breaks causing a relaxation of the DNA making the DNA extend towards the anode, leading to the formation of the "tail" of the "comet"[20].

The stained microscope slides were observed in an Olympus BX60 Fluorescence microscope. For each microscope slide, 100 cells were randomly selected and a visual score corresponding to DNA damage was attributed to the cells. This score ranged from 0 (no damage) to 4 (extreme damage) arbitrary units. A cell with a 0 would have its entire DNA on the "head" of the comet and no "tail" (0 in Figure 3). On the other hand, if the DNA was mostly in the "tail", we would give that cell a score of 4 arbitrary units (4 in Figure 4). Figure 3 shows an example of the 5 classes used in visual scoring. The possible score of each slide could range from 0 to 400 arbitrary units.

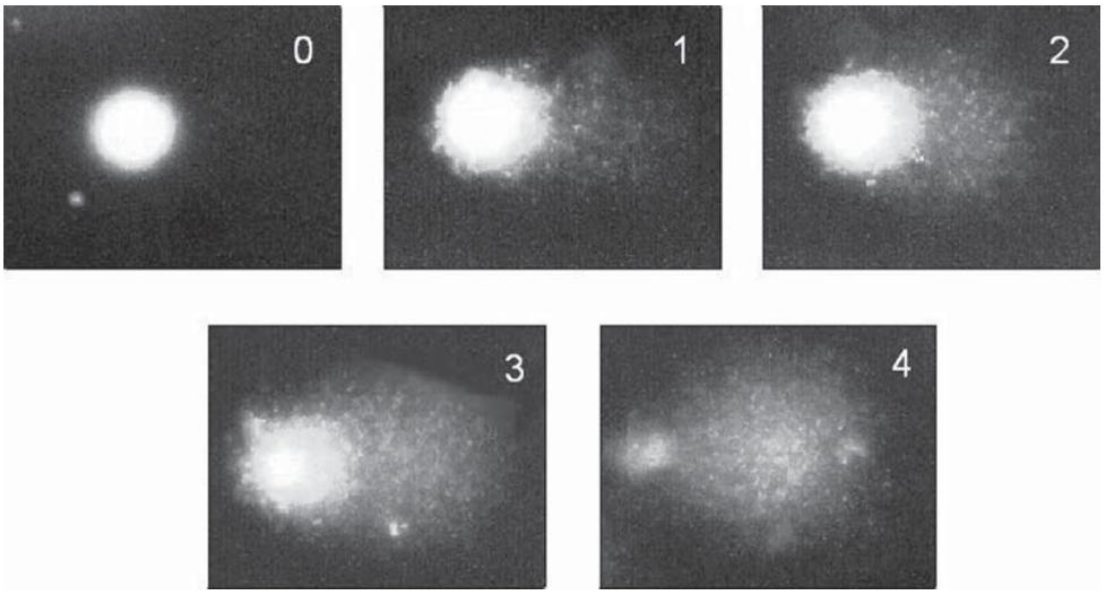

Figure 3: The five classes used in the visual scoring of comets. The 0 represents cells with no damage and 4 represents cells with extreme damage in DNA. The 1 to 3 classes represent intermediary values of DNA damage[20].

2.6. Biochemical analysis

On the biochemical analysis of the biomarkers related oxidative stress, 96 well flat bottom plates (Figure 4) were used and examined with a plate reader (Figure 5) and the appropriate software. Previous to the analysis, the samples were prepared and the necessary dilutions for each of the tested biomarker were determined. The determination of the protein content on each sample was necessary to express the biomarkers in function of this value.

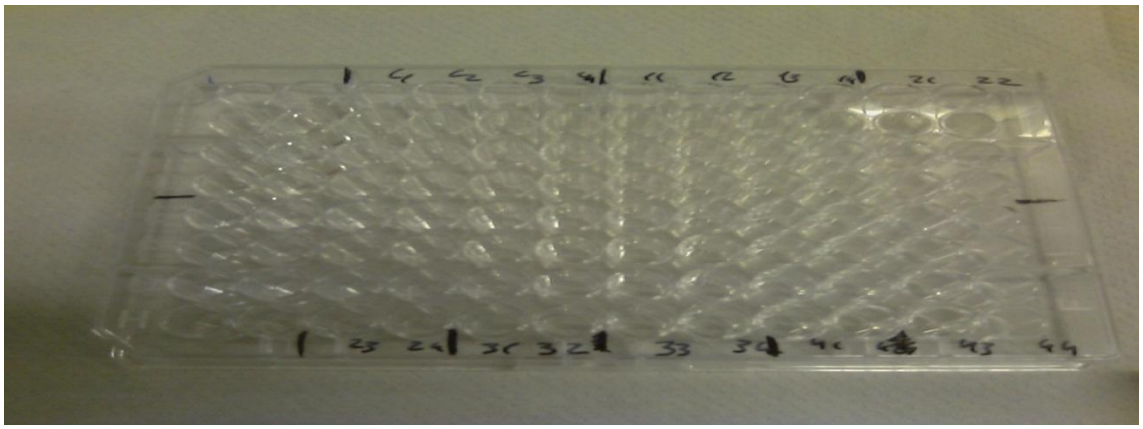

Figure 4: Representation of the 96 well flat bottom plates used in the analysis of the biomarkers.

Figure 5: Illustration of the Microplate reader used during the analysis of the biomarkers.

2.6.1. Preparation of the samples

The earthworms from each sample were homogenized on phosphate buffer (50 mM, pH= 7.0 with 0.1% Triton X-100). Homogenates were centrifuged at 4 ºC and 15000 g for 10 minutes (Figure 6). After that, the supernatant was distributed between five Eppendorfs (four for the biochemical analysis and another one as an extra) that were stored for later use at -80ºC (Figure 7).

Figure 6: Centrifuge used in the centrifugation of the homogenates.

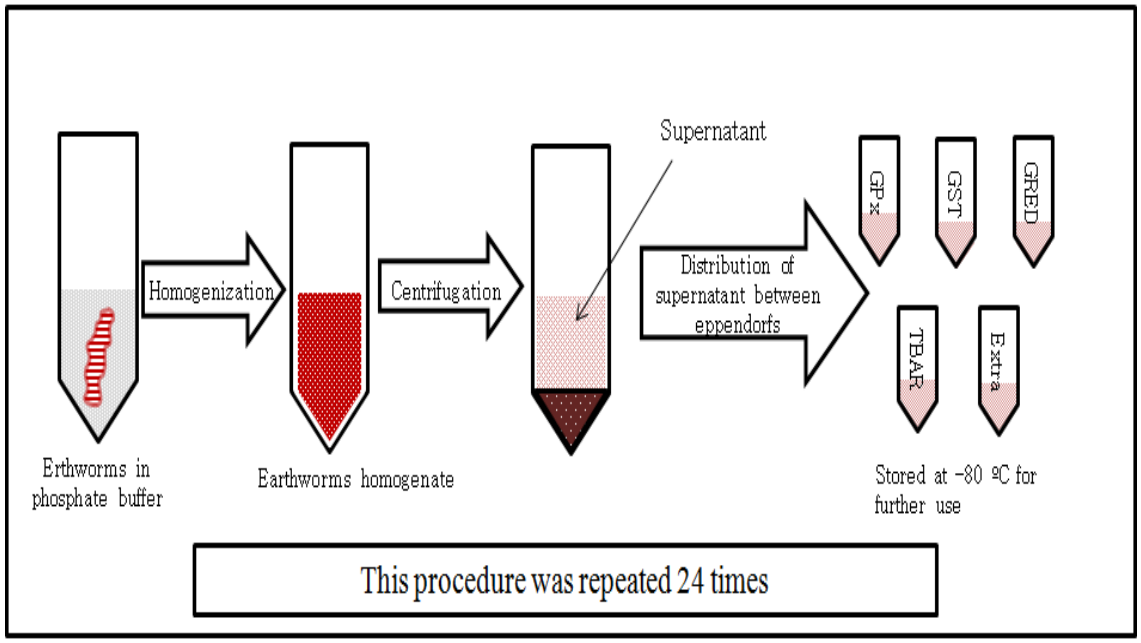

Figure 7: A simple scheme of the steps used in the preparation of the samples for the biochemical analysis.

2.6.2. Protein quantification

To find the concentration of the protein in the tested samples in the microplates, an adapted method from Bradford[27] was used. The reagent from Bradford BioRad forms a complex with the proteins that allows their quantification at 595 nm. Bovine γ-globuline was used as a standard. On each sample, the protein quantity was determined four times (four wells used on the microplate).

2.6.3. Determination of lipid peroxidation

To determine the lipid peroxidation, the quantity of Thiobarbituric Acid Reactive Substances (TBARS) was measured at 535 nm, using a modification of the protocol described by Buege and Aust[28]. The results are expressed as nmol of peroxidation products per mg of sample protein.

