Ecotoxicity of CdTe and its functional groups on *Enchytraeus albidus*

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Summary

Laboratory toxicity tests are used worldwide to assess the acute and chronic toxicity of specific pollutants to contribute towards the calculation of environmentally safe concentrations for nanomaterials (NMs). The field of nanotechnology is rapidly growing and the use of manufactured NMs in commercial products is increasing, however very little is known about the environmental effects of these materials. Soils are the end depository so it is essential to have an understanding of how the materials will affect soil organisms.

The aim of this study was to assess the acute and chronic ecotoxicity of cadmium/tellerium (Cd/Te) Quantum Dots (QDs) utilizing *Enchytraeus albidus* as a test organism. The first objective was to determine the lethality of the NMs on *Enchytraeus albidus*, determine the reproductive success using sub lethal concentrations, assess avoidance behaviour and biomarker responses, the approach was to compare the toxicity of the three functional groups (Polyethylene glycol (PEG), Carboxylic acid (COOH) and Ammonia (NH_3)) and lastly determine the uptake and distribution of the nanomaterial within the test organism. The nanomaterial was characterized by measuring the hydrodynamic size distribution by using Dynamic Light Scattering. Transmission electron microscopy was used to measure the diameter of QDs. Low dissolution rates (<23.7%) were found for the QDs coated with three different functional groups. The mean particle size showed that the NH_3 group exhibited the smallest particle size of the three functional groups.

Range-finding exposures were used to determine the concentration range for the definitive test using a standard Organization for Economic Co-operation and Development (OECD) guideline (220). The prepared exposure soil was characterised by oven-drying the soil and digesting with 10 mL of HNO_3 for metal content following a three-week exposure and compared to the corresponding bulk metals $CdCl_2$ and $TeCl_4$. In comparison with the bulk metals after a three-week exposure, the QDs displayed considerably less Cd and Te of the same nominal concentration. Transport of NMs in soil was conducted utilizing a flow-through system. Results indicated that when a stock concentration of QDs in Milli-Q water was added to the top of soil, the highest metal content was found in the eluted water and eluted clay fraction with the third highest in the top layer of the soil. When the NMs were homogenously mixed into the soil the highest metal content was again found in the eluted water and eluted clay fraction, but the metal content in the soil column increased towards the lower level of the soil. CytoViva Dark field imaging illustrated internalization of QDs in the intestine and ICP-MS analysis of whole worm tissue indicated metal uptake.

The Lethal Concentration (LC_{50}) values could not be calculated from the acute toxicity test because no mortality was observed. Only QD-COOH could determine the Effective Concentration (EC_x) value for reproduction, with the EC_{10} and EC_{20} for COOH, calculated as

124.1 mg/kg and 720.6 mg/kg respectively. For QD-PEG and QD-NH_3 no EC_x values could not be determined. Avoidance behaviour of *E. albidus* was assessed and no significant behaviour was observed compared to the control, whereas the corresponding bulk metals caused avoidance at the highest concentrations (500 mg/kg). A series of biomarkers (Catalase (CAT), superoxide dismutase (SOD), protein carbonyl (PC), Malondialdehyde (MDA), Acethylcholinesterase (AChE), lipid fractions and protein content) were utilized to determine sub-lethal effects on the enchytraeid. Biomarker responses indicated that oxidative damage occurred after a three-week exposure. Inhibition of CAT and SOD occurred indicating no defence mechanisms could be activated to counter the stress of QDs, which can be explained by the low lipid fraction and high MDA content. Protein carbonyl content compared to the control, indicate protein damage has occurred. AChE displayed significant inhibition of QD-PEG 100 mg/kg is related to the high avoidance response to QD-PEG 100 mg/kg.

In conclusion, the acute lethality tests showed no toxicity on the survival of the enchytraeids. However, chronic toxicity tests indicate that NM internalisation does occur and the QDs do have a sub-lethal biomarker response on the worms although survival, reproduction and avoidance response were not affected.

Keywords: Nanomaterials; Cd/Te; Quantum Dots; Functional Groups; PEG; NH_3; COOH; Enchytraeus albidus; Flow-through system; Mortality; Reproduction; Avoidance & Biomarkers

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General Introduction

1.1. Soil:

Soil is defined by the International Organization for Standardization (ISO) as “the upper layer of the earth crust composed of mineral parts, organic substance, water, air and living matter” (ISO, 2015). This underlines the importance of soil organisms as they play an important role in: organic matter decomposition, regulating of microbial activities, nutrient cycles, affect soil pH through denitrification and nitrification, improving soil porosity as well as soil formation (Cortet *et al.*, 1999; Beck *et al.*, 2005; Novais *et al.*, 2010). Soil can be described as the interface between the lithosphere and the atmosphere as well as the fresh and salt water bodies (the hydrosphere) and sustains growth for all terrestrial life (Hillel, 1980; White, 2006). Soil is a very complex system consisting of mineral (45%) and organic (5%) (solid components) content as well as water (25%) and air (25%). It is the soft material that covers the surface of the earth and forms part of the biosphere through sustaining plant growth and animals (White, 2006; Parker, 2010). Soil can be defined as a provider and without soil in the ecosystem many plants won’t survive because soil provides food and water for plants and is the home for a variety of small animals (Parker, 2010). It is not just water and air that is important for natural ecosystems, soil also provide a basis for ecosystems (Parker, 2010).

The entire system is barely ever in a state of steadiness, as it consistently swells and shrinks, wets and dries, separates and flocculates, contract and crack, exchanges ions, precipitates and re-dissolves salts and occasionally freezes and defrosts (Hillel, 1998; White, 2006; Parker, 2010). Over the years, soil as a habitat for organisms has been increasingly recognized as they are contributing to important processes within a soil system (Jänsch *et al.*, 2005; Hönemann and Nentwig, 2009). Soil, which acts as a biological habitat and gene reserve for a variety of species, has an immense diversity of soil organisms that have a number of influences such as plant growth, hydrology and nutrient cycles and lastly the occurrence and richness of pathogens in agricultural crops (Beck *et al.*, 2005; Blum, 2005; Jänsch *et al.*, 2005; Lavelle *et al.*, 2006). Invertebrates microfauna (enchytraeids), mesofauna (Protozoans and Nematoda) and macrofauna (Mollusca, annelids, Crustacea as well as arthropods) are immensely diverse and can represent as much as 23% of the total and local diversity of living organisms in some ecosystems may exceed the number of above-ground species (Cortet *et al.*, 1999; Lavelle *et al.*, 2006).

Soil organisms are responsible for ecosystem processes such as the cycling of nutrients and the decomposition of soil organic matter (Beck *et al.*, 2005). With soil being part of the most significant parts in the natural environment, it performs a large number of key social, environmental and economic functions (Blum, 2005). Ecological functions include biomass production, protection of the environment and humans, gene reservoir and the non-ecological functions include physical basis of human activities, geogenic and cultural heritage and raw materials (Blum, 2005). Soils are critical for terrestrial ecosystems due to the key functions they play in fertility, decomposition processes, nutrient and energy flows, soil provides regulating services by influencing organic matter dynamics and extensive effects on soil physical properties (Sochová *et al*., 2006; Lavelle *et al*., 2006; Parker, 2010). Soil is not only important for the above-mentioned processes but for remediation as well, it aids as a recycling factory for a number of waste products (Hillel, 1980).

The functioning of soil is imperative for terrestrial ecosystems and subsequently for human activity (Didden and Römbke, 2001). Human as well as animal life is sustained through functions such as biomass production, ensuring food, fodder, raw materials and renewable energy all of which are part of the ecological functions of soil (Blum, 2005). Therefore, soil is fundamental and irreplaceable as it governs plant productivity of terrestrial ecosystems, maintains biogeochemical cycles because of microorganisms that degrade in soil (Nannipieri *et al.*, 2003).

Soil quality can be defined as the “capacity of a specific kind of soil to function, within natural or managed ecosystem boundaries, to sustain plant and animal productivity, maintain or enhance water and air quality, and support human health and habitation” (Karlen *et al.*, 1997; Beck *et al.*, 2005). The quality and health of soil has generated quite a lot of interest because it is a vital component to the earth’s atmosphere as it functions not only in producing food but in managing local, regional and global environmental quality (Doran and Zeiss, 2000).

The criteria used to indicate good or bad soil quality and health relate mostly to their effectiveness in defining ecosystem processes and incorporate physical, chemical and biological processes, their sensitivity to management and climatic deviations, and their availability and effectiveness to policy makers, agricultural specialists and conservationists (Doran, 2002). There are eight key threats to soil namely: decline in soil biodiversity, floods and landslides, compaction, sealing, erosion, soil contamination, decline in organic matter and salinization (Blum *et al*., 2004). With the human population increasing, agricultural land to develop is decreasing and to meet the food demands of the human population the crop yield needs to be doubled. The increase in food demands that agricultural production be increased which in effect opens up the opportunity for environmental pollution through agricultural chemicals (i.e. pesticides and insecticides) as well as fertilizers. The use of pesticides,

insecticides and herbicides by farmers enabled them to secure high crop yields at low costs (Doran, 2002; Chen, 2007).

1.2. Nanomaterials:

The term 'nanometer' was first introduced by Richard Zsigmondy in 1925 (a Nobel Prize – winner in chemistry). He defined the term nanometer specifically for characterizing particle size and he was the first to quantify the particle size of gold colloids using a microscope (Hulla *et al*., 2015). The rise of nanoscience started in 1959, when Richard Feynman (a Nobel Prize winner for physics in 1965), gave a lecture about the fact that "there is plenty of room at the bottom" (Fanfair *et al*., 2007; Nouailhat, 2008). During this lecture he presented a concept about the manipulation of matter at the atomic level and with this idea, new ways of thinking was demonstrated and Feynman's hypotheses since then have been proven correct. It is for these exact reasons that he is considered to be the father of modern nanotechnology (Hulla *et al*., 2015).

The field of nanotechnology is rapidly growing and the use of manufactured NMs in commercial products is increasing (Lowry *et al*., 2012). Nanotechnology can be seen as technology that involves the use of nano-scale (1–100 nm) materials in various applications (Crane *et al*., 2008; Stone *et al*., 2010). The rate at which nanotechnology is expanding, becoming more and more promising in the 21st century is alarming. Nanotechnology is a relatively new field of science that requires more ecotoxicological research due to concerns over the potential release of NMs into the environment and potential negative impacts (Crane *et al*., 2008; Handy *et al*., 2008; Lin *et al*., 2010). An article published by BCC Research in 2016 stated the global market value for nanotechnology in 2016 would be $39.2 billion and should reach $90.5 billion by the year 2021 (BCC, 2016).

Nanomaterials have a greater reactivity than most of the conventional materials, with their interfaces and surfaces providing a substrate for biological, chemical as well as physical reactions including industrial catalysis (i.e. producing gasoline). With their unique optical and electronic properties, these properties can be personalized for specific applications (i.e. blue lasers) (Navrotsky, 2000). The potential benefits of nanotechnology and NMs are immense as they are used in several applications, for example, environmental monitoring, nano-drug delivery, biorobotics, nanoarrays as well as in medicine (Crane *et al*., 2008). Nanomaterials can occur as dust in the air, in soil, in volcanic ash, technological applications that ranges from ultra-tough ceramics to microelectronics as well as paints, cosmetics, medicines, food and suntan lotions and can find their way into our bodies (Navrotsky, 2000; Stone *et al*., 2010).

Two types of NMs exist namely: natural NMs and manufactured NMs. Natural NMs result from natural processes as well as anthropogenic impacts, for example, in acid mine drainage the aggregation of nanometer-scale metal oxides (Lowry *et al*., 2012). Manufactured NMs are is

specifically manufactured for specific products, for example, semiconductors like quantum dots, gold NMs, nanosilver, zinc oxide, titanium dioxide and iron oxide (Lowry *et al.*, 2012; Tourinho *et al.*, 2012). Metal and metal oxide-based NMs have been used in many products with different purposes, such as consumer products like sunscreens and cosmetics (ZnO and TiO_2), detergents and antibacterials (Ag), paints (TiO_2 and Ag), printer inks (Ag and Au) as well as textiles (Ag & TiO_2). Other applications include sporting goods (carbon nanotubes & nanoclay particles), catalysts, explosives (aluminium powders), coatings (TiO_2 & custom nanocomposites), filtration (made of nanofibers – for example aluminium fibers), alloys and metals (nano-crystalline nickel, nano-crystalline aluminium alloys), non-metallic components (carbon nanotubes), lubricants (nanodiamonds) (Tourinho *et al.*, 2012; Khan, 2014).

