Beatriz Isabel Manso de Matos

Licenciada em Bioquímica

Study of Quantum Dots (CdS, ZnS) toxicity in *Danio rerio*, *Daphnia magna* and *HepG2* cell line.

Dissertação para obtenção do Grau de Mestre em
Bioquímica

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“Why it's simply impassible!
Alice: Why, don't you mean impossible?
Door: No, I do mean impassible. (chuckles) Nothing's impossible!”

Lewis Carroll

AGRADECIMENTOS

A minha passagem pela FCT tem sido muito rápida, mas também muito feliz. Rápida porque olho para trás e parece que foi há uns dias que aqui cheguei, e feliz porque sinto que tive a sorte de ao longo do meu percurso terem surgido as pessoas e as oportunidades certas.

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À Leonor e à Madalena por despertarem a criança que há em mim e não a deixarem desaparecer!

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ABSTRACT

The increasing use of nanoparticles in recent decades led to a growing concern about the possible consequences for the environment.

The present study aims to evaluate the toxicity of the Quantum Dots (QDs) (ZnS and CdS), using *Danio rerio*, *Daphnia magna* and HepG2 cell line. Both organisms were exposed to different concentrations of QDs (10, 100 and 1000 µg/L), singly and combined, to assess toxicity. Several biochemical analyses were performed: antioxidant enzymes (Catalase, Glutathione-S-transferase and Superoxide Dismutase), total antioxidant capacity (TAC), ubiquitin, heat shock proteins (HSPs) and lipid peroxidation (MDA content). An HepG2 cell line was also exposed to the same QDs concentrations, and cell viability was assessed. QDs were characterized by Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) and Dynamic light scattering (DLS). The results from exposure assays showed that the concentrations of QDs tested did not cause mortality in fish but resulted in high mortality rates in *Daphnia magna* exposed to higher QDs concentrations (100 and 1000 µg/L). Regarding HepG2 assay, QDs were not able to cause significant cytotoxicity. Overall, the results from fish and Daphnids assays showed sub-lethal effects following exposure to the different concentrations of QDs. *D. rerio* exposure assays showed increased levels of antioxidant enzymes, HSP70 and Ubiquitin in fish exposed to 100 µg/L QDs (ZnS). *D. magna* exposure assays revealed decreased levels of antioxidant enzymes and increased levels of Ubiquitin according to QDs concentrations tested.

Was investigated the trophic transfer of QDs from *D. magna* (previously exposed to QDs) to *D. rerio.* The results from Inductively coupled plasma atomic emission spectroscopy (ICP-AES) analysis indicate that the QDs (and/or their ions released into the aqueous medium) were ingested by Daphnia and then transferred to fish via ingestion as well.

Keywords: Quantum Dots; *Danio rerio; Daphnia magna*; HepG2; Oxidative stress; Toxicity

RESUMO

O crescente uso de nanopartículas nas últimas décadas levou a uma crescente preocupação com as possíveis consequências para o meio ambiente.

O presente estudo tem como objetivo avaliar a toxicidade de Quantum Dots (QDs) (ZnS e CdS), utilizando *Danio rerio*, *Daphnia magna* e linha celular HepG2. Os organismos foram expostos a diferentes concentrações de QDs (10, 100 e 1000 µg/L), isoladamente e combinados, para avaliar a toxicidade. Diversas análises bioquímicas foram realizadas: enzimas antioxidantes (catalase, glutationa-S-transferase e superóxido dismutase), capacidade antioxidante total (TAC), ubiquitina, proteínas de choque térmico (HSPs) e peroxidação lipídica (conteúdo de MDA). A linha celular HepG2 também foi exposta às mesmas concentrações de QDs para avaliar a viabilidade celular. Os QDs foram caracterizados por Microscopia Eletrónica de Varrimento (SEM), Microscopia Eletrónica de Transmissão (TEM) e dispersão dinâmica de luz (DLS). Os resultados dos ensaios de exposição revelaram que as concentrações de QDs testadas não causaram mortalidade nos peixes, mas resultaram em taxas de mortalidade elevadas em *Daphnia magna* expostas a concentrações maiores de QDs (100 e 1000 µg/L). Em relação ao ensaio HepG2, os QDs não causaram citotoxicidade significativa. No geral, os resultados dos ensaios com peixes e dáfnias indicaram efeitos sub-letais após a exposição às diferentes concentrações de QDs. Os ensaios de exposição a *D. rerio* revelaram níveis aumentados de enzimas antioxidantes, HSP70 e Ubiquitina em peixes expostos a 100 µg/L de QDs (ZnS). Os ensaios de exposição à *D. magna* revelaram níveis diminuídos de enzimas antioxidantes e níveis aumentados de Ubiquitina de acordo com as concentrações de QDs testadas

Foi também investigada a transferência trófica de QDs de *D. magna* (previamente expostas a QDs) para *D. rerio*. Os resultados da análise de espectrometria de emissão atômica por plasma acoplado indutivamente (ICP-AES) indicam que os QDs (e / ou iões libertados no meio aquoso) foram ingeridos por dáfinas e depois transferidos para peixes por ingestão.

Palavras-chave: Quantum Dots; *Danio rerio; Daphnia magna*; HepG2; Stress oxidative; Toxicidade

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ABREVIATIONS

ABTS	2,2′-Azino-bis (3-ethylbenzothiazoline-6-sulfonic acid)
BSA	Bovine Serum Albumin
CAT	Catalase
CDNB	1-Chloro-2,4-Dinitrobenzene
CdS	Cadmium Sulphide
DLS	Dynamic light scattering
DMEM	Dulbecco's Modified Eagle Medium
DTNB	5,5’-Dithio-bis-2-nitrobenzoic Acid
EDTA	Ethylenediamine Tetraacetic Acid
ELISA	Enzyme-Linked Immunosorbent Assay
FBS	Fetal Bovine Serum
FCS	Fetal Calf Serum
GSH	Reduced Glutathione
GST	Glutathione-S-Transferase
HSP	Heat Shock Protein
ICP	Inductively coupled plasma
LPO	Lipid peroxidation
MDA	Malondialdehyde
MTT	3-[4,5dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide
NADPH	Nicotinamide Adenine Dinucleotide Phosphate
NBT	Nitro Blue Tetrazolium
PBS	Phosphate-buffered saline
PNPP	4-Nitrophenyl Phosphate Disodium Salt Hexahydrate
Purpald	4-Amino-3-hydrazino-5mercapto-1,2,4-triazole
QD	Quantum Dot
ROS	Reactive oxygen species
SDS	Sodium Dodecyl Sulphate
SEM	Scanning Electron Microscopy
SOD	Superoxide Dismutase
TAC	Total Antioxidant Capacity
TBA	Thiobarbituric acid
TBARS	Thiobarbituric Acid Reactive Substances
TCA	Trichloroacetic Acid
TEM	Transmission Electronic Microscopy

TROLOX	6-Hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid
XOD	Xanthine Oxidase
ZnS	Zinc sulphide

Chapter 1

Introduction

1 Introduction

In the last century, the development of technology and industry led to an increase in the human population mostly in large urban areas. This growth resulted in increased pollution, namely aquatic pollution. Sources like atmospheric deposition, wastewater effluents and leaching from soils, contributed to this type of pollution[1] . However, one of the most important sources is related to the use of chemicals that are intentionally or not, released into the environment. Some of these chemicals are heavy metals such as cadmium (Cd) and lead (Pb) [2] or organic compounds (e.g. PCBs, PAHs, pesticides and herbicides) [3]. Thus, the anthropic activities have become a major threat to aquatic ecosystems, also promoting the degradation of water quality which is a serious ecological problem worldwide. The unknown behaviour and fate of these contaminants are one of the main causes of stress on aquatic ecosystems that are highly vulnerable to external aggression[3]. Therefore, toxicology studies are fundamental since there are many compounds that need a toxicity assessment. With the new discoveries in the field of Nanotechnology and the growing use of nanoparticles, it is difficult to avoid exposure to this type of contaminants. In fact, aquatic environments seem to be very vulnerable to nanomaterials due to their ability to mix and disperse in the environment very fast. Moreover, several issues regarding bioavailability, degradability, reactivity and toxicity of nanomaterials are still to understand. In addition, with the global concern about the ecological problem, nanoparticles such as Quantum Dots (QDs) have been the target of several ecotoxicity studies to evaluate if their presence in the ecosystems represents a risk for the biota.

1.1 Nanotechnology

Nanotechnology is a new field of science and technology that involves the production and application of new materials operating at a nanoscale range [1,4]. A nanoparticle is usually defined as a type of nanomaterial with at least two dimensions between 1 and 100 nm [5]. In the field of chemistry, this range of sizes is also related with colloids, micelles, polymer molecules, and lately, other structures such as carbon nanotubes, silicon nanorods and QDs-semi-conductors, which have been recently discovered as an interesting class of nanomaterials [6]. Furthermore, materials show a unique behaviour and have different properties when at nanoscale. Some of these properties are their small size, chemical composition, surface structure, solubility and shape, allowing them to be used in many areas such as pharmaceutics, medicine, textile or electronic industries [7].

1.1.1 Nanomaterials

Every year different types of nanomaterials are being discovered for different uses (e.g. drug delivery, cancer diagnosis and therapeutic, optical imaging, antibacterial agents, screens, sunscreen products, textiles, cosmetics, coating, food preservation, and others)[4,8] and making these products more efficient and low-cost. However, the unique properties that make nanomaterials attractive, can also be responsible for adverse effects on organisms (e.g. microorganisms, algae, fish, humans, etc.) [9]. Although nanoparticles have always existed in the environment from natural sources, the anthropogenic sources are becoming an increasing concern.[5] For instance, many types of nanoparticles made by natural processes like photochemical reactions, volcanic eruptions, forest fires, and erosion are present in the environment. [8] These occurrences, even if unintentional, can produce large quantities of materials which, when dispersed can reach water, soil, and the air compartments where they can stay for long periods of time. If no biodegradation occurs, then they can bioaccumulate in the food chain and be an ecotoxicological hazard. [7]

Nanowaste is the term commonly used to refer to the release of nanomaterials into the environment as nanofabrication residues or during their use in biomedical applications, industrial processes, and diverse domestic uses. When nanowastes are released into the environment they can lead to soil pollution and contaminated sludge, water or sediments. An example of nanowaste was the discovery in 2010 of silver nanoparticles (Ag NPs) in wastewater and platinum nanoparticles (Pt NPs) released from automotive exhaust converters. [7,10]

A major challenge in the field of nanotechnology is the development of regulatory legislation for the management of environmental and public health implications. Some improvements in this field are the development of instruments for the assessment of contaminated air and water, the development and validation of methods for toxicity evaluation and mathematical models to predict the potential impacts on the environment. [10]

1.1.2 Quantum Dots

The Quantum Dots (QDs) are a type of nanoparticles that have several potential applications in diverse industrial and biomedical areas and are the most used and well-characterized nanomaterials. These nanoparticles are widely used as therapeutic agents and fluorescent dyes and are useful in the electronic field as is used in LED flat panel displays and in photovoltaic solar cells [11,12]. QDs are semiconductor crystals with nano dimensions, containing 200-10,000 atoms[13], and are composed of semiconductor metals core (e.g. CdSe, CdTe, ZnSe) of groups II-VI and are often encapsulated by a shell or a "cap"(eg. ZnS, DHLA, BSA, polyacrylate) [1,14] The shell improves their optical and electronic properties and prevent core metal leaching events, which are a major cause of toxicity. [12,14] QDs shows unique features as luminescence, a wide and continuous absorption spectrum, narrow emission spectra and high light stability. These properties are easily achieved by modulating their size and chemical composition. Additionally, with only a few hundred atoms, QDs can emit only one wavelength of light when they are excited, and the colour of this emission is determined by the size of the nanoparticle [15]. Therefore, QDs development was one of the first nanotechnologies used in biological sciences. Moreover, an increase in its use in several domains of biological research like cancer detection and targeted drug delivery is expected in the future [16].

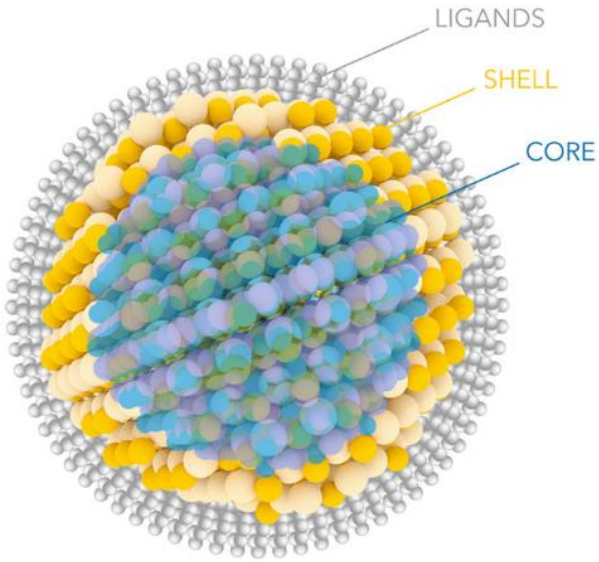

Figure 1.1 - Structure of a quantum dot.
Source: avsforum. Quantum Dot Structure. [ONLINE] Available at: https://www.avsforum.com/electroluminescent-quantum-dots-are-coming-sooner-than-you-think/. [Accessed 19 September 2019]

QDs have unique and remarkable properties, however, little is known regarding their toxicity. Recent studies indicate that the detection of nanoparticles, namely QDs, in wastewater is increasing, mostly in places like hospitals (due to their biomedical applications) and near industries (e.g. electronic and textile). [13] The main concern about the use of QDs are the ions released from the metal core that can induce the generation of reactive oxygen species (ROS)[17]. When QDs are released into the environment, several oxidative phenomena may occur, like air or photo-oxidation with UV-light increasing the leaching of metal ions. However, diverse storage and coating strategies can be employed to avoid these events: the shell removal following the release of QDs into the environment by natural phenomena's or after intake by organisms. If QDs are internalized by cells through endocytosis they are then trafficked to acidic and oxidative environments for degradation resulting in the production of harmful substances or elements like free toxic ions[12]

Thus, it is still necessary to continue researching in the QDs areas, mainly by conducting more toxicity studies since there are no guarantees that the cap remains intact to prevent the release of metal (e.g. Cd, Zn, Ag, Al) ions which, in elevated concentrations can pose a threat to the ecosystems.

1.1.3 Quantum Dots Characterization

The growing need to study the toxicity of nanomaterials, as QDs, led to the development of novel techniques or the use of various characterization techniques as Scanning Electron Microscopy (SEM), Transmission Electron Microscopy (TEM) or Dynamic Light Scattering (DLS), among others. Due to the small size of nanoparticles, optical microscopy is not used for characterizing nanoparticles. Instead, it is commonly used the electron microscopy[18].This technique uses a microscope with a beam of accelerated electrons as source of illumination and it is capable to achieve higher resolutions (size range: 0.5 nm – 1 mm)[19]. On the other hand, SEM is usually used to obtain information regarding size, size distribution and shape of the nanomaterials. TEM uses a more powerful beam of electron being capable to offer more detailed information than SEM, as crystal structure and granularity of the samples. However, the samples are dried for analysis, which is a disadvantage since it doesn't allow to see nanoparticles dispersed in aqueous solutions. To overcome this difficulty DLS analysis is as a complement to the electron microscopy since this technique works in aqueous solutions. DLS uses a laser light beam that is scattered off the nanoparticle solution and correlates the Brownian diffusion of the nanoparticles and the Stokes–Einstein equation to the hydrodynamic diameter of the nanoparticle (size range: 0.7 nm – 7 µm)[18,19]. DLS is very useful to assess the state of aggregation of the nanoparticles in aqueous solution. However, for the characterization of QDs, using both techniques can be helpful to solve ambiguities that may occur when using only one technique.

1.1.4 Nanotoxicology

Nanotoxicology is a new interdisciplinary field that combines different areas like chemistry, physics, biology, medicine, and toxicology. This new scientific area goes hand in hand with the development of new nanomaterials, and it operates by developing nanotoxicology studies. [20] To date, the results from several studies were enough to suggest that nanoparticles present new and unusual risks, however, these same studies give little information on how these risks can be identified, assessed and controlled. Nonetheless, there are many new nanomaterials being manufactured every year and appearing in the environment. [21] The hazard effects of nanoparticles are likely to occur in very different circumstances and they have greater potential to enter and travel through the organism than other materials or larger particles. [22] The properties that make nanoparticles so unique are the same that require a closer look to avoid harmful consequences to organisms. In fact, there is no size cut-off below which nanoparticles become unsafe and this is due to the surface area of the nanoparticle and its reactivity. It is easy to comprehend that as smaller is the particle, more atoms are available at the surface increasing the energy to react and consequently rising the toxicity to living organisms. [22,23] It must be noticed that properties like size, chemical composition, shape, state of aggregation, solubility, and surface properties affect biocompatibility and potentiate harmful effects. [24,25] Exposure of organisms to nanomaterials can occur in diverse moments as during their development, manufacture, use or following disposal. Nanoparticles can enter the organism mainly by three main routes: inhalation, skin or by ingestion.

The respiratory system is the most usual entrance because nanoparticles can travel great distances in the air. It is known that nanoparticles have a natural tendency to aggregate which can be a reason that limits their entrance in the organisms. Several studies have shown that nanoparticles deposited in the lungs gain access to the rest of the body traveling through the circulatory system into the liver, spleen, heart and secondary organs. This is a cause for concern because these studies prove that there is a direct link between inhaled nanoparticles and several diseases. [24] Other studies have concluded that neuronal translocation via nasal sensory neurons is a potential route of CNS (Central Nervous System) exposure in humans with hazardous consequences such as loss of cell viability., induction of oxidative stress and dopamine depletion. [24,26]

Another route of exposure to nanoparticles is dermal exposure and is mainly due to the increasing uses of new materials containing nanoparticles as sunscreens and cosmetics. However, many studies are inconclusive although particles like QDs and fullerenes seem to enter the skin, depending on their size and surface coatings. [26] Moreover, it is important to refer that intact skin, when healthy, is an important barrier able to prevent nanoparticles from entering the body. [24]

The last route of exposure is ingestion, which can occur in organisms following ingestion of food or water, drugs or direct swallowing of nanoparticles. Subsequently, absorption occurs by the gastrointestinal tract, and it seems to be regulated by the size and surface properties of the

nanoparticles. Thus, particles that are small, hydrophobic and with neutral charge are preferably absorbed [24]. However, more studies in this scientific area are required to draw more conclusions.

After entering the body by these routes, nanoparticles can promote their harmful effects by chemical or physical mechanisms. The chemical mechanisms are commonly the production of reactive oxygen species (ROS), dissolution and release of toxic ions, disturbance of the electron/ion cell membrane transport activity, oxidative damage through catalysis, lipid peroxidation or surfactant properties. All these processes, but mainly the ROS production can lead to secondary processes that can cause cell damage and compromise cell integrity and, in more severe cases can result in cell death. Physical mechanisms are due to particle size or surface properties. These two mechanisms can lead to a biological response that may occur before or after the internalization of the nanoparticles. [26]

Thus, researchers and manufacturers are trying to keep the balance between the pros and cons of the nanoparticles regarding their unique properties and their potential toxicity. Therefore, the creation of this new field called “Nanotoxicology” is crucial to help them to follow the developments in these materials and answer the questions that are still unanswered to keep this industrial revolution as safe as possible to human health but also to wildlife.

1.2 Biological Models

Aquatic organisms are widely chosen as biological models since they are more sensitive to exposure to toxic agents than terrestrial organisms including mammals. Besides, many aquatic organisms are easy to maintain and to breed providing a suitable model to evaluate toxicity and other adverse effects of pollutants. [27]

1.2.1 Zebrafish (*Danio rerio*)

The zebrafish (*Danio rerio*) is the preferred vertebrate model in many scientific studies. Recently, it´s value in toxicology has been recognized and several studies were carried out in different scientific areas such as developmental biology, reproductive studies, teratology, genetics, neurobiology, environmental sciences and behavioural studies.[1,28,29] *D. rerio* is a freshwater fish native from the asian continent and belongs to the family *Cyprinidae* and to the order *Cypriniformes*[30,31]. Zebrafish can live in water with a temperature ranging from 6.7°C to 41.7°C and it can be classified as eurythermal (tolerance to wide temperature ranges). The ideal temperature value for zebrafish in the wild has not been set, however, for zebrafish culture the ideal temperature is about 28.5°C. The pH value is also an important factor for zebrafish housing. They can tolerate a pH range of 6 to 9.5, however, to meet the best culture conditions and promote good health the pH values must be between 7 to 8. [32] Their diets are based on insects, zooplankton and inorganic material [33] and the lifespan is about 3-5 years. [34]

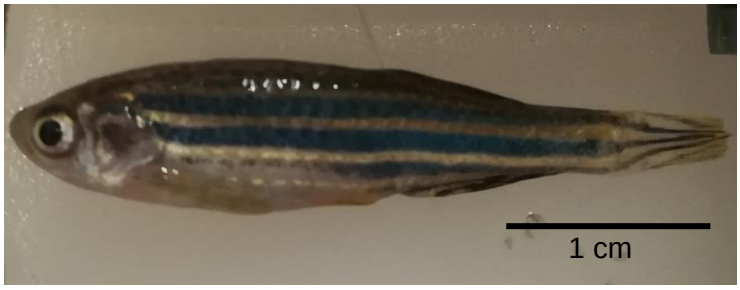

Figure 1.2 Adult zebrafish *(Danio rerio).*

Zebrafish is an excellent biological model to compare experimental research performed on vertebrates (it's more comparable to human than to invertebrate models) and its use has many benefits. [33] Thus, the main advantages in the use of Zebrafish are related to their small size, husbandry, early morphology, transparent embryo, short life circle, and well-characterized genetics.[28,33] Several human genes have homologues in Zebrafish and functional domains of proteins (Kinase ATP biding domains share 100% of identity, for example). [34] Also, cardiovascular, nervous and digestive systems of the Zebrafish are similar to human systems. [20] In addition, zebrafish species measure about 3 to 5cm in adults, which minimize costs both for the maintenance of the animals and for chemicals tested (e.g. drugs and pollutants). Moreover, is relatively easy to perform several assays with many fishes. One pair of adult fish can lay about 200-300 eggs in one morning and if properly sustained they can provide this amount of eggs every 5-7 days. [28] The unique optical properties of the transparent embryos allow the study of all stages of the development in great detail in vivo. [28,29] With all these features is easy to understand why zebrafish is one of the most successful model and although zebrafish does not replace other mammalian models, represents the first stage experiments and have already contributed for a huge development in the nanotoxicology field. [20,34] Cell culture assay is another approach for the assessment of toxicity. In fact, there are some zebrafish cell lines that can be used for cytotoxicity assays (e.g. liver, muscle).