2.6.4. Determination of the activity of Glutathione Reductase

The activity of this enzyme was determined using a modified protocol from Carlberg and Mannervik[29]. Spectophotometry was used to monitor the oxidation of Nicotinamide Adenine Dinucleotide Phosphate (NADPH) by GRED at 340 nm. The activity was expressed in μmol per minute per mg of sample protein.

2.6.5. Determination of the activity of Glutathione S-Transferases

The determination followed the protocol of Habig et al.[30]. In this assay, the activity of the GST can be determined by the increase of the absorbance at 340 nm due to the conjugation (catalyzed by the enzyme) of the substrate 1-chloro-2,4-dinitrobenzene (CDNB) with glutathione and the formation of a thioter. The activity was expressed in nmol per minute per mg of sample protein.

2.6.6. Determination of the activity of Glutathione Peroxidase (selenium and non-selenium dependent)

The enzyme Glutathione Peroxidase (GPx) is responsible for the oxidation of glutathione. The oxidated glutathione is then reduced by glutathione reductase using NADPH. According to Flohé and Günzler[31], the activity of GPx can be monitored following the oxidation of NADPH at 340 nm. In this assay, two substrates used independently by the GPx in the oxidation of glutathione were used – hydrogen peroxide and cumenehydro peroxide. Selenium dependent GPx only uses hydrogen peroxide, cumenehydro peroxide can be used by GPx selenium and non-selenium dependent. The activity of both enzymes was expressed in nmol per minute per mg of sample protein.

2.7. Statistical analysis

To perform the statistical analysis, the software SigmaPlot 11.0 (United States, Systat Software) was used. The objective of the study was to find if there were statistically significant differences between the control group and the groups exposed to different concentrations of $TiSiO_4$-NPs. The data obtained from the DNA damage quantification and the biochemical analyses were tested for normality (Shapiro-Wilk test) and homogeneity of variance. One way analysis of variance (ANOVA) was used to test the differences between the treatments and Dunnett's tests were used to determine what treatments were different from the control. A level of significance of 0.05 was chosen for rejecting the null hypothesis.

3. Results

After the 30 days exposure, no mortality was found among the earthworms exposed to different $TiSiO_4$ treatments. During the depuration step, the earthworms from one of the replicas with the concentration 3 (444.4 mg/Kg) escaped from the flask, leaving only 3 replicas for this tested concentration. Even on the highest concentrations, it was possible to find earthworms cocoons in the soil, meaning that the presence of NPs did not inhibit the reproduction of the earthworms.

3.1. Effects of $TiSiO_4$-NPs in the DNA of earthworms

The results obtained in the DNA damage quantification of the earthworms exposed to different concentrations of $TiSiO_4$-NPs are shown in Figure 8. A first look at Figure 8 reveals an increase of DNA damage with the elevation of the concentration of the NPs tested. At the highest concentration, the mean damage score has doubled in comparison with the control. Statistically significant differences ($F = 12.77$, $df1 = 12$, $df2 = 22$, $p < 0.001$) were found between earthworms exposed to the control and to concentrations equal or superior to 444 mg/kg (concentrations 3, 4 and 5). No differences were found between the control and the concentrations 1 and 2.

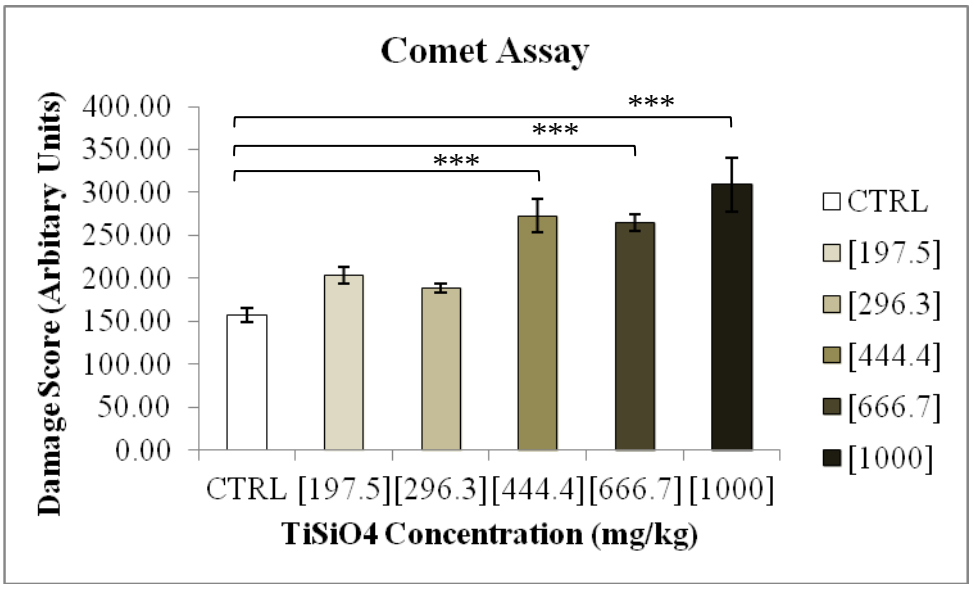

Figure 8: Mean DNA damage score, show in arbitrary units, for each treatment. A *** above the bar indicates statistically significant differences between the treatment and the control ($F = 12.77$, $df1 = 12$, $df2 = 22$, $p < 0.001$). The error bars represent the standard error.

3.2. Biochemical Analysis

No statistically significant differences were found on the content of the TBARS (Figure 9), suggesting that there were no alterations on the lipid peroxidation. Although there was a

tendency to the increase of the GRED activity, this growth was not significant for any of the tested concentrations (Figure 10). Also, no significant differences were found for the activity of the other tested enzymes: GST (Figure 11), GPx selenium-dependent (Figure 12) and total GPx (Figure 13). There was a decrease in the activity of the GPx selenium-dependent with the increase of the $TiSiO_4$ concentration, but it was not statistically significant.

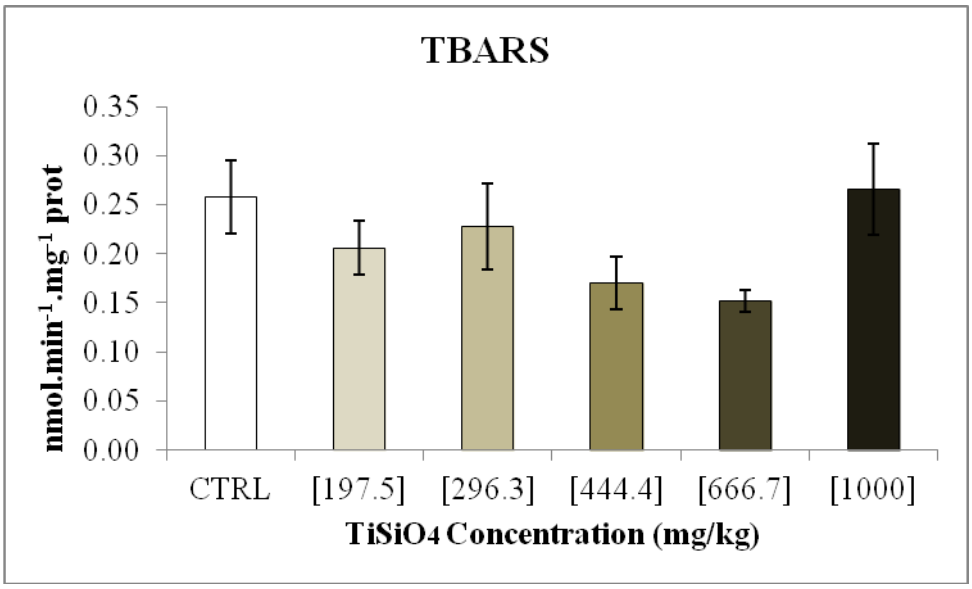

Figure 9: Mean content of TBARS for each treatment. The error bars represent the standard error.