In medicine, nanotechnology has a major role to play, for instance NMs or nanotubes can be used in medical devices such as biosensors, microarrays, nanobarcodes, Lab-on-a-chip, imaging, therapy and regenerative medicine (Filipponi and Sutherland, 2012). Biosensors use nano-sieves, carbon nanotubes, nanowires (silicon nanowires), quantum dots, silica NMs, metallic NMs, magnetic beads as well as fullerenes (Filipponi and Sutherland, 2012). There are still many NM products currently being developed to be used in batteries, fuel cells, solar cells, light sources, display technologies, electronic storage media, biodetectors and bioanalysis, drug delivery and medical implants (Khan, 2014).

1.3. Soil ecotoxicology:

Soil ecotoxicology can be described as the study in which ecology and toxicology are studied to determine the effect of chemicals (Forbes & Forbes, 1994). The first soil ecotoxicological papers date back to the 1960s, where observations on the negative effects of pesticides on soil invertebrates had been made (van Gestel, 2012). Ecotoxicology was first defined by Prof. R. Truhaut in the late 1960s as a discipline which describes the toxicological effects of various chemicals on living organisms, particularly on populations and communities within ecosystems (Connell *et al.*, 1999). Ecotoxicology assesses the effect of chemicals on species or ecosystems in order to protect those (Hoffman *et al.*, 2003). By its nature, ecotoxicology is multidisciplinary which combines mechanisms, responses, toxicology, chemistry, pharmacology, ecology and epidemiology through the idea of understanding the sources and fate of pollutants in the environment, resulting in the increasingly need to regulate human and industrial activities which can lead to environmental pollution (Eijsackers *et al.*, 1994; Connell *et al.*, 1999). The aim of ecotoxicological tests is to generate data that will predict the outcome of environmental stress and conclude the effect concentrations that will be safe for populations and communities (Holloway *et al.*, 1997; van Gestel, 2012). Therefore, risk assessments as well as risk management can be brought into the ecotoxicological equation (Connel *et* al., 1999).

In risk assessments of chemicals, the assessment evaluates the likelihood of adverse effects as a result of one or more stressor (Hoffman *et al*., 2003). One of the key principles in risk assessments is to identify the appropriate endpoints for the assessment (Solomon, 1996). The main task for ecotoxicology is to identify a concentration level at which the risks to a predefined percentage of the population are not exceeded. This is accomplished through toxicity tests in which the organisms are exposed to a range of concentrations and then the effects are measured for the specific concentration (Van Straalen, 2002). The one common approach in soil-ecotoxicology is determining the effective concentration level for substances and the effective concentrations include EC_{10} (10% effective) or EC_{50} (50% effective) is identified (Van Straalen, 2002). Ecotoxicology (scientific discipline) will continue to be indispensable in order to guarantee that the management practices linked with potentially toxic materials are well understood (Hoffman *et al*., 2003).

1.4. Nano-ecotoxicology:

Research in the field of nano-ecotoxicology has started back in the early 1990s and the first scientific papers on the effects of ultrafine particles were published in Web-of-Science of Thompson Scientific (Kahru and Dubourguier, 2010). In the last few years the field of nano-ecotoxicology is growing rapidly as a result of the continuous development of nanotechnology and should be researched in order to understand the potential hazardous effects prior to the use in products and release into the environment (Kahru & Dubourguier, 2010; Sigg *et al*., 2014). Nano-ecotoxicology aims at understanding the effects of NMs on species and their environment, conducting toxicological studies at different dose ranges to determine the effect on different organs such as the kidneys, liver, lungs and the spleen (Sigg *et al*., 2014; Hedge *et al*., 2015; Pachapur *et al*., 2015). Environmental concerns on the ecotoxicity of NMs only started to rise later as the first papers was published in 2006 (Kahru & Dubourguier, 2010).

Nano-ecotoxicology remains limited to aquatic environments, mainly freshwater, while studies on sediments and non-aquatic environments remain scarce. Daphnids (*Daphnia magna*, *Daphnia pulex* and *Ceriodaphnia dubia*) are the most studied aquatic organisms and fullerenes and metal oxides are the most studied materials, comprising of 70% of available literature (Cattaneo *et al*., 2009). Quantum dots are among the NMs that receive special interest due to their applications in medicine, molecular biology and information technology (Ju-Nam & Lead, 2008).

The toxicity of NMs is not well-known and has gained interest over the past few years, especially quantum dots (Ju-Nam & Lead, 2008; Kurwadkar *et al*., 2014). Only a few ecotoxicological studies have been done on the effects of NMs on environmentally relevant species such as algae, bacteria, plants, crustaceans and fish (Santos, 2009). When assessing the effects that NMs have on the environment, an understanding about the quantities released

into the environment is required as well as their distribution through the environment looking at the exposure rate and their threat to the environment (Oberdörster *et al*., 2007; Sigg *et al*., 2014). Currently, there is a lack of knowledge about which environments and which organisms are the most likely to be exposed to NMs as the toxicological studies on a variety of organisms has only started (Oberdörster *et al*., 2007). Assessing NMs in the environment is difficult because certain tools such as reliable measure units, analytical chemical procedures, sample-related certified standards are lacking (Cattaneo *et al*., 2009).

The imminent release of manufactured NMs needs to be assessed because of the huge forecasted increase in NMs and exposure to these NMs is likely to be increased and lead to environmental as well as human health impacts (Lowry *et al*., 2012; Nowack & Bucheli, 2007). Nanotechnology applications are so diverse that NMs can enter the environment through many pathways: i.e. accidental spills, emissions which lead to deposition in soil and water, agriculture, solid wastes, transport, storage and soil and water remediation technologies and environmental remediation projects (Lin *et al*., 2010; Tourinho *et al*., 2012; Kurwadkar *et al*., 2014). With the ever-increasing purchasing of nanoproducts, it is evident that it will soon become a serious pollutant and carry a big risk of toxicity to living organisms (Jośko & Oleszczuk, 2013).

Products containing NMs are becoming available at an alarming rate and therefore the associated risk of environmental spills, waste release and product lifetime release are increasing as well. It is important to determine the health, safety and environmental risk of NMs to assist in predicting associated risks should NMs be released into the environment.

1.5. Research aims and objectives:

The aim of the study was to assess the acute and chronic ecotoxicity of cadmium/tellerium quantum dots (Cd/Te QDs) utilizing an enchytraeid (*Enchytraeus albidus*) as test organism.

The specific objectives of the study were:

1. To determine the acute toxicity of QDs using the standard enchytraeid mortality test (OECD, 2015) in spiked artificial OECD soil.
2. To determine the chronic toxicity of QDs using the standard enchytraeid reproduction and avoidance tests (OECD, 2015) and oxidative stress biomarker responses in spiked artificial OECD soil.
3. To compare the acute and chronic toxicities of three different functional groups (i.e. PEG, COOH and NH_3) QDs.
4. To determine the uptake and distribution of the different functionalized QDs in the enchytraeids.

1.6. Chapter outline:

This study was divided into 6 chapters

- ✓ Chapter 1: provides the rationale for the study as well as the aims and objectives of the study.
- ✓ Chapter 2: provides the literature overview for the study.
- ✓ Chapter 3: introduces the materials and methods used in the exposures. Materials and Methods include the characterization of the nanomaterials, Enchytraeid Reproduction Test (ERT), Avoidance behaviour, biomarkers, TEM and SEM and lastly ICP-MS.
- ✓ Chapter 4: provides all the results from the ERT, Avoidance, biomarkers, TEM and SEM and ICP-MS done in the study.
- ✓ Chapter 5: includes the discussion based on the results found from the exposures including the ERT, biomarker analysis, TEM and SEM, Avoidance behaviour and ICP-MS.
- ✓ Chapter 6: provides the conclusions and recommendations for the study.
- ✓ The references used for the different chapters are provided at the end of this dissertation.

Literature Review:

2.1. Soil:

Soil is made up of a diverse range of organic material and minerals and forms the thin outer layer of the terrestrial system (Wall *et al*., 2013). It provides vital natural resources to living organisms and plays a fundamental role in supporting life on earth, therefore deserves special importance as it regulates the environment (Plaster, 2013; Paul, 2015; Schoonover & Crim, 2015). Figure 2.1 displays soil's ability to act as a filter for anthropogenic waste, therefore helps with detoxifying, purifying and counteracting toxic elements that can be harmful to the environment (Blum, 2005; Huang *et al*., 2011). Soil forms the foundation for an ecosystem as the productivity of soil will determine what plant and animal life can be supported by an ecosystem, for example in cultivated fields, soils play a vital role in determining crop yield (Schoonover & Crim, 2015). Plaster (2013) stated that the human population will grow to 9 billion people by the year 2050, therefore soil will become even more important. Only about 7% of the world's soil is suitable for agriculture and that is going to decrease due to urbanization and degradation (Plaster, 2013).

Soil contains one third of all life on earth and has the ability to support terrestrial life as well as providing a habitat for it (Wall *et al*., 2013; Paul, 2015; Mishra *et al*., 2016). Terrestrial organisms are good indicators to determine quality and health of soil due to their role in processes that are globally important such is nutrient cycling, organic matter and energy, physical and chemical properties of soil and plant growth (Doran and Zeiss, 2000; Beck *et al.*, 2005; Menta *et al*., 2006; Stirling *et al*., 2016). Anthropogenic activities have a strong influence on the diversity of soil biota E.g. the use of pesticides and inorganic fertilizers that lead to a decline of a variety of groups of terrestrial invertebrates (Beck *et al.*, 2005).

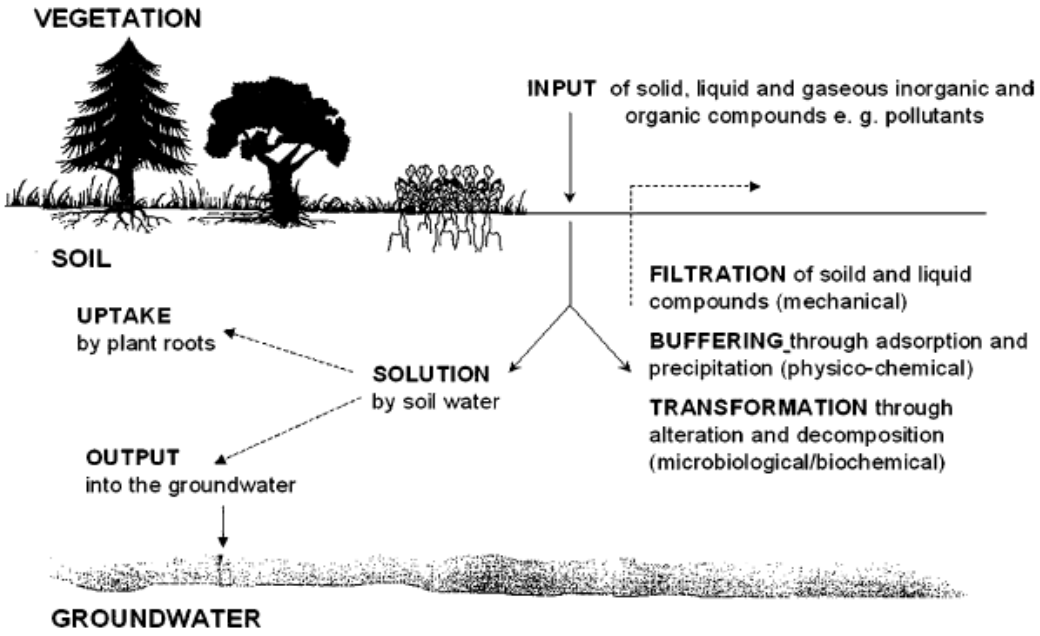

Figure 2.1: Soil acting as a filter, buffer and transformation system for hydrosphere, biosphere and atmosphere protect (adapted from Blum, 2005).

2.2. Cadmium & Tellurium:

Metals that are of environmental, human and animal health significance include e.g. Cd, Cr, Pb, Cu, Hg and Zn (Hooda, 2010). Human activities such as traffic, farming and irrigation are the major causes for releasing metals back into the environment and are posing a long-term risk as they are retained in soil causing the environment to deteriorate (Hooda, 2010; Hu *et al.*, 2013; Su *et al*., 2014).

Cadmium has an atomic number of 48, mass of 112.4 g/mol, a density of 8.65 g cm^{-3}, a melting point of 320.9ºC and a boiling point of 765ºC (Wuana & Okieimen, 2011). Although cadmium is extremely rare, human activities such as mining and smelting, sewage sludge, fertilizers and pesticides have led to more Cd-exposure to soil (Shahid *et al*., 2016). It gets taken up easily by plants because Cd is very mobile within the soil-plant system (Shahid *et al*., 2016). This metal together with two other metals, Pb and Hg, are the three major toxic heavy metals (Wuana & Okieimen, 2011). The chemical characteristics of the soil, such as pH, soil particle size, cation exchange capacity and temperature play a key role in the fate and toxicity of cadmium in soil-plant system (Shahid *et al*., 2016).