1.2.2 *Daphnia magna*

Daphnia magna, usually referred as water flea, has been used for many years as a biological model in aquatic tests, more namely in chronic and acute tests which are the most common toxicity tests. [35,36] Daphnia are widely distributed in natural ecosystems and are well adapted to such fluctuations: low oxygen levels, high pH and wide ranges of salinity and temperatures. This organisms belongs to the family *Daphniidae,* order *Cladocera* and measures about 5-6mm in length. [37] It can reproduce asexually or sexually, depending on environmental factors, but in both cases, eggs are produced in the ovary. The sexual reproduction is mediated by external stimuli (high density of population, scarcity of food), haploid oocytes are fertilized. In

the asexual reproduction, parthenogenic oocytes remain diploid and embryogenesis happens deprived of fertilization. [38,39] In fact, *D. magna* is a key species in lake ecosystems since they have a central role in the freshwater food web and because they feed on phytoplankton and are prey to vertebrate and invertebrate predators. Moreover, they are filter feeders and can filter about 400ml of water per day. [40] Several studies used *Daphnia* as a biological model inareas such as evolution, ecology, ecotoxicology, predator-induced polyphenisms, and genomics. [38]

Figure 1.3 *Dapnhia magna.*
Source: Fineartamerica. 2019. Daphnia magna. [ONLINE] Available at: https://fineartamerica.com/featured/10-water-flea-daphnia-magna-ted-kinsman.html [Accessed 19 September 2019].

The main factors that make Daphnia a good biological model are the short life cycle, parthenogenic reproduction, easily cultured in the laboratory using little space and it is sensitive to a broad range of aquatic contaminants[35,36]. Since *Daphnia sp.* has a huge contact with the surrounding medium, it has a great potential to bioaccumulate pollutants (e.g. heavy metals, chemicals, nanoparticles) present in the aquatic medium. The contaminated Daphnia can increase toxicity in the organisms at higher trophic levels if they use the *daphnids* as food source[40].

1.2.3 Human hepatoma cell lines (HepG2)

Another approach in toxicology studies is through *in vitro* cell models. Since the liver is the principal organ responsible for metabolism, its hepatocytes contain most of the enzymes required for the metabolic reactions. For this reason, hepatocytes and more properly human primary hepatocytes are considered a model for drug metabolism and toxicity of xenobiotics .[41] HepG2 (Fig. 1.4) is a human liver cancer cell line derived from the liver tissue of well-differentiated hepatocellular carcinoma of a young boy. These cells are easy to maintain in an appropriate

culture medium and incubation at 5% CO_2, and are widely used for risk assessment since they are capable to maintain several liver functions.[42].

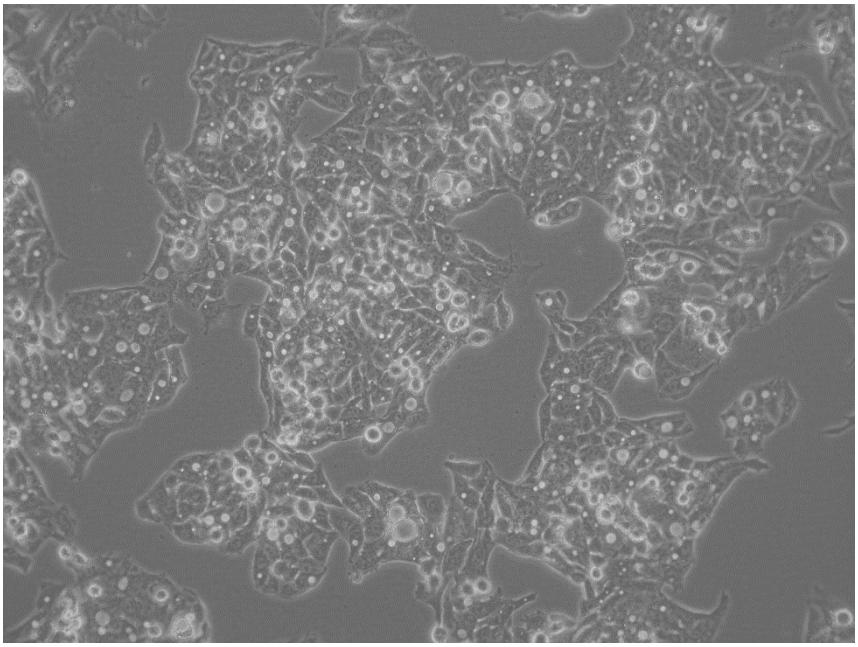

Figure 1.4 Representative image of Human hepatoma cells (HepG2). Image acquired using a inverted phase contrast microscope (Olympus, Japan) Magnification: 100x magnification.

1.3 Toxicity Assays

The increase in pollution, mainly aquatic, due to anthropogenic activities have become a global concern. Aquatic organisms are exposed to several toxic agents such as pesticides, heavy metals, nanoparticles, and others. These compounds compromise the health of thr marine ecosystems and if biomagnification occurs, the pollutants accumulated in the tissues of aquatic organisms are transferred across trophic levels of the food chain[43,44]. Thus, the increasing need for the evaluation of contaminants leads to the study and development of indicators of the biological effect(s) of these compounds in the organisms. When the natural conditions to the welfare of the organisms are compromised or change, they respond by getting sick or in the worst scenario, they can die. These changes can be due to natural or anthropogenic sources and can induce responses at the biochemical, histological, immunological or physiological level. The responses lead us to the concept of biomarker which is defined as a biological response to an environmental compound that can be measured in body fluids, cells, etc. and indicates the presence of contaminants or the magnitude of the host response. [43] Biomarkers can be antioxidant enzymes (e.g. Catalase, Glutathione-S-Transferase and Superoxide Dismutase), lipid peroxidation (MDA content), Ubiquitin and HSPs production and by measuring of the Total Antioxidant Capacity (TAC), among other markers.

All living organisms are continuously generating, transforming and consuming oxygen reactive species (ROS), which are essential to the physiological control of the cell function. Free radicals are one of the products produced from basic cellular metabolism in aerobic organisms [45]. Several mechanisms such as cellular respiration, exposure to microbial infections and the action of pollutants like cigarette smoke, alcohol, ionizing and UV radiations, pesticides, and ozone produce free radicals. However, if overproduction occurs, it can lead to oxidative stress [27,46,47]. Although reactive species can be harmful, they have essential biological effects as well, and the maintenance of “redox homeostasis” is essential for the good function of the organisms[47].

The most biological relevant ROS are molecular oxygen (O_2), superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), nitric oxide (NO^-), peroxynitrite ($ONOO^-$) and ozone. Sometimes, during the metabolic processes, a small amount of those may escape from the protective shield and cause oxidative damage in cells [27,46,47]. The formation of these ROS results from several phenomena. The superoxide anion is a nucleophile oxidizing agent and is generated by various oxidases, and it can also be formed in the mitochondrial electron transport chain for the ATP production. This anion is capable to react with hydrogen donors and dismutate molecular oxygen and hydrogen peroxide. Thus, the hydrogen peroxide comes from superoxide systems and can produce highly reactive radicals when reacting with metal ions, being stable at physiological pH and can cross membranes easily. In order to maintain the balance, hydrogen peroxide is converted in water by catalase and glutathione peroxidase [47,48] The hydroxyl radical is known to cause oxidative damage to some biomolecules. It is highly reactive but has a short lifetime (nanoseconds). Its main targets are membrane lipids, proteins and DNA. [27] Nitric oxide has unpaired electrons and is synthesized from L- arginine, oxygen, and NADPH by nitric oxide

synthase. Peroxynitrite when occurring in the protonated form is another strong oxidizing agent that has a similar action as hydroxyl by promoting oxidative damage, doing depletion of sulfhydryl groups. Ozone itself is not a reactive species but has the power to oxidize biological components in a direct form. [47,49]

Thus, there was a growing need, during the organism´s evolution, to develop suitable defence mechanisms against ROS overproduction, ie development of antioxidant mechanisms. Thus, an antioxidant is any substance present in the organism, that intercepts, delays or inhibits the oxidation of the reactive radicals. These antioxidants could be classified by several parameters, as primary and secondary, preventative or repair-systems, non-enzymatic or enzymatic, endogenous and exogenous, hydrosoluble and liposoluble and natural or synthetic [47]. Primary antioxidants are chain breakers which can scavenge radical species by hydrogen donation. The secondary ones are singlet oxygen quenchers, peroxide decomposers, metal chelators, oxidative enzyme inhibitors or UV radiation absorbers. Preventative systems block and capture radicals that are being formed and are present in call compartments. When there are damaged biomolecules, then repair systems intervene to remove damaged compounds or repair them with specific enzymes (phospholipases, peroxidases, acyltransferases). For instance, vitamin E (α-tocopherol), vitamin C (ascorbic acid), flavonoids, glutathione, selenium, chlorophyllin, plasma proteins, and β-carotene belong to the nonenzymatic category of antioxidants. The group of enzymatic antioxidants has as its main characteristic the inducibility of enzymes under oxidative stress conditions. [50] Superoxide dismutases, catalases, ascorbate peroxidases and glutathione peroxidases belong to this group of antioxidants[51]. Thus, when moderate oxidative stress occurs, there is an increase in the amounts of enzymatic antioxidants. It ss this characteristic that makes the enzymatic antioxidants, in many cases, good biomarkers. [27,47].

1.3.1 Catalase

Catalases are ubiquitous enzymes that catalyse the dismutation of hydrogen peroxide (H_2O_2) to oxygen and water and this way prevent the oxidative damage. Catalases are classified as oxidoreductases and are mainly located in peroxisomes. Structurally, are a homotetramer, that is, have four subunits and each of one contains a porphyrin heme group at the active site [50,52,53]. The heme groups in a non-polar pocket with hydrophobic strait channels that confers hydrogen peroxide selectivity [48]. Are these structural features that allow catalases to react with hydrogen peroxide as the following reaction (1):

(1) $$2H_2O_2 \rightarrow 2H_2O + O_2$$

As previously referred, catalases are one of the most important enzymes during the oxidative stress because they are the principal scavenger of hydrogen peroxide that can damage cells. When occurs an increase in catalase activity, can be assumed that is an adaptative response to overcome the injury in tissues, reducing toxic levels of hydrogen peroxide.

Accordingly, in toxicity assays, a high concentration of the catalase levels can be an indicator of high concentrations of oxygen peroxide levels. [50]

1.3.2 Glutathione-S-Transferase (GST)

Glutathione Transferases (GST) are a large group of proteins with great importance in detoxification of xenobiotics and endogenous molecules in nature. This enzyme is a dimer composed of two identical subunits that contains the active site (one in each subunit). [54] The Glutathione-S-Transferase is present in all type of organisms (e.g. mammals, plants, yeast, insects and bacteria) and is usually found in the liver tissues that have an important role in detoxification processes. The reaction catalysed by GST is the conjugation of glutathione (GSH) with a hydrophobic co-substrate with an electrophilic centre. These reactions can be substitutions, additions or isomerizations according to the enzyme and the type of the second substrate. [55] These substrates can be mainly, by-products of oxidative stress and polycyclic aromatic hydrocarbon epoxides that come from actions of phase 1 cytochrome P450. After this reaction, molecules become more soluble and less reactive, which becomes the excretion process easier [54,56,57]. In toxicity assays, is common to measure GST activity which indicates the effects of xenobiotics in the organisms.

1.3.3 Heat Shock Proteins (HSPs)

Heat shock proteins (HSPs) are a family of proteins with an important role during oxidative stress. Under normal physiologic conditions, HSPs are expressed at low levels but in oxidative stress scenarios like hyperthermia, oxidative damage, physical injury or chemical stressors, its expression increases abruptly [58]. The main function of HSPs is acting like chaperones preventing protein aggregation and targeting inappropriately folded proteins to degradation pathways. HSPs are subdividing among five families according to their molecular weight, amino acid sequence homologies and functions [59]. The HSP70 family has proteins with a molecular weight between 68 KDa and 75 KDa and is the most well-studied and characterized class of heat shock proteins. Members of this family are usually located in the cytosol, endoplasmic reticulum, and mitochondria and are the first to be induced under stress conditions [59]. Thus, HSPs act as a good biomarker due to its inducibility of expression (relevant increase) during oxidative stress conditions.

1.3.4 Lipid Peroxidation

One major concern regarding the overproduction of free radicals is lipid peroxidation. Lipid peroxidation is a harmful chain of reactions that results from oxidative stress. The main targets of these reactions are some components of the biological membranes that become damaged [46,60]. Lipid peroxidation gives rise to several products that are unstable and tend to degrade quickly in toxic secondary products. Malondialdehyde (MDA) is a three-carbon, low molecular weight aldehyde with two or more methylene-interrupted double bonds and is the main product of lipid peroxidation. [46,61] Due to its toxicity it should be considered as more than a biomarker because it is highly reactive and capable to react with biological molecules inside and outside the cell. As a biomarker, the measurement of the quantity of the MDA is a good indicator of the level of lipid peroxidation. TBARS method is the simplest and most frequently used method for MDA quantification [46,60,61].

1.3.5 Superoxide Dismutase (SOD)

Superoxide dismutases (SOD) are a group of metal-containing enzymes that have an important role in detoxification of reactive oxygen species, more properly, the superoxide anion. SOD catalyses the breakdown of superoxide anion into molecular oxygen and oxygen peroxide. Superoxide dismutases could use copper, zinc or manganese as a cofactor and are widely distributed prokaryotic and eukaryotic cells [62,63]. The reaction carried out by SOD is the following (2):

(2) $$2O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$$

This reaction is preventive, that is, prevents the formation of a compound more aggressive like peroxynitrite or hydroxyl radical, and after that, the reaction catalysed by catalase can occur.[64] Therefore, the action of both enzymes needs to be concerted to maintain balance in the organism´s body and keep it safe from reactive oxygen species. Therefore, superoxide dismutases are part of the first line of defence against oxidative stress and can serve as an earlywarning biomarker for pollution. [63,64]

1.3.6 Ubiquitin

Ubiquitin is a polypeptide with 76 amino acids that can be found in cells either free or covalently bonded with other proteins. [65,66] Ubiquitins are involved in the regulation of cellular signalization and homeostasis, i.e. ubiquitin marks damaged proteins, through a covalent bond, for degradation in a ubiquitin-proteasome system [67]. Proteasome is the preferential machinery for degradation of normal and damaged proteins. Several types of oxidized cells and tissues are

degraded in a selective way. Thus, the ubiquitin-proteasome pathway (UPP) constitutes a secondary defence mechanism against oxidative stress, degrading abnormal proteins. During the oxidative stress, the amount of ubiquitin conjugates increases, and it could be useful as a biomarker. [65]

1.3.7 Cell viability assay – MTT

MTT assay is a widely used method to measure viable cells without need the process of counting cells. The principle of this assay is the dehydrogenases reduction of MTT (3-[4,5dimethylthiazol-2-yl]-2,5diphenyl tetrazolium bromide) in MTT formazan according to the reaction (3)

(3) MTT (yellow) —dehydrogenases→ MTT fromazan (purple)

In this reaction, the change of colour (yellow to purple) occurs due to the mitochondrial activity in living cells. This activity, and the change of colour is usually constant, so if any increase or decrease in colour is detected it could be linearly related to its activity. Only living cells are capable to carry this reaction; thus, the number of viable cells can be detected by measuring formazan concentration reflected by the absorvance at 540 nm. When the absorvance at 540nm decreases it reflects a reduction on cell viability.

Chapter 2

Objectives

2 Objectives

In recent decades, developments in the technological and industrial fields have raised concerns about exponential production of nanomaterials and the consequent discharge of industrial effluents, containing these materials, into the aquatic environment, posing a serious threat to aquatic ecosystems. Thus, the main objectives of the present study are: (i) evaluation of the sublethal effects in *D. rerio*, *D. magna* and human cell lines (HepG2) after exposure to different concentrations of QDs (ZnS and CdS), singly and combined; (ii) the study of trophic transfer through zebrafish ingestion of daphnia previously exposed to different concentrations of QDs.

Therefore, to assess the effects of exposure to QDs and to evaluate the trophic transfer, several biomarkers will be analysed in organism´s tissues: catalase, GST, LPO (MDA content), SOD, HSP70, Ubiquitin and TAC.

An additional goal of the present work is to obtain information on the targeted QDs toxicity to the aquatic biota allowing to adopt future strategies to minimize the risk to aquatic ecosystems.

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Chapter 3

Materials and Methods

3 Experimental Procedure

3.1 Quantum Dots (QDs) synthesis

The QDs synthesis was carried out in the Cenimat research laboratory by the researcher "David Sousa". To synthesize the Zinc Sulphide QDs and the Cadmium Sulphide QDs, zinc acetate and cadmium acetate were added, respectively, to 1-dodecanethiol, and heated in a microwave reactor up to the constant temperature of 300ºC for 25 minutes. The suspension was then centrifuged (7,000×g), and the pellet washed with ethanol 96%. Afterwards, the resultant powder was washed with chloroform and stored in the dark.

3.2 Preparation of QDs stock solutions

The Stock solutions (1 g/L) were prepared by weighing 10 mg of QDs using an Analytical balance (Nimbus, Adam Equipment USA), in a microtube followed by the addition of 1 mL of Chloroform 100% (Panreac, USA). Then the QDs solution was centrifuged (Elmi Centrifuge & Vortex, Latvia) at 2,000 ×g for 1 minute at room temperature, and the supernatant was removed. Then, 1.0 mL of distilled water was added to wash out the chloroform remaining. The washing steps were repeated and then the QDs solution was transferred to falcon tubes and volume adjusted to 10 mL. Finally, the Stock Solutions of each QD type were ultra-sonicated (10 min, room temperature) using an ultrasonic bath (J-P Selecta Ultrasons H-D, Spain). The Stock Solutions were stored in the dark at 4°C until further use.

3.3 Quantum dots (QDs) characterization

The quantum dots' size distribution and morphology were analysed by scanning electron microscopy (SEM), transmission electron microscopy (TEM) and dynamic light scattering (DLS).

3.3.1 Scanning electron microscopy (SEM)

For the SEM analysis, dispersions of all the QDs (CdS and ZnS) were applied on a carbon-coated adhesive, dried at room temperature and examined with a 1.00 to 2.00 keV field; scanning electron microscope (Microscope Carl Zeiss AURIGA). SEM images were obtained at the CENIMAT facilities at FCT-NOVA, Portugal.

3.3.2 Transmission electron microscopy (TEM)

For transmission electron microscopy (TEM), samples were dispersed in ethanol, dried on a carbon membrane coated grid and examined on a JEOL 100SX instrument at 100 kV using a photographic plate. Then, plates were scanned, and the image analysis was performed using image J software (NIH, USA) after optimizing the parameters (area of analysis = 4–200 nm^2; circularity = 0.4–1). The TEM analyses were performed at Egas Moniz – Cooperativa de Ensino Superior electron microscopy facilities.

3.3.3 Dynamic light scattering (DLS)

The instrument used for DLS characterization was NANO PARTICLE ANALYZER SZ-100 from HORIBA (Japan). The Laser specifications were: Laser Diode of Showa Optronics Co., Ltd., model JUNO10G-HO with an Output of 10 mW (wavelength: 532 nm) and at 25ºC. Measurements obtained were processed using HORIBA SZ-100 software, furnished by HORIBA (Japan).

The same nominal concentrations of QDs used for conducting the exposure tests (10, 100 and 1000 μg/l QDs), were re-dispersed and prepared in a volume of 1.0 ml of Milli-Q. water to directly measure by DLS. Then, ZnS and CdS (QDs), singly and combined were re-dispersed using an ultrasonic bath (Selecta, Spain) to obtain the same dispersions used in exposure tests. In general, all samples showed aggregation behaviour when compared to the sizes determined by SEM and TEM. Due to their surface nature ZnS and CdS (QDs), singly and combined, the analysed QDs tend to aggregation when using water as re-dispersion medium, however, this trend to aggregate was expected.

Other re-dispersion mediums were explored to achieve better characterization such as chloroform, SDS, SDS + methanol, and dilutions of them. Dried samples of both QDs species were weighted and re-dispersed in water:SDS, water:SDS:methanol and chloroform (the synthesis medium). With the aid of the ultrasonic bath Selecta, Spain) for 10 minutes at 25ºC, the samples were re-dispersed and measured by DLS.

3.4 *In vivo* assays

3.4.1 Test Organisms

The selected biological models used to o perform the experiments were a freshwater fish (*D. rerio*) and a freshwater crustacean (*D. magna*).

The fish were obtained from commercial suppliers (Aquaplante, Portugal), transported to the laboratory facilities and acclimated before the beginning of the exposure assays.

The daphnids were obtained from Aquário Vasco da Gama (Portugal), transported to the laboratory for acclimation and reproduction before being used in exposure tests.

3.4.2 Acclimation

Before the beginning of the assay *D. rerio* were acclimated for one week in a closed-circuit system with filtered de-chlorinated tap water, at a pH of 7.4 ± 0.2, temperature of 20 ± 1ºC, photoperiod: 12hL:12hD, with continuous aeration enough for keeping the dissolved oxygen always higher than 6 mg/L. Daphnids were acclimated for one week in a closed system with artificial water with minerals made in laboratory (for 5L of distilled water was added 989.35 mg of $CaCl_2$ (Sigma-Aldrich, USA), 411mg of $MgSO_4$ (LABCHEM, Portugal), 324mg $NaHCO_3$ (Panreac, Spain), 29mg of KCl (Sigma-Aldrich, USA) and 0.01mg of Na_2SeO_3 (Sigma-Aldrich, USA)), at a pH of between 7.5 and 8.2, the temperature of 25 ± 1ºC, photoperiod: 12hL:12hD and with continuous aeration enough for keeping the dissolved oxygen always higher than 6 mg/L. The physical and chemical parameters were checked using an ammonia test kit (API, USA), a dissolved O_2 and pH meter (Hanna Instrumentation, USA), and a manual thermometer.

3.4.3 Exposure assays

Bioassay 1: Exposure of *Danio rerio* to QDs (ZnS, CdS), singly and combined

After the acclimation period, *D. rerio,* (N=50; 0.27 ± 0.15 g; 2.74 ± 0.37 cm standard length), of both sexes, with less than one year of age, were randomly distributed into 1000 mL volume glass containers, in groups of four fishes per tank. Fish were exposed for seven days to different concentrations of ZnS QDs and CdS QDs (10 µg/L, 100 µg/L and 1000 µg/L) singly or combined. A control tank containing filtered and de-chlorinated tap water was used as control. A scheme of the bioassay is shown in figure 3.1.

The fish were tested under a constant temperature of 20 ± 1ºC, pH of 7.5 ± 0.2, photoperiod: 12hL:12hD and continuous aeration. Some physical and chemical parameters as pH, TºC, ammonia and dissolved oxygen were monitored during the assay. The experiment conditions in each tank were renewed every 48 hours, and the assay had a duration of seven days (according to OECD guidelines)[68]. During the experiment period, fish were daily fed *ad libitum* with commercial dry food (Eco vita). Glass containers were monitored constantly for the counting of dead fish.