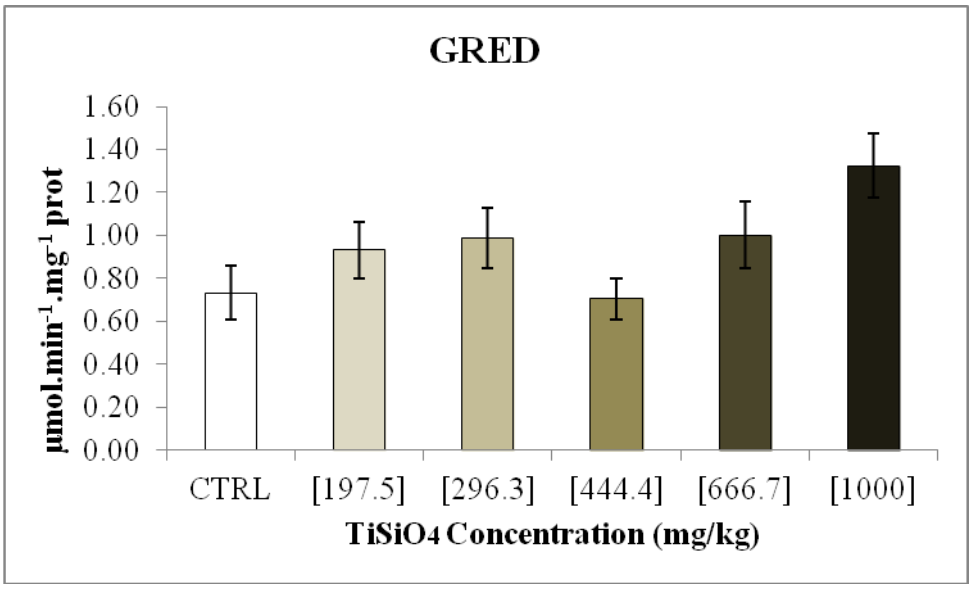

Figure 10: Mean activity of GRED for each treatment. The error bars represent the standard error.

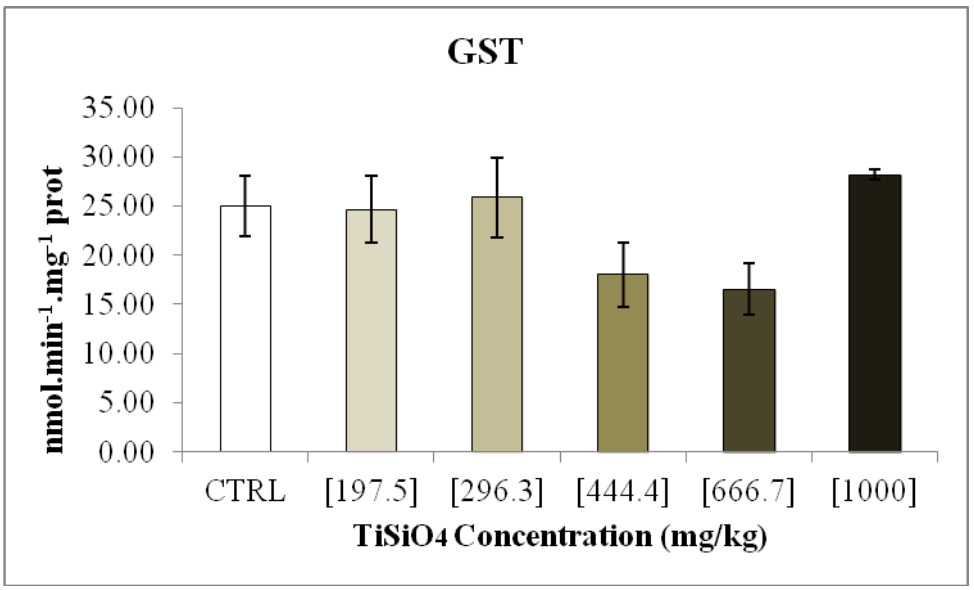

Figure 11: Mean activity of GST for each treatment. The error bars represent the standard error.

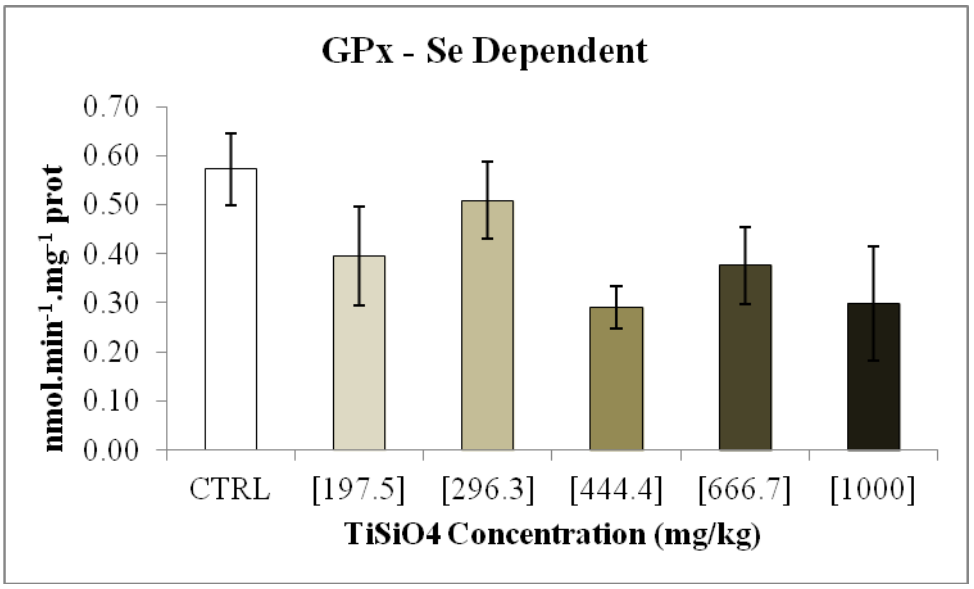

Figure 12: Mean activity of selenium-dependent GPx for each treatment. The error bars represent the standard error.

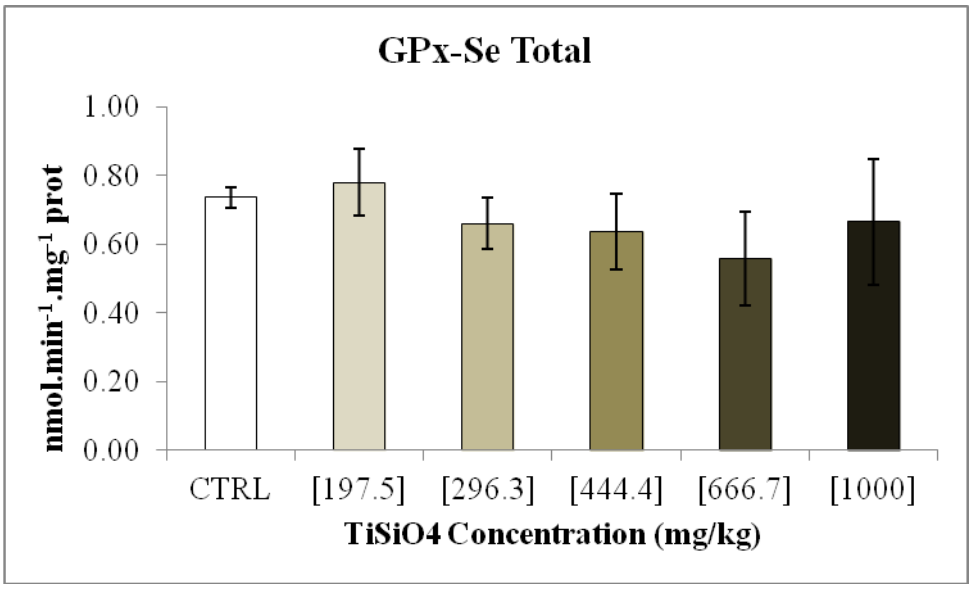

Figure 13: Mean activity of total GPx for each treatment. The error bars represent the standard error.

4. Discussion

Although in the last years there has been a growth in the NPs industry[9] and in the release of studies about the potential toxic effects of these materials[11], not much information is available about $TiSiO_4$-NPs. Most of the studies available are related to genotoxicity of silver and TiO_2 NPs[19].

There is a lack of information about the way the NPs exert they genotoxicity. The indirect damage of DNA by ROS is believed to be the main mechanism of NPs genotoxicity[32]. ROS are needed to maintain physiological homeostasis but an increase in ROS can lead to damage to proteins, lipids and DNA[21]. Studies about TiO_2 and other NPs have shown that these particles can lead to an increase of ROS and consequent DNA damage[19]. The increase in ROS production is countered with an antioxidant system. Part of this system is revolved around a molecule called glutathione and the enzymes GRED, GST and GPx[21]. During the augment of stress by the production of ROS the lipid peroxidation of the cell membranes also increases giving arise to products like malondialdehyde (MDA)[32]. If the NPs could reach the nucleus or interact with it they could possibly cause direct damage to the genetic material or indirect damage by interacting with nuclear proteins or by interfering with the cell cycle[19]. In our work, we used the DNA damage of the cells as biomarkers of genotoxicity and also oxidative stress biomarkers like the alterations of the activity of antioxidant enzymes and products of lipid peroxidation like MDA.