Excessive cadmium concentrations can have major consequences on an organism and is well known to cause increased production of reactive oxygen species (ROS). An increase in ROS leads to a variety of damages to DNA, RNA, enzyme inhibition, lipid peroxidation (LP) and covalent modifications of proteins (Shahid *et al.*, 2016). The critical problem of cadmium is the fact that it has a persistent long lifecycle, thus once absorbed by an organism it stays active in the body therefore making it very biopersistent (Wuana & Okieimen, 2011).

Tellurium was first discovered in 1782 by Müller and is frequently associated with gold, silver, bismuth and lead (Cerwenka & Cooper, 2013). Tellurium gets produced through the anode slime from electrolytic copper refining and has a variety of applications such as rubber, metallurgy, copper alloys, ferrous and electronics like generators and thermoelectric cooling systems (Taylor, 1997; Cerwenka & Cooper, 2013). Together with other metals such as selenium (Se), arsenic (As) and antimony (Sb), Te inhibits the same properties related to these metals and is used in a variety of important industrial applications but is not used as much as these metals (Taylor, 1996). It improves hardness and resistance to corrosion because of Te being part of a component of special composites (Taylor, 1996). Tellurium displays the same properties of the same metals that are toxic to humans but the toxic effects of tellurium are less known (Taylor, 1996; Cerwenka & Cooper, 2013). With the increase in industrial processes that involves the application of Te its toxicity effect on physiology and methods of monitoring tellurium in the environment and in species needs to be understand and considered (Taylor, 1997).

2.3. Enchytraeidae:

Oligochaetes are nonselective, burrowing feeders that consume plant-derived detritus, sediment-bound bacteria and microphytobenthos and can be found in almost all sediments, from intertidal and sub-tidal, marine and freshwater to terrestrial soil. They are also a good energy source for nektonic predators (Worsfold, 2003; Gillett *et al*., 2007). Oligochaetes form part of the class Clitellata (annelids developing clitellum), and are dioecious hermaphrodites (Pinder & Ohtaka, 2012).

According to Esser and Simpson, (1994) enchytraeids are classified as follows:

Phylum: Annelida

Class: Clitellata

Order: Oligochaeta

Family: Enchytraeidae

Genus: *Enchytraeus*

Species: *albidus* (Henle, 1837)

Enchytraeids are whitish, small oligochaetes that can be found in almost all soil types, marine habitats as well as freshwater (Schmelz *et al*., 2000; OECD, 2015). Worldwide, there are about 900 species described that appear in a variety of soils with high seasonal fluctuating abundances ranging from a few thousand up to more than 100 000 individuals m^{-2} (Hönemann and Nentwig, 2009). Enchytraeids are known to contribute to fundamental environmental processes such as regulating organic matter and improving the soil pore structure (Hedlund and

Augustsson, 1995; Amorim *et al.*, 2011; Novais *et al.*, 2012). One worm can live on average between 2-9 months, they reach their sexual maturity between 5-7 weeks after an egg has hatched (Hönemann and Nentwig, 2009). Embryos develop over a period of 12 days and the juveniles over 21 days with the total development cycle over 33 days (Römbke & Moser, 2002).

Most enchytraeids measure 2 - 40 mm in length, *E. albidus* being one of the largest (Didden *et al*., 1997; Römbke, 2003; Jänsch *et al*., 2005; Jeffery *et al*., 2010; OECD, 2015). Because of their slender body diameter (0.05 -1.5 mm), they are categorized as soil mesofauna and they are found in virtually all soil types (Didden *et al*., 1997; Jeffery *et al*., 2010). According to Römbke (2003) they usually reproduce sexually, but asexual fragmentation is possible. They feed on decomposed plant residues and micro-organisms. According to Jänsch *et al*. (2005) some of the species can feed on largely non-decomposed litter. An enchytraeid's body is composed of a variety of generally matching segments with a glandular girdle (clitellum) located at the end of the first third of the body (Jänsch *et al*., 2005). The skin of an enchytraeid is plain and always moist, which is used for respiration (Jänsch *et al*., 2005).

The individuals of *E. albidus* can reproduce quickly, can be kept in various substrates and can also be fed with different foods as well (Römbke, 2003). These worms are tolerant to temperature, but most of the individuals of the species prefer temperatures between 5 and 28°C, thus enchytraeid populations are unlikely to be influenced by temperature regimes (Didden *et al*., 1997). Enchytraieds are hermaphrodites, but some of the species can reproduce through parthenogenesis or self-fertilization (Didden *et al*., 1997; Jänsch *et al*., 2005). Another form of reproduction exists which is known as fragmentation. This happens when an individual autonomously breaks up into several parts, each of which regenerates into a complete new individual (Jänsch *et al*., 2005).

Hedlund & Augustsson (1995) stated that enchytraeids play an important part in the decomposition of organic material and the turnover of plant nutrients and according to Silva *et al*. (2013) they promote soil structure. The activities of enchytraied worms lead to a rise in plant growth and that their role in the decomposer food-web is regularly better than other groups (Cole *et al*., 2001). "In acidic forest soils, where soil mixing earthworms are absent, enchytraeids play a dominant role in litter degradation" (Jeffery *et al*., 2010).

Enchytraeids have been recognized to be suited for ecological soil assessments due to the fact that they have high ecological relevance in many soils: they occur in almost any soil habitat; an association exists between community composition and specific soil properties; the number of species at one site is scarcely too small to be identified and it is never too high to be managed; and lastly they are easy and fast to quantify together with applying and standardization of sampling methods (Jänsch *et al*., 2005). Enchytraeids fulfill five of the criteria that makes them a good indicator: they play a key role in the functioning of the soil ecosystem; they are present in a wide range of ecosystems, where comparisons can be made between their ecosystems; they are plentiful in ecosystems; they are easy to be used in the laboratory and field conditions and lastly they can come in contact with a variety of stress factors, via the soil solution, solid phase and gaseous phase in the soil and can therefore be considered as suitable candidates as indicator organisms (Didden & Römbke, 2000).

Enchytraeids, especially *E. albidus*, have been used in toxicity tests for over 30 years. Since the draft for ecotoxicity tests using enchytraeids have been released by ISO, they have gained acceptance from ecotoxicologists (Kuperman *et al*., 2006). The test has been specifically developed for relevant annelids of the genus *Enchytraeus* and was originally intended to be used with OECD artificial soil. Enchytraeids have been used in a variety of toxicological studies of different chemicals and metals such as lead, copper, zinc, nickel and cadmium being the most tested (Spurgeon *et al*., 1994; Novais *et al*., 2011; González-Alcaraz & Van Gestel, 2016; Zhu *et al*., 2008; Lock & Janssen, 2002 a & b; Novais *et al*., 2013; Castro-Ferreira *et al*., 2012). Enchytraeids have also been used in nanotoxicity studies on coated silver (Topuz & van Gestel, 2015), copper NMs (Amorim & Scott-Fordsmand, 2012; Amorim *et al*., 2012; Gomes *et al*., 2012a; Gomes *et al*., 2012b; Gomes *et al*., 2015), gold NMs (Voua Otomo *et al*., 2014), silver NMs (Ribeiro *et al*., 2015; Gomes *et al*., 2013; Bicho *et al*., 2016; Gomes *et al*., 2015), titanium dioxide and zirconium dioxide (Gomes *et al*., 2015).

The Enchytraeid Reproduction Test (ERT) is not the only test that can be done to assess the toxicity of a chemical (Table 2.1). The ERT with a duration of 4-6 weeks, is a long exposure test with a long waiting period which is not suitable when results are expected immediately (Kobetičová *et al*., 2009). A suitable alternative to the ERT is avoidance tests and the avoidance behaviour of earthworms is well established with a standard protocol (ISO 17512, 2008; Amorim *et al*., 2008; Kobetičová *et al*., 2009). The test has a duration of 48 hours, which is significantly shorter than the ERT and is a rapid screening method (Amorim *et al*., 2008). *Enchytraeus albidus* described by Henle (1837) was the first species to be recommended during the test with duration, pH, soil moisture and validity of test (mortality and reproduction), but since then other species of the genus *Enchytraeus* have also been used as alternatives given that the reason for the specific use for the species been given (Kuperman *et al*., 2006). Species include

Enchytraeus crypticus Westheide & Graefe (1992), *Enchytraeus bucholzi* Vejdovsky (1879) and *Enchytraeus luxuriosus* Schmelz & Collado (1999) (Kuperman *et al*., 2006).

Table 2.1: Different toxicity tests for Enchytraeids (adapted from Van Gestel, 2012).

Test organism	**Species**	**Duration of exposure (days)**	**Endpoint**	**Guideline**
Enchytraeids	*Enchytraeus albidus,* other *Enchytraeus* species	21 (+21)	Survival, Reproduction	ISO 16387 OECD 220
		48 hours	Avoidance	No standard protocol

Avoidance behaviour can be assessed with enchytraeids because they possess chemoreceptors, which is highly sensitive to unsuitable conditions. The energy budget of the worms is also affected and contributes indirectly to soil structure changes through worm movement (Amorim *et al*., 2008a; Amorim *et al*., 2008b; Kobetičová *et al*., 2009). There are still a few issues regarding the use of enchytraeids for avoidance behaviour as it is under development. Some of the issues include: the duration of the test and non-specific influence of soil properties (Kobetičová *et al*., 2009). The changes that the compound bioavailability undergoes during exposure may alter the enchytraeid response and this still remains unclear (Kobetičová *et al*., 2009). The induced behaviour contributes to increase predation of the worms and can have an impact on the food web (Amorim *et al*., 2008). By avoiding the contaminated area, worms can find refuge in deeper soil layers or out of range of the contaminated area, thus avoidance behaviour affects animal communities (Amorim *et al*., 2008).

Avoidance tests has been successfully used for evaluating earthworm's behavioural changes in heavy metals (Langdon *et al*., 2001; Lukkari & Haimi, 2005) and pesticides (Reinecke *et al*., 2002; Garcia *et al*., 2008). This test can be seen as a useful complement to acute and chronic tests (Amorim *et al*., 2008).

Soil pollution has three ways in which they affect soil animal communities (1) directly – causing mortality, decreasing the chance of reproduction, and influencing feeding habits, (2) indirectly – a decrease in the predator populations or the plant and microbial communities and (3) avoidance behaviour of organisms by resisting toxicants, looking for refuge in the deeper layers of soil or moving outside the contaminated area (Amorim *et al*., 2008). Enchytraeid avoidance behaviour has been studied when exposed to heavy metals such as copper, zinc and cadmium

(Amorim *et al*., 2008). For NMs, there are currently no studies that have been performed using *E. albidus*.

2.4. Nanomaterials:

Nanomaterials can be described to have at least one dimension between 1 and 100 nm (Figure 2.2); nano-objects have two dimensions that is less than 100 nm and then nanoparticles can be defined as particles that has three dimensions of less than 100 nm, and materials that is included is nanofilms (one dimension), nanowires and nanotubes (two dimensions) or nanoparticles (three dimensions) (Handy *et al*., 2008; Stone *et al*., 2010). Nanomaterials occur naturally in the environment in the form of volcanic ash, forest-fire smoke, ocean spray, clay and clouds (Shah, 2010). Manufactured NMs, differ from natural occurring NMs in the sense that they have distinctive surface properties and chemistry, and they are designed in a specific way to achieve particular physico-chemical properties that relate to the specific product application and due to the fact that they are so small, they are very useful for nanotechnology. The small particle size generally results in higher reactivity and changed surface properties (Handy *et al*., 2008; Stone *et al*., 2010; Tourinho *et al*., 2012).

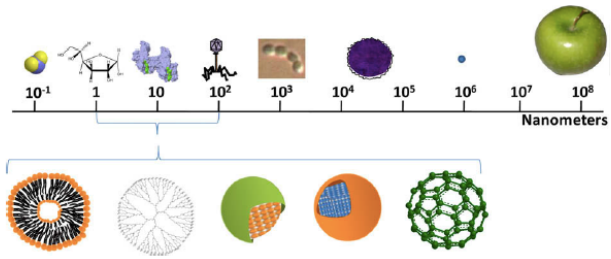

Figure 2.2: Comparison between nanoscale and macroscale.

The properties of manufactured NMs will also determine what happens with the particles in soil, for example, NMs can break through the soil matrix or can be retained by soil particles to reach groundwater (Lin *et al*., 2010). Figure 2.3 gives a schematic illustration of the fate and transport of manufactured NMs. Transformation and degradation can happen in the environment through dissolution in water, oxidation and reduction reactions, sulfidations, adsorption and aggregations (Lin *et al*., 2010; Nowack *et al*., 2012; Lowry *et al*., 2012). Lin *et al*. (2010) explained that uptake can occur through organisms where they can degrade and cleanse the released NMs.