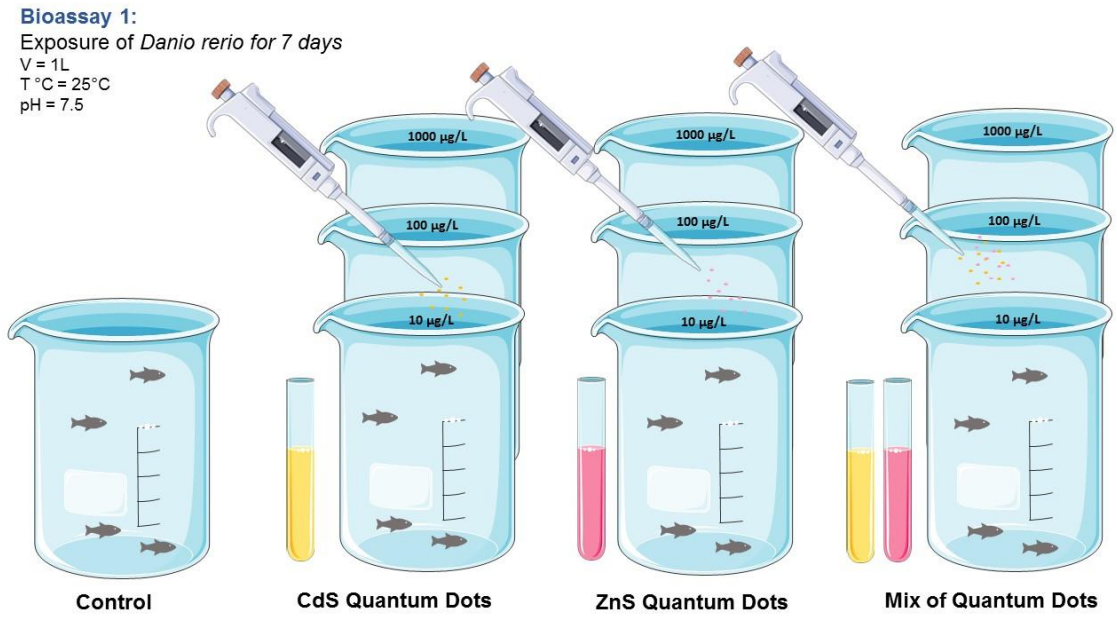

Figure 3.1 Schematic representation of Bioassay 1.

Bioassay 2: Exposure of *Daphnia magna* to QDs (ZnS and CdS), singly and combined

After the acclimation period, *D. magna,* (N=120), of both sexes, with less than one month of age, were randomly distributed into 0.1L volume glass containers, in groups of 10 daphnids per container. Daphnids were then exposed for 21 days to different concentrations of ZnS QDs and CdS QDs (10 µg/L, 100 µg/L and 1000 µg/L) singly or combined. A control tank containing clean water was used as control. A scheme of the bioassay is shown in figure 3.2.

The daphnids were tested under a constant temperature of 20 ± 1ºC, pH of 7.9 ± 0.2, photoperiod: 12hL:12hD and continuous aeration. Some physical and chemical parameters as pH, temperature, ammonia and dissolved oxygen were monitored during the assay. The experimental conditions in each tank were renewed every seven days, and the assay had a duration of 21 days (according to OECD guidelines)[69]. During the assay, daphnids were fed every two days with a mixture of chlorella (Shine), bread yeast (Levital) and blood gnat (Ocean Nutrition, Europe). Glass containers were monitored constantly for the counting of dead daphnia.

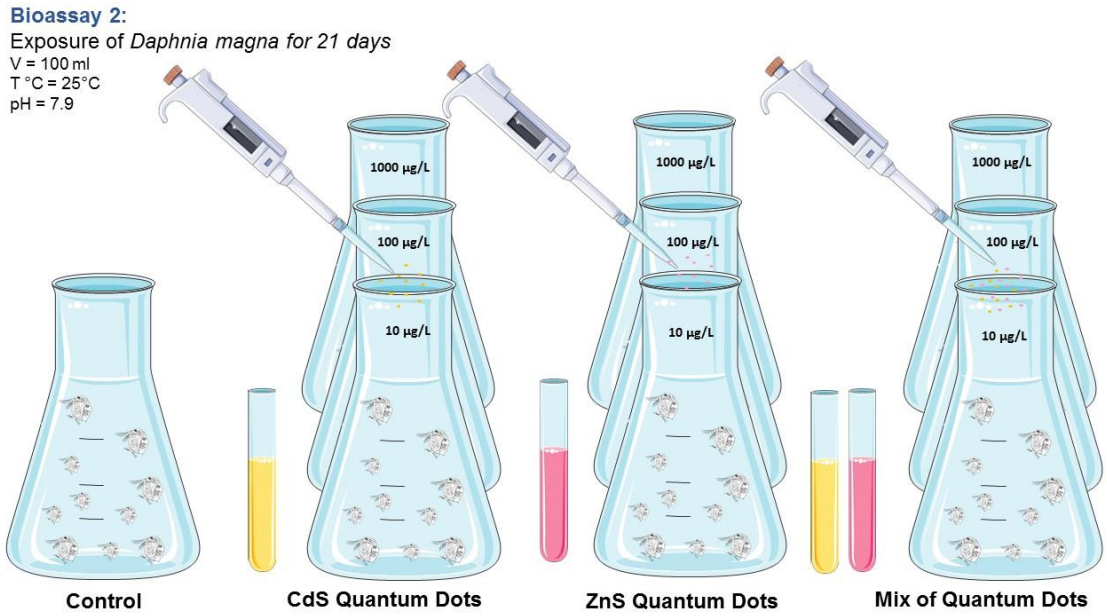

Figure 3.2 Schematic representation of Bioassay 2.

Bioassay 3: Trophic transfer of QDs: *Danio rerio* ingestion of exposed *Daphna magna* to QDs (ZnS and CdS) singly and combined

First, after the acclimation period, adult *Daphnia magna,* (N=240), of both sexes, with less than one month of age, were randomly distributed into 0.25L capacity glass tanks, in groups of 20 daphnids per tank. Then, Daphnids were exposed for 96 hours to different concentrations of ZnS and CdS (QDs) (10 µg/L, 100 µg/L and 1000 µg/L), singly or mixed. A tank containing clean water was used as control. A scheme of the bioassay is shown in figure 3.3.

The daphnia were tested under a constant temperature of 20 ± 1ºC, pH of 7.9 ± 0.2, photoperiod: 12hL:12hD and continuous aeration. During the assay, daphnids were fed on the first day with a mixture of chlorella (Shine), bread yeast (Levital) and blood gnat (Ocean Nutrition, Europe). Tanks were monitored constantly for the counting of dead daphnia.

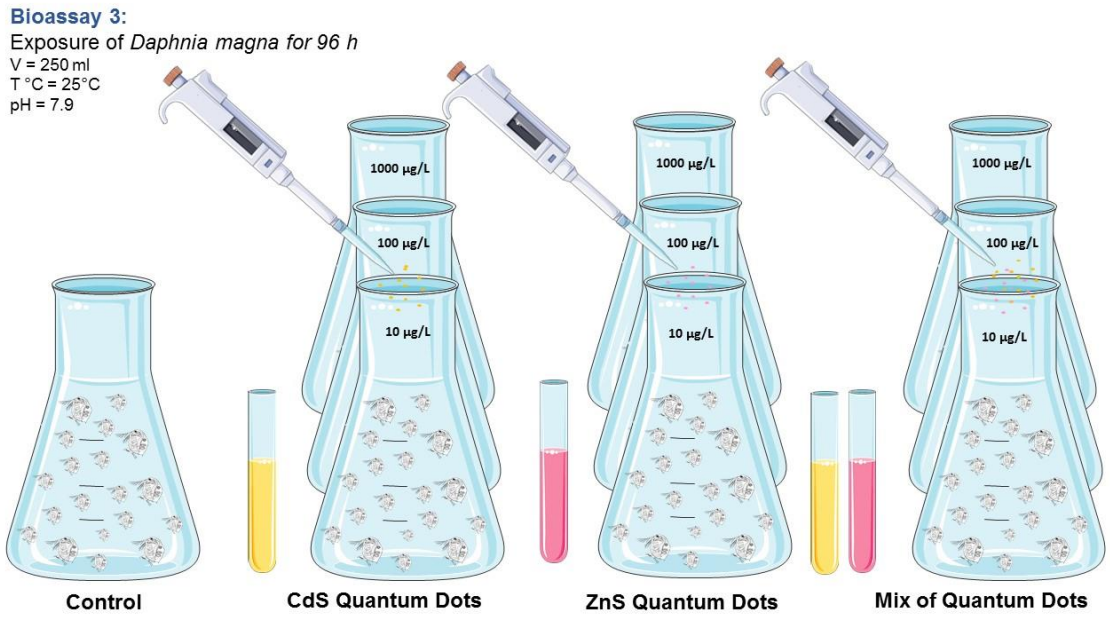

Figure 3.3 Schematic representation of bioassay 3: Exposure of *D. magna* for 96h.

Previously acclimated *Danio rerio,* (N=40; 0.215 ± 0.084 g; 2.788 ± 0.289 cm standard length), of both sexes, with less than one year of age, were randomly distributed into 0.3L capacity glass tanks, one per tank and four per group. Fish were exposed for 48 hours to the Daphnids, previously exposed to the different concentrations of QDs (singly and combined). A scheme of the bioassay is shown in figure 3.4.

The *Danio rerio* exposure assays were performed for 48h, under a constant temperature of 20 ± 1ºC, pH of 7.5 ± 0.2, photoperiod: 12hL:12hD and continuous aeration. The lasted for 48 hours. In the first day of the experiment, Zebrafish were fed with three contaminated daphnids. Tanks were monitored constantly for the counting of dead zebrafish or the number of ingested daphnids. An additional sub-sample of Daphnia (n=4) was collected for further biochemical analyses.

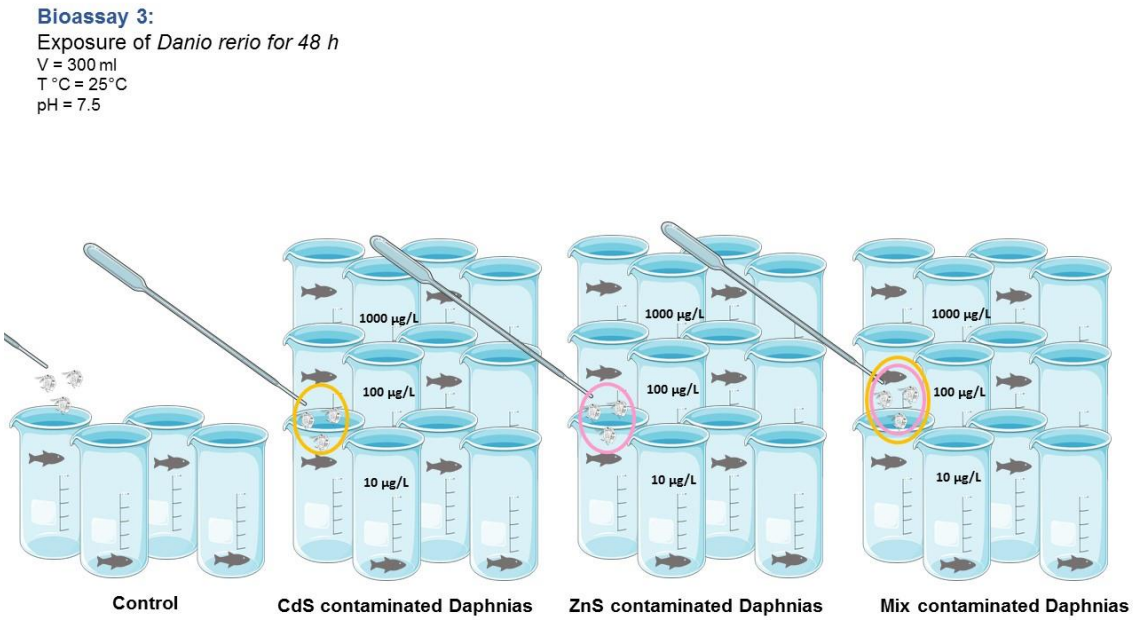

Figure 3.4 Schematic representation of bioassay 3: Exposure of *D. rerio* for 48h

3.4.4 Samples collection and processing

3.4.4.1 *Danio rerio*

Fish were sampled after 7 days of exposure (bioassay 1) or after 48h of exposure (bioassay 3) and were sacrificed by freezing at - 80° for 5 minutes. The fish were weighed and measured (see annexes). The whole fish was homogenized (Ika T10 basic, Germany) on ice-cold conditions with 3.0 mL of phosphate buffered saline solution (PBS; 140 mM NaCl, (Panreac, Spain); 10 mM Na_2HPO_4, (Sigma-Aldrich, USA); 3 mM KCl, (Merck, Germany); 2 mM KH_2PO_4, pH= 7.40, (Sigma-Aldrich, USA). Tissue homogenates were centrifuged in 1.5 mL microtubes, for 15 min at 10,000 ×g, at 4 ºC (VWR, model CT 15RE from Hitachi Koki Co., Ltd, Japan) and frozen at -80 ºC until further analyses. All biochemical analyses were performed at least in duplicate.

3.4.4.2 *Daphnia magna*

Daphnids were sampled after 21 days of exposure (bioassay 2) or after 96h of exposure (bioassay 3) and were euthanized by freezing at - 80°C. The Whole Daphnids were homogenized (Ika T10 basic, Germany) two specimens per microtube (1.5 mL) on ice-cold conditions with 450 µL of phosphate buffered saline solution (PBS; 140 mM NaCl, (Panreac, Spain); 10 mM Na_2HPO_4, (Sigma-Aldrich, USA), 3 mM KCl, (Merck, Germany); 2 mM KH2PO4, pH= 7.40, (Sigma-Aldrich, USA). Tissue homogenates were centrifuged in 1.5 mL microtubes, for 15 min at 10,000×g, at 4 ºC (VWR, model CT 15RE from Hitachi Koki Co., Ltd, Japan) and frozen at -80 ºC until further analyses. All biochemical analyses were performed in duplicate.

3.4.5 Bradford Assay

The Bradford Assay was performed according to the Bradford method [70]. First, BSA (NzyTech, 98%) protein standards were prepared. It was prepared a BSA stock solution (4 mg/mL) and serial dilutions were done with PBS to attain a range of concentrations from 0 to 4 mg/mL. Then, in a 96 well microplate (Greiner bio-one, Germany), it was added 190 µL of Bradford reagent into each well and 10 µL of BSA Standard in the standard wells to make the calibration curve and 190 µL of Bradford reagent and 10 µL of the sample in the sample wells. Finally, the absorbance was read at 595 nm in a microplate reader (Synergy HTX, Biotek, USA). With a calibration curve constructed with BSA protein standards the total protein concentration was determined for each sample (see annex figure 8.3). The total protein results were later used for biomarkers normalization.

3.4.6 Catalase assay

The catalase (CAT) activity was determined according to procedures first described by Aebi and adapted for 96-well microplates [71]. The method is based on the reaction of the enzyme with methanol in the presence of an optimal concentration of H_2O_2. The formaldehyde produced is measured colourimetrically with 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (Purpald) as the chromogen. Purpald specifically forms a bicyclic heterocycle with aldehydes, which upon oxidation changes from colourless to a purple colour.

The assay was performed in a 96 well microplate. The formaldehyde standards were prepared from a 4.25 mM formaldehyde (Sigma-Aldrich, USA)) stock solution to obtain to obtain a range of concentrations from 0 to 75 µM. Then, it was added 100 µL of Assay Buffer, 30 µL of methanol (Scharlab, Spain) and 20 µL of formaldehyde standard or sample, per well. This step was performed in duplicate. The reaction was initiated by adding diluted Hydrogen Peroxide (30%) (Sigma-Aldrich, USA) to all the wells and then the microplate was incubated for 20 minutes on a shaker at room temperature. The previous reaction was stopped with the addition of 30 µL of diluted Potassium Hydroxide 10 M (Chem-Lab, Belgium) and then 30 µL of Purpald (Sigma-Aldrich, USA) was added to each well. The microplate was covered aluminium foil and then incubated for 10 minutes on the shaker at room temperature. Afterwards, 10 µL of potassium periodate (Sigma-Aldrich, USA) was added to each well and then allowed to incubate during 5 minutes on a shaker at room temperature. The absorbance was read in a microplate reader (Synergy HTX, Biotek, USA), at 540 nm. With the formaldehyde standards, a calibration curve was built, and the total enzyme activity was determined by the ratio of total formaldehyde concentration to the reaction time (4).

$$(4) \quad Catalase\ activity\ (nmol\ min^{-1}mL^{-1}) = \frac{Formaldehyde\ (sample)\ \mu M}{tr}$$

tr – reaction time – 20 minutes

Results are expressed in relation to the total protein concentration of the sample.

3.4.7 Glutathione-S-transferase assay

GST activity was determined following the procedure described by Habig *et al*. [72] and optimized for 96-well microplates. This assay utilizes 1-chloro-2,4-dinitrobenzene (CDNB) as a substrate which is suitable for the broadest range of GST isozymes. Upon conjugation of the thiol group of glutathione to the CDNB substrate, there is an increase in the absorbance at 340 nm.

To perform this assay, a reaction mixture solution (10ml) was prepared by adding 9.8 ml of a substrate solution (PBS, phosphate buffered saline solution), 100 µL of 200 mM glutathione reduced (Sigma-Aldrich, USA) and 100 µL of 100 mM CDNB (Sigma-Aldrich, USA). Then, in a 96 well microplate, 20 µL of sample and 180 µL of the mix solution, were added to each well. The absorvance was read at 340 nm in a microplate reader (Synergy HTX, Biotek, USA), every minute for 6 minutes to determine the total enzyme activity. The change in the absorvance per minute was then estimated, and the reaction rate was calculated using a CDNB extinction coefficient of 5.3 mM^{-1} according to the equation below (5).

$$(5)\ \ldots\ldots GST\ specific\ activity\ (nmol\ mL^{-1}min^{-1}) = \frac{\text{Abs } 340/\text{min x V (mL) x dil}}{\varepsilon\ (\text{CDNB}) \text{x Vsample(mL}} x1000$$

dil – the dilution factor of the original sample; **εmM ($mM^{-1}cm^{-1}$)** – extinction coefficient for CDNB conjugate at 340 nm for test in Sigma 96-well plate = 5.3 mM^{-1} (path length -0.552 cm); **V** – Reaction volume: 0.2 ml; **Vsample** – the volume of the sample tested.

Results are expressed in relation to the total protein concentration of the sample.

3.4.8 Heat Shock Protein (HSC/HSP) assay

The heat shock response (HSR) was assessed from heat shock protein 70 (HSP70) which was quantified through Enzyme-linked Immunosorbent Assay (ELISA) based on a protocol from Njemini *et al.* [73].

The ELISA assay was performed using a 96-well microplate (Greiner bio-one, Austria). Purified HSP70 active protein (OriGene Technology, USA) was used to prepare standards ranging from 0 to 2.0 µg/ml by successive dilutions.

Then, 50 µL of each sample or standard were added to the microplate and left incubating overnight at 4 ºC. Then, the microplates were washed three times with a 0.05% PBS-Tween-20 solution (Sigma-Aldrich, USA) and 200 µL of blocking solution, composed by PBS with 1% BSA (Nzytech, Portugal)) was added to each well and left incubate at 37 ºC for 90 minutes in the incubator (Labnet, USA). After that, the microplates were washed again (3X with PBS and 0.05% of Tween-20 solution) and 50 µL of primary antibody solution (anti-HSP70, Santa Cruz Biotechnology; diluted to 1 µg/mL in a 1% BSA solution) was added to each well and incubated overnight at 4 ºC. After another washing step, 50 µL of secondary antibody solution (anti-mouse IgG Fc specific - alkaline phosphatase, Sigma-Aldrich, Germany; diluted to 1.0 µg/mL in 1% BSA solution) were added to each microplate well and incubated for 90 min at 37 ºC. After another wash, 100 µL of alkaline-phosphatase substrate (composed by 100 mM NaCl (Panreac, Spain), 100 mM Tris-HCl (Sigma-Aldrich), 50 mM MgCl2 (Sigma-Aldrich, USA) and 27 mM PnPP (4-Nitrophenyl Phosphate disodium salt hexahydrate, pH 8.5, Sigma, Germany), were added to each microplate well and incubated for 30 min at room temperature. Finally, 50 µL of stop solution (3M NaOH, Panreac, Spain) were added to each microplate well and the absorbance measured at

405 nm, using a microplate reader (Synergy HTX, Biotek, USA). With the HSP70 standards, a calibration line was made, and the HSP70 content in each sample was determined. Results are expressed in relation to the total protein concentration of the sample.

3.4.9 Lipid Peroxidation assay

The lipid peroxidation assay followed the thiobarbituric acid reactive substances (TBARS) protocol [74].

Then, 5 µL of sample or MDA standard, 45 µL of phosphate buffer (pH 7.0 – 7.4), 12.5 µL of sodium dodecyl sulfate (8.1%) (Merk, Germany), 93.5 µL of trichloroacetic acid (Panreac, Spain) (20% , pH 3.5), 93.5 µL thiobarbituric acid (1 %) (Sigma-Aldrich, USA), 50.5 µL MilliQ ultrapure water were added to a 1.5 mL microtube. Each tube was then centrifuged for 1 min at 2,000 ×g, the lids were then punctured with a needle and microtubes were incubated in boiling water for 10 min. After that, the microtubes were placed on ice for a few minutes for cooling. Next, 62.5 µL of MilliQ ultrapure water was added to the microtubes. Then, 150µL of each microtube content was added into a 96-well microplate wells and absorbance was read at 530 nm (Synergy HTX, Biotek, USA) in a microplate reader. A calibration curve was prepared with MDA standards ranging from 0 to 0.1 µM for MDA quantification. Results are expressed in relation to the total protein concentration of the sample.

3.4.10 Superoxide Dismutase assay

The Superoxide dismutase assay followed the nitroblue tetrazolium (NBT) method [75]. In this method, superoxide radicals ($\cdot O_2^-$) are generated by the reaction of xanthine with xanthine-oxidase (XOD) that reduces NBT to formazan, which is assessed spectrophotometrically at 560 nm. SOD competes with NBT for the dismutation of $\cdot O_2^-$, inhibiting its reduction. The percentage of inhibitions used as a measure of SOD activity.