Our results showed that the exposition of earthworms to concentrations superior to 444 mg of $TiSiO_4$-NPs per kg of soil can lead to significant increase of DNA damage, suggesting that this NP is genotoxic as shown before by Pereira et al.[13]. For the higher concentration (1000 mg of $TiSiO_4$-NPs per kg of soil), the damage score almost doubled. Although the indirect damage of DNA by ROS production is the main mechanism of the NPs genotoxicity, our study showed no significant alterations on the tested antioxidant enzymes and the content of TBARS, suggesting that the damage on the genetic material might not related to oxidative stress, going against the conclusions of Salvaterra et al.[15], at least for invertebrate species. The absence of alterations in these oxidative stress biomarkers seems to indicate that the $TiSiO_4$-NPs interact directly or indirectly with the DNA causing the observed genotoxicity.

In our study we used NPs with a size smaller than 50 nm. However we have to take in consideration that all the previous studies done with $TiSiO_4$-NPs, authors noted a high tendency to the formation of $TiSiO_4$-NPs aggregations bigger than 100 nm in aqueous suspensions[14]. Although we contaminated the soil directly with the NPs, deionized water was added immediately, leading most likely to the formation of aggregations. These aggregations may cause a reduction in the NPs potential to exert their toxicity since 100nm seems to be the limit for many of the NPs to exert their negative influences[33]. Although size seems to matter when talking about the toxicity, the chemical and other physical properties of the NPs also play an important role[33]. The size of aggregates was suggested by Lopes et al.[14] as one of the possible reasons for the lack of genotoxicity of the $TiSiO_4$-NPs in the Ames assay. The same test with different conditions, e.g. the exposure medium, has led to different results, suggesting that the medium were the NPs are inserted is important for their genotoxicity (e.g. alterations the in the size of aggregates)[13].

To be able to effectively damage the DNA the NPs need to be internalized first. It is thought that some NPs can enter the cell by endocytosis, smaller particles can enter the cell through passive diffusion and larger ones can cause deformations in the membrane and enter the cell[34]. Two studies performed by Novak. et al.[35], [36], using the invertebrate *Porcellio scaber*, reported that feeding the test species with food containing more than 1000 µg of TiO2-NPs per gram, leads to the destabilization of the digestive gland epithelium cells membrane. At the highest tested concentration, the NPs were internalized. The authors suggest that the membranes need to be first destabilized for the internalization of the NPs to occur[35], [36]. The study done by Valent et al.[37], using the same test species and similar experimental conditions, found that the observed destabilization resulted mainly by the direct interaction of the NPs with the cell membrane and not exclusively by the action of oxidative stress.

When inside the cell, smaller NPs (<10 nm) can enter the nucleus using nuclear pores and larger ones (15 to 60 nm), like our NP, may access the DNA during mitosis when the nuclear membrane is dissolved (if no aggregates were formed)[19]. Although aggregates make harder the access of the NPs to nucleus and the DNA, finding these particles in the nucleus is not impossible has the study done by Hackenbertg et al.[38] has shown. In their study, normal and large aggregates of TiO_2-NPs could be found in the nucleus of human nasal epithelial cells (4% of the cells had TiO_2-NPs in the nucleus) but

no genotoxicity was found[38]. Once inside the nucleus, the NPs could interfere with the cell cycle by chemical binding or by mechanical interaction with the genetic material, leading to different kinds of damage: strand break, loss of chromosomes and interference with the DNA replication and transcription[19]. We also have to take in consideration that the NPs can cause indirect genotoxicity by interacting with proteins inside the nucleus involved in the normal functioning of the cell cycle[19]. Some NPs can even be genotoxic without entering the nucleus as the study done by Di Virgilio et al.[39] using Chinese hamster ovary cells has shown. Aggregates of TiO_2-NPs formed vesicles that did not enter the nucleus but interacted with it and modified its shape causing damage to the genetic material[39]. In Figure 14 we have an illustration of the cellular uptake of NPs and the possible routes that these particles use to exert their genotoxicity.

In our study, we used coelomocytes in the genotoxicity tests. These cells are extremely important for the immunity system of earthworms being involved in a diverse range of functions like encapsulation, inflammation and phagocytosis[40], and thus, performing the macrophages functions[41]. The uptake of NPs by the cells differ accordingly to their type, being the macrophages one of the cell types with larger uptake (related with their phagocytosis capacity)[42]. The genotoxicity observed using the comet assay in our work might be related to this bigger capacity to take more NPs in the cell. Once inside the cell, the NPs could cause the observed damage in the genetic material. Cells with lower uptake capacity could possibly be immune to the genotoxic effects of our tested NPs.

In our work we only used a maximum concentration of 1000 mg of NPs per kg of soil. The other two works available that make use of soil and these NPs used a much higher concentration. None of them reported toxicity. Genotoxicity was found in Pereira et .al[13] study using a concentration of 5000 mg per kg of soil and after a exposure time of 30 days. Our results show that $TiSiO_4$-NPs can be genotoxic at even lower concentrations.

Our work shows that independently of the way that the DNA is damaged by the $TiSiO_4$-NPs, these particles can be genotoxic and they should be dealt with care. Testing different types of cells would also be interesting to see if genotoxicity is also observable in other kind of tissues. Although we did not use mortality has a toxicity indicator, like the standard toxicity tests, it is interesting to see that no mortality was found. Regular tests that do not make use of genotoxic biomarkers would not found the potential harmful effects of

these NPs. In the future, it would be interesting to find how these TiSiO4-NPs damage the genetic material, what are the concentrations present on the environment, and if they are relevant and potentially genotoxic.

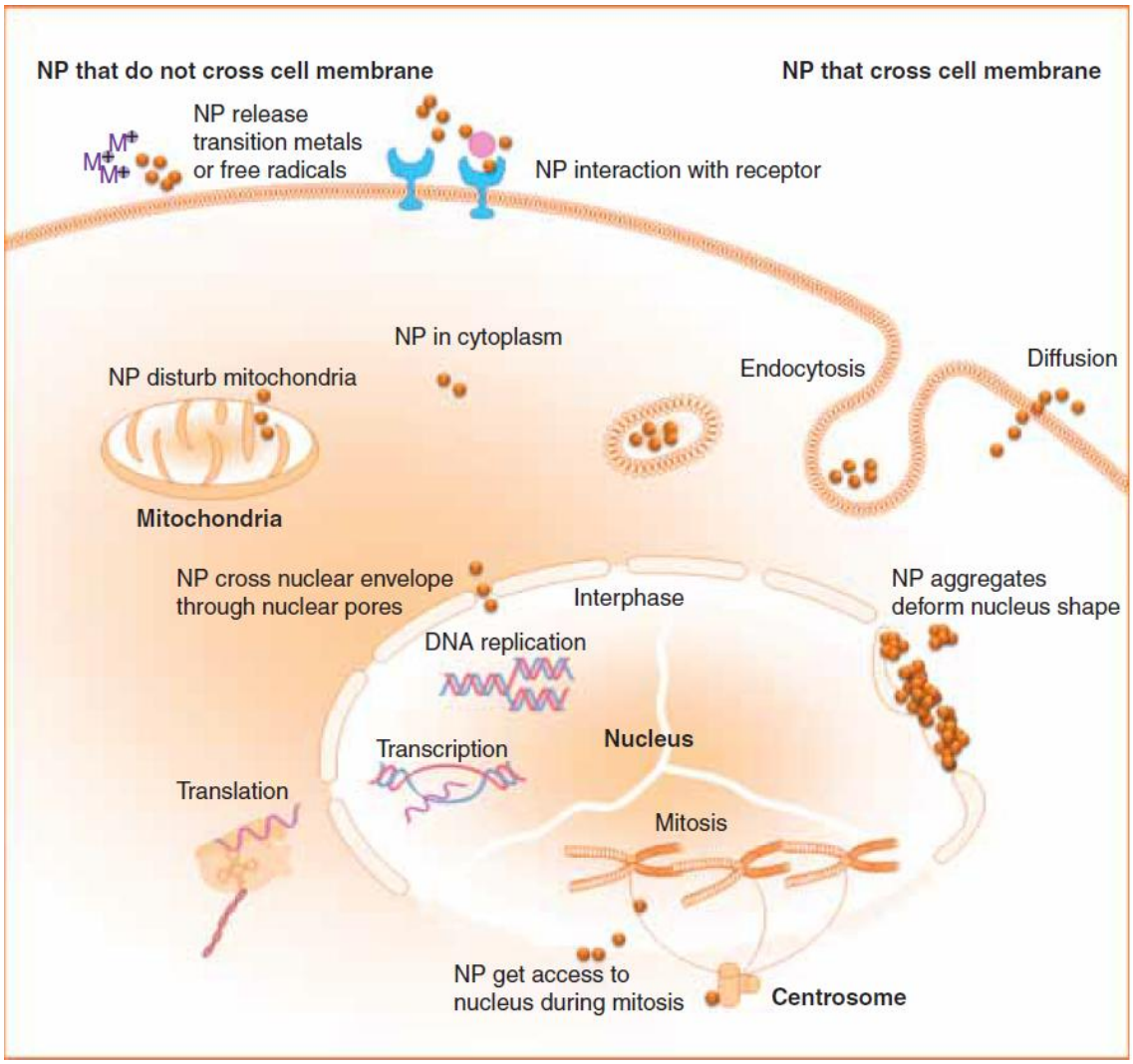

Figure 14: Cellular uptake of nanoparticles and possible routes of genotoxicity[19].