The physico-chemical properties of manufactured NMs are specific to the type of product application (Handy *et al*., 2008). To date manufactured NMs has been used in a large range of diverse products because of a variety of different types of chemical composition, shapes, sizes and their ability to disperse in solution (Handy *et al*., 2008). Therefore, it is very important in studying their behaviours in environmental systems as these functions will function as the surface chemistry of NMs, the presence of any “coatings”, the composition of the NMs,

dissolution of the NMs and the presence of any soluble substances in the preparation of the NMs (Handy *et al*., 2008).

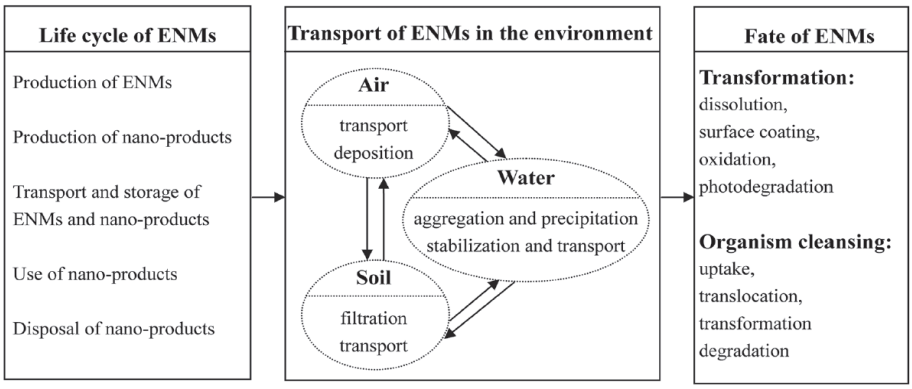

Figure 2.3: Fate and transport of engineered nanomaterials (ENM) in their life cycle (adapted from Lin et al., 2010).

Thus, the physico-chemical behaviour of NMs would suggest that these particles can adsorb or aggregate to any surface (Handy *et al*., 2008). The behaviour of manufactured NMs in water, sediments and soils is likely to involve a variety of processes that can influence the toxicity of these particles (Handy *et al*., 2008). These processes include aggregation and the NM's ability to form stable dispersions liquids, the effects of particle size and shape, surface area and surface charge on aggregation and ecotoxicity, adsorption of manufactured NMs on surfaces, this includes organisms and lastly abiotic factors (pH, water hardness, salinity, temperature and the presence of dissolved organic matter and so forth) could alter the toxicity of manufactured NMs (Handy *et al*., 2008). The particle shape, surface area and size of NMs need to be measured to confirm the structure of the material being used and particle surface area is a superior metric to explain dose-response relationship rather than concentrations (Handy *et al*., 2008). Literature states that particle shape and size is critical to uptake and toxicity (Handy *et al*., 2008).

The toxic effects of natural NMs are well known but there exists a gap between the toxicity and behaviour of manufactured NMs (Shah, 2010). Nanomaterials possess large surface area per unit of volume which give them unique properties in relation to conventional chemicals and with the increase in use of NMs, the potential release into the environment increases (Brar *et al*., 2010). The discharge of manufactured NMs will transport and transfer NMs into environmental media (soil, water and air) causing them to be taken up by organisms or be removed by organisms (Lin *et al*. 2010). Manufactured NMs can behave like an aerosol and be transported over long distances releasing them on land and water bodies. Aggregation occurs after NMs entered the aqueous environment and then precipitate to the sediments but they can also stabilize in the water flow, depending on the properties of the NMs as well as the pH, dissolved organic matter and ionic strength of the water (Lin *et al*., 2010).

The number of products in the market containing NMs or nanofibers now exceeds 1628 products (Figure 2.4) and is rapidly growing (Kahru & Dubourguier, 2010; Lahive *et al*., 2014). Carbon is the most common element in NM with 29 products, silver is second element with 25 products, silica has 14 products, titanium dioxide and zinc oxide with 8 products and lastly cerium oxide has only one product (Kahru & Dubourguier, 2010). Nanomaterials can be found in environmental application such as remediation of contaminated groundwater through nanoscale iron, personal-care products – titanium dioxide and zinc oxide in toothpaste, sunscreen, beauty products and textiles (Kahru & Dubourguier, 2010).

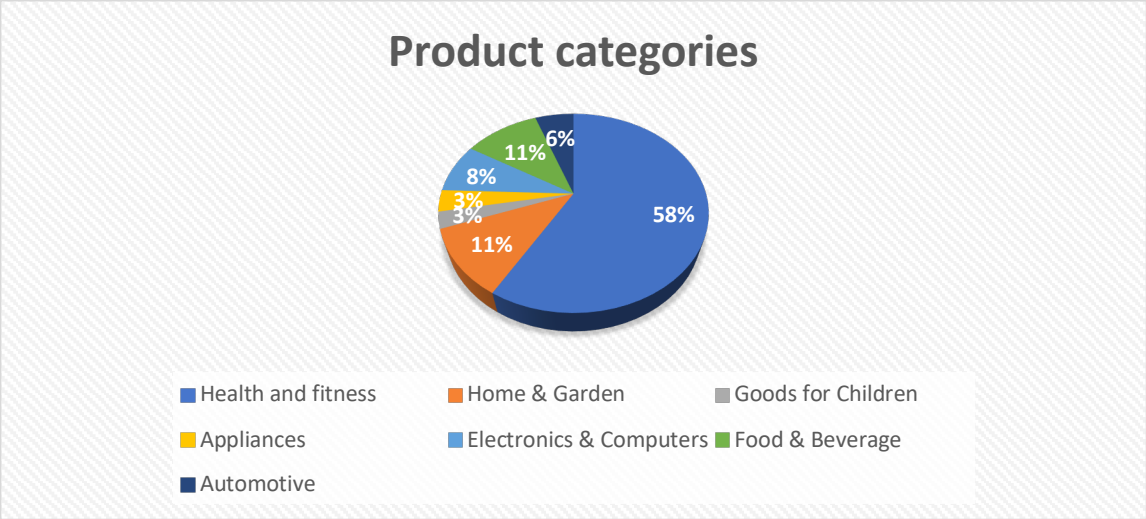

Figure 2.4: Available products containing NMs (adapted from Singh et al., 2009).

The exponential growth of the development of NMs has resulted in that manufactured NMs can be divided into different classes for example, carbonaceous, metal oxides, semiconductor materials (quantum dots), zero-valent metals (silver, iron and gold) and nanopolymers (dendrimers) (Klaine *et al*., 2008). With the preparation of NMs, it can either take a synthetic route which yields particles into nanosize range or through grinding and milling in order to reduce the size of a macroparticulate product (Klaine *et al*., 2008).

The diverse applications of nanotechnology can lead to a variety of ways (Figure 2.5) for NPs to be released into the environment (Tourinho *et al*., 2012; Pachapur *et al*., 2015). In order to understand the environmental and health impacts of NMs, it requires an understanding of the routes and toxic effects through acute and chronic exposures (Lowry *et al*., 2012). With industrial processes and transportation, spills can occur. Emissions to the atmosphere can result in deposition in soil and water from a variety of ways (for example, remediation of contaminated water) (Tourinho *et al*., 2012; Pachapur *et al*., 2015). Nanomaterials can be released onto the environment through remediation processes and agriculture such as fertilizers. Manufactured NMs can enter the environment unintentionally through packaging, consumer products, clothing, food, sporting equipment, tires, health-care products, paints, and detergents. All of these different products have different lifecycles causing a variety of

concentrations in the environment (Santiago-Martín *et al*., 2015). The release of consumer products depends greatly on the behaviour of the products such as washing habits and the use of the product, and environmental conditions such as pH, temperature and rainwater (Santiago-Martín *et al*., 2015).

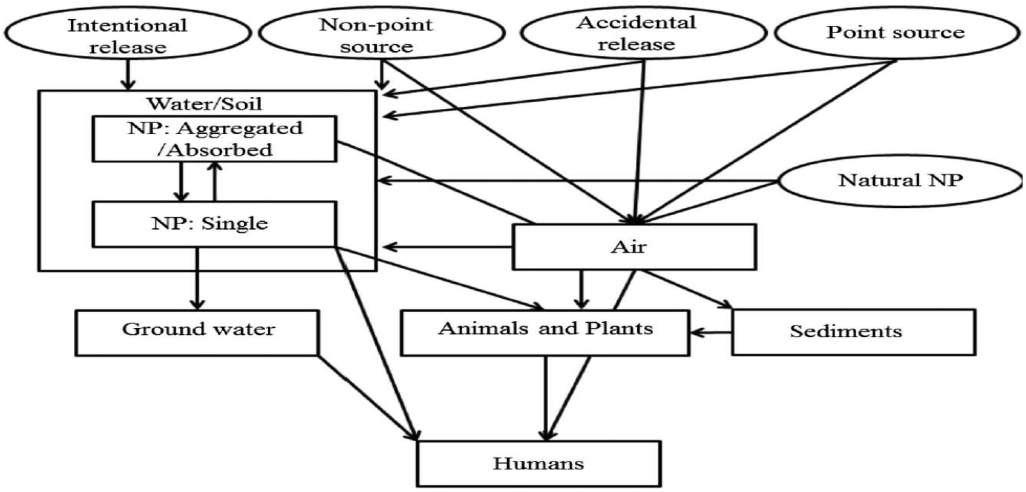

Figure 2.5: Pathways by which NMs are released into the environment with animals and humans final phase (adapted from Pachapur et al., 2015).

Nanomaterials can be deliberately released into the environment through agricultural chemicals such as nanofertilizers, naopesticides, seed treatment, and so forth (Santiago-Martín *et al*., 2015). Manufactured NMs can be released with remediation products containing NMs (Santiago-Martín *et al*., 2015). Nanomaterials can contaminate soil when wastewater sludge is concentrated with NMs and during the clarification processes it can lead to the soil (Santiago-Martín *et al*., 2015). Although the concentrations of NMs released into the soil can be low, it should not be neglected due to the fact that NMs can bioaccumulate in soils over a long period leading to unpredictable consequences (Santiago-Martín *et al*., 2015). Nanomaterials that reach the terrestrial environment have the probability of contaminating soil and migrating into the surface and groundwater and then interacting with the biota (Klaine *et al*., 2008). Nanomaterials that are in direct discharges, solid wastes or accidental spills can be transported to aquatic systems through rainwater runoff or by wind (Klaine *et al*., 2008). The biggest environmental risk associated with NMs is through spillage and the transportation of manufactured NMs (Klaine *et al*., 2008).

Studies have recently identified the environmental exposure and the route of uptake into organisms, while bio-imaging tools have provided a clearer picture of their fate and distributions (Figure 2.6) (Schultz *et al*., 2015). Assessments in ecotoxicology have shown that important parameters such as size, core chemistry, surface charge, shape, oxidation state and crystallinity have an influence on the manufactured NMs exposure, toxicity and assimilation (Schultz *et al*., 2015). Agglomeration of NMs has received the most attention to date since size distributions

and sedimentation rates are affected by agglomeration. It remains an important factor in governing potential exposure to soil, water and air (Schultz *et al*., 2015).

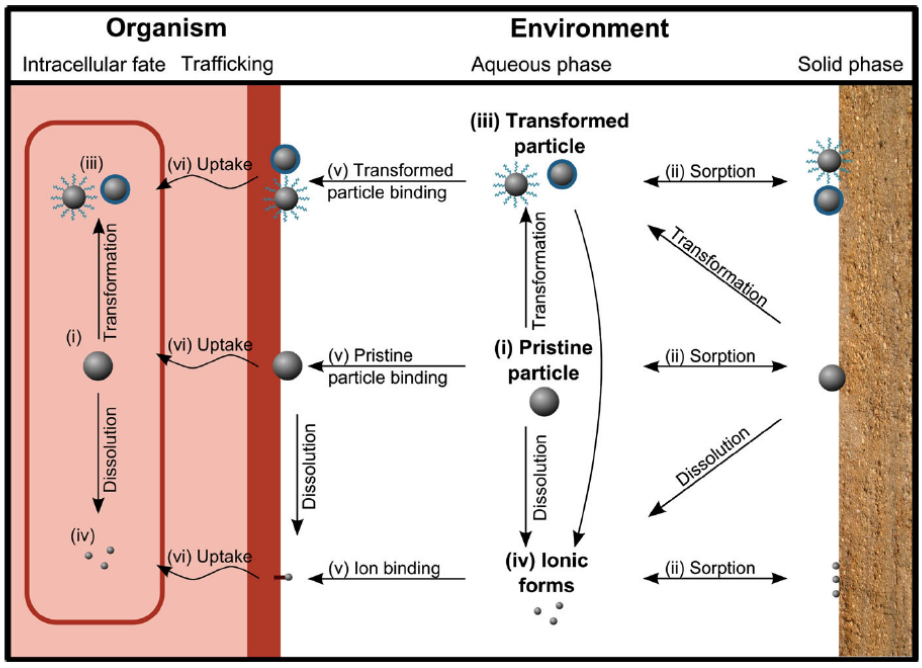

Figure 2.6: Behaviour and transformation of NMs (grey spheres) entering the environment (Adapted from Schultz et al., 2015).