The assay was performed using a 96-well microplate where 200µL of 50mM phosphate buffer (pH 8.0) (Sigma-Aldrich, USA) was added to each well, followed by 10 µL of 3 mM EDTA (Riedel-Haën, Germany), 10 µL of 3 mM xanthine (Sigma-Aldrich, USA), 10 µL of 0.75 mM NBT (Sigma-Aldrich, USA) and 10 µL of sample. The reaction was initiated by the addition of 10 µL of XOD (Sigma-Aldrich, USA) and the absorbance at 530 nm was recorded every 5 min, using a plate reader (Synergy HTX, Biotek, USA) until reach 15 minutes. Negative controls included all components except sample, producing a maximal increase in absorbance at 560 nm, which allowed determining the inhibition percentage per minute, caused by SOD activity. The SOD results are expressed as a percentage (%) of inhibition that was calculated by the following equation (6).

$$(6)\ \%\ of\ inhibition = \frac{Abs\ per\ minute\ (negative\ control) - Abs\ per\ minute\ (sample)}{Abs\ per\ minute\ (negative\ control)}\ x\ 100$$

Results were expressed in relation to the total protein concentration of the sample

3.4.11 Total Antioxidant Capacity (TAC) assay

Total antioxidant capacity (TAC) of the samples was determined using a modified protocol according to the Trolox equivalent antioxidant capacity principle, firstly reported by Miller *et al.* [76] The assay measures the ability of antioxidants to scavenge the stable radical cation ABTS+ (2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid)), a blue-green chromophore that decreases in its intensity in the presence of antioxidants.

To perform this assay, 10 µL of the sample was mixed with 10 µL of myoglobin (Sigma-Aldrich, USA) at 90 µM and 150 µL of ABTS (Alfa Aesar, Germany) at 600 µM. Afterwards, 40 µL of hydrogen peroxide at 500 µM was added to initiate the reaction. Trolox (Sigma-Aldrich, USA) standards were prepared using a concentration range from 0 to 0.330 mM to construct a calibration curve for TAC quantification. The microplate was incubated for 5 minutes at room temperature and then the absorvance was measured at 415 nm.

Results are expressed in relation to the total protein concentration of the sample.

3.4.12 Ubiquitin assay

Ubiquitin was quantified through indirect ELISA as described by Njemini *et al*. and adapted to a 96-well microplate as previously described by Rosa *et al.* [73,77]

The assay was performed in a 96 well microplate (Greiner bio-one, Austria). In this assay, 50 µL of each sample or standard were added to the microplate wells and left incubating overnight at 4 ºC. Then, the microplates were washed three times with a 0.05% PBS-Tween-20 solution (Sigma-Aldrich, USA) and 200 µL of blocking solution containing PBS with 1% BSA (Nzytech, Portugal) was added to each well and incubated at 37 ºC for 90 minutes (Labnet, USA). Afterwards, the microplates were washed again (3X with PBS and 0.05% of Tween-20 solution) and 50 µL of primary antibody solution (Ub (P4D1) Sc-8017, mouse monoclonal IgG, Santa Cruz Biotechnology, Portugal; diluted to 1 µg/mL in a 1% BSA solution) was added to each well and incubated overnight at 4 ºC. After another washing step, 50 µL of secondary antibody solution (anti-mouse IgG Fc specific - alkaline phosphatase, Sigma-Aldrich, Germany; diluted to 1 µg/mL in 1% BSA solution) were added to each microplate well and incubated for 90 min at 37 ºC. After

another washing stage, 100 µL of alkaline-phosphatase substrate (composed by 100 mM NaCl (Panreac, Spain), 100 mM Tris-HCl (Sigma-Aldrich), 50 mM MgCl2 (Sigma-Aldrich, USA) and 27 mM PnPP (4-Nitrophenyl Phosphate disodium salt hexahydrate, pH 8.5, Sigma, Germany), were added to each microplate well and incubated for 30 min at room temperature. Finally, 50 µL of stop solution (3M NaOH, Panreac, Spain) were added to each microplate well and the absorbance measured at 405 nm, using a microplate reader (Synergy HTX, Biotek, USA). Ubiquitin standards of purified ubiquitin active protein (Santa Cruz Biotechnology, Portugal), were prepared using concentration range from 0 to 0.8 µg/ml by successive dilutions to build a calibration curve. The Ubiquitin content in each sample was then quantified. Results are expressed in relation to the total protein concentration of the sample.

3.5 *In vitro* assays

3.5.1 Culture of HepG2 cells

Human hepatoma cells (HepG2) were a kind gift from Dr. Maria João Silva ((Instituto Nacional de Saúde Doutor Ricardo Jorge -INSA). Cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) (MERK, Germany) and Ham´s F-12 nutrient mix (Thermo Fisher Scientific, USA) medium (1:1) supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich, USA) and 1% (v/v) penicillin (10,000 U/ml)/ streptomycin (10,000 μg/ml) mixture (Sigma-Aldrich, USA). Cells were maintained at 37°C in 5% CO_2 humidified incubator.

3.5.2 Cell viability assay

Cell viability was determined by the MTT assay as described by Monsmann [78] and modified by Carvalho et al. [79]. Briefly, cells were seeded into 96-well plates at a density of $5x10^3$ cells per well, in 200µl of culture medium and allowed to attach for 12 h before the addition of quantum dots. Cell were then exposed to different concentrations (10 µg/L, 100 µg/L and 1000 µg/L – made in Milli-Q water) of ZnS, CdS quantum dots and the combination of both nanoparticles (was added the same volume of each QD to the culture medium), for 24h and 48h. A positive control (Triton 5%) and a negative control (cells with culture medium) were also included in the assay. Quintuplicates of each treatment were used and 2 independent experiments were performed.

After the exposure, 50µl of sterile MTT (2.0 mg/ml) pre-heated at 37°C (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, USA) were added to each well and let incubate for 2h at 37°C. After the incubation period, MTT dye was removed, and plates were frozen at -20°C overnight to allow mechanic lysis to occur. In the next morning, the plates were defrosted, and the formazan crystals were dissolved in 150µl of DMSO/glycine buffer (pH 10.5)/ (VWR, EUA) (4:1) mixture. The plates were shaken for 20 minutes and cell viability was assessed by measuring formazan absorption at 550 nm using a microplate reader (Synergy HTX, Biotek, USA).

3.6 Inductively coupled plasma mass spectrometry (ICP-AES) analysis

The concentrations of the QDs´s elements (Zn and Cd) in water samples collected from the exposure bioassays were determined by inductively coupled plasma atomic emission spectrometry (ICP-EAS) using an Horiba-Jobin Yvon, model Ultima) apparatus.

With respect to water samples, 1.0 ml of water from each tank of the bioassays was placed into a microtube and 50 µl of HNO_3 (Merck, Germany) was added to digest any organic matter present in samples.

Fish samples (homogenates) were transferred to microtubes (1.5 mL) and dried in an oven at 70 °C for 48h and weighed and then digested with 500 µL of HNO_3 (Merck, Germany) and 10 µL of H_2O_2 (Sigma-Aldrich, USA) for 48h.

3.7 . Statistical Analysis

The statistical analysis of the results was carried out by using the analysis of variance (ANOVA), followed by the post-hoc Tukey (Unequal N HSD) test to compare pairs of means and detect significant differences. Data were checked for normality and variance homogeneity using Levene's test, and if necessary, appropriately transformed before analysis. When the assumptions required for ANOVA were not accomplished, then nonparametric tests were performed using the Mann-Whitney U test. Statistical analyses were performed with a significance level of 5%, using STATISTICA TM software 8.0 (StatSoft Inc., USA). All data were expressed as mean ± standard deviation (mean ± SD).

Chapter 4

Results

4 Results

4.1 Quantum Dots Characterization

4.1.1 TEM analysis

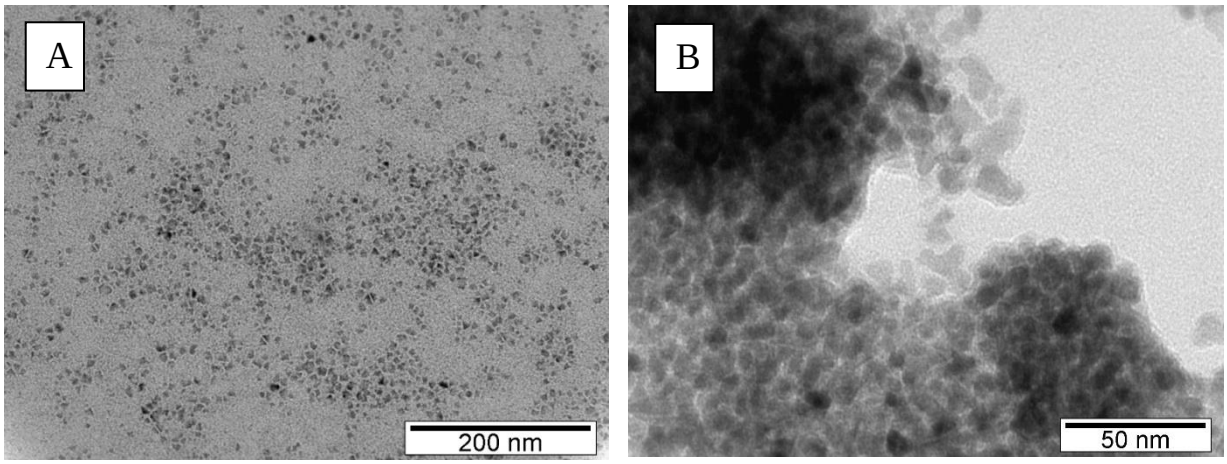

Figure 4.1 Representative TEM images of the tested QDs. Legend: A – ZnS (8 – 12 nm); B – CdS (6 – 10 nm). Note: characterization and images were performed by the researcher David Sousa from CENIMAT.

Representative images from the TEM analysis of the QDs (CdS and ZnS) are shown in figure 4.1. TEM images showed allowed to analyse the size and shape of the QDs which are presented in Table 4.1.

Table 4.1 Size determined from TEM observations and colour of QDs.

QDs	Size (nm)	Shape	Colour
ZnS	10 ± 2	Irregular	Withe/pink
CdS	8 ± 2	Spherical	Orange

4.1.2 SEM analysis

Representative images from the SEM analysis of the QDs (CdS and ZnS) tested are shown in figure 4.2. The SEM images showed the presence of large QDs aggregates (white irregular/spherical dots).

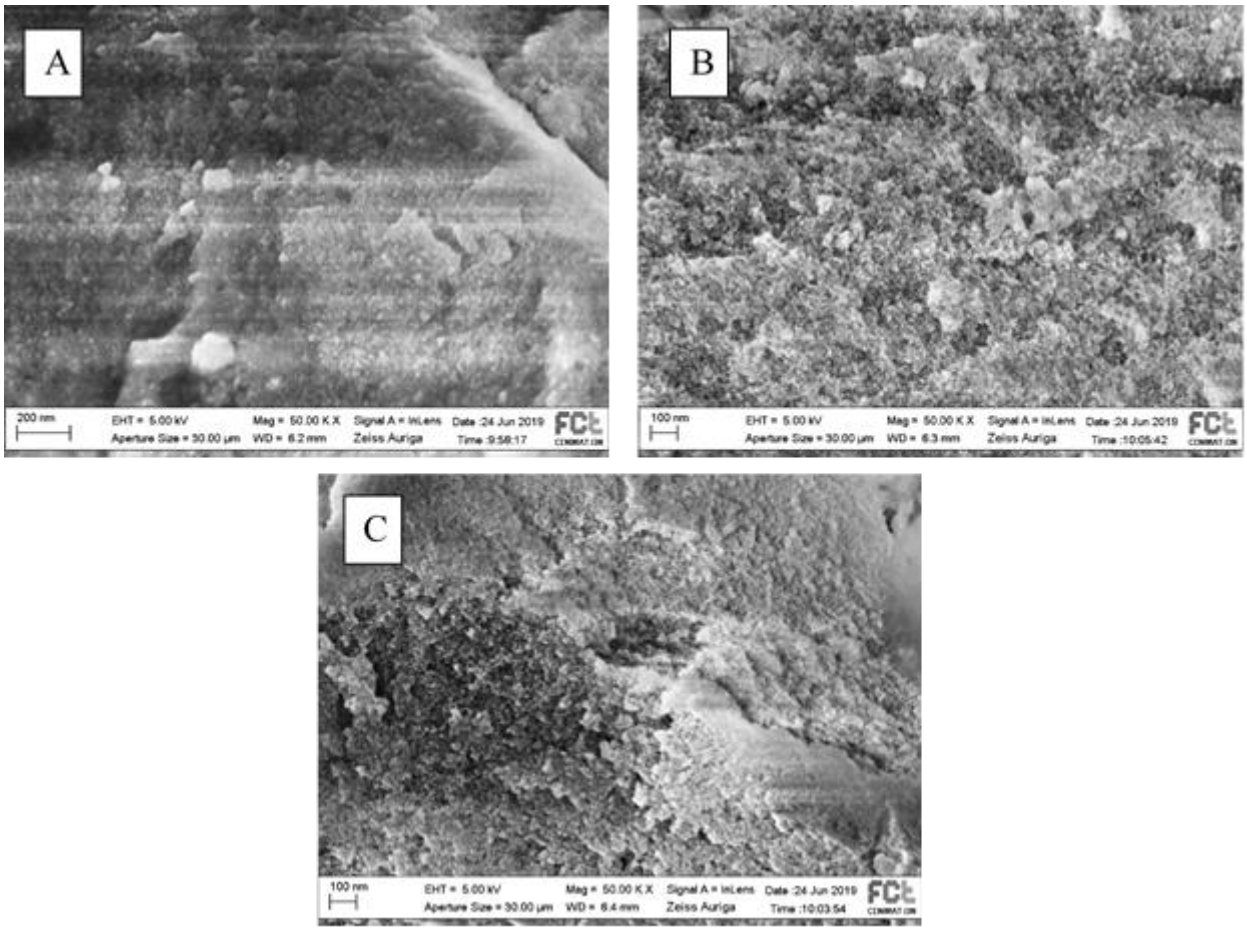

Figure 4.2 Representative SEM images of the QDs tested. Legend: A: ZnS; B : CdS.; C : Mixed QDs.

4.1.3 DLS analyses

Results from DLS analysis are shown in table 4.2. The results confirmed that both QDs (ZnS and CdS) show a trend to aggregate in water. Results from the other re-dispersion mediums tested (chloroform and water:SDS:methanol) showed that chloroform was the best dispersant medium (annexes figures 8.1 and 8.2).

Table 4.2 Dynamic Light Scattering (DLS) results for the QDs concentrations tested. *D.Max. (nm): diameter of QDs with higher frequency on DLS measurement.

	Zn-QDs		Cd-QDs		Mixture	
Concentration (µg/L)	Z-Average (nm)	D.Max (nm)	Z-Average (nm)	D.Max. (nm)	Z-Average (nm)	D.Max (nm)
10	2202.4	837.07	87.5	945.74	567.6	1068.52
100	380.5	454.69	1140.7	2837.04	2008.2	749.89
1000	1082.4	740.89	1544.6	246.98	2971.7	402.44

4.1.4 ICP analyses

The results for the different QDs (ZnS and CdS) concentrations tested (singly and combined) determined in the water samples by ICP-AES are presented in table 4.3. The results show that the real concentrations of QDs in water are much lower than the nominal concentrations tested.

Table 4.3 Results from ICP-AES analysis of QDs (ZnS and CdS) in water samples from bioassays.

Assay	Element	
	Zn (µg/L)	Cd (µg/L)
Zn 10 µg/L	9.23 ± 0.46	n.d
Zn 100 µg/L	11.15 ± 0.55	n.d
Zn 1000µg/L	44.86 ± 2.24	n.d
Cd 10 µg/L	n.d	6.97 ± 0.35
Cd 100 µg/L	n.d	18.38 ± 0.92
Cd 1000 µg/L	n.d	155.95 ± 7.79
Mix 10 µg/L	2.73 ± 0.14	11.57 ± 0.58
Mix 100 µg/L	6.04 ± 0.30	23.67 ± 1.18
Mix 1000 µg/L	12.03 ± 0.60	99.76 ± 4.98

n.d: not detected. LD: Zn (0.5 µg/L); Cd (0.5 µg/L)

The results for the different concentrations of QDs (ZnS and CdS) tested (singly and combined) determined in the fish tissues (bioassay 3) by ICP-AES are presented in table 4.4. The results confirmed the presence of the metals in fish tissue, that suggest a trophic transfer of Zn and Cd from *D. magna* to *D. rerio*.

Table 4.4 Results from ICP-AES analysis of ZnS and CdS (QDs) in tissues from *D. rerio* that ingested *D. magna* exposed to the different concentrations of QDs tested.

Assay	Element	
	Zn (µg/mg dry weight)	**Cd (µg/mg dry weight)**
Zn_Control	39.45 ±10.34	n.d
Zn 10 µg/L	44.37 ± 9.32	n.d
Zn 100 µg/L	59.90 ± 12.17	n.d
Zn 1000 µg/L	48.80 ± 18.70	n.d
Cd_Control	n.d	0.07 ± 0.02
Cd 10 µg/L	n.d	0.07 ±0.02
Cd 100 µg/L	n.d	0.11 ± 0.06
Cd 1000 µg/L	n.d	0.12 ± 0.05
Mix_Control	34.09 ± 0.23	0.03 ± 0.01
Mix 10 µg/L	128.48 ± 27.23	0.21 ± 0.01
Mix 100 µg/L	331.92 ± 51.50	0.48 ± 0.15
Mix 1000 µg/L	31.45 ± 6.64	0.05 ± 0.01

n.d: not detected. LD: Zn (0.5 µg/L); Cd (0.5 µg/L)

4.2 Mortality rate and physical and chemical parameters

No mortality was observed for the assays performed using *D. rerio* (bioassay 1 and bioassay 3). The results from mortality rate for the different bioassays performed with *D. magna* (bioassay 2) are presented in figure 4.3.

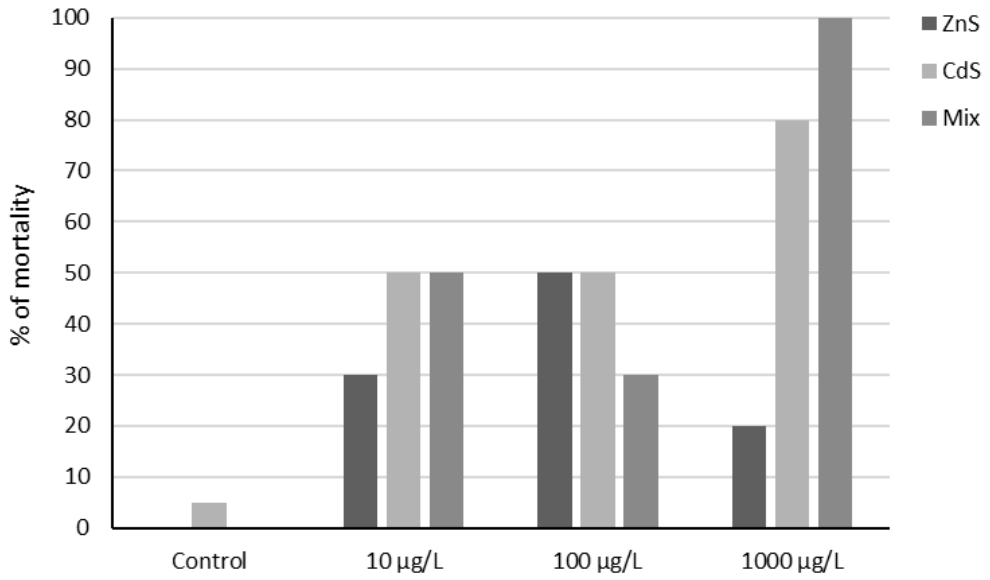

Figure 4.3 Mortality rate of *D. magna* for the bioassay 2.

For ZnS QDs, the percentage (%) of mortality observed was between 0% and 50%, being the highest mortality rate found in organisms exposed to 1000 µg/L QDs (CdS and combined). Concerning CdS, the mortality rate was between 50% and 80% with the highest value being observed in organisms exposed to 1000 µg/L. Mortality rates of 80% and 100% were registered in Daphnids exposed to 1000 µg/L QDs (CdS combined).

With respect to the physical and chemical parameters monitored during the assays (pH 7.5 ± 0.1, temperature 20 ± 1 °C), they remained constant at normal levels until the end of the assay. Ammonia and dissolved oxygen were also monitored and were within acceptable values for freshwater organisms, namely fish.

4.3 **Bioassay 1:** Exposure of *Danio rerio* to QDs (ZnS and CdS), singly and combined

4.3.1 Antioxidant Enzyme Activities

4.3.1.1 Catalase (CAT)

The results from CAT activity determined in fish exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.4. The highest average levels of CAT activity (17.42 ± 4.62 nmol/min/mg total protein) were found in fish exposed to 100 µg/L Zns QDs whereas the lowest average levels (7.85 ± 1.61 nmol/min/mg total protein) were measured in fish exposed to 100 µg/L QDs combined.

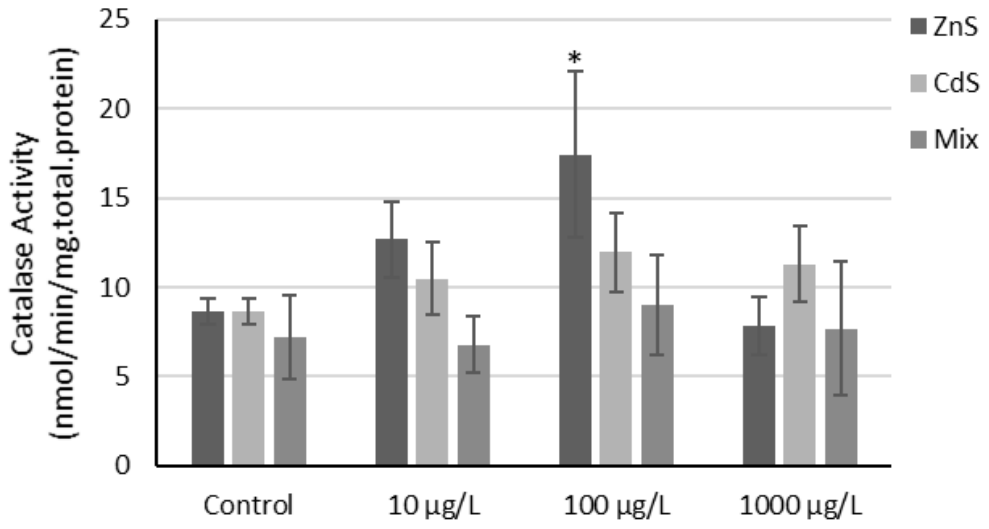

Figure 4.4 CAT activity (mean ± SD) determined in *D. rerio* exposed to the different QDs concentrations, singly and combined. The experiment was performed in duplicate. Asterisk means significant differences ($p<0.05$) in comparison to the respective controls.

The statistical analysis showed a significant increase ($p<0.05$) in fish exposed to 100 µg/L ZnS QDs, while for the other concentrations tested no significant differences were found ($p> 0.05$). A significant decrease ($p< 0.05$) was also detected between fish exposed to 100 µg/L and those exposed to 1000 µg/L ZnS QDs. With respect to the others QDs and the different tested concentrations, singly or combined, no significant differences ($p> 0.05$) were found.