5. Conclusions

Not much information is available about the toxicity of TiSiO4-NPs. This work has shown that soils contaminated with concentration equal or higher than 444 mg of TiSiO4-NPs per kg of soil can lead to alteration in the DNA increasing its damage. For the tested oxidative

stress biomarkers and the conditions used in our work, the DNA damage found might not be related with this stress condition as no alterations were found in antioxidant enzymes or products of lipid peroxidation. This could suggest that the genetic material damage might not be caused by ROS but instead by the direct interaction of the NPs with the DNA or by any other mechanism of genotoxicity.

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Chapter 3

Evaluation genotoxic effect and antioxidant response to vesicles composed of sodium dodecyl sulphate/didodecyl dimethylammonium bromide in *Eisenia andrei*

Evaluation genotoxic effect and antioxidant response to vesicles composed of sodium dodecyl sulphate/didodecyl dimethylammonium bromide in *Eisenia andrei*

Abstract

Sodium Dodecyl Sulfate/ Didodecyl Dimethylammonium Bromide (SDS/DDAB) vesicles result from the mixture of an anionic with a cationic surfactant. These vesicles, with sizes on the nanoscale, are very stable and their application for drug delivery and gene therapy is being studied. Not much information is available about these nanoparticles (NPs). Their potential harmful effects if they ever get released in the environment are still unknown. The objective of our work was to determine if different concentrations of SDS/DDAB–NPs are genotoxic and also if there is an antioxidant response in terrestrial organisms. The selected species for this work was the earthworm *Eisenia andrei.* The so called "engineers of the soil" are essential for the maintenance of soil properties and have been used for more than 30 years in a large range of ecotoxicity tests. For this purpose, earthworms (weight: 300 - 600mg) were exposed for 30 days to the OECD artificial soil contaminated with different concentrations of SDS/DDAB-NPs. After the exposure, coelomocytes were extracted from earthworms and DNA damage was assessed by comet assay. In addition the activity of antioxidant enzymes (e.g. glutathione peroxidase, glutathione reductase and glutathione-S-Transferase) was assessed, as well as lipid peroxidation. Earthworms exposed to the highest concentration of the contaminant (1000 mg of NPs per kg of soil) had statistically significant damage in the DNA, when compared to the control. In our work we could not find statistically significant alterations on the antioxidant enzymes or lipid peroxidation. With our tested concentrations and conditions, the results indicate that the genotoxicity found, might not be related with the activity of reactive oxygen species. The harmful observed effects could have resulted from the direct interaction of the NPs with the genetic material or by other mechanism.

Keywords: Earthworms, comet assay, DNA damage, SDS, DDAB, antioxidant enzymes, oxidative stress, organic nanoparticles

1. Introduction

Catanionic mixtures results from the mixture of a cationic and an anionic surfactant[1]. Sodium Dodecyl Sulfate (SDS) is an anionic surfactant, used in diverse applications like cosmetics and cleaning products, that mixed with a cationic surfactant like Didodecyl Dimethylammonium Bromide (DDAB) lead to the formation of stable vesicles[2]. This catanionic vesicles have interesting properties as they seem to form spontaneously (thermodynamically stable system) and remain stable for long periods of time[3]. The potential of these vesicles is being studied and they could be used in the future in diverse areas like microreactor chemistry, medicine (gene therapy), cosmetics, and drug delivery[2]–[4]. Vesicles of diverse sizes can be found ranging from 10 - 50 nm to 1 - 50 µm[3]. With a size on the nanoscale, vesicles can be considered NPs and, in this case, organic NPs.

In the last years, there has been a significant growth in the nanotechnology industry[5]. Most of the products available that make use of nanotechnology are related to health and fitness which include areas like cosmetics and personal care[6]. The potential uses of SDS/DDAB-NPs make them good candidates to be inserted in this last category, increasing the risk of their introduction in the environment. Not much information is available about the potential toxic and genotoxic effects of this NP[2], [7]–[9]. Table 1 has a brief description of what is known about the toxicity and genotoxicity of these particles.

To test the potential genotoxicity of these SDS/DDAB-NPs on terrestrial ecosystems, OECD soil was contaminated with five different concentrations of SDS/DDAB-NPs. Earthworms from the species *Eisenia andrei* were exposed to this soil for 30 days. Standard guidelines to test contaminants in soil using earthworms have been developed and used for more than 30 years[10]. Some characteristics like a ubiquitous worldwide distribution, great tolerance for different temperatures and moistures, easiness to handle, and short life cycles make these earthworms a recommended test species for toxicity tests in soil[11].

The increase of the production of ROS directly or indirectly caused by the NPs is believed to be the main genotoxicity mechanism. Even though this is the principal tool of genotoxicity, the DNA may also be damaged by direct action of the NPs[12]. In our work, we used genotoxicity biomarkers like DNA damage. The DNA damage was measured

using the comet assay. This assay is currently the most used method to access the genotoxicity of NPs in organisms[12]. The large number of advantages, like its “simplicity, sensitivity, versatility, quickness, and its relative cheapness” has recruit a big number of followers[13]. With the help of spectrophotometry, alterations in oxidative stress biomarkers, like the activity of enzymes as Glutathione Reductase (GRED), Glutathione S-Transferase (GST) and Glutathione Peroxidase (GPx) implied in the antioxidant response, and the determination of Thiobarbituric Acid Reactive Substance (TBARS) that results from an increase of lipid peroxidation during stress conditions were also used[14]. The objective was to see if DNA damage is found and if it is related with alterations in the oxidative stress biomarkers.

Table 1: Summary of the information available about the toxicity of SDS/DDBA nanoparticles indicating the used organism(s), exposure medium, exposure duration, concentration of SDS/DDBA in medium, and results.

Reference	Organism(s)	Exposure medium	Exposure duration	Concentration of SDS/DDBA in medium	Results
[7]	*Vibrio ficheri* *Salmonella typhimurium*(Strain TA98 and Strain TA100)	Soil contaminated with aqueous suspension of SDS/DDBA	2 hours and 30 days	1.7g/kg	Microtox assay (*Vibrio ficheri*) revealed that the NPs were very toxic. The Ames (*Salmonella typhimurium)* assay revealed genotoxicity after 30 days in strain TA98.
[2]	*Vibrio ficheri* *Salmonella typhimurium*(Strain TA98 and Strain TA100)	$TiSiO_4$ suspended in two aqueous media	N/A	6.8g/l	Microtox assay (*Vibrio ficheri*) revealed that suspensions were toxic using both aqueous media. The Ames (*Salmonella typhimurium)* assay revealed genotoxicity to strain TA98.
[8]	Soil microbial community	Soil contaminated with aqueous suspension of SDS/DDBA	30 days	1.7g/kg	Alterations in the structural diversity of the soil microbial community were found.
[9]	White-rot fungi (*Trametes versicolor, Lentinus sajor caju, Pleurotus ostreatus, Phanerochaete chrysosporium)*	Aqueous suspension of SDS/DDBA-NPs spread in agar.	Until control Petri dishes were totally covered by the respective fungi mycelium.	3.3, 3.9, 4.7, 5.8 and 60 g/L	All the tested concentration inhibited the growth of the fungi.

2. Material and Methods

2.1. Test soil

The standard artificial *Organization for Economic Co-operation and Development (*OECD) soil was used in this work[10]. This soil has approximately the following constitution: 75% industrial sand, 20% kaolin and 5% of sphagnum peat (5 mm sieved). The pH of the soil was adjusted to 6.0±0.5 using calcium carbonate. To assess the Water Holding Capacity (WHC), samples of the soil were inserted in flasks with the bottom removed and replaced with filter paper. These flasks were immersed for two to three hours in water. After the immersion, the weight of the soil was assessed. After that the soil was dried for 24 hours at 105ºC and the weight was measured again. To determine the WHC the difference between the two weights was used[15].

2.2. Test organism

Earthworms of the species *Eisenia andrei* were used on this study. The organisms were obtained from laboratorial cultures under controlled conditions: temperature of 21ºC and photoperiod of 16 hours of light and 8 hours of dark. Once a week, the earthworms in culture were feed horse manure or oatmeal. From the cultures, 240 earthworms with a body mass between 300 and 600 mg were washed with deionized water and let to acclimatize in containers containing OECD soil for 24 hours.

2.3. Tested nanomaterial

In this work, vesicles of SDS/DDAB supplied by Sigma-Aldrich were used. The preparation of these organic NPs in an aqueous suspension followed the indications of Antunes et al.[16] and the expected NPs average size was 30 nm.