One feature that can play an important role in the fate and toxicity of manufactured NMs is dissolution rate (Schultz *et al*., 2015). It determines the life time of the NM and can produce potentially toxic ionic species. Chemistry, particle size, shape and surface area are what drive dissolution rate (Schultz *et al*., 2015). With metals to be known to be toxic in the ionic form, it remains an important early determinant in manufactured NMs toxicity (Schultz *et al*., 2015).

With the rapid expansion of the use of NMs in industry, the development of toxicity methods using soil organisms needs special attention (Handy *et al*., 2012). There are already several toxicity methods testing the effects of metals and other contaminants using soil organisms such as the acute 14-day earthworm test (OECD, 207), earthworm reproduction test (OECD, 2015), ERT (OECD, 2015). Tests using *Caenorhabditis elegans* are under development as these species are used extensively for both terrestrial and aquatic toxicity test (Handy *et al*., 2012). Recent studies have shown that manufactured NMs are filtered in natural soils during the transport, particularly if the ionic strength of the clay content is elevated (Handy *et al*., 2012).

The bioavailability for soil organisms will differ between natural and artificial soil because the method of dosing these soils will change the bioavailability, dosing artificial soil will result in higher bioavailability for manufactured NMs as the NMs are not properly incorporated in the soil structure (Handy *et al*., 2012). Nanomaterial research in soils needs further strengthening to improve the understanding of the tests with soil organisms as the bioavailability of manufactured NMs to soil organisms is largely unknown (Handy *et al*., 2012).

There are a lot of protocols available to test the ecotoxicity of manufactured NMs, but there are still a lot of issues regarding the preparation, the dosing, characterization of NMs, selection of species, the endpoints to be analysed and the inclusion of controls for manufactured NMs (Handy *et al*., 2012). With preparation of media the NMs can be added to the soil as a dry powder and then mixed into the soil or the NMs can be added in a liquid form (Handy *et al*., 2012). Both approaches have its advantages and disadvantages and have been done before with dry powder (Hu *et al*., 2010) and in suspension (Scott-Fordsmand *et al*., 2008; Johansen *et al*., 2008; Rohr *et al*., 2009). Dry mixing the powder will ensure that the NMs are homogenously mixed with the soil but adding the NMs in a liquid form is more environmentally relevant (Handy *et al*., 2012).

2.5. Cd/Te Quantum dots (QDs):

Quantum dots (QDs) are NMs that can be synthesized using a variety of approaches such as polyolhydrolysis, chemical precipitation, electron beam irradiation, photochemical synthesis, y-radiation or microwave-assisted aqueous synthesis (Kominkova *et al*., 2014). Quantum dots are colloidal semiconductor nanocrystals with a size that range from 1.5 to 12 nm and the primary Cd source for QDs is Cadmium/Selenium (CdSe) and CdTe that is encapsulated in a variety of coatings (Figure 2.7) (Rzigalinski & Strobl, 2009; Singh *et al*., 2009; Gomes *et al*., 2011; Rosenthal *et al*., 2011; Luo *et al*., 2013). They can be manufactured for a variety of applications, for example, drug delivery, bioimaging and infrared giving them the potential to detect cancer (Luo *et al*., 2013). The optical and electronic properties of QDs are controlled by their size, coating and morphology (Gomes *et al*., 2011).

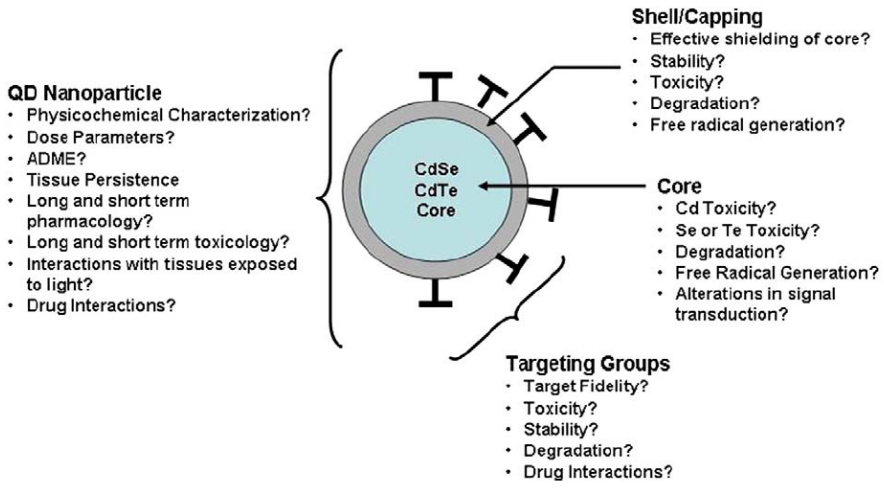

Figure 2.7: The core and capping of QDs (adapted from Rzigalinski & Strobl, 2009).

There are five distinctive properties which make QDs unique: (1) they are small ranging from 1.5 to 12 nm; (2) multiplexed experiments can be performed with QDs due to their narrow, size-tunable light emission; (3) they are exceptionally bright because of their substantial ability to absorb and they have high fluorescent quantum yields; (4) dynamic imaging is enhanced

because they are photochemically tough due to the fact that they are inorganic and lastly (5) biological function at molecular level is enhanced because QDs have the ability to ensure that a single dot event can be seen, which can translate into the observation of a single protein (Qi & Gao, 2008; Rosenthal *et al*., 2011). Quantum dots are well known for their exceptional optical properties making them excellent for a lot of different applications because are strong in fluorescence without photobleaching (Qi & Gao, 2008; Zhang *et al*., 2008).

Quantum dots are comprised of a variety of toxic metals such cadmium (Cd), selenium (Se), tellurium (Te), and lead (Pb) (Singh *et al*., 2009). Under an oxidative environment, these materials could be dangerous as these toxic elements could be released in a cellular oxidative environment (Singh *et al*., 2009). Quantum dots are designed with a shell or cap that surrounds the metallic core and functional groups or coatings such as PEG are utilized to enhance the bioactivity and biocompatibility (Singh *et al*., 2009). More coatings exist that can be used to further enhance QDs specific bioactivities for therapeutic and diagnostic purposes (Singh *et al*., 2009). The one problem that exists with these coatings is the stability of these functional groups. As they get broken down and degraded, the metal core of the QD are exposed which can make them more toxic (Singh *et al*., 2009). Cadmium-based QDs release free Cd^{2+} ions which causes its cytotoxicity as several researchers have shown the formation of free Cd^{2+} in the QD solution which is in relation to their cytotoxicity (Cho *et al*., 2007).

Their small size, make QDs unique in terms of their optical and electronic properties. This gives the nanoparticle a bright, highly stable fluorescence (Rzigalinski & Stroble, 2009). Their large surface areas together with their small size make them readily able to be functionalized for site-directed activity (Rzigalinski & Strobl, 2009). Their toxicity is dependent on size, core composition, charge and the stability of outer layers and the discharge of toxic heavy metals in colloidal form (Cho *et al*., 2007; Gagné *et al*., 2008; Rzigalinski & Strobl, 2009; Luo *et al*., 2013). The primary source for toxicity in QDs, is cadmium that resides in the core (Rzigalinski & Strobl, 2009). The outer coating acts as a shield for the inner metal core in order to enhance the solubility and quantum yield and when the outer coating degenerates due to oxidation or low pH, then toxic metals can leak form the QDs (Luo *et al*., 2013). The leaked toxic core has been reported to generate reactive oxygen species (ROS), which causes cellular damage, lipid peroxidation and can cause oxidative damage to cellular proteins and DNA which could lead to cell death (Gagné *et al*., 2008; Luo *et al*., 2013). When QDs are uncoated it is believed to be associated with increased ROS production which damages the mitochondria, membranes and nucleus whereas coated QDs generate free radicals which cause oxidative stress (Singh *et al*., 2009).

Quantum dot toxicity to rodents, cell cultures, aquatic invertebrates and fish have been tested but the use of soil organisms has been very limited (Stewart *et al*., 2013). The reproductive effects of quantum dots on nematodes have been investigated by exposing them to QDs in their

growth media (Stewart *et al*., 2013). Studies about the bioaccumulation of quantum dots have yet to be published. In terms of reproduction, earthworms have shown to be tolerant to doses of cadmium (40 μg/g), but for quantum dots, acute toxicity tests are not relevant to assess expected environmental concentrations which would be much lower than cadmium concentrations (Stewart *et al*., 2013). The bioaccumulation of QDs has been observed in other organisms, but is yet to be determined in earthworms and potworms (Stewart *et al*., 2013).

2.6. Biomarkers:

In pollution research, biomarkers have been used for over 35 years (Handy *et al*., 2003). A biomarker can be defined as "biochemical, cellular, physiological or behavioural variations that can be measured in tissue or body fluid samples, or at the level of whole organisms, to provide evidence of exposure and/or effects from one or more contaminants" (Depledge, 1994). With the use of biomarkers, it serves as a measure of chemical contaminants (Handy *et al*., 2003). Biomarkers are based on the causal relationship between contamination of environment by any pollutant (PAHs, metals, pesticides etc.) stress and biological changes of the polluted environment (Pauwels *et al*., 2013). It can be divided into two groups: biomarkers of effect and biomarkers of exposure (Lionetto *et al*., 2012). The latter indicates the exposure the organism has experienced for a time period, which gives an early indication of exposure to pollutants (Lionetto *et al*., 2012). Biomarkers of effect (SOD, CAT, MDA, PC) particularly indicates the action of a specific toxicant and are well developed to show the degree of adverse effect (Lionetto *et al*., 2012). Biomarkers of exposure (AChE) provides the ability to measure short lifecycle chemicals as well as chemical analysis, giving a more relevant warning of exposure (Lionetto *et al*., 2012).

Biomarkers are often used to measure oxidative stress through lipids, proteins, carbohydrates and DNA (Halliwell & Poulsen, 2014). This can aid in detecting the biological impact of the pollutant and focuses on the effects of bioavailable fraction of environmental pollutants to help describe the effects in the Ecological Risk Assessment (ERA) (Pauwels *et al*., 2013). It is well known that enzymes can bind to both oxygen and their substrate to bring them in close together in order for a chemical reaction to occur and the energy produced are stored as Adenosine triphosphate (ATP) for other use and for later in the cell (Halliwell & Poulsen, 2014). Sies (1985, 1986) described oxidative stress as "a disturbance in the pro oxidant-antioxidant balance in favour of the former, leading to potential damage." This results in a severe imbalance between oxidation and antioxidants (Halliwell & Poulsen, 2014). Oxidative stress results from the formulation of reactive oxygen species (ROS) and biomarkers for oxidative stress can be used for environmental monitoring programs (Pandey *et al*., 2003). Organisms that are under increased physiological stress produces ROS and are both related to metabolism and environmental factors. Examples of ROS are hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\bullet}OH$),

singlet oxygen ($^1O_2$), superoxide anion radical ($O_2^{\bullet-}$), nitric oxide (NO), alkoxyl ($RO^{\bullet}$), peroxinitrite ($ONOO^-$) and peroxyl ($ROO^{\bullet}$) radicals to name a few (De Almeida *et al*., 2007).

In a healthy cell, prooxidative products and ROS are detoxified by antioxidant defense that are comprised of water and lipids that are soluble (for example, Reduced Glutathione (GSH) and specific antioxidant enzymes (Figure 2.8) (Howcroft *et al*., 2009).

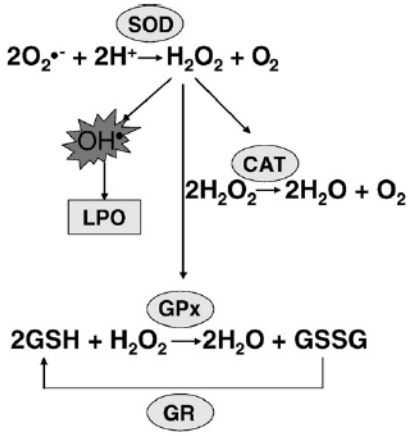

Figure 2.8: SOD, CAT, LPO and GSH response to oxidative stress (adapted from Howcroft et al., 2009).

The increased formulation of ROS can be associated with the biotransformation of organic xenobiotics, since that substantial amounts of $O_2^{\bullet-}$ and H_2O_2 could be generated from the biotransformation processes (De Almeida *et al*., 2006). Increased ROS can lead to oxidative stress as they overcome the antioxidant defense (Howcroft *et al*., 2009). Over time, organisms have adapted their antioxidant defense mechanisms that helps with intercepting and preventing ROS and repair mechanisms for oxidized components (De Almeida *et al*., 2007). There are three major antioxidant enzymes namely, catalase (CAT) that decomposes H_2O_2 to molecular oxygen and water, superoxide dismutase (SOD), which decomposes $O_2^{\bullet-}$ to H_2O_2 and glutathione peroxidase (GPx), associated with glutathione (GSH) oxidation, which decreases lipid hydroperoxides and H_2O_2 (De Almeida *et al*., 2007).