4.3.1.2 Glutathione-S-Transferase (GST)

The results from GST activity measured in fish exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.5. The highest average levels of GST activity (0.043 ± 0.013 µmol/min/mg total protein) were found in fish exposed to 100 µg/L ZnS QDs whereas the lowest average levels (0.014 ± 0.003 µmol/min/mg total protein) were measured in fish exposed to 1000 µg/L QDs combined. GST activities in fish exposed singly to 10 and 100 µg/L QDs (ZnS and CdS) show a similar trend, with a general increase in enzyme activities followed by a decrease in fish exposed to the highest concentrations.

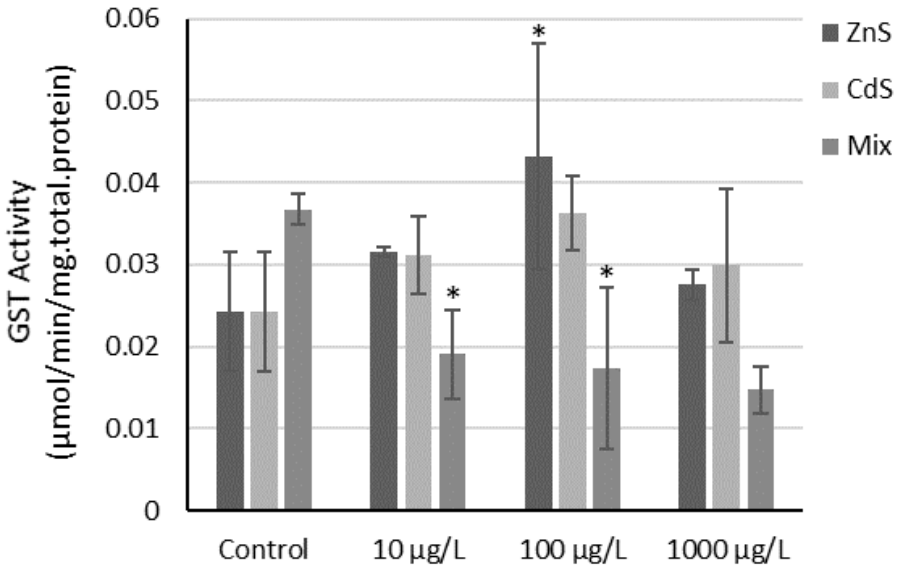

Figure 4.5 GST activity (mean ± SD) determined in *D. rerio* exposed to the different QDs concentrations, singly and combined. The experiment was performed in duplicate. Asterisk means significant differences ($p<0.05$) in comparison to controls.

Statistical analysis showed a significant increase ($p<0.05$) in fish exposed to 100 µg/L ZnS QDs and a significant decrease ($p<0.05$) was found in fish exposed to 10 and 100 µg/L QDs combined, in comparison to the respective controls.

4.3.1.3 Superoxide Dismutase (SOD)

The results from SOD expressed as percentage (%) of inhibition of NBT-diformazan in fish exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.6. The highest average levels of SOD (18.59 ± 5.02 % inhibition/mg.total.protein) were found in fish exposed to 100 µg/L ZnS QDs and in fish exposed to 1000 µg/L CdS QDs (18.89 ± 3.55 % inhibition/mg.total.protein), whereas the average lowest levels (7.85 ± 1.61 % inhibition/mg.total.protein) were measured in fish exposed to 10 µg/L QDs combined.

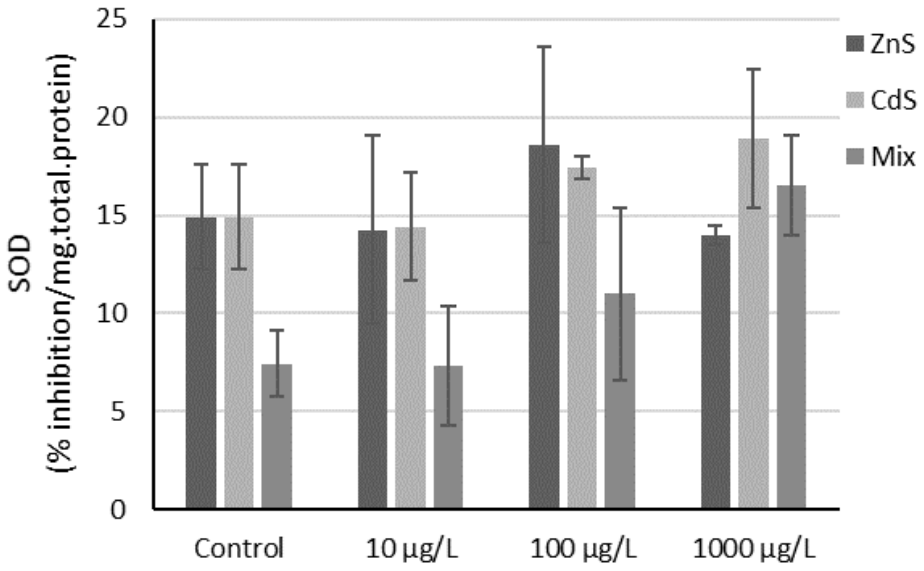

Figure 4.6 SOD (% inhibition/mg.total.protein) (mean ± SD) determined in *D. rerio* exposed to the different QDs concentrations, singly and combined. The experiment was performed in duplicate.

Although no statistical differences ($p > 0.05$) were found for all concentrations tested, singly and combined, a general trend to increase of SOD (% inhibition/mg.total.protein) can be observed in accordance with the tested QDs concentrations.

4.3.2 Lipid Peroxidation (LPO)

The results from LPO (MDA content) determined in fish exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.7. The highest average levels of MDA (0.0164 ± 0.0018 µM/ mg of total protein) were found in fish exposed to 10 µg/L CdS QDs whereas the average lowest levels (0.0026 ± 0.0012 µM /mg total protein) were measured in fish exposed to 100 µg/L CdS QDs.

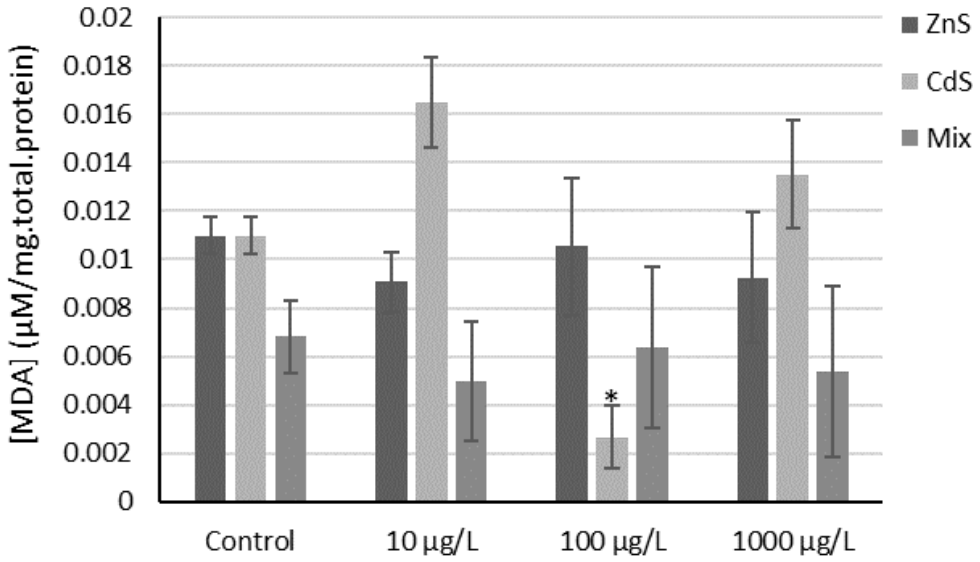

Figure 4.7 MDA concentration determined in *D. rerio* (mean ± SD) exposed to the different QDs concentrations, singly and combined. The experiment was performed in duplicate. Asterisk means significant differences ($p<0.05$) in comparison to the respective controls

Statistical analysis revealed a significant decrease ($p<0.05$) between controls and fish exposed to 100 µg/L CdS QDs, whereas no significant differences were found for all the other tested QDs concentrations, singly and combined, in comparison to the respective controls. Was verified that the other two concentrations were increased related to control.

4.3.2 Total Antioxidant Capacity (TAC)

The results from TAC assay in fish exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.8. The highest average levels of TAC (0.092 ± 0.013 µM/mg total protein) were found in fish exposed to 1000 µg/L combined QDs, whereas the lowest average levels (0.036 ± 0.009 µM/mg total protein) were measured in fish exposed to 1000 µg/L of ZnS QDs.

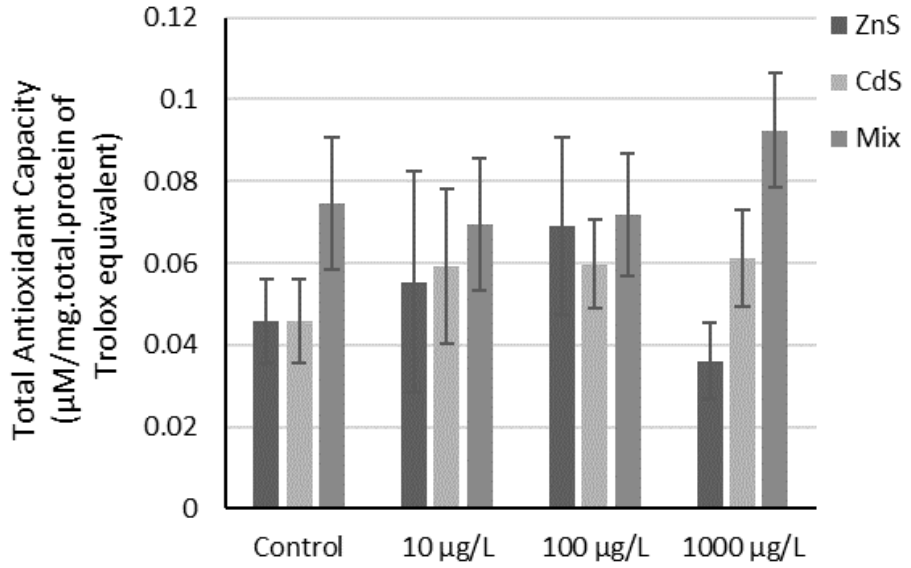

Figure 4.8 TAC mean ± SD) determined in *D. rerio* exposed to the different QDs concentrations, singly and combined. The experiment was performed in duplicate.

The statistical analyses showed no significant differences ($p>0.05$) for all the different tested QDs concentrations, singly and combined. However, the results suggest a slight trend to increase in accordance with the QDs tested concentrations.

4.3.3 HSC70/HSP70

The results from HSP70 assay in fish exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.9. The highest average levels of HSP70 (0.0346 ± 0.0081 µg/mg total protein) were found in fish exposed to 100 µg/L ZnS QDs, whereas the lowest average levels (0.0042 ± 0.0019 µg/mg total protein) were measured in fish exposed to 1000 µg/L ZnS QDs.

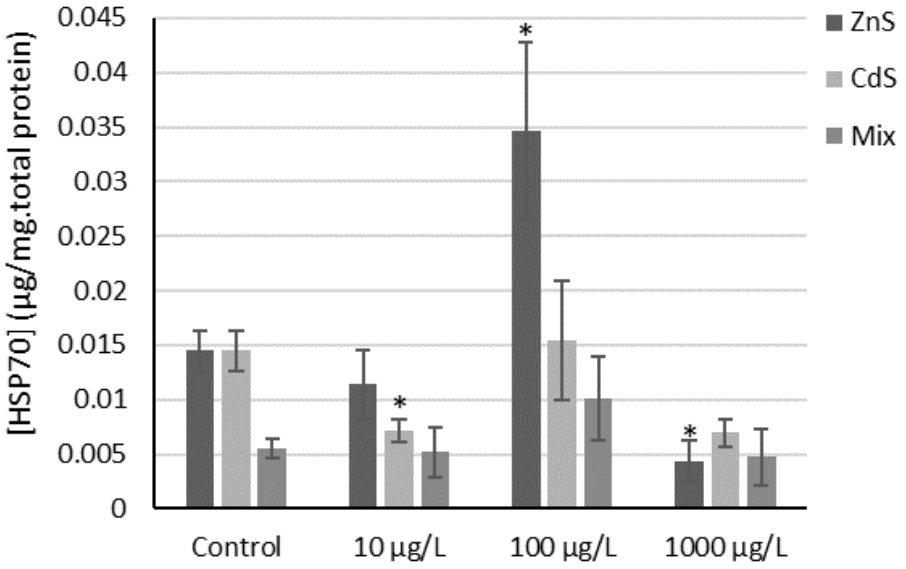

Figure 4.9 HSP70 concentrations (mean ± SD) determined in *D. rerio* exposed to the different QDs concentrations. The experiment was performed in duplicate. Asterisk means significant differences ($p<0.05$) in comparison to the respective controls.

The statistical analysis showed a significant decrease ($p< 0.05$) in HSP70 production in fish exposed to 10 µg/L CdS QDs and exposed to 1000 µg/L ZnS QDs in comparison to the respective controls. A significant increase ($p<0.05$) in HSP70 concentration was also detected in fish exposed to 100 µg/L ZnS QDs.

4.3.4 Ubiquitin

The results from Ubiquitin assay determined in fish exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.10. The highest average levels of Ubiquitin (0.0111 ± 0.0050 µg/mg total protein) were found in fish exposed to 100 µg/L ZnS QDs, whereas the lowest average levels (0.0052 ± 0.0005 µg/mg total protein) were measured in fish exposed to 1000 µg/L CdS QDs and QDs combined.

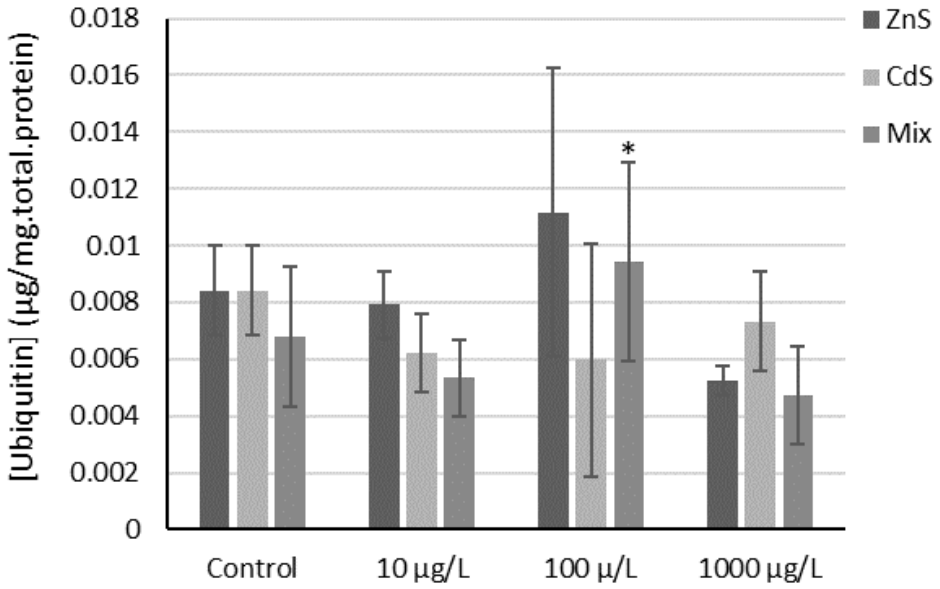

Figure 4.10 Ubiquitin concentrations (mean ± SD) in *D. rerio* exposed to the different concentrations of QDs, singly and combined. The experiment was performed in duplicate. Asterisk means significant differences ($p<0.05$) in comparison to the respective controls.

The statistical analysis showed a significant increase ($p<0.05$) in fish exposed to 100 µg/L QDs combined, whereas no significant differences ($p> 0.05$) where found for the other QDs tested concentrations, singly and combined. However, the results show a trend to decrease in fish exposed to 1000 µg/L QDs, singly or combined.

4.4 **Bioassay 2:** Exposure of *Daphnia magna* to QDs (ZnS and CdS), singly and combined

4.4.1 Antioxidant enzymes

4.4.1.1 Catalase (CAT)

The results from CAT activity in Daphnids exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.11. The highest average levels of CAT (22.971 ± 0.0052 nmol/min/mg total protein) were measured in Daphnids exposed to 10 µg/L QDs combined, whereas the lowest average levels (0.446 ± 0.162 nmol/min/mg total protein) were measured in Daphnids exposed to 1000 µg/L ZnS QDs.

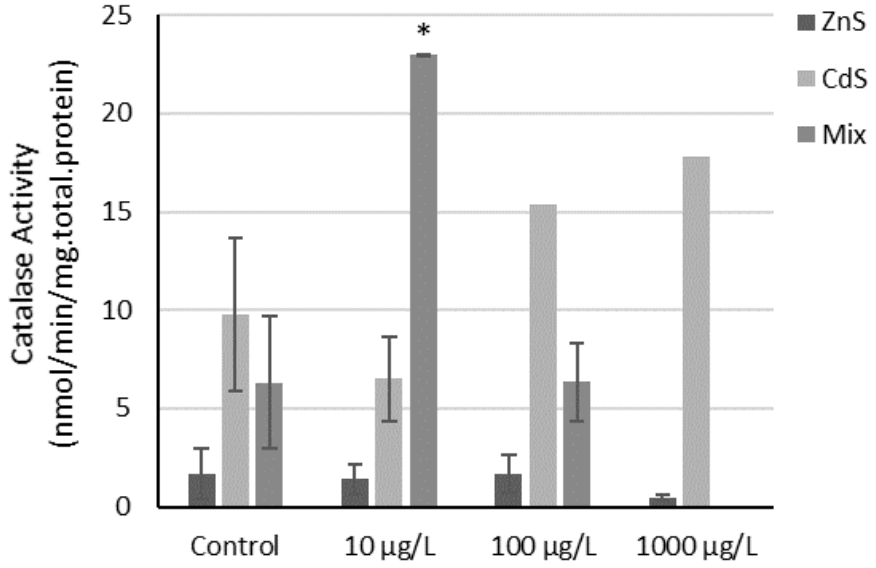

Figure 4.11 CAT activity (mean ± SD) in *D. magna* exposed to the different QDs concentrations. The experiment was performed in duplicate. Absence of bars means 100% of mortality rate. Absence of standard deviation means just one individual analysed.

The statistical analysis showed significant differences ($p < 0.05$) in daphnids exposed to 10 µg/L QDs combined. The results show an increase in CAT activity measured in fish exposed to 100 and 1000 µg/L CdS QDs, however, this increase was based in the analysis of a single individual.

4.4.1.2 Glutathione-S-Transferase (GST)

The results from GST activity in Daphnids exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.12. The highest average levels of GST (0.4835 µmol/min/mg total protein), although based in the analysis of a single individual, were measured in organisms exposed to 1000 µg/L CdS QDs, whereas the lowest average levels (0.065 ± 0.018 004 µmol/min/mg total protein) were determined in Daphnids exposed to 10 µg/L ZnS QDs.

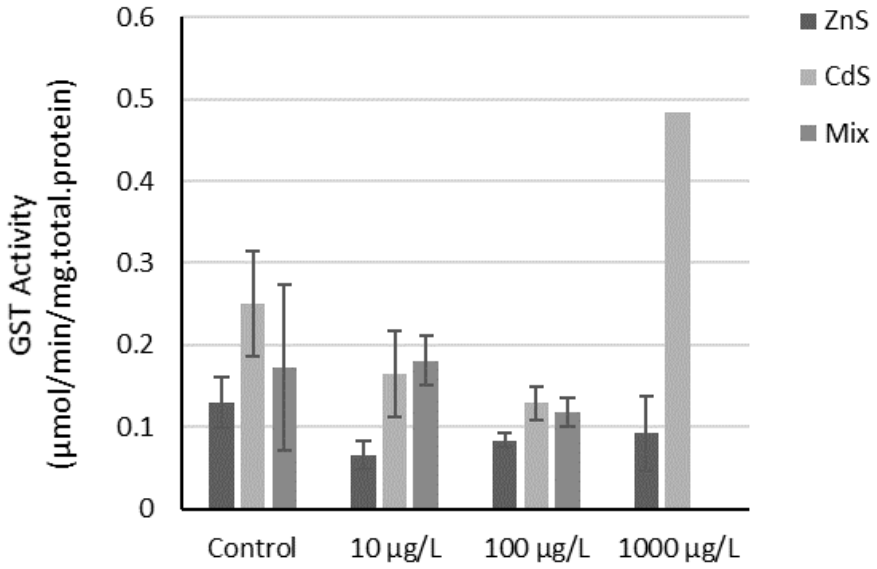

Figure 4.12 GST activity (mean ± SD) in *D. magna* exposed to the different QDs Asterisk means significant differences ($p<0.05$) in comparison to the respective controls. The experiment was performed in duplicate. Absence of bars means 100% of mortality rate. Absence of standard deviation means just one individual analysed.

The statistical analysis showed no significant differences ($p>0.05$) for all the tested QDs concentrations, single and combined. However, a great increase in GST levels was observed in organisms exposed to 1000 µg/L QDs combined but corresponding to the analysis of a single individual. In addition, the GST activities show a trend to decrease in Daphnids exposed to CdS QDs according to the different tested concentrations.

4.4.1.3 Superoxide Dismutase (SOD)

The results from SOD assay (% inhibition of NBT-diformazan) in Daphnids exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.13. The results show high variability. The highest average levels of SOD inhibition (3.574 % inhibition/mg.total.protein) were determined in control organisms and the lowest average levels (0.528 % inhibition/mg.total.protein) were also determined in control organisms.

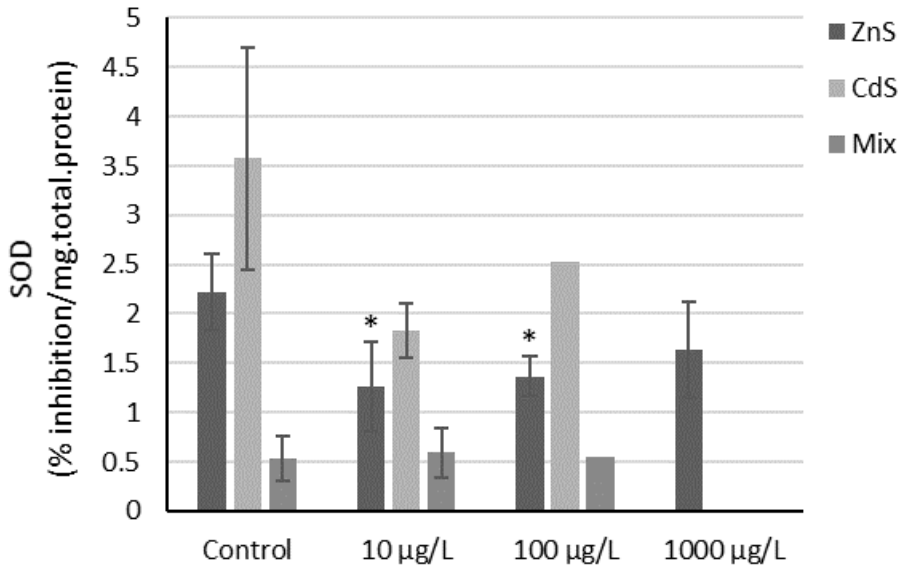

Figure 4.13 SOD mean ± SD) expressed as (%) inhibition/mg.total.protein in *D. magna* (exposed to the different QDs concentrations. The experiment was performed in duplicate. Asterisk means significant differences ($p<0.05$) in comparison to the respective controls. Absence of bars means 100% of mortality rate. Absence of standard deviation means just one individual analysed.