2.4. Test procedure

In this study five concentrations of SDS/DDAB-NPs were tested: 246.9, 370.4, 555.6, 833.3, and 1000.0 mg/kg. We named the concentrations respectively as 1, 2, 3, 4, and 5. A control with only OCDE soil was also used. For each concentration and control, four replicas were used. For the exposition, 24 buckets with pierced lids with an approximate volume of 0.6 L were used. In each bucket, 500 g of OECD soil were added. To obtain the desired concentration of NPs in the soil, a total volume of 41 ml (x ml of SDS/DDAB-NPs suspension + y ml of water) corresponding to 40% of the calculated WHC were added to each bucket. After this, the soil was homogeneously mixed. The 240 earthworms previously acclimatized were cleaned with deionized water. For each bucket ten earthworms with a mass between 300 and 600 mg were added. Five earthworms were used for the DNA damage quantification and the other ones for the biochemical analysis. In Figure 1 we have a quick illustration of this procedure. The 24 buckets with the 240 earthworms were stored for 30 days in controlled conditions (temperature of 21ºC and a photoperiod of 16hours of light and 8 hours of dark). Once a week, oatmeal was used to feed the earthworms. After the 30 days exposure the earthworms were removed from the soil and washed with deionized water. The earthworms from each exposition bucket were transferred and depurated for 24 hours on smaller recipients (making a total of 24 smaller recipients) with filter paper in the bottom embedded with deionized water (Figure 2). Earthworms used for the DNA damage quantification were used on the same day and the other ones were frozen in nitrogen and stored at -80 ºC.

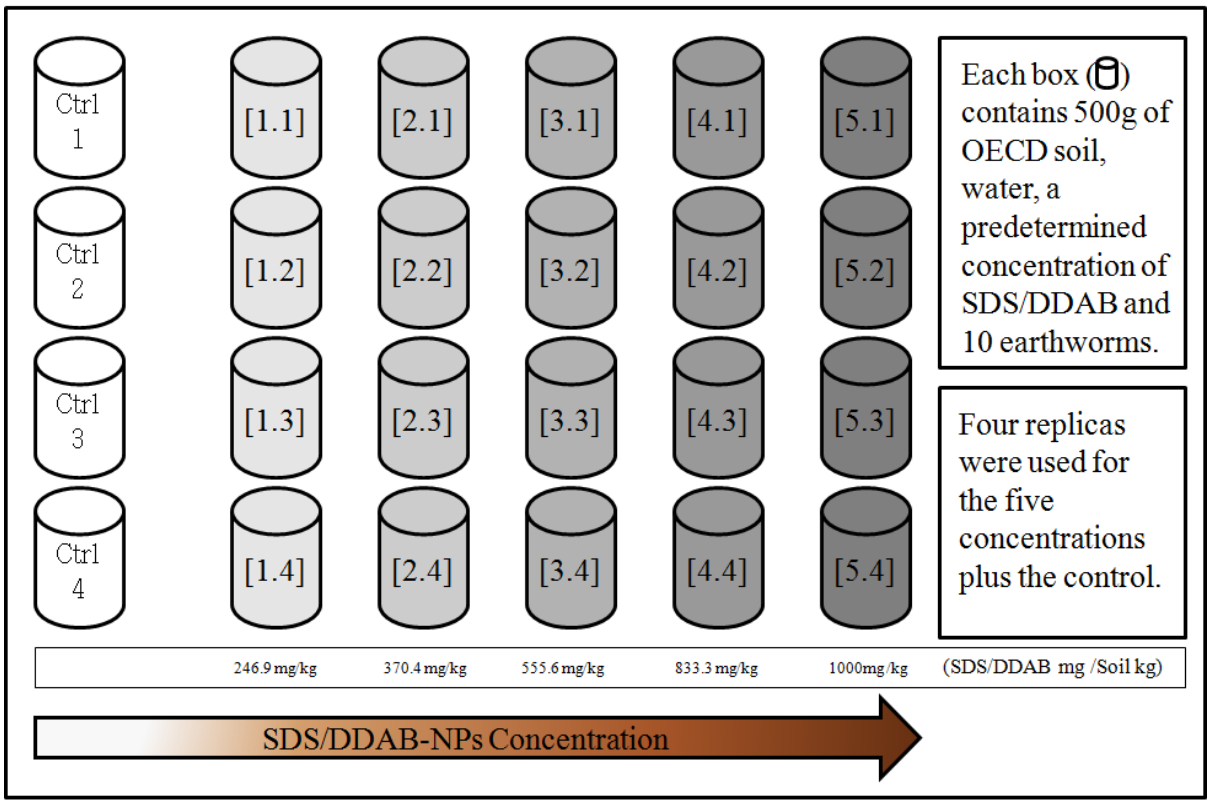

Figure 1: Schematic representation of the test procedure used in the exposition of the earthworms to SDS/DDAB nanoparticles.

Figure 2: Flask with earthworms inside being depurated.

2.5. DNA damage quantification

The DNA damage quantification used on this chapter follows that same line of work done in Chapter 2.

2.6. Biochemical analysis

The biochemical analyses of the biomarkers related to oxidative stress used on this chapter follows that same line of work done in Chapter 2.

2.7. Statistical analysis

To perform the statistical analysis the software SigmaPlot 11.0 (United States, Systat Software) was used. The objective of the study was to find if there were statistically significant differences between the control group and the groups exposed to different concentrations of SDS/DDAB. The data obtained from the DNA damage quantification and the biochemical analyses were tested for normality (Shapiro-Wilk test) and homogeneity of variance. One way analysis of variance (ANOVA) was used to test the differences between the treatments and Dunnett's tests were used to determine what treatments were different from the control. A level of significance of 0.05 was chosen for rejecting the null hypothesis.

3. Results

After the 30 days of exposure, no mortality was found among the earthworms exposed to different SDS/DDBA treatments. It was possible to find earthworms cocoons in every tested concentration and control, meaning that the presence of NPs did not inhibit the reproduction of the earthworms.

3.1. Effects of SDS/DDAB nanoparticles in the DNA of earthworms

The results obtained in the DNA damage quantification of the earthworms exposed to different concentrations of SDS/DDBA-NPs are show in Figure 3. Statistically significant difference (F = 4.403, df1 = 5, df2 = 18, P = 0,009) was found between the earthworms exposed to control and concentration 5 (1000 mg/kg). No significant differences were found between the control and concentrations 1, 2, 3, and 4.

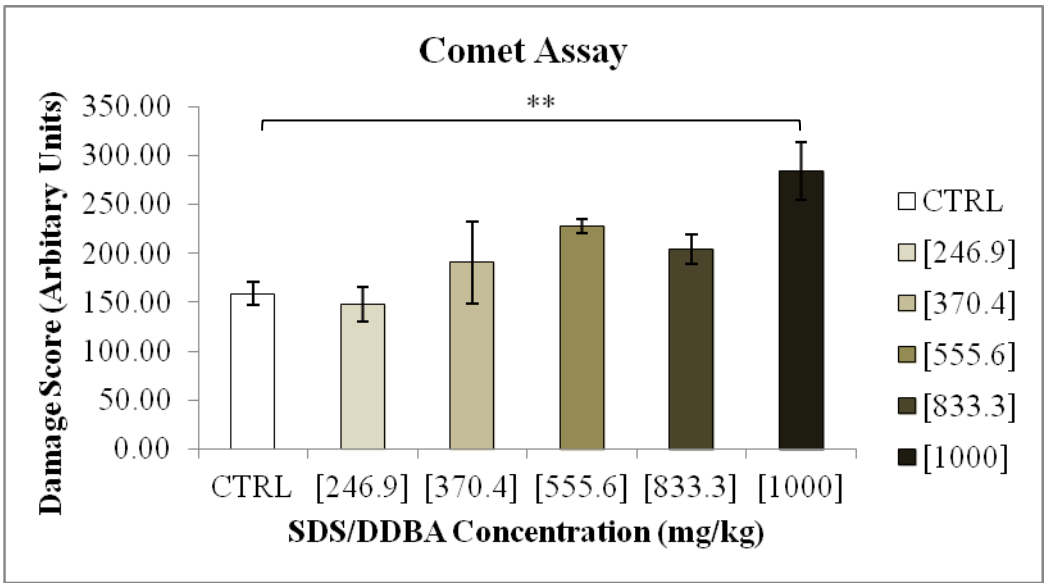

Figure 3: Mean DNA damage score, show in arbitrary units, for each treatment. A ** above the bar indicates statistically significant differences between the treatment and the control (F = 4.403, df1 = 5, df2 = 18, P = 0,009). The error bars represent the standard error.