Biomarkers can be used for example, to measure the concentration of metal binding protein metallothionein in tissues rather than focussing on metal concentrations in biota, water or sediments (Handy *et al*., 2003). This is a great method in detecting sub-lethal effects or to indicate specific-toxicant concentration (De Coen & Janssen, 2003). An organism's metabolism undergoes changes due to sub-lethal stress and because an organism uses most of its energy for growth, reproduction and metabolism, an increase in energy use in metabolism to combat toxic stress will lead to a decrease in reproduction and growth (De Coen & Janssen, 2003). Biomarkers provide knowledge into both the factors causing the hazard and the consequences, depending on the specific biomarker (Lionetto *et al*., 2012).

One fundamental issue based on experimental approaches is the characterization of the effects of the contaminant on the biology of the organism (Pauwels *et al*., 2013). The hazardous nature of a chemical needs better understanding and this can be done through acute or chronic toxicity tests, aiding in the prediction to which degree the contaminant has a toxic effect or is harmless (Pauwels *et al*., 2013). Fundamental parameters that aids in the better understanding of the contaminant, on the characteristics of the environment, the development of the exposed organism, the physical and chemical properties of the pollutant, route of exposure and duration of exposure (Pauwels *et al*., 2013). With biomarkers detecting sub-lethal responses, it helps quantifying the bioactive part of a contaminant, which helps with exposure or effects of a contaminant (Šuteková & Hofman, 2011). Biomarkers can serve as an early indicator when there is a link between biomarker response and changes to the organisms or reproductive success (Šuteková & Hofman, 2011).

There are some advantages when it comes to applying biomarkers to help with the understanding of pollution (Handy *et al*., 2003). First advantage is that a biomarker can indicate whether the biological available contaminant is present, rather than in its inert form. Contaminants that are not expected can be revealed through using a standard suite of biomarkers. Biomarker responses can pick up the exposure of a contaminant long after it has been degraded or if it is non-detected through monitoring. It is usually much easier to perform and less expensive than instrumental chemical analysis (Handy *et al*., 2003). Thus, biomarkers show that organisms have been exposed to contaminants and/or if the exposure is deteriorating the condition of the organism (Handy *et al*., 2003).

Soil invertebrates are great indicators because of their role that they play in an ecosystem and in ecotoxicology. There are a variety of biomarkers (ranging from molecular to organism level) to be used using soil invertebrates to assess a toxic contaminant (Šuteková & Hofman, 2011; Gao *et al*., 2015). These biomarkers include DNA alterations, stress proteins, oxidative stress, metallothioneins or other metal binding proteins, enzymes, cholinesterase, behavioural changes, immunological and histopathological responses and many more (Šuteková & Hofman, 2011).

2.6.1. Biomarkers of effect:

2.6.1.1. Superoxide dismutase (SOD) and Catalase activity (CAT):

It is known that SOD and CAT provide the first line of defense mechanism against oxygen toxicity (Pandey *et al*., 2003; Lushchak *et al*., 2009). The focus of SOD is the catalyzing of the superoxide anion radical to H_2O and H_2O_2 and detoxifies the CAT activity decreasing H_2O_2 to water (Pandey *et al*., 2003; Bocchetti *et al*., 2003). One-unit SOD activity is the amount of enzyme (per protein milligram), that inhibits 50% of the oxidation reaction (Lushchak *et al*., 2009). Superoxide dismutase and CAT play equal roles in the antioxidant system meaning that

when SOD activity is increased, the production of superoxide anion radical is higher which could attribute to low levels of CAT activity and inhibit CAT activity due to the excess production of superoxide anion radical (Pandey *et al*., 2003). Catalase activity gets inactivated by the superoxide anion and SOD through H_2O_2 (Lushchak *et al*., 2009). It is well reported that metals can change the activity of several enzymes through the binding of their functional groups or by replacing the metal associated with the enzyme (Vieira *et al*., 2009).

Lionetto *et al*. (2003) reported that metals are known to inhibit CAT activity. They also showed that although CAT activity is low, oxidative stress can still occur through other enzyme activities (Lionetto *et al*., 2003). An induction response is usually observed when exposed to contaminants (Pandey *et al*., 2003).

Enchytraeids have been used as an indicator species for SOD and CAT analysis in previous studies by Howcroft *et al*. (2009) testing the effect of phenmedipham and copper; Novais *et al*. (2011) investigating the biochemical responses of *E. albidus* to zinc and cadmium; Novais *et al*. (2014) investigated the CAT response of *E. albidus* on altrazine, dimethoate and carbendazim. Gomes *et al*. (2012) analyzed the effect of copper NMs versus copper salt.

2.6.1.2. Protein Carbonyl (PC) and Malondialdehyde content (MDA):

Protein Carbonyl is one of the biomarkers that illustrates oxidative stress to organisms exposed to contaminants (Parves & Raisuddin, 2005). Oxidative stress biomarkers illustrate the damages done by ROS to proteins, lipids and carbohydrates (Parves & Raisuddin, 2005). The other biomarker that is a good indicator for oxidative stress is MDA. Malondialdehyde is a good indicator of free radical production, tissue damage and is a result because of lipid peroxidation (LP), where the membrane lipids are attacked by ROS and initiates an autocatalytic oxidation process (Choudhary *et al*., 2007; de Almeida *et al*., 2007; Lushchak *et al*., 2009). Choudhary *et al*. (2007) found that the MDA content increases as the heavy metal concentration increases, which suggests that the toxicity of heavy metals are affected through free radical production.

Lipid peroxidation disrupts membrane function and MDA can be used to measure LP which is affected by different factors namely: fluctuation in environmental factors such as temperature and the physical status of the organism (Parihar *et al*., 1996). Increase in LP is indicative of increased $O_2^{\bullet-}$ production which disrupts the balance of SOD (Parihar *et al*., 1996). Malondialdehyde can be seen as an important biomarker due to the fact the adverse effects could arise because of high LP levels (Parihar *et al*., 1996). Protein carbonyls are formed when changes to proteins of amino acids occur due to oxidative stress (Parves & Raisuddin, 2005). This assay provides a convenient technique to detect and quantify oxidative changes to proteins (Parves & Raisuddin, 2005). In the process of oxidative stress, hydroxyl radical ($OH^{\bullet}$), is generated and is considered to be the most liable for the production of carbonyl groups in

proteins (Parves & Raisuddin, 2005). Measuring the induction of protein carbonyls may provide insight into contaminants causing oxidative stress (Parves & Raisuddin, 2005).

In terms of MDA, lipid peroxidation studies have been done using enchytraeids. The effect on lipids has been studied by Novais *et al*. (2011); Phenmediphan and copper was studied by Howcroft *et al*. (2009). With the focus on NMs, Gomes *et al*. (2012) tested the effect of copper NMs on lipids using *E. albidus*.

2.6.2. Biomarkers of exposure:

2.6.2.1. Acetylcholinesterase activity (AChE):

The AChE enzyme is responsible for forming choline and acetic acid through the hydrolysis of acetylcholine (ACh) and considered to be a suitable biomarker for detecting pollution caused by neurotoxic compounds such as heavy metals (Lionetto *et al*., 2003; Pfeifer *et al*., 2005). The compounds mentioned above results in the inhibition of this enzyme, thus acetylcholine is accumulated; the nervous transmission is interrupted and the postsynaptic membrane remains hyperpolarized (Pfeifer *et al*., 2005). Neurotoxic compounds could lead to serious consequences such as behavioural changes, paralysis and death (Pfeifer *et al*., 2005). Natural factors have to be taken into account when interpreting AChE activities, since environmental variables can have a direct or indirect impact on the AChE activity (Pfeifer *et al*., 2005). This enzyme activity is a great indicator for pollution via carbamates, organophosphates, similar neurotoxins as well as metal pollution (Wepener *et al*., 2005; Tu *et al*., 2009).

Novais *et al*. (2014) investigated the ChE response of *E. albidus* on altrazine, dimethoate and carbendazim. Howcroft *et al*. (2011) tested the effects of phenmedipham and copper by studying the ChE activity of *E. albidus*.

2.6.3. Cellular Energy Allocation (CEA):

The method for Cellular Energy Allocation (CEA) assessment has been developed to assess the effect of a toxin of the energy budget on organisms (De Coen & Janssen, 1997b). This is a short-term assay that focusses on the changes in energy reserves (proteins, lipids and carbohydrates) and energy consumption (electron transport activity – ETS) (De Coen & Janssen, 1997b). The difference between energy available (E_a – measuring the lipid, protein and carbohydrate content) and the energy consumption (E_c – is measured through ETS at the mitochondrial level) represents overall net-energy budget value (De Coen & Janssen, 2003). Through combining the energy available and the energy consumption a new biomarker was developed, CEA, reflecting the energy of the organism at cellular level (De Coen & Janssen, 2003). The energy available is determined separately and then converted into energetic equivalents (De Coen & Janssen, 1997b).

The energy allocation was assessed in enchytraeids by testing two different NMs (Cu & Ag), by Gomes *et al*. (2015). Novais *et al*. (2013) analyzed Cd and Zn with the focus on changes in the cellular energy allocation in *E. albidus*.

2.7. Uptake and distribution of nanomaterial in *E. albidus*:

Heavy metals are a serious problem worldwide affecting plant growth, soil organisms as well as human health (Maboeta *et al*., 1999; Demirevska-Kepova *et al*., 2004). Soils are a basin for heavy and trace metals and are of great concern as high concentrations are found in agricultural soils (Kinkle *et al*., 1994; Zimmerman and Weindorf, 2010). Heavy metal toxicity can be enhanced through the food chain (soil-plant-animal or soil-plant-human) and it is difficult to remediate once found in soils (Wuana and Okieimen, 2011; Su *et al*., 2014). Regardless of its origin, heavy metals can have major impacts on the soil quality, reduce the quality of agricultural products and crop yield and have a negative impact on the ecosystem, animals and humans (Hu *et al*., 2013).

In terms of environmental pollution, soils are running the greatest risk of pollution as they act as a sink (Wuana & Okieimen, 2011; Ashraf *et al*., 2014). Soil has the ability to adsorb metal ions from aqueous solution and has major significances for agriculture (soil fertility) and for the environment (waste deposition and remediation) (Bradl, 2004). Adsorption is a major activity that results in the accumulation of heavy metals in soil, therefore needs to be studied in order to understand how heavy metals move through liquid phase to solid phase (Bradl, 2004). The solubility as well as bioavailability of heavy metals depends on a variety of factors such as clay, iron oxides, organic matter and pH, for example in clay and more calcareous soils the solubility is low but acidic soils make considerable amount of heavy metals available for uptake (García-Sánchez *et al*., 1999; Lock *et al*., 2000; Bradl, 2004).

Most metals occur naturally in the environment as rare elements and soil biota require some of the metals, but exposure to high concentrations can lead to adverse effects (Tyler *et al*., 1989). Therefore, with the additional release of these metals by human activities, it can seriously damage or alter the ecosystems (Tyler *et al*., 1989). The toxicity and uptake by soil organisms will not only depend on the concentrations in soil, but on soil conditions as well (Spurgeon *et al*., 2006). In order to get a more accurate prediction about metal toxicity in soil, it is necessary to understand the influence that soil properties have on the chemical toxicity (Spurgeon *et al*., 2006). The effect of toxicity on organisms is better understood when assessing the total content present in the organism rather than assessing the total content that is found in the environment (Lock & Janssen, 2001). Important parameters that are used to assess the metal bioavailability in the environment is the chemical concentration in the organism (measured through uptake, elimination and bioaccumulation factors) divided by the chemical concentration in soil are found to be independent from the total metal concentration found in soil (Lock & Janssen, 2001).

There are a number of identified sources of environmental NM contamination such as anthropogenic sources like air pollution, the use of NMs in agriculture products, water-related products for water treatment (Handy *et al*., 2008). Remediation using NM products is one source that deliberately releases NMs into soils and water and currently, there is no monitoring NM programs available so there is a need for understanding the expected fate and transport of these particles and the potential uptake by organisms (Handy *et al*., 2008). Literature about the possible uptake and accumulation of manufactured NMs is rare, but the mechanisms of uptake are still the focus in terms of research (Klaine *et al*., 2008). Not many toxicity tests have been performed in detail to understand the distribution, absorption, excretion and metabolism of the particles and the parameters are fundamental in understanding the distribution in the environment and uptake by organisms (Handy *et al*., 2008). With biological uptake, absorption is the first step as any substance is adsorbed to the exterior surface of an organism and the physico-chemical properties of the material will determine the aggregation and precipitation of NMs onto the exterior of the organism (Handy *et al*., 2008). Investigating the uptake across the cell membrane could also help in determining the dissolution of metal NMs, endocytosis of NMs or aggregates (Handy *et al*., 2008; Klaine *et al*., 2008). Because the particles are much larger than ions, it makes it impossible for them to be ion transported across the cell membranes (Handy *et al*., 2008). The distribution and target organs in organisms are largely unknown because the solubility depends on the solution chemistry of the body fluids and the behaviour of the NMs in the body fluids (Handy *et al*., 2008).