The statistical analysis showed a significant decrease of SOD ($p<0.05$) in organisms exposed to 10 and 100 µg/L ZnS QDs. Moreover, a trend to decrease in SOD (% inhibition/mg.total.protein) can be observed in organisms exposed to CdS QDs.

4.4.2 Lipid Peroxidation (LPO)

The results from LPO (MDA content) determined in Daphnids exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.14. The highest levels of MDA (0.0518 ± 0.0165 µM/ mg total protein) were found in Daphnids exposed to 100 µg/L CdS QDs whereas the lowest levels (0.009 µM /mg total protein) were measured in organisms exposed to 1000 µg/L CdS QDs (although based in the analysis of a single individual).

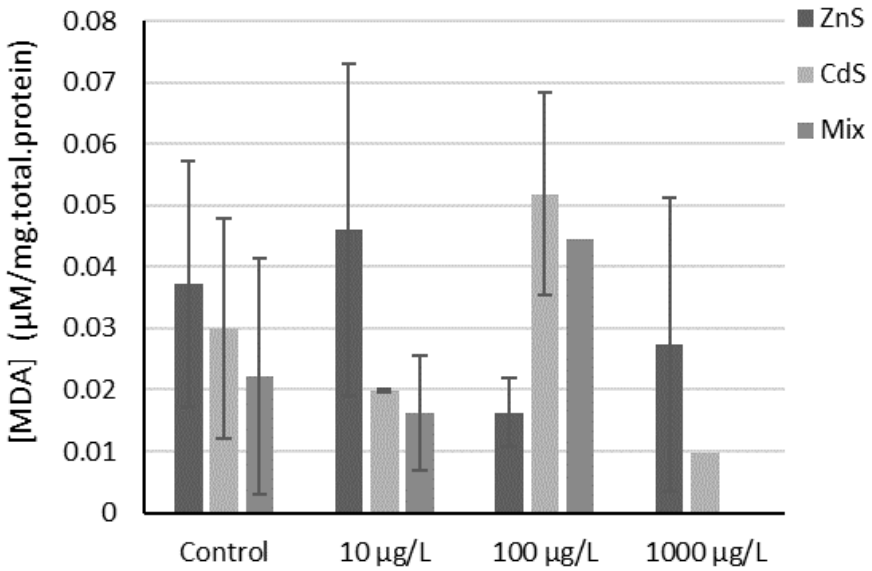

Figure 4.14 MDA concentrations (mean ± SD) in *D. magna* exposed to the different QDs concentrations. The experiment was performed in duplicate. Absence of bars means 100% of mortality rate. Absence of standard deviation means just one individual analysed.

The statistical analysis revealed no significant differences ($p>0.05$) for all the QDs concentrations tested, singly and combined. The LPO (MDA content) results show high variability for all tested QDs.

4.4.3 Total Antioxidant Capacity (TAC)

The results from TAC assay in Daphnids exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.15. The highest average levels of TAC (1.261 ± 0.522 µM/mg total protein) were found in control organisms, whereas the lowest average levels (0.391 µM/mg total protein) were measured in Daphnids exposed to 100 µg/L CdS QDs, however, this value corresponds to the analysis of a single individual.

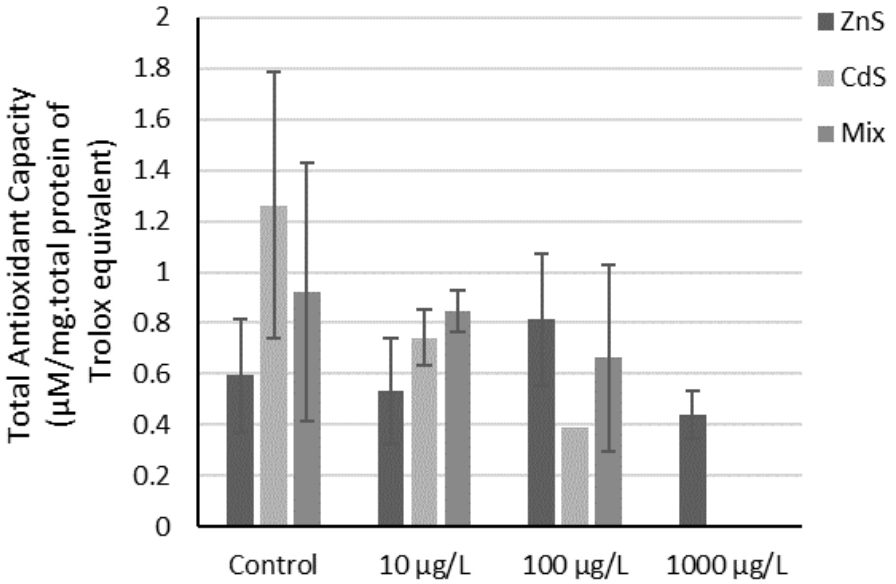

Figure 4.15 TAC concentrations (mean ± SD) determined *D. magna* exposed to the different QDs concentrations. The experiment was performed in duplicate. Absence of bars means 100% of mortality rate. Absence of standard deviation means the presence of just one individual.

The statistical analysis revealed no significant differences ($p > 0.05$) for all tested QDs concentrations, singly and combined. Nonetheless, there is a trend to decrease in TAC levels for all tested concentrations in comparison to controls.

4.4.4 Ubiquitin

The results from the Ubiquitin assay determined in Daphnids exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.16. The highest average level of Ubiquitin (0.112 µg/mg total protein) was found in a single individual exposed to 1000 µg/L CdS QDs, whereas the lowest average level (0.016 ± 0.006 µg/mg total protein) was determined in organisms exposed to 10 µg/L ZnS QDs.

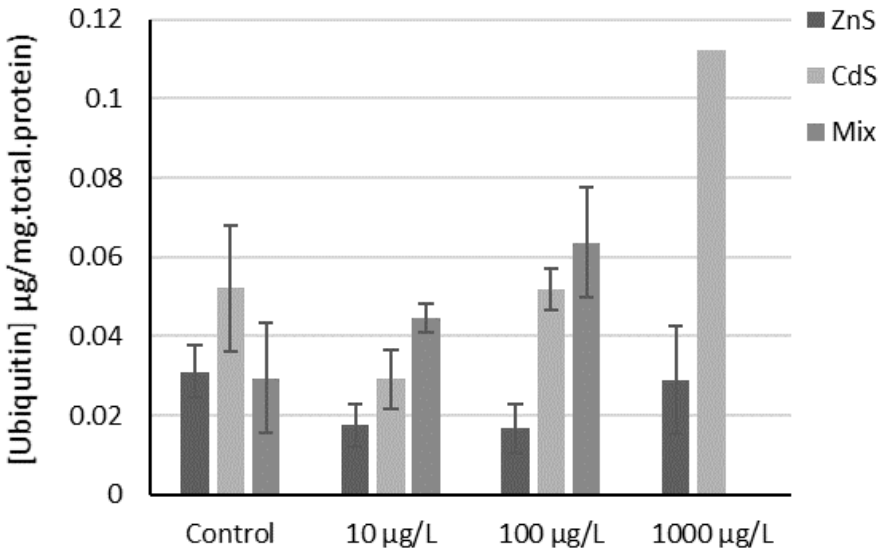

Figure 4.16 Ubiquitin concentrations (mean ± SD) determined in *D. magna* exposed to the different QDs concentrations. The experiment was performed in duplicate. Absence of bars means 100% of mortality rate. Absence of standard deviation means just one individual analysed.

The statistical analysis showed no significant differences ($p>0.05$) for all tested QDs concentrations, singly and combined, in comparison to controls. However, the results suggest a trend to increase in Ubiquitin levels for Daphnids exposed to CdS QDs and to QDs combined.

4.5 **Bioassay 3:** Trophic transfer of QDs assay: *Danio rerio* ingestion of *Daphnia magna* exposed to QDs (singly and combined)

4.5.1 *Daphnia magna*

4.5.1.1 Antioxidant Enzymes activities, Lipid peroxidation, Total antioxidant capacity and Ubiquitin content

The results from antioxidant enzymes activities, lipid peroxidation, total antioxidant capacity and ubiquitin content in Daphnids exposed for 96h to the different concentrations of QDs (single and combined) are presented in table 4.5. The Daphnids form this experiment were used to later feed *D. rerio* in a trophic transfer assessment of the QDs. It should be noticed that some individuals died during the exposure period (96h) of Daphnids to QDs. Statistical analysis showed no significant differences ($p>0.05$) for all the assays.

Table 4.5 Antioxidant enzymes activities (CAT, GST, SOD (%inhibition)), Lipid peroxidation (MDA content), Total Antioxidant Capacity and Ubiquitin (mean ± SD) in *D. magna* exposed to the different QDs concentrations, singly and combined. * means just one individual analysed. ** means 100% of mortality rate.

Sample	CAT (nmol/min/mg total protein)	GST (µmol/min/mg total protein)	SOD (% inhibition)	LPO [MDA] (µM/mg total protein)	TAC [Trolox] (µM/mg total protein)	Ubiquitin [Ubiquitin] (µg/mg total protein)
ZnS Control	61.961 ± 45.859	0.139 ± 0.034	4.467 ± 1.756	0.256 ± 0.251	1.613 ± 0.557	0.303 ± 0.030
ZnS 10 µg/L	37.786 *	0.196 ± 0.040	5.530 ± 0.158	0.551 ± 0.024	1.387 ± 0.056	0.225 ± 0.006
ZnS 100 µg/L	11.248 ± 12.800	0.209 ± 0.016	8.220 ± 2.324	0.144 ± 0.076	4.389 ± 1.440	0.249 ± 0.133
ZnS 1000 µg/L	21.308 ± 14.617	0.397 ± 0.307	7.576 ± 0.458	0.175 ± 0.006	3.176 ± 0.213	0.182 ± 0.027
CdS Control	48.563 *	0.426 *	19.611 *	0.107 *	4.375 *	0.592 *
CdS 10 µg/L	**	0.805 *	34.678 *	0.248 *	28.515 *	0.592 *
CdS 100 µg/L	49.48 *	0.966 *	15.497 *	0.324 *	5.735 *	0.483 *
CdS 1000 µg/L	**	**	**	**	**	**
Mix Control	20.356 ± 25.194	0.315 ± 0.085	13.202 ± 3.148	0.0446 ± 0.035	2.763 *	0.303 *
Mix 10 µg/L	5.716 *	0.126 *	6.471 *	0.038 *	3.298 *	0.319 *
Mix 100 µg/L	52.926 ± 0.840	0.544 ± 0.098	15.165 ± 0.268	0.060 ± 0.016	7.043 ± 0.098	0.438 ± 0.249
Mix 1000 µg/L	**	**	**	**	**	**

The results from CAT activity showed that the highest average levels of CAT activity (61.961 ± 45.859 nmol/min/mg total protein) were measured in control organisms, whereas the lowest average levels (5.716 nmol/min/mg total protein) were determined in Daphnids exposed 10 µg/L QDs combined QDs (although based in the analysis of a single individual). The results show a trend to decrease for ZnS QDs and the opposite trend for QDs combined.

The results from GST activity showed that the highest average level of GST activity (0.966 µmol/min/mg total protein) were determined in Daphnids exposed to 100 µg/L CdS QDs but corresponding to the analysis of a single individual. The lowest average levels (0.126 µmol/min /mg total protein) were found in Daphnids exposed to 10 µg/L QDs combined but also

corresponding to the analysis of a single individual. A general trend to increase of GST activity was found in accordance with the tested QDs concentrations.

The results from SOD assay (% inhibition of NBT-diformazan) showed that the highest average levels of SOD inhibition (34.678 % inhibition) were determined in organisms exposed to 10 µg/L CdS QDs (although based in the analysis of a single individual). The lowest average levels (4.467 ± 1.756 % inhibition) were measured in control organisms. A trend to increase for SOD (% inhibition) was found in organisms exposed to the different QDs concentrations, singly or combined.

The results from LPO (MDA content) showed that the highest average levels of MDA (0.551 ± 0.0024 µM/mg total protein) were determined in Daphnids exposed to 10 µg/L ZnS QDs whereas the lowest average levels of MDA (0.038 µM/mg total protein) were found in organisms exposed to 10 µg/L QDs combined but corresponding to the analysis of a single individual. The results present high variability and for the QDs combined results show lower average values.

The results from TAC assay showed that the highest average levels of TAC (28.515 µM/mg total protein) were measured in Daphnids exposed to 10 µg/L CdS QDs (although based in the analysis of a single individual). The lowest average levels (1.387 ± 0.056 µM/mg total protein) were found in organisms exposed to µg/L ZnS QDs. The TAC results show high variability for all tested QDs.

The results from the ubiquitin assay showed that the highest average levels of ubiquitin (0.592 µg/mg total protein) were measured in control organisms and in organisms exposed to 10 µg/L CdS QDs (but both corresponding to the analysis of a single individual). The lowest average levels (0.182 ± 0.027 µg/mg total protein) were determined in Daphnids exposed to 1000 µg/L ZnS QDs. A trend to increase for Ubiquitin levels was noticed in individuals exposed to the different QDs concentrations (singly) and an opposite trend was observed for the different concentrations of QDs tested combined.

4.5.2 *Danio rerio*

4.5.2.1 Antioxidant Enzyme Activities

4.5.2.1.1 Catalase (CAT)

The results from CAT activity in fish that ingested Daphnids exposed to QDs (singly and combined) are presented in figure 4.17. The highest average levels of CAT (15.281 ± 5.288 nmol/ml/min) were measured in fish that ingested Daphnids exposed to 10 µg/L QDs combined, whereas the lowest average levels (9.097 ± 3.621 nmol/ml/min) were measured in in fish that ingested Daphnids exposed to 100 µg/L QDs combined.

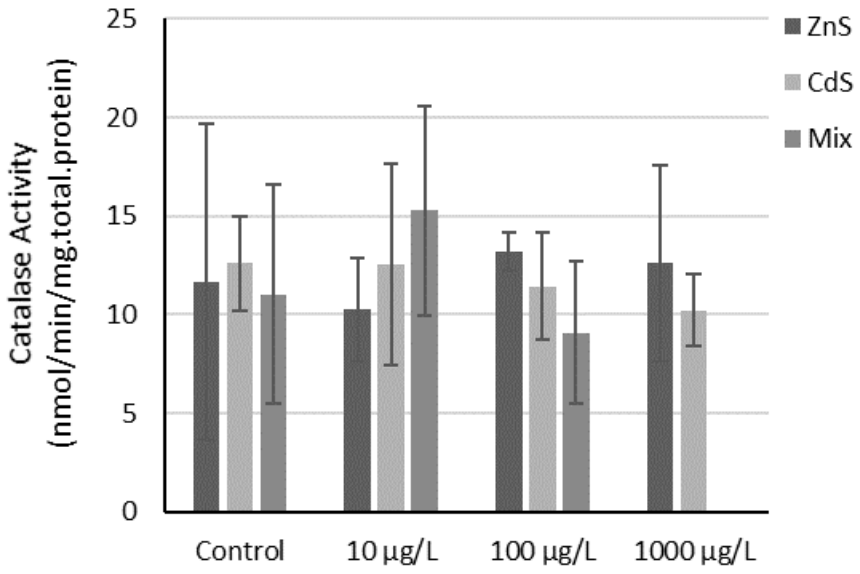

Figure 4.17 CAT activity (mean ± SD) in *D. rerio* that ingested *D. magna* exposed to the different concentrations of QDs, singly and combined. The experiment was performed in duplicate.

The statistical analysis showed no significant differences ($p>0.05$) for all the tested concentrations of ingested Daphnids, in comparison to the respective controls. The results were variable, and no trend was observed.

4.5.2.1.2 Glutathione-S-Transferase (GST)

The results from the GST activity in fish that ingested Daphnids exposed to QDs (singly and combined) are presented in figure 4.18. The highest average levels of GST (0.0485 ± 0.0115 μmol/ml/min) were measured in fish that ingested Daphnids exposed to 10 μg/L QDs combined, whereas the lowest average levels (0.033 ± 0.005 μmol/ml/min) were measured in fish that ingested Daphnids exposed to 1000 μg/L CdS QDs.

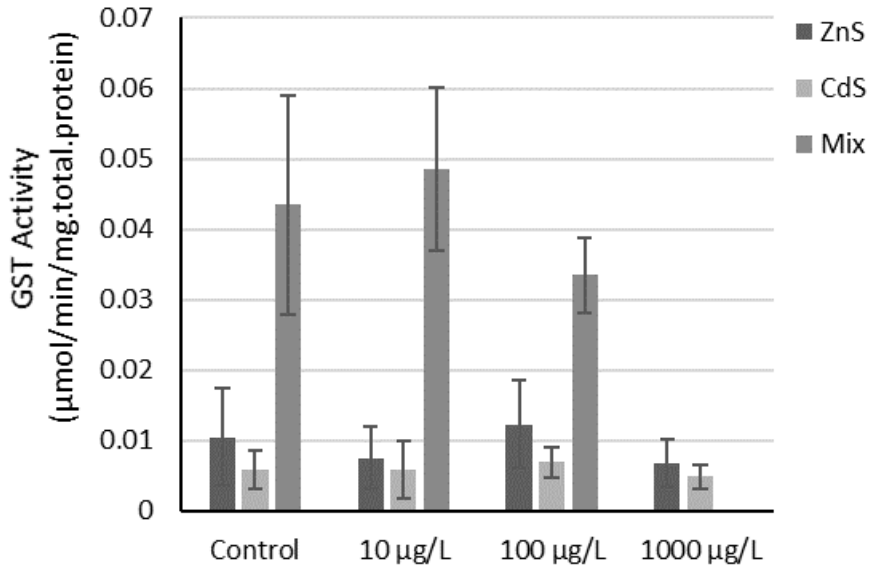

Figure 4.18 GST activity (mean ± SD) in *D. rerio* that ingested *D. magna* exposed to the different QDs concentrations, singly and combined. The experiment was performed in duplicate.

The statistical analysis showed no significant differences ($p>0.05$) for all the tested concentrations of ingested Daphnids, in comparison to the respective controls.

4.5.2.1.3 Superoxide Dismutase (SOD)

The results from SOD assay (% inhibition of NBT-diformazan) in fish that ingested Daphnids exposed to QDs (singly and combined) are presented in figure 4.19. The highest average levels of SOD inhibition (30.978 ± 4.570 % inhibition/mg.total.protein) were determined in control fish, whereas the lowest average levels (8.112 ± 4.036 % inhibition/mg.total.protein) were measured in in fish that ingested Daphnids exposed to 10 µg/L QDs combined.

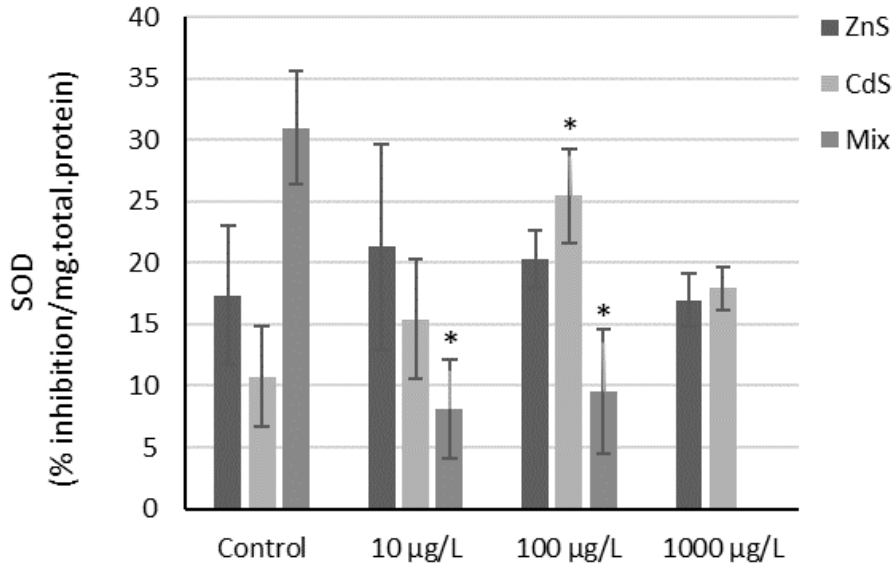

Figure 4.19 SOD (mean ± SD) express as (%) inhibition/mg.total.protein in *D. rerio* that ingested *D. magna* exposed to the different QDs concentrations, singly and combined. The experiment was performed in duplicate. Asterisk means significant differences ($p<0.05$) in comparison to the respective controls.

The statistical analysis revealed a significant decrease ($p<0.05$) of SOD (% inhibition/mg.total.protein) in fish that ingested Daphnids exposed to the different concentrations of QDs combined. A significant increase ($p<0.05$) was observed in fish that ingested Daphnids exposed to 100 ug/L CdS QDs.

4.5.2.2 Lipid Peroxidation (LPO)

The results from the LPO assay (MDA content) in fish that ingested Daphnids exposed to QDs (singly and combined) are presented in figure 4.20. The highest average levels of MDA (0.0334 ± 0.0148 µM/mg total protein) were determined in fish that ingested Daphnids exposed to 1000 µg/L ZnS QDs, whereas the lowest average levels (0.0043 ± 0.0015 µM/mg total protein) were measured in control fish.

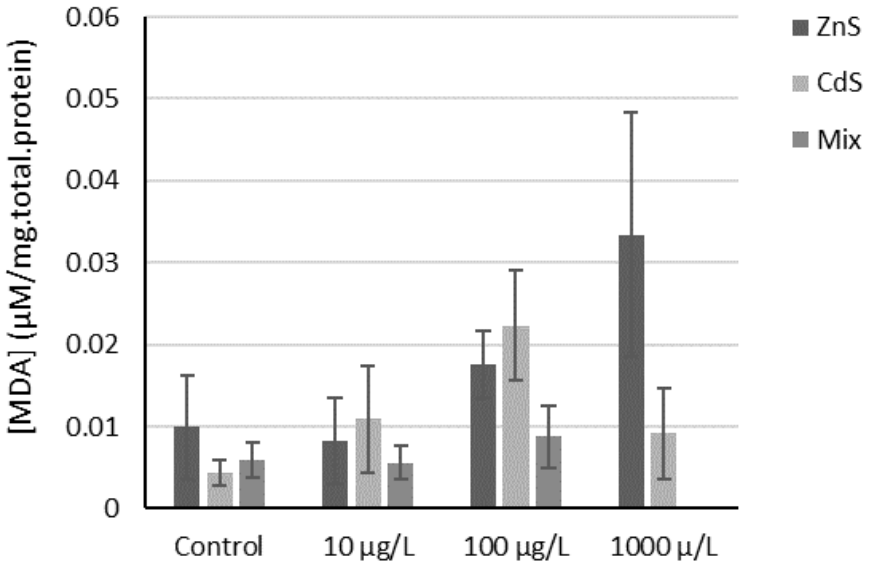

Figure 4.20 MDA concentration (mean ± SD) measured in *D. rerio* that ingested *D. magna* exposed to the different QDs concentrations, singly and combined. The experiment was performed in duplicate.