3.2. Biochemical Analysis

No statistically significant differences could be found on any of the oxidative stress biomarkers. Although it was possible to observe an increase on the TBARS with the raise of the SDS/DDAB concentration, this increase was not significant, and thus no alterations could be found on the lipid peroxidation (Figure 4). Lower values of GRED activity were found on concentration 2, 3 and 4 when compared with the control (Figure 5). No alterations on the activity of GST were found (Figure 6). A problem with the microplate reader left us without information about the activity of this enzyme on the highest concentration (no values were recorded). No significant differences were found on the activity of GPx selenium-dependent (Figure 7) and total GPx (Figure 8).

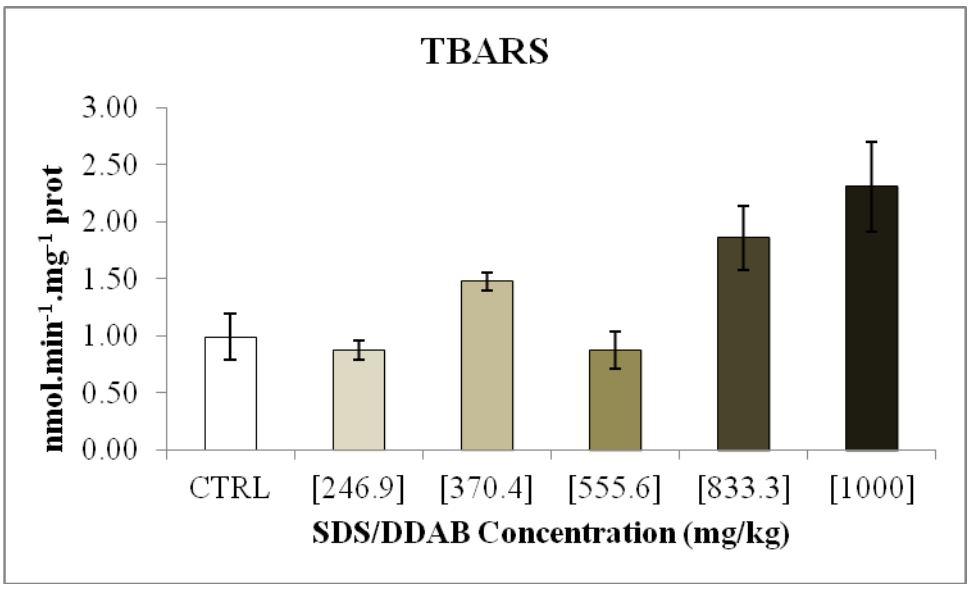

Figure 4: Mean content of TBARS for each treatment. The error bars represent the standard error.

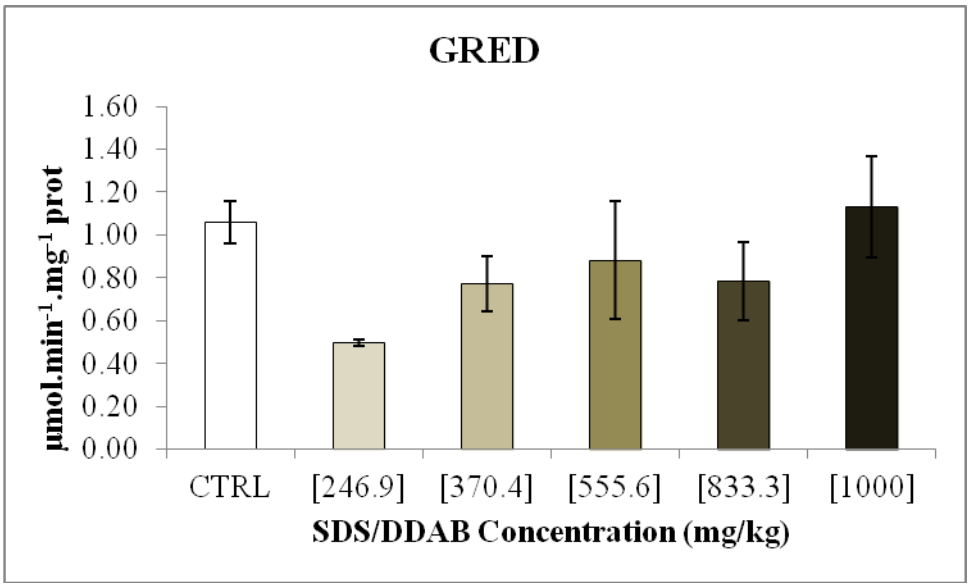

Figure 5: Mean activity of GRED for each treatment. The error bars represent the standard error.

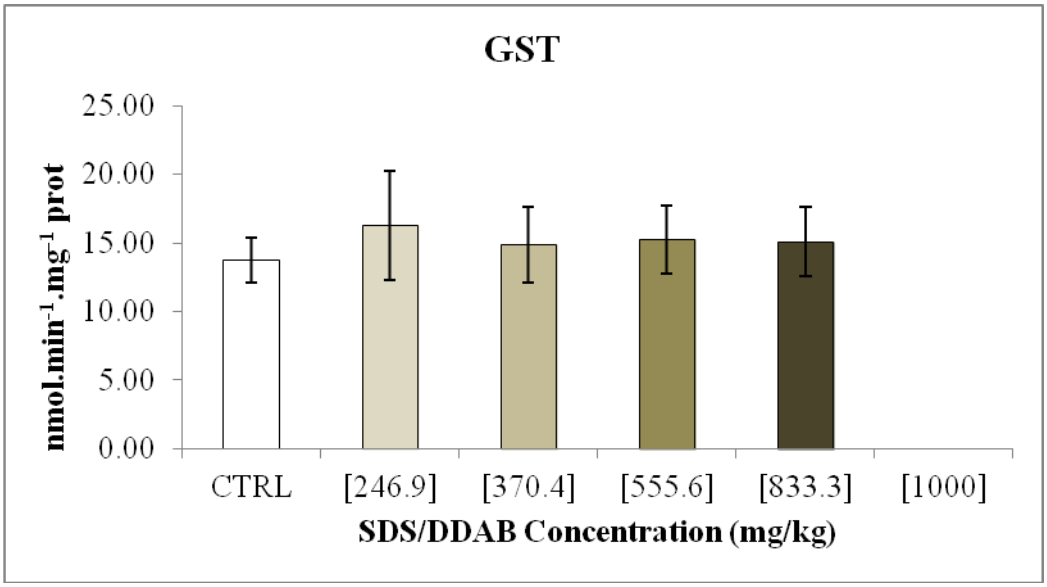

Figure 6: Mean activity of GST for each treatment. The error bars represent the standard error.

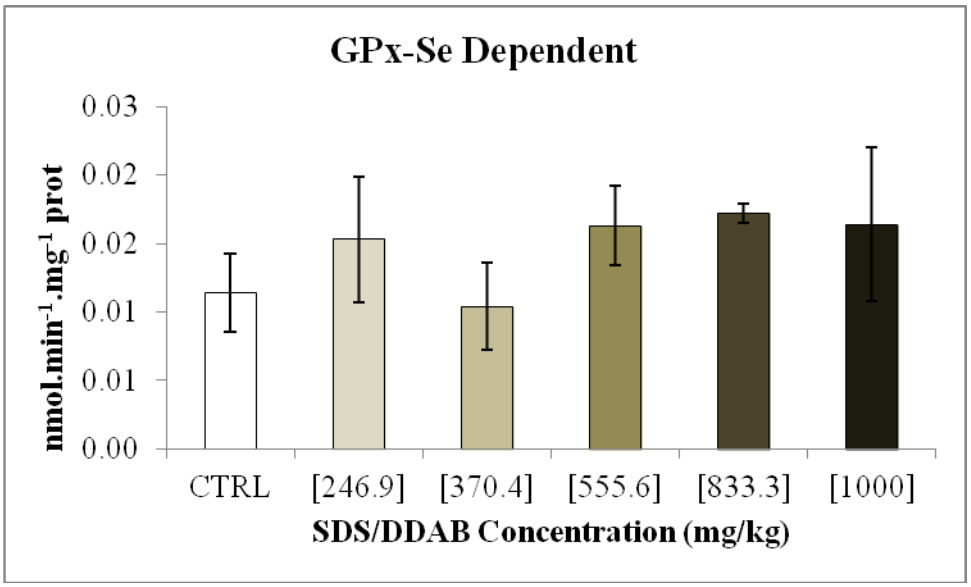

Figure 7: Mean activity of selenium-dependent GPx for each treatment. The error bars represent the standard error.