By identifying the metal concentrations in different parts of an organism, one can identify the target organ of the organism (Handy *et al*., 2008). One method of identifying the particles in the tissue is through an electron microscope, but this process is time consuming and aggregation objects can be a problem during the processing of the tissue (Handy *et al*., 2008). Another method to identify metal content in tissues is through inductively coupled plasma mass spectrometry (ICP-MS) or similar acid digesting techniques.

It is reported that organisms that live in a NM exposed area will inevitably get exposed to the materials via the gut with the possibility of translocating them through the body to other regions (Klaine *et al*., 2008). Nanomaterials can enter organisms through the diffusion across cell membranes, endocytosis and adhesion. Quantum dots have been specifically designed to react with cell membranes, proteins and nucleic acids because they are employed in drug delivery (Klaine *et al*., 2008). For most NMs, the toxicity mechanisms have yet to be explained. Some possibilities include the disruption of membranes, genotoxicity, oxidation, disrupting the energy allocation, the formation of ROS and lastly the release of toxic elements (Klaine *et al*., 2008). The effectiveness of NMs has a big influence on the toxicity mechanisms (Klaine *et al*., 2008). There are some NMs that have shown to disrupt the integrity and function of cell membranes by attaching to the cell surface (Klaine *et al*., 2008). By generating ROS, NMs have the ability to

cause indirect damage to the cell membranes through the process of lipid peroxidation (oxidation of fatty acids tails membrane phospholipids). Because of the permeability of the membranes, it makes uptake more susceptible to osmotic stress (Klaine *et al*., 2008). Fatty acids that are peroxidized can lead to more damage to cell membrane and DNA damage because of generating free radicals (Klaine *et al*., 2008). Certain NMs target bacterial cells causing toxicity through releasing harmful elements such heavy metals or ions (Klaine *et al*., 2008). Quantum dots contain transition metals such as CdSe, CdSeTe, CdTe, ZnSe, PbSe or InAs in their core and in their shell, ZnS or CdS and organic coatings (Klaine *et al*., 2008). Thus, QD toxicity could lead to the accumulation of toxic metals within the cells of the organism and with their long lifecycle they may cause adverse effects in processes such as reproduction and growth (Klaine *et al*., 2008).

Materials & Methods:

3.1. Characterization of nanomaterials

3.1.1. Characterization of Cd/Te QDs (Functional groups PEG, COOH and NH_3):

Cd/Te QD powder with different charged functional groups (neutral – PEG, negative – COOH and positive – NH_3) was supplied by PlasmaChem GmbH Rudower Chaussee 29, D-12489 Berlin (Lot# YF140402). The $CdCl_2$ was provided by Kanto Chemical Co. Inc., product code JIS K 8120, batch number (303A5864) and $TeCl_4$ was purchased from Sigma Aldrich Chemistry (99%) 205338-25G (Lot# MKBL8016V). The nanomaterial suspension was prepared by mixing 10 mg nanomaterial powder with 100 mL of Milli-Q water (Milli-Q Simplicity® UV). The solution was then sonicated (Scientech, Ultrasonic Cleaner) at 25 V in a bath filled with 900 mL of H_2O for an hour to ensure that all of the particles are dispersed throughout the MilliQ water. Characterization of Cd/Te QDs was done by measuring the hydrodynamic size distribution by using Dynamic Light Scattering (Malvern Zetasizer Nano series, NanoZS). Nominal exposure concentrations of stock solutions (100 mL) were acidified (10 µL 65% HNO_3 per 10 mL) and measured using inductively coupled plasma atom emission spectroscopy (Botha *et al*., 2016). Transmission electron microscopy (TEM) (FEI Tecnai G2) was used to measure the diameter of Cd/Te QDs. The TEM was also used to illustrate particle aggregation patterns. The Cd/Te QD medium (one drop) was allowed to settle for a few minutes when dropped into a carbon coated copper grid. A filter paper was used by gently touching the each of the droplet to remove any excess water. After the water was removed, the grid was allowed to dry to be examined at high resolution (200 kV) and images were taken using a digital micrograph FEI company. Material dissolution rate data were obtained from the Nanosolutions project data sheets since they were exactly the same batches that were used in this study (Nanosolutions, 2013). For the dissolution, 100 mg/L of each nanomaterial stock solution were prepared. Dialysis bags were used containing 700 µl of the appropriate stock solution. Then the samples were dialysed in falcon tubes which contained 44.3 mL Milli-Q water. Each material was analysed in duplicates and samples were collected at 1, 3 and 24 h. To agitate the solutions, a shaker plate (IKA Labortechnik, KS250 basic) was used and the metal concentration was measured by inductively coupled plasma optical emission spectrophotometry (ICP-OES, ICAP 7400) or inductively coupled plasma optical mass spectrophotometry (ICP-MS, Thermoscientific, X Series 2).

3.2. Exposure substrate:

Artificial OECD (OECD, 2015) soil, which is commercially available, was used for the Enchytraeid Reproduction Test (ERT) with a composition of 10% sphagnum peat, 20% kaolin clay, approximately 0.3 to 1.0% calcium carbonate to obtain pH of 6.0 ± 0.5 and approximately

70% air-dried quartz sand. The dry constituents of this soil were mixed thoroughly and stored for two days in order for the acidity to equilibrate/stabilize. The maximum water holding capacity was calculated and adjusted to 60% and the pH of the soil was determined (ISO, 2008).

The maximum water holding capacity (WHC) of the soil was determined (OECD, 2015) by placing 5 g of the test soil in an auger tube. This tube was covered with a piece of filter paper (Whatman water separation, silicon treated filters, 75 mm) at the bottom and then placed in a water bath. Water was allowed to gradually move through the soil to above the top of the soil for three hours; the tubes were left for two hours upright onto a bed of 100% silica to drain. Following this period, the 100% WHC was determined, soil was weighed and dried at 105°C (OECD, 2015). Moisture content is adjusted to 60% of the maximum WHC by the addition of the test substance solution and/or by adding deionised water (OECD, 2015).

The formula that was used to calculate the Water Holding Capacity:

$$100\%\ \text{WHC} = \frac{(\text{g})\ \text{of soil x}\ (\%\text{Moisture})}{100}$$

$$20\ \text{g} = \chi$$

$$\chi = \frac{20\ \text{x WHC}}{(\text{g})\ \text{of soil}}$$

20 g [100% WHC] = ...mL

$$60\% = \chi$$

$$\chi = \frac{60\ \text{x}\ldots.\text{mL}}{100}$$

The pH was determined through a mixture of soil and 1 M potassium chloride (KCl) or 0.01 M calcium chloride ($CaCl_2$) in a 1:5 solution (OECD, 2015). Samples were adjusted using $CaCO_3$ for acidic substances while more solution was added for samples which were too basic (OECD, 2015).

The duplicate NM stock suspensions were prepared by mixing 10 mg nanomaterial powder with 100 mL of Milli-Q water (Milli-Q Simplicity® UV). The solution was then sonicated (Scientech, Ultrasonic Cleaner) in a bath filled with 900 mL of H_2O for an hour at 25 V to ensure that all of the particles are dispersed throughout the milli-Q water. After the sonication, the appropriate amount of solution together with the 60% WHC was pipetted into the test vessels. Prior to exposure, 20 g of dry weight OECD soil was weighed and placed into the test vessel. All the test vessels were spiked and mixed individually in order to ensure that the NM suspension was homogenously mixed in the soil. Each vessel was mixed for 2 min to ensure that the NMs were mixed through the soil. For the bulk metal exposures, the metal salts ($CdCl_2$ and $TeCl_4$) were used to obtain the specific exposure concentrations. The molecular mass was used to calculate the appropriate mass of the metal salt to obtain the desired metal salt control exposure

concentrations of 100 and 500 mg/kg Cd and 500 and 1000 mg/kg Te. These concentrations were selected based on sub-lethal responses reported in the literature (USEPA Ecotox database).

3.3. Nanomaterial transport in soils using a flow through system:

In order to understand the fate and toxicity of NMs in soil, the transport of the NMs in soil was studied using a flow-through system (Figure 3.1 & 3.2). Two different nanomaterial transport tests were conducted. For the first test the nanomaterial stock suspension in Milli-Q water was applied to the surface of the flow-through system and allowed to elute through the column. In the second test the NM suspension was homogenously mixed into the soil substrate. Milli-Q water was then added to the surface of the column and eluted.

To determine the fate and mobility of NMs in soils, glass soil core vessels with stoppered openings to allow for sampling within the core, were constructed (Figure 3.1). Although the bottom of the core tube was open, a sintered glass filter (A in Figure 3.1) prevented soils and clays from flowing out of the tube. Only the eluted water and eluted clay was able to pass through the glass filter and was collected in a beaker at the tapered end of the tube (Figure 3.1B). The glass core vessels were filled with 100 g artificial soil. All artificial soils used in the flow through testing systems were pre-moistened to 100% saturation with Milli-Q. This was to ensure that water would elute through the soil and not be retained.

For the first test, 60 mL of 100 mg/L nanomaterial stock suspension was applied to the top of the exposure vessel. The total volume was not added at once but in 10 mL aliquots over a period of 120 s to prevent compression of the soil. The top of the tube was covered with Parafilm to prevent evaporation and left to equilibrate for 24 h in an environmental room (20 ± 2°C). After 24 h, subsamples of approximately 8 g were collected from the top, middle and bottom and analysed for Cd and Te concentrations. The water and clay that had eluted through the core were collected in the beakers at the outflow. The eluent was retained for metal analysis using inductively coupled plasma mass spectrometer (ICP-MS) techniques described below. The experiment was conducted in triplicate for each of the three nanomaterial functional groups.

For the second test (Figure 3.2), the NMs were mixed into the soils at a concentration of 100 mg/kg. The nanomaterial stock suspension was suspended in Milli-Q water since the stock was used to pre-moisten the soil to 100% saturation. Soils and NM suspensions were mixed thoroughly to ensure homogenous distribution of the materials in the soils. Following the mixing 100 g of soil was transferred to each tube. Milli-Q water (60 mL) was poured on top and in 10 mL volume units over a period of 120 s. The tube was covered with Paraflim to avoid evaporation and the set-up was left for 24 h in an environmental room (20 ± 2°C). After 24 h, subsamples (± 8 g) were collected from the top, middle and bottom and stored in 50 mL Falcon tubes at -80°C. The eluted clay and water fraction were also collected in the beakers at the

outflow and stored in 50 mL falcon tubes in at -80°C. The soil samples were prepared for analysis as described in section 3.3.1. Concentrations of Cd and Te were measured using standard ICP-MS techniques. The experiment was conducted in triplicate for each of the three NM functional groups.

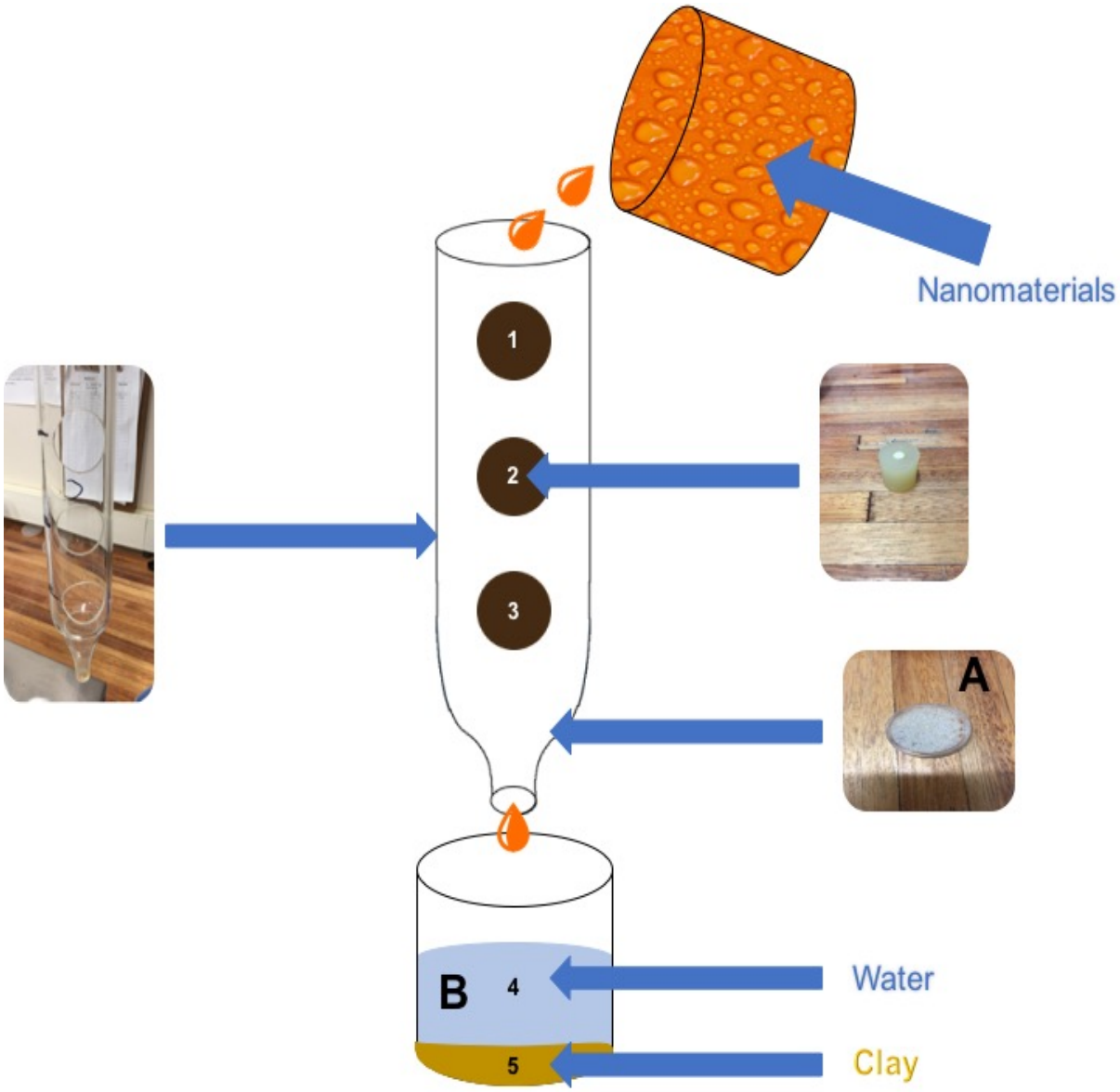

Figure 3.1: Graphic illustration of experiment one and sampling areas being 1) Top, 2) Middle, 3) Bottom, 4) Eluted Water & 5) Eluted Clay fraction.

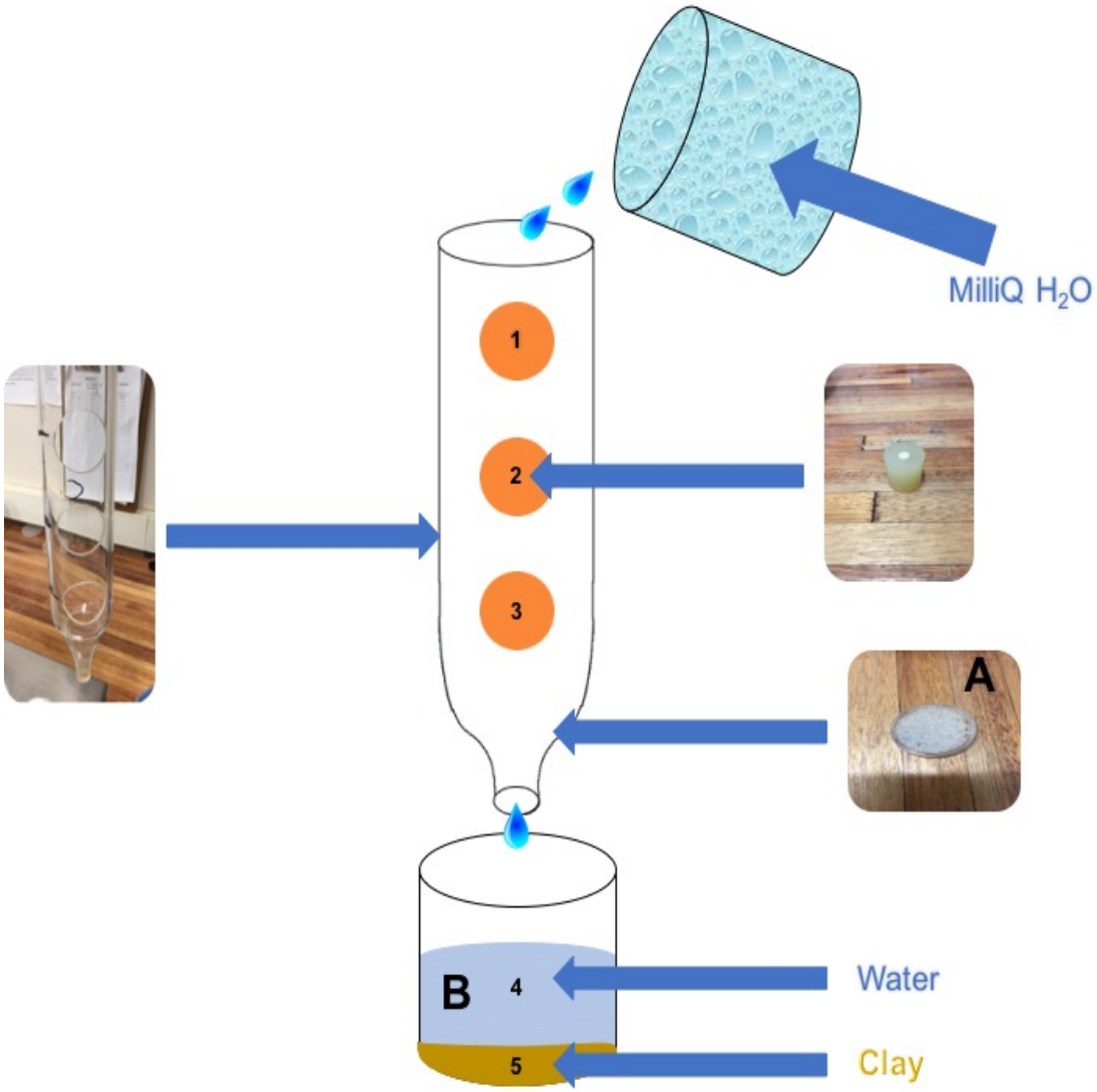

Figure 3.2: Graphic Illustration of experiment two together with the five different sampling areas. 1) Top, 2) Middle, 3) Bottom, 4) Eluted Water & 5) Elute Clay fraction.

3.3.1. Metal analysis (chemical characterization) of the exposure soils and soil transport tests:

Soil samples from the exposure experiments, i.e. 5, 30, 100 and 500 mg/kg NM (QD-PEG, QD-COOH and QD-NH_3) exposure and the two soil transport tests were oven dried to a constant weight. Approximately 200 mg soil was added to Teflon digestion vessels. Ten mL 65% HNO_3 was added and the samples were digested using an Ethos Easy MAXI-44 Microwave Digestion System for 50 min. Following digestion, the samples then filtered (Cellulose Nitrate Filter; pore size: 0.45 μm) to remove any excessive soil particles. Metal concentrations (Cd and Te) in soils were determined using an ICP-MS (Agilent 7500CE). Analytical efficiency was ensured by using a certified reference material (CRM) by preparing the CRM the same way as the samples (Table 3.1). The CRM that was used was NCS DC 73310 Stream Sediment from the China National Analysis Centre for Iron & Steel. For metal analysis in eluted water samples they were acidified with 1 mL 1 % HNO_3 and then filtered through 0.45 μm filter. The samples were not digested but analysed directly using the ICP-MS described above.

Table 3.1: Certified reference material for Cd and Te with its % recovery.

CRM (NCS DC 73310)	Reference	Measured	% Recovery
Cd	4.0	4.42	110.49%
Te	0.29	0.37	128.24%

3.4. Enchytraeid Reproduction Test:

The Enchytraeid Reproduction Test (ERT) was performed using the OECD Guideline (220, 2015) for the testing of chemicals. It has been designed to assess the toxicity of chemicals based on the reproductive success of *E. albidus* (potworms) in artificial soil (OECD, 2015).

Adult potworms were exposed to a series of concentrations of Cd/Te QDs and Cd and Te metal salts that were mixed into the artificial soil. This test was divided into two steps namely:

(a) a range-finding test (QDs – 5, 10, 25 & 100 mg/kg) where mortality was the main endpoint being assessed after a period of 14 days and

(b) a definitive test (QDs – 0.5, 1, 5, 15, 30, 100 & 500 mg/kg, Cd – 100 & 500 mg/kg and Te – 500 & 1000 mg/kg) where the reproductive success (42 days), by counting the number of juveniles hatched from the cocoons, was assessed (OECD, 2015).

The breeding cultures were maintained within an environmental chamber (18-21ºC) and fed twice a week. *Enchytraeus albidus* was used for all testing within the study. The duration of the definitive was six weeks in total. After an exposure duration of three weeks, the adult worms were removed and mortality was calculated; exposures were left for an additional three weeks to determine reproductive success (OECD, 2015). In order to calculate the no-observed effect concentration (NOEC) and/or the EC_x (i.e. EC_{10} or EC_{50}) the reproduction success of the exposed groups were compared to that of the control(s) (OECD, 2015). Tests were only considered valid if adult mortality did not exceed 20% and at least 25 juveniles per vessel at the end of the test in the control. Lastly the coefficient of variation around the mean number of juveniles should not be higher than 50% at the end of the reproduction test (OECD, 2015).

All the organisms used in the exposures were clitellate adults of similar size (OECD, 2015). For this experiment, twelve treatments, each with five replicates, were used. Ten adults per treatment were exposed to 20 g wet weight of QDs, positive and negative control. The adults were removed from the breeding colonies using a tweezer, hook or loop and then placed in a deionised water filled Petri dish then placed into the test vessel (OECD, 2015). These replicates were kept in a climate chamber at a temperature of between 18 and 21ºC and fed twice a week with autoclaved rolled oats.

Mortality was determined by gently placing the soil of the vessel in a sieve and then checked using a small needle. The surviving adults were rinsed with deionised water and put into 2 mL Eppendorph tubes with General Homogenizing Buffer (GHB) [40 mM Tris HCl; 10 mM b-mercapto-ethanol; 1 mM EDTA; 1 mM BSA] and stored at -80°C until biomarker analysis was conducted. Worms for metal bioaccumulation determination were placed into Eppendorph tubes and immediately frozen at -80°C pending analysis. After the surviving adults were removed, the soil was placed back into the vessel and left for another three weeks.

At the end of the definitive test (six weeks), the replicate soils in the vessels were fixed with 5 mL ethanol. After the ethanol has been added, a few drops (400 µL) of Bengal Red (1% solution in ethanol) was added and then mixed carefully. The worms were stained a red colour after twelve hours and could then be easily counted.

At the end of the test, surviving adult worm samples were collected for biomarkers and metal bioaccumulation as described above. Additional worms were also collected for determination of body distribution of particles using dark field hyper-spectral imaging with the CytoViva system (described in Section 3.6).

3.5. Avoidance test:

The main endpoint being assessed during this test was the avoidance behaviour of the worms (ISO, 2008). This rapid test was adapted for use with *E. albidus* species and was done by Amorim *et al*. (2008b), Kobetičová *et al*. (2008) and Novais *et al*. (2010). For the test to be valid, the number of missing or dead worms must be lower than 10% per treatment.

The NM suspension was sonicated before spiking the soil and was mixed for two minutes to ensure that the substrate was homogenously mixed (QDs – 0.5, 1, 5, 15, 30, 100 & 500 mg/kg, Cd – 100 & 500 mg/kg and Te – 500 & 1000 mg/kg). The control substrate was placed into the vessel and then a divider was introduced and the exposed soil was added to ensure that the substrates were separated from one another. After the soil was added the divider was removed and ten adult *Enchytraeus albidus* worms were introduced into the middle of the vessel of the test substrate presenting the worms the opportunity to select between which substrate they preferred. The vessel was covered with lids containing holes and kept in a climate chamber at a temperature of between 18 and 21ºC. Five replicates per concentration was done for avoidance testing.

After 48 h, the divider was inserted again to separate the soils and to ensure that the test soil and control soil weren't mixed when taking out the soil. The number of the worms present was determined for both sections. The worms that were damaged when the divider was inserted were counted as 0.5 independent of the length of the remaining body section. Missing worms were considered to have escaped the vessel or have not survived.

At the end of the test, the mean (± standard deviation, SD) number of individuals in the test soil for each treatment was determined (ISO, 2008). The results were then presented as the number of individuals in the test soil per test vessel. In a single concentration test, the mean number of individuals at the end of the test in the test substrate was