The statistical analysis showed no significant differences ($p>0.05$) for all the tested concentrations of ingested Daphnids, in comparison to the respective controls. Nonetheless, the results show a trend to increase according to the different tested concentrations of ingested Daphnids.

4.5.2.3 Total Antioxidant Capacity (TAC)

The results from TAC assay in fish that ingested Daphnids exposed to QDs (singly and combined) are presented in figure 4.21. The highest average levels of TAC (0.039 ± 0.047 µM/mg total protein) were determined in fish that ingested Daphnids exposed to 100 µg/L CdS QDs, whereas the lowest average levels (0.007 ± 0.002 µM/mg total protein) were determined in fish that ingested Daphnids exposed to 100 µg/L QDs combined.

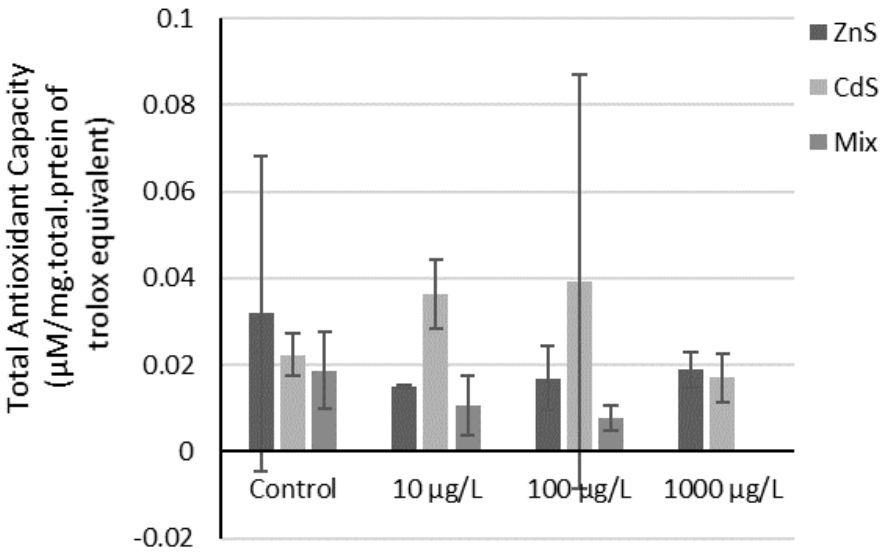

Figure 4.21 TAC concentrations (mean ± SD) determined in *D. rerio* that ingested *D. magna* exposed to the different QDs concentrations, singly and combined. The experiment was performed in duplicate.

The statistical analysis showed no significant differences ($p>0.05$), for all the tested concentrations of ingested Daphnids, in comparison to the respective controls. The results for TAC presented a high variability in fish that ingested Daphnids previously exposed to 100 µg/L CdS QDs.

4.5.2.4 HSC70/HSP70

The results from HSP70 assay in fish that ingested Daphnids exposed to the different concentrations of QDs (singly and combined) are presented in figure 4.22. The highest average levels of HSP70 (0.0528 ± 0.0291 µg/mg total protein) were determined in fish that ingested Daphnids exposed to 100 µg/L QDs combined, whereas the lowest average levels (0.007 ± 0.0009 µg/mg total protein) were determined in fish that ingested Daphnids exposed to 1000 µg/L CdS QDs.

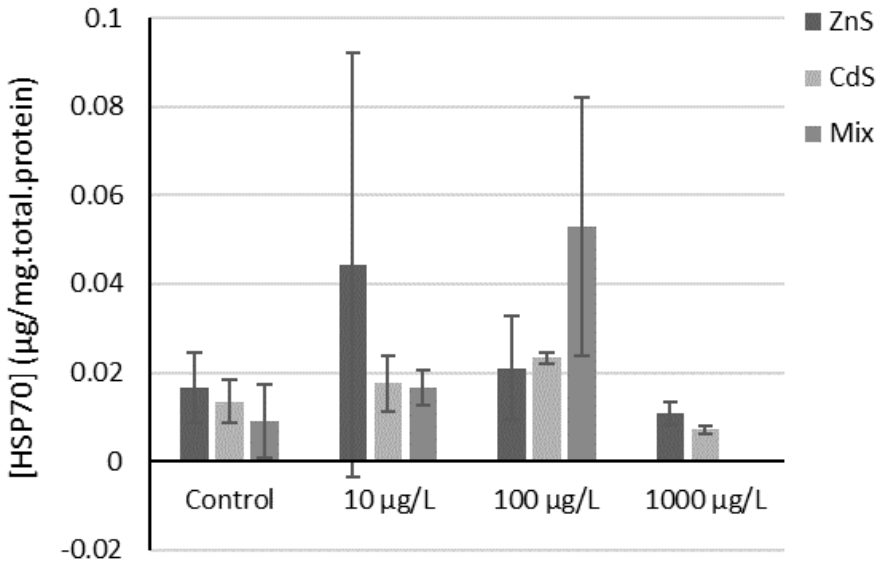

Figure 4.22 HSP70 concentration (mean ± SD) determined in *D. rerio* that ingested *D. magna* exposed to the different QDs concentrations, singly and combined. The experiment was performed in duplicate.

The statistical analysis showed no significant differences ($p>0.05$), for all the tested concentrations of ingested Daphnids, in comparison to the respective controls. Nonetheless, a trend to increase in fish that ingested Daphnids exposed to 10 µg/L ZnS QDs and to 100 µg/L QDs combined can be noticed, followed by a decrease of HSP70 production in fish that ingested Daphnids exposed to 1000 µg/L QDs (ZnS and CdS).

4.5.2.5 Ubiquitin

The results from the Ubiquitin assay in fish that ingested Daphnids exposed to QDs (singly and combined) are presented in figure 4.23. The highest average levels of Ubiquitin (0.0178 ± 0.0039 µg/mg total protein) were determined in fish that ingested Daphnids exposed to 100 µg/L CdS QDs, whereas the lowest average levels (0.0054 ± 0.0012 µg/mg total protein) were determined in fish that ingested Daphnids exposed to 1000 µg/L CdS QDs.

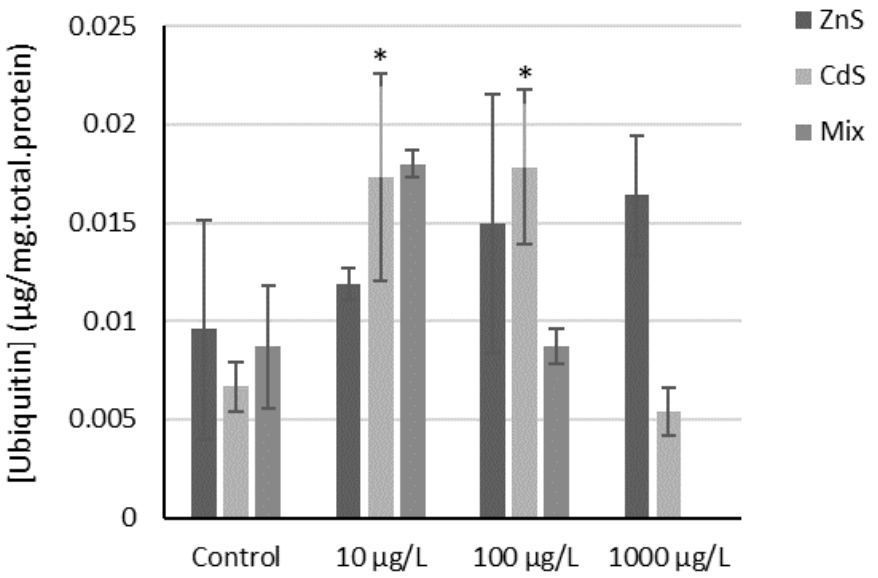

Figure 4.23 Ubiquitin concentration (mean ± SD) determined in *D. rerio* that ingested *D. magna* exposed to the different QDs concentrations. The experiment was performed in duplicate. Asterisk means significant differences ($p<0.05$) in comparison to the respective controls.

The Statistical analysis showed a significant increase ($p<0.05$) in fish that ingested Daphnids exposed to 10 and 100 µg/L CdS QDs, whereas for the other concentrations tested of ingested Daphnids no significant differences were found. Nonetheless, a trend to increase can be observed for the fish that ingested Daphnids exposed to the different concentrations of QDs, singly and combined, although a decrease in Ubiquitin levels was found in fish ingested Daphnids exposed to 1000 µg/L CdS QDs.

4.6 Cell Viability

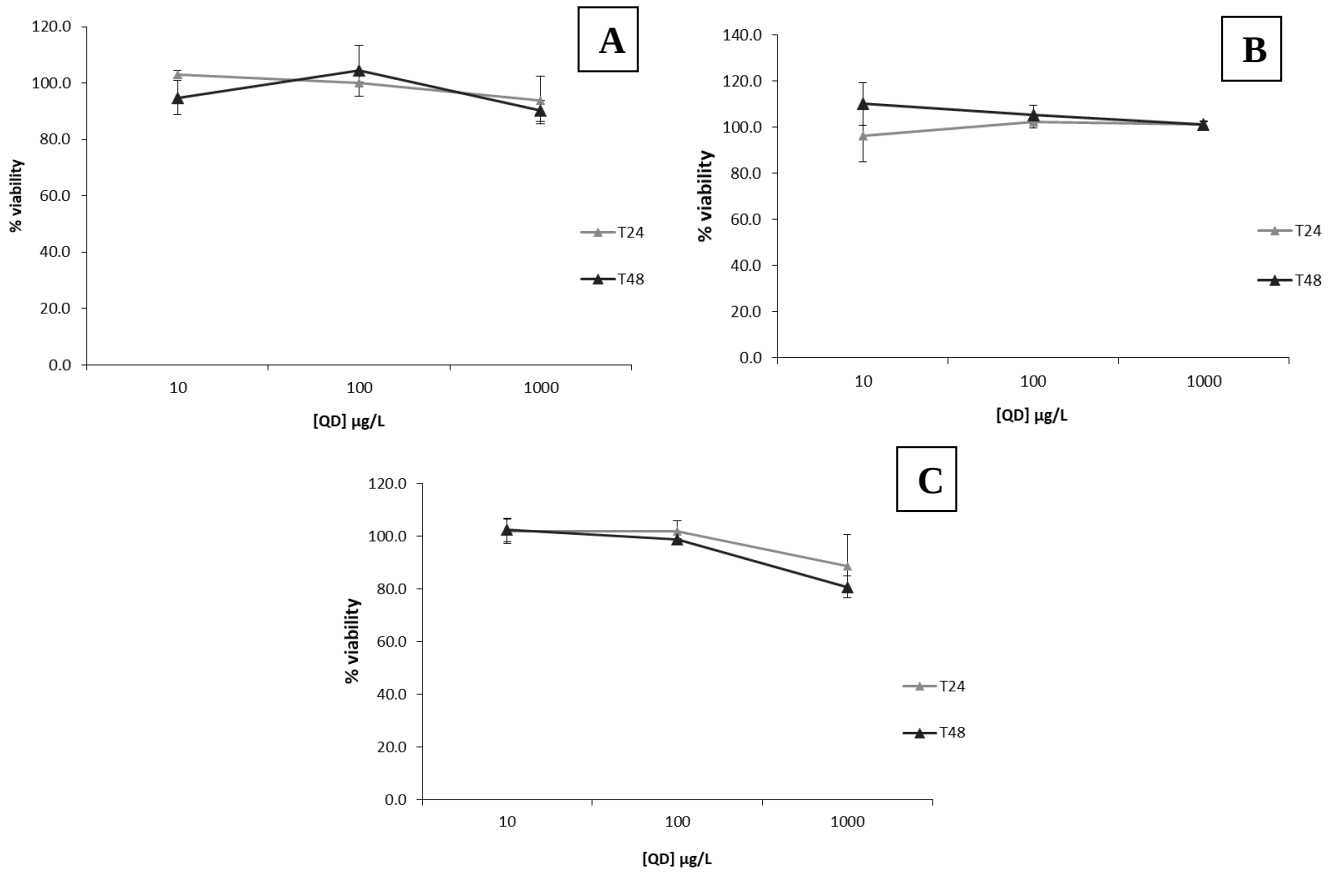

Figure 4.24 Cell viability (% of control) measured by the MTT assay on HepG2 cells exposed to 10, 100, 100 µg/L of Quantum Dots (A- ZnS, B- CdS, C- Mix) for 24 h and 48 h. An OD value of control cells (unexposed cells) was taken as 100% viability (0% cytotoxicity). Data were reported as mean ± SD of two independent experiments performed in quintuplicate. The relative cell viability related to control was calculated by [OD] test/ [OD] control ×100.

The MTT results are shown in figure 4.24. Statistical analysis showed no significant differences ($p>0.05$) between the different quantum dots treatments.

The cell viability obtained after the exposure to ZnS and CdS QDs singly varied between 90% and 110% and no significant differences were obtained between the QD concentrations and time of exposure.

Similarly, no cytotoxicity was observed in cells exposed to combined ZnS and CdS QDs and cell viability varied between 81%- 102%. The lower cell viability was obtained at the highest QD concentration at 48h, although without statistical significance.

Chapter 5

Discussion

5 Discussion

With the great development of nanotechnology that has occurred in recent decades, there has been a new type of contamination that affects the aquatic environment - contamination by engineered man-made nanomaterials. Additionally, with new discoveries emerging almost daily in the field of nanotechnology and the increasing use of nanomaterials, the threat to aquatic life is increasing. In fact, the release of nanomaterials into the environment constitute a serious hazardous to living organisms, namely to the aquatic biota because of their potential to mix and disperse in the environment. Moreover, it is known that nanoparticles are capable to enter organisms following exposure and be distributed through organisms' organs and tissues.

Furthermore, different sizes and shapes added to the fact that there are nanomaterials of a different nature (e.g. metal oxide nanoparticles, carbon nanoparticles, etc.), leading to largely unknown impacts on the environment. Aware of this problem the scientific community has conducted many nanomaterials (e.g. nanoparticles, nanotubes, QDs) toxicity studies with the aim of assessing the risk to human health and the environment [23,80–82].

Regarding QDs toxicity, it is less known and several questions about its toxicity remain unanswered. Therefore, the present studies, reports the assessment of QDs toxicity using three different biological models: *Danio rerio, Daphnia magna* and an HepG2 cell line. Additionally, another assay was conducted to evaluate the trophic transfer of QDs from *D. magna* to *D. rerio*. It should be noted that some controls from the assays where the QDs were tested combined showed significant higher levels of some enzymes. This can be attributed to a lot of fish that was acquired later to perform the tests and possibly had high stress levels due to transport or other unknown factor.

The size of nanoparticles is highly dependent on aggregation since nanoparticles tend to aggregate when in suspension due to attractive forces. It is known that in buffered or biological solutions, nanoparticles tend to aggregate very quickly, so the results can be interpreted in this context. [19]

Another important issue regarding QDs behaviour in aqueous medium is the fact that they can release ions from its core causing oxidative stress in living organisms following exposure. The ICP-AES analysis confirmed the presence of QDs (CdS and ZnS) in the water samples from the bioassays but at lower concentrations than nominal concentrations, suggesting that just a smaller fraction is dispersed in the water and most of the QDs are aggregated at the bottom of the glass containers. We can also hypothesize that some of the Cd and Zn analysed are metal ions released from the QDs core. Similar results were also obtained by Benavides et al.[63] after exposing *C. auratus* to AlO_3 and ZnO nanoparticles.

Quantum dots characterization

With respect to QDs characterization, the results from electron microscopy (TEM and SEM) confirmed the average size of the QDs (~5 to 10 nm for CdS and ~8 to 12nm for ZnS) used to conduct the biological assays. However, the results obtained from DLS analyses revealed that in solution (H_2O) they tend to form large aggregates meaning that organisms are mostly exposed to micro-aggregates composed of QDs instead of the single QDs.

Bioassay 1: Exposure of Danio rerio to QDs (ZnS, CdS), singly and combined

No significant mortality occurred during the exposure period (7 days), meaning that none of the different QDs concentrations tested were high enough to cause death in fish. Nonetheless, the biochemical results showed sub-lethal effects. These effects can be, in part, due to the exposure to metal ions released by QDs. Several studies reported the release of metal ions from metal oxide nanoparticles [63,83,84]. In fact, the released metal ions can cause ROS overproduction leading lead to oxidative stress. Consequently, the release of metal ions from QDs (e.g. ZnS and CdS) may play an important role in organisms' toxicity.

The most biological relevant ROS species are the molecular oxygen (O_2), superoxide anion (O_2), hydrogen peroxide (H_2O_2), hydroxyl radical (OH^-), nitric oxide (NO^-), peroxynitrite ($ONOO^-$) and ozone.[85] Thus, organisms, during the evolution process, had developed suitable defence mechanisms against the overproduction of ROS, which are usually referred as antioxidant mechanisms. Some of this important group of antioxidant enzymes include superoxide dismutase (SOD), which converts O_2^- in H_2O_2; catalase (CAT) that remove H_2O_2 avoiding its accumulation in cells and tissues; glutathione-S-transferase (GST) which transform xenobiotics into other conjugates to excretion).[51]

In general, the results from Bioassay 1 suggest that the tested QDs concentrations, singly and combined, are capable to cause sub-lethal effects in *D. rerio* as supported by some results from the antioxidant enzymes (e.g. CAT, GST), HSP70 or Ubiquitin results. The results from the biochemical analyses suggest that fish exposed to the lowest QDs concentration produce reduced or no toxicity effects. The observed increase in some antioxidant enzymes may indicate an adaptative response to oxidative stress. Moreover, the results also indicate that the tested QDs concentration of 100 µg/L apparently produces more adverse effects than the highest concentration. Moreover, the results also suggest that ZnS QDs seems to be slightly more toxic to fish than CdS QDs, which was unexpected since Cd toxicity is well known.

SOD and CAT are the first enzymes acting sequentially against ROS and can be used as an oxidative stress signal for environmental pollution [63,85]. Generally, these enzymes work together and when SOD activity increases it will transform some ROS into H_2O_2, then the CAT activity increases as well to transform H_2O_2 into H_2O and O_2. In effect, the results showed an increase, although not significant, of SOD activity in fish exposed to 100 µg/L and 1000 µg/L of

QDs (ZnS and CdS), while for the exposure of QDs combined, a slight but not significant increase of SOD was observed in fish exposed to 1000 µg/L QDs. CAT results showed a trend to increase in fish exposed to ZnS QDs with a significant ($p < 0.05$) increase detected in fish exposed to 100 µg/L QDs (ZnS). The same trend was observed for CAT results in fish exposed to CdS QDs. However, no trend or significant changes were detected in CAT activities for fish exposed to 1000 µg/L QDs, singly or combined. These results are in also agreement with SOD results. A possible explanation for the CAT activity reduction in fish exposed to 1000 µg/L is that the highest concentration caused cell´s exhaustion due to oxidative stress or another possibility is that other defence mechanisms were triggered to avoid oxidative stress as glutathione enzymes like Glutathione-Peroxidase [85]. We cannot exclude as well the possibility of larger aggregates (microparticles) being formed at higher concentrations leading to diminishing QDs bioavailability [86–88].

The GST is an enzyme (Phase II biotransformation system) which plays a major role in the biotransformation of xenobiotics facilitating its excretion. This enzyme catalyses the conjugation of GSH with contaminants making them easier to eliminate by the organisms. Results from GST activity in *D. rerio* showed a trend to increase for ZnS and CdS QDs, with a significant increase ($p < 0.05$) detected in fish exposed to 100 µg/L ZnS QDs. These results suggest a negative impact of the tested QDs concentration (ZnS) on fish but also indicate an activation of the defence mechanisms to respond to oxidative stress due to the exposure to these compounds. However, GST results from the tested QDs combined showed an opposite trend, where a significant decrease ($p<0.05$) of GST activity was observed in fish exposed to 10 µg/L and 100 µg/L. These results may suggest the suppression of GST activity due to a decrease in GSH levels [63,89].

ROS have the capacity to cause lipid peroxidation, causing damage in cell membranes and leading to cell to death. The MDA content in an organism is usually used as an indicator of lipid peroxidation due to oxidative stress in cells.[60,63] The results from LPO assay (MDA content) observed in exposed *D. rerio* showed variable values for the different QDs concentrations tested. However, it seems that CdS QDs were able to cause a more pronounced effect than ZnS QDs or when tested combined as shown by increased levels of MDA in fish exposed to 10 µg/L and 1000 µg/L CdS QDs. This can suggest a harmful effect on membrane lipids. The reduction in MDA levels in fish exposed to 1000 µg/L CdS QDs can be associated with an antioxidant system response to fight oxidative stress in fish exposed to 100 µg/L CdS QDs. With respect to fish exposed to ZnS QDs and QDs combined no significant changes ($p>0.05$) on MDA levels were observed which can mean that the antioxidant system is responding to fight oxidative stress, which is also in agreement with SOD and CAT results since these two enzymes work together to remove the ROS. Thus, MDA results may suggest that CdS QDs caused more damage to membrane lipids in fish exposed to this type of QDs than ZnS QDs.

Since, total antioxidant capacity (TAC) is a global measurement of antioxidant status, i.e., the capacity to inhibit the oxidation process and give a global idea about the response to oxidative

stress. Regarding, TAC results, although no significant changes ($p>0.05$) were detected in fish exposed to the different concentrations of QDs (singly and combined), a global trend to increase was observed which is in agreement with the previous antioxidant enzyme results where a general increase was also found.

The results from HSP70 production in *D. rerio* exposed to the different QDs concentrations tested (singly or combined) showed an increase in fish exposed to 100 μg/L QDs, in comparison to the respective controls, while for the other tested QDs concentrations a decrease was observed. Since HSP70 increase is commonly related to thermal stress it was not expectable to detect significant increases following QDs exposure. However, since HSP70 acts also as a chaperone to protect cells, the rise detected in fish exposed to 100 μg/L QDs can be associated with this protective role. Nonetheless, the HSP70 results may suggest that the tested QDs concentrations (singly and combined), with an exception for the tested 100 μg/L concentration, which did not triggered HSP70 production, possibly due to the action of the antioxidant system fighting oxidative stress.