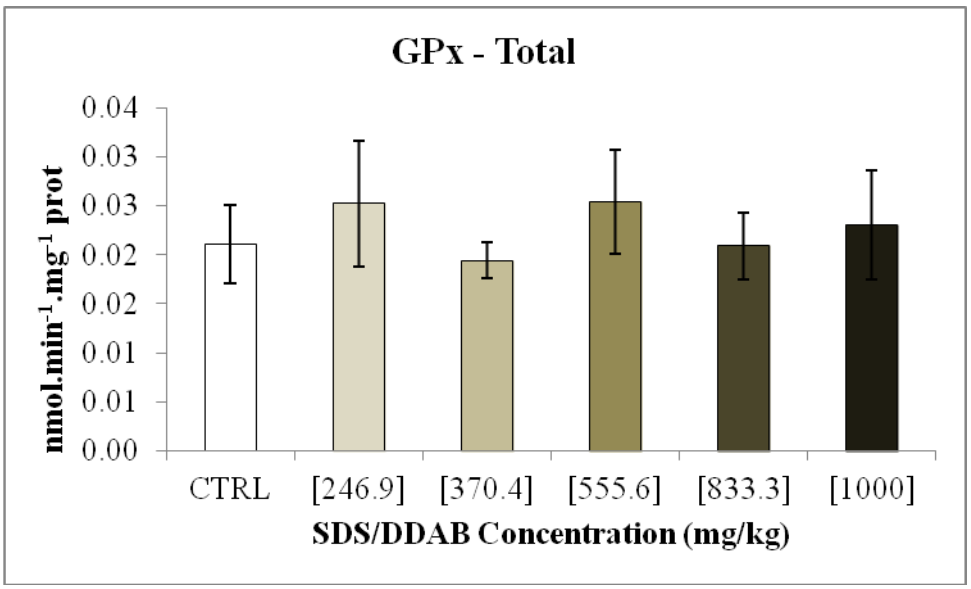

Figure 8: Mean activity of total GPx for each treatment. The error bars represent the standard error.

4. Discussion

The stable vesicles resulting from catationic mixtures of SDS and DDAB are extremely promising and can be used in the future in diverse areas of the nanotechnology industry, especially in pharmacology and medicine for drug delivery and gene therapy[2], [3]. Since these are relatively new NPs, not much is known about them and their possible harmful effects on the ecosystems when released unintentionally. The few available studies point out the high toxicity and genotoxicity of these NPs. The fact that these particles are very stable reduces the probability of formation of aggregates when in aqueous suspensions allowing them to maintain a smaller size (± 30 nm)[2], [7]–[9]. It is known that NPs with smaller sizes tend to be more toxic than larger ones[17]. Since these NPs are organic, their degradation by the microbial community into simpler forms, that can be toxic or nontoxic, is possible[7].

Even though there has been a growth in the number of published studies related to the NPs toxicity[18], the mechanism by which they exert their genotoxicity is not very well understood[12]. ROS production occurs naturally in the cells and are essential for the maintenance of homeostasis[14]. An increase in the production of ROS by direct or indirect action of NPs is believed to be the main mechanism of genotoxicity[19]. The increase of ROS and the incapacity of antioxidant enzymes to deal with them leads to a condition called oxidative stress, which may conduct to DNA damage[14]. Other mechanisms causing damage to the genetic material also exist, like the direct interaction of the NPs with the DNA or other related molecules[12].

In our work we used DNA damage as a genotoxicity biomarker and oxidative stress biomarkers like alterations of the antioxidant enzymes activity and products of lipid peroxidation. The aim was to find if the NPs are genotoxic (using the comet assay) and if this genotoxicity was related to the oxidative stress.

The results of our work showed that an increase of the concentration of SDS/DDAB NPs in the soil lead to an increase of DNA damage in the tested coelomocytes of the earthworms. For the higher concentration (1000 mg per kg of soil), this increase of genetic material damage was statistically significant indicating that these NPs can be genotoxic to earthworms. These results support the data gathered by Pereira et al.[6] and Lopes et al.[2] with the bacterium *Salmonella typhimurium*. Using the information

available, it would be expected that this damage could be related to a possible increase in the oxidative stress in the cells. Our results did not support this idea. An increase in the TBARS could be found, but it was not significant, the same happened for the tested antioxidant enzymes. Since we could not find alteration in oxidative stress biomarkers in our work, the NPs might have damaged the genetic material toxicity by either interacting directly with the genetic material or indirectly using other molecules related with the DNA.

Since our tested NPs have an high stability, variations in the size of these particles are not expected and other previous works reported an observed size of ± 74 nm[2], [7].

To exert their toxicity the NPs need to gain access to the cell. The main mechanism of cellular uptake is endocytosis, but other mechanisms also exist, like plasma membrane diffusion[20]. Not all the cells have the same NPs uptake capability. For example, macrophages, capable of phagocytosis, have a higher capacity to take NPs and thus can be more exposed to the effects of these particles[21]. The used cells in our study, coelomocytes, are part of the earthworms immunity system, performing functions often associated with macrophages (e.g. phagocytosis)[22], [23]. By having the capacity to take more NPs, they are more likely to suffer from their dire effects.

Once inside the cell and depending on their nature and the mechanism of their uptake, NPs can be found in different locations: the cell membrane, cytoplasm, mitochondria, next to the nuclear membrane, or inside the nucleus were it can damage the DNA[17]. Particles size can influence the way by which a NP reaches the nucleus. Smaller particles can use nuclear pores or cross the membrane while bigger NPs may only have access to the nucleus during mitosis when there is no nuclear envelope[12]. Size does not seem to be the only important factor for the nuclear uptake of the NPs, as suggested by Garcia-Garcia et al.[24]. In their work, two NPs with similar size but different properties (e.g. surface composition) were used and only one was found in the nucleus. More available studies have reported the access of NPs to the nucleus (e.g. [25], [26]). A review on NPs genotoxicity by Magdolenova et al.[12] suggests diverse mechanisms by which NPs can exert their toxicity, after gaining access to the nucleus. They said that NPs can interact directly with the DNA by mechanical or chemical binding. This interaction can result in the break or loss of chromosomes, alteration in the conformation of the genetic material, and changes in transcription and replication of the DNA. Another way of DNA

damage is by the interaction of the NPs with molecules involved in the cycle of the cell[12].

Since these vesicles are being studied for gene delivery and drug delivery, it is expected to some degree, a non-harmful relation with the genetic material present in the cell and the vesicles. This fact can be the reason why no oxidative stress was found or only DNA damage was encountered at the highest concentration[4].

Our work shows that SDS/DDAB-NPs are genotoxic and their use in the future should be done with care. More studies must be performed to find the exact way by which these particles exert their toxicity and how they interact with the soil compartment. Moreover, the dangerous concentrations to the ecosystem must be intensively analyzed.

5. Conclusions

The information about SDS/DDAB-NPs is very scarce since these are fairly new particles, still being in development for future applications. The results from our work show that when SDS/DDAB-NPs are present in the soil with concentrations equal to 1000 mg per kg of soil, they can be genotoxic. No alterations were found on the oxidative stress biomarkers suggesting that the DNA damage resulted from the direct or indirect interaction of the NPs with the genetic material.

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Chapter 4

General discussion and conclusions

General discussion and conclusions

In our work, the genotoxicity and antioxidant response of earthworms to two promising NPs were accessed. For this, genotoxicity and oxidative stress biomarkers were used.

The comet assay was used successfully in our work to detect DNA damage in coelomocytes of the earthworms exposed to the tested NPs. Having these results in consideration, the $TiSiO_4$-NPs needed a lower concentration than SDS/DDAB-NPs to exert their toxicity, suggesting that the tested inorganic NPs could be more toxic than the organic ones. By having in consideration the information available about the toxicity of NPs, it was expected, that the discovered genotoxicity would be related with alterations on oxidative stress biomarkers. Such expectation was not met, because our results reported no statistically significant alterations in these biomarkers. Although the results were negative, some tendencies were found in our data. For $TiSiO_4$-NPs, there was an increase in GRED activity and a decrease in GPx selenium dependent activity with the increase of the NP concentration. With SDS/DDAB-NPs, an increase in TBARS products was found. The data gathered with the analysis of the oxidative stress biomarkers should be dealt with care, because samples within the same concentration presented high variability. The variability causes were not entirely clear, thus the experiment should be repeated to better understand it. Nonetheless, the variability may be associated with technical problems during the experiment.

The soil compartment is extremely complex, having a large diversity of living organisms and different properties. It is difficult to predict how our tested NPs would interact with different types of soil and living organism and how they would be available to exert their genotoxicity. More studies are needed to understand the interaction between NPs and the soil. Also, more studies could be done using other organisms, probably some organisms are more sensible than others to the effects of NPs.

Some standardization of the techniques use to access the toxicity of NPs and other contaminants can already be found (e.g. standard toxicity tests for earthworms), but additional standardized procedures would be useful because it is very common to find contradictory information about certain NPs using similar test conditions.

Further research is needed to understand the mechanisms that these NPs use to exert their genotoxicity. The interaction of the particles with the diverse available matrixes, organisms, and other molecules could also be interesting to investigate.