Ubiquitin results in fish exposed to the different concentrations of QDs followed a similar pattern found for HSP70 results. Where a general trend to increase was detected in fish exposed to 100 μg/L QDs, singly and combined. As mentioned previously, this is the single concentration that caused an increase in ubiquitin levels possibly linked to an increase in oxidative stress in fish exposed to this concentration Since ubiquitin is usually induced when damaged proteins increase, probably at the other concentrations the damaged proteins were in small amount, what can suggest that for the other exposures the antioxidant enzymes were capable to deal with oxidative stress.

In general, enzymatic activities, lipid peroxidation and concentrations of HSP70 and ubiquitin revealed a trend to increase in fish exposed to 100 μg/L QDs singly or combined. These results can partially be explained by QDs aggregation in water leading to diminished bioavailability. This is a phenomenon already reported in numerous studies (Diniz, M. S. *et al.* Phenrat *et al*, Ramsden *et al.)* [86,88,90] and whose occurrence was also confirmed in the present study as confirmed by the results obtained from DLS analyses. These results also are in accordance with the idea that size can influence nanoparticles toxicity, due to the relationship between mass and surface area [19,63]. In this study, the tested concentration of 100 μg/L QDs is an intermediate value, where, due to the concentration, the nanoparticles tend to be more aggregated than 10 μg/L but less than 1000 μg/L. According to Bian *et al.* [91] it is possible that at an exposure concentration of 10 μg/L QDs fewer aggregates exist due to dissolution processes, however, at this lower concentration, the results suggest lower toxicity. In fact, studies from Bian *et al*.[91] showed that the dissolution rate for ZnO nanoparticles is proportional to the particle surface area and consequently small particles should dissolve faster than larger ones.

On the other hand, in fish exposed to a concentration of 1000 μg/L QDs, more aggregation occurs and the increase in particles size will reduce their bioavailability making them more difficult to be uptake by organisms and consequently aren't able to cause damage because

the dissolution process occurs in a smaller extension. Regarding the tested QDs concentration of 100 μg/L possibly, nanoparticles are in enough concentration to cause some damage in organism´s cells since they in some extent may suffer dissolution allowing the release of metal ions and aggregation is lower than at a higher concentration as 1000 μg/L.

With respect to the QDs nature, ZnS QDs seems to be more harmful than CdS QDs. The higher toxicity of Zn nanoparticles was reported before and was associated to the higher release rate of metal ions [63,83,91] Several studies were performed using ZnO nanoparticles and revealed that zinc ions, Zn (II), released from nanoparticles to water are capable to cause harm to the organisms even if zinc is an essential element. The results from this bioassay using ZnS QDs are in accordance with these previous studies since the fish exposed to ZS QDs revealed higher values of oxidative stress than those exposed to CdS QDs. This can suggest that the Cd ions dissociation occurred in a less extension reaction, and probably Cd ions exist in aqueous solution in small quantity, which was not enough to cause more pronounced effects in organisms. This is also in agreement with studies by Benavides that assessed Al and Zn nanoparticles toxicity, singly and combined [63]. Even if these results suggest that the release of ions from the core of QDs in the aqueous medium is the principal cause for their toxicity. We can also hypothesize that the dissociation process can occur inside the cells of the fish and cause injury. In water, fish exposed to QDs can ingest them depending on their bioavailability, whereas inside organisms, nanoparticles can cross the cell´s membrane according to their size and shape by endocytosis for further degradation. During the elimination process, nanoparticles are trafficked to acid and oxidative conditions that will improve the process of dissociation into toxic components that will compromise the cell viability [12]. As referred previously, the aggregation will then be determinant to QDs toxicity.

Bioassay 2: Exposure of *Daphnia magna* to QDs (ZnS and, CdS) singly and combined

Following the exposure period (21 days), the results showed high mortality rates for the tested QDs concentrations (singly and combined), with organisms exposed to 1000 μg/L QDs (combined) registering 100% mortality. This suggests that *D. magna* are sensitive for the range of the tested QDs concentrations, particularly for the highest tested concentration.

CAT activities show higher levels in individuals exposed to 100 and 1000 μg/L QDs (CdS) and combined, in comparison to the controls. The results suggest that this increase can be linked to the higher tested QDs concentrations which caused an increase in CAT activity possibly because organism´s cells are attempting to fight oxidative stress[85,92]. This toxicity increase is also in agreement with the mortality rates. Whereas when testing QDs combined, a significant increase was detected for fish exposed to 10 μg/L followed by a decrease. Although SOD values are below 5% inhibition a trend to decrease was observed in comparison to controls which can mean that other mechanisms (e.g. enzymes, antioxidants) are also acting to oppose oxidative stress. The GST activity results showed a decrease following *D. magna* exposure to the different

concentrations of tested QDs, except for a single individual that survived after exposure to 1000 μg/l CdS QDs which presented the highest levels of GST. Once again, a possible hypothesis is that other antioxidant mechanisms are acting to prevent oxidative stress and thus in some extent are protecting cells from injury [93]. Another possibility can be related to an, inhibition by CdS and ZnS since it is known that these metals can inhibit GST activity. [85,94,95]

Since MDA is a product of the reaction between free radicals and unsaturated fatty acids in cellular membranes it is used to measure the magnitude of the damage [46,92]. Thus, results revealed high variability of MDA levels determined in organisms exposed to the different tested QDs concentrations, which suggest that the exposure to QDs affected differently, but not significantly, the exposed organisms.

Since TAC is a measurement of antioxidant status, is possible to infer about the global status of antioxidant defence of the organism. In general, it was found a decrease of TAC levels in organisms exposed to the different QDs concentrations, singly and combined, which can be due to a depression of the antioxidant mechanisms.

Ubiquitin is a protein responsible for labelling damaged proteins for degradation by the ubiquitin-proteasome system. Normally, it is observed an increase in the number of ubiquitin conjugates during the oxidative stress [65,96]. In effect, the activity of ubiquitin conjugation and proteasome depends on the severity of oxidative stress and the concentration of ubiquitin conjugates can increase (sustained oxidative stress) or decrease (severe oxidative stress).[65,67] The results showed a trend to increase in Ubiquitin levels of the organisms exposed to QDs combined, which is in agreement with an oxidative stress event. The increase observed after organism´s exposure to CdS QDs (represents a single individual that survived) and the observed mortality rate is also in accordance with this hypothesis. In fact, the high mortality rates observed after exposure to the different QDs concentrations, namely to 1000 μg/L, supports that Daphnids are under oxidative stress and that the highest tested concentration is lethal to this species.

The results presented in this work are in agreement Shaw et *al.*[97] that studied Zn and Cd toxicity, singly and combined, in different Daphnia species, including *D. magna.* The authors concluded that *D. magna* were more tolerant to the exposure to metals singly than when combined, suggesting and additive interaction between the metals. The same authors. observed an additive effect (90% of mortality after *D. magna exposure* to LC50 of Zn and Cd) which is also in accordance with our results.

Bioassay 3: Trophic transfer of QDs (ZnS and CdS) from *D. magna* to *D. rerio*

Overall, the antioxidant enzymes and the other biomarkers results (TAC, Ubiquitin, LPO) showed that *D. magna* were under oxidative stress caused by the exposure (for 96h) to the different tested concentrations of QDs. The ICP-AES results from fish tissues indicate that fish ingested Daphnia which in turn bioaccumulated QDs (ZnS and CdS, singly and combined) suggesting that there is QDs (and or their metal ions) transfer from Daphnids to fish. Nonetheless, the results were variable which can be attributed to the fact that the animals did not eat the same amount of Daphnids. Biomarkers results of fish following ingestion of exposed daphnids showed no significant differences for CAT, SOD, GST, MDA, TAC and HSP70. Moreover, the results for these biomarkers presented high variability, making an explanation difficult. The results variability can be related to the fact that the amount of daphnia ingested by each fish is unknown. Nonetheless, LPO results (MDA content) show a general trend to increase for fish that ingested daphnids exposed to ZnS QDs, while fish that ingested daphnids exposed to CdS QDs presented a trend to increase followed by a decrease in fish that ingested daphnids exposed to the highest concentration (1000 μg/L). These results indicate some effects after daphnids ingestion by fish. In fact, it may suggest that exposed daphnids ingestion by fish lead, to some extent, to oxidative stress [98].

With respect to Ubiquitin levels, it was found an increase in fish that ingested daphnids exposed to the different tested QDs concentrations followed by a decrease in fish that ingested daphnids exposed to 1000 μg/L CdS QDs. This increase can be indicative of oxidative stress conducting to a rise in organism´s damaged proteins, and consequently augmenting ubiquitin concentration and so that damaged proteins are degraded by the proteasome system [65,67].

Overall, results suggest that fish were under some oxidative stress following uptake of the exposed daphnids to the different concentrations of tested QDs.

Toxicity assessment of QDs using HepG2 cell lines

This type of nanoparticles is widely used for biomedical applications in humans, so their cytotoxicity must be low. In accordance, the results obtain in the present study, showed that ZnS and CdS QDs exposures, isolated or combined, were not able to cause cytoxicity in HepG2 cells In fact, these results are in accordance with Peng *et al.*[99], Guo *et al.*[100] Smith *et al.*[101] studies that obtained similar viability percentages but with QDs coated with different type of molecules. Also, another approach could be done to conclude about the behaviour (aggregation state) of quantum dots in cell culture medium. Like was explained above, quantum dots had a trend to aggregate in water leading to a slower release of toxic ions to the cell medium, which may explain the absence of QD cytotoxicity in HepG2 cells. Despite that, some authors (Sabuncu et *al.,* Allouni et *al.*, Maiorano et *al.* [102–104]) stated that, the albumin present in cell culture medium containing FBS or

Fetal Calf Serum (FCS) promotes the dispersion of nanoparticles and decreases the occurrence of the aggregation phenomena. So, a DLS assay should be done to infer this hypothesis.

Chapter 6

Conclusions

6 Conclusions

In the present study, the exposure to the different concentrations of QDs tested, alone or combined, was not lethal for *Danio rerio*. However, high mortality rates were found for *Daphnia magna* following the exposure to the different concentrations of QDs. Yet, Daphnia were exposed for 21 days and fish were exposed for seven days, which making hard any comparison. With respect to HepG2 cell line, no apparent cytotoxicity was observed for the same QDs concentrations.

Furthermore, the biochemical results revealed that the exposure to the different concentrations of QDs caused sub-lethal effects on *D. rerio* and *D. magna* causing oxidative stress (as indicated by induction of antioxidant enzymes activity, increase in lipid peroxidation and ubiquitin and HSP contents).

The characterization's techniques (SEM, TEM and DLS) showed a trend of QDs to aggregate, in aqueous medium. In fact, the results suggest that this phenomenon could decrease the QDs bioavailability and consequently the toxicity of QDs to fish.

The results from Biomarkers' revealed that the most severe effects were observed in fish exposed to 100 µg/L QDs (singly and combined), suggesting that at this concentration the QDs and or their ions the effects in fish are more pronounced. Regarding the type of QDs tested, the results suggest that ZnS was more harmful than CdS, even considering that it is an essential element.

With respect to *Daphnia magna it* was found that after exposure, to 1000 µg/L of QDs combined, all the individuals died showing that after 21 days of exposure the highest concentrations were lethal to this species.

The results from the trophic transfer assay revealed that *Danio rerio* were under oxidative stress conditions following the ingestion of the previously exposed daphnids to the different concentrations of QDs. ICP-AES results indicate the presence of Zn and Cd ions in the fish tissues suggesting that trophic transfer occurred from Daphnia to fish.

Toxicity assessment of QDs using HepG2 revealed that the concentrations of QDs tested were not able to cause cytotoxicity in this cell line. These results are good indicators since QDs are widely used in humans for biomedical applications.

All these results together may suggest that QDs may pose a potential risk for aquatic ecosystems even if they do not cause cytotoxicity in HepG2 cells. Other studies should be performed to examining the route from the factory to the ecosystems to assess the state of the capping during this process.

Moreover, there are still many unanswered questions about nanotechnology and its consequences to wildlife. Thus, more studies must be carried out since knowing the toxicity of

these nanomaterials must be a priority for the development of new strategies to minimize the risk for the aquatic ecosystems.

7 References

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8 ANNEXES

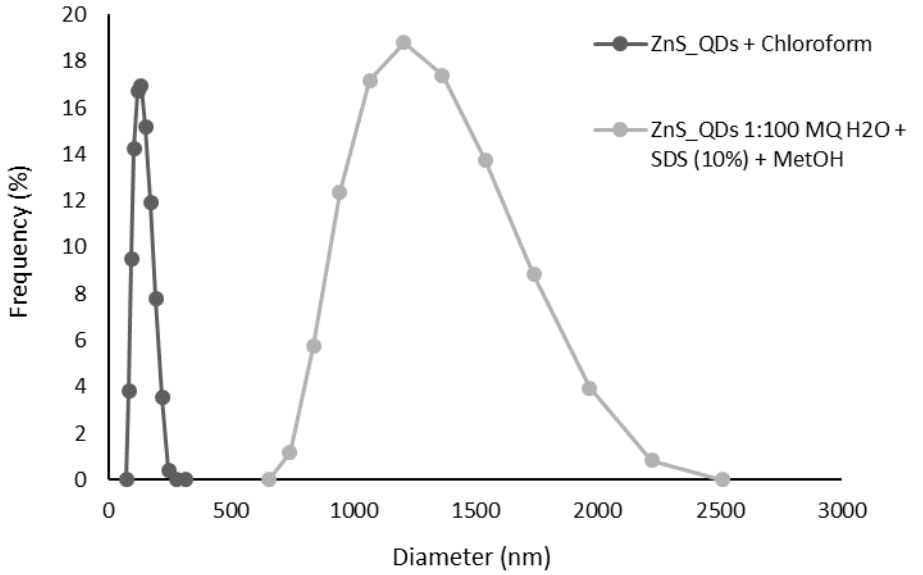

Figure 8.1 Graphic representation of the results obtained. ZnS_QDs re-dispersed in chloroform (black). ZnS_QDs re-dispersing in water, with the aid of SDS (surfactant) and methanol (grey).

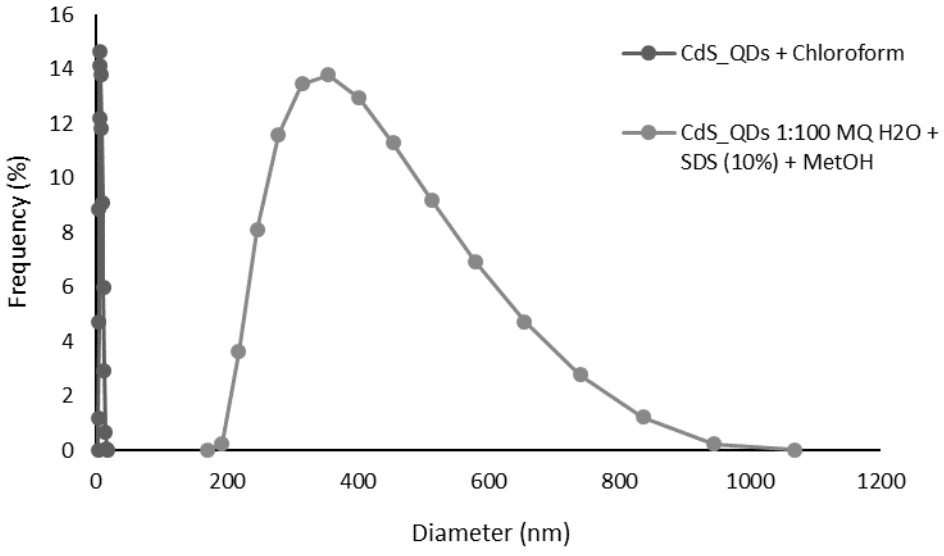

Figure 8.2 Graphic representation of the results obtained. CdS_QDs re-dispersed in chloroform (black). CdS_QDs re-dispersing in water, with the aid of SDS (surfactant) and methanol (grey).

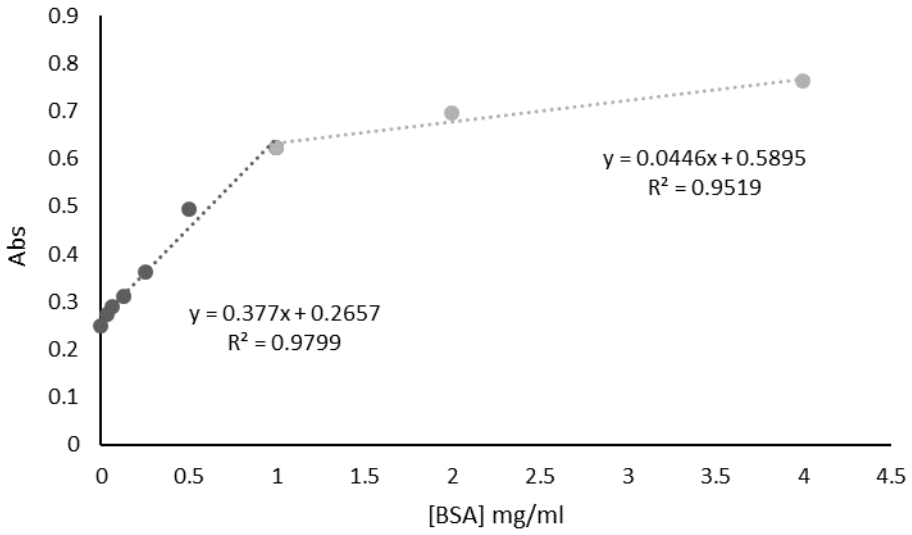

Figure 8.3 BSA calibration curve to estimate the total protein concentration for further normalization of the assays.

Table 8.1 Biometric data (Weight (g) and size (cm)) of Danio rerio exposed to ZnS QDs (Bioassay 1).

Sample	Weight (g)	Size (cm)
Control_1	0.201	2.5
Control_2	0.127	2.6
Control_3	0.225	2.5
Control_4	0.162	2.4
10 µg/L_1	0.239	2.6
10 µg/L_2	0.211	2.7
10 µg/L_3	0.263	2.7
10 µg/L_4	0.271	2.4
100 µg/L_1	0.209	2.5
100 µg/L_2	0.185	2.7
100 µg/L_3	0.153	2.4
100 µg/L_4	0.072	2.1
1000 µg/L_1	0.168	2.5
1000 µg/L_2	0.172	2.1
1000 µg/L_3	0.275	2.7
1000 µg/L_4	0.216	2.5
Average	0.197	2.5
STDEV	0.054	0.2
Total	16	

Table 8.2 Biometric data (Weight (g) and size (cm)) of Danio rerio exposed to CdS QDs (Bioassay 1).

Sample	Weight (g)	Size (cm)
Control_1	0.201	2.5
Control_2	0.127	2.6
Control_3	0.225	2.5
Control_4	0.162	2.4
10 µg/L_1	0.134	2.7
10 µg/L_2	0.147	2.4
10 µg/L_3	0.151	2.5
10 µg/L_4	0.217	2.6
10 µg/L_5	0.171	2.5
100 µg/L_1	0.238	2.9
100 µg/L_2	0.200	2.8
100 µg/L_3	0.124	2.7
100 µg/L_4	0.158	2.4
100 µg/L_5	0.183	2.3
1000 µg/L_1	0.275	2.9
1000 µg/L_2	0.071	2.5
1000 µg/L_3	0.231	2.9
1000 µg/L_4	0.107	2.6
1000 µg/L_5	0.074	2.1
Average	0.168	2.6
STDEV	0.056	0.2
Total	19	

Table 8.3 Biometric data (Weight (g) and size (cm)) of Danio rerio exposed to QDs combined (Bioassay 1).

Sample	Weight (g)	Size (cm)
Control_1	0.397	3.0
Control_2	0.453	3.3
Control_3	0.594	3.0
Control_4	0.139	2.5
10 µg/L_1	0.600	3.2
10 µg/L_2	0.528	3.7
10 µg/L_3	0.473	3.8
10 µg/L_4	0.352	2.8
10 µg/L_5	0.450	2.8
100 µg/L_1	0.568	3.0
100 µg/L_2	0.611	3.4
100 µg/L_3	0.244	2.8
100 µg/L_4	0.338	3.1
100 µg/L_5	0.340	2.7
1000 µg/L_1	0.307	3.1
1000 µg/L_2	0.294	3.0
1000 µg/L_3	0.379	3.2
1000 µg/L_4	0.456	3.1
1000 µg/L_5	0.300	3.0
Average	0.412	3.1
STDEV	0.131	0.3
Total	19	

Table 8.4 Biometric data (Weight (g) and size (cm)) of D. rerio that ingested contaminated *D. magna* with ZnS QDs (Bioassay 3)

Sample	Weight (g)	Size (cm)
Control_1	0.483	3.5
Control_2	0.227	3.0
Control_3	0.132	2.5
10 µg/L_1	0.246	3.0
10 µg/L_2	0.119	2.4
10 µg/L_3	0.220	2.7
100 µg/L_1	0.119	2.4
100 µg/L_2	0.103	2.1
100 µg/L_3	0.217	2.8
100 µg/L_4	0.126	2.2
1000 µg/L_1	0.152	2.6
1000 µg/L_2	0.134	2.8
1000 µg/L_3	0.204	2.8
1000 µg/L_4	0.211	3.0
Average	0.192	2.7
STDEV	0.097	0.4
Total	14	

Table 8.5 Biometric data (Weight (g) and size (cm)) of D. rerio that ingested contaminated *D. magna* with CdS QDs (Bioassay 3)

Sample	Weight (g)	Size (cm)
Control_1	0.148	2.6
Control_2	0.237	2.6
Control_3	0.124	2.5
Control_4	0.298	3.0
10 µg/L_1	0.161	2.5
10 µg/L_2	0.198	2.8
10 µg/L_3	0.180	2.6
10 µg/L_4	0.231	3.0
100 µg/L_1	0.227	2.9
100 µg/L_2	0.166	2.7
100 µg/L_3	0.221	2.8
100 µg/L_4	0.128	2.7
1000 µg/L_1	0.176	2.5
1000 µg/L_2	0.245	2.6
1000 µg/L_3	0.212	2.8
1000 µg/L_4	0.186	2.9
Average	0.196	2.7
STDEV	0.047	0.2
Total	16	

Table 8.6 Biometric data (Weight (g) and size (cm)) of *D. rerio* that ingested contaminated *D. magna* with QDs combined (Bioassay 3)

Sample	Weight (g)	Size (cm)
Control_1	0.228	2.6
Control_2	0.178	2.8
Control_3	0.407	3.3
10 µg/L_1	0.174	2.9
10 µg/L_2	0.266	3.0
10 µg/L_3	0.173	2.9
10 µg/L_4	0.228	3.0
100 µg/L_1	0.244	2.8
100 µg/L_2	0.401	3.3
100 µg/L_3	0.371	3.4
100 µg/L_4	0.415	3.2
1000 µg/L_1	0.22	2.7
1000 µg/L_2	0.19	2.6
1000 µg/L_3	0.191	2.9
1000 µg/L_4	0.161	2.8
Average	0.256	2.6
DESVPAD	0.094	0.7
Total	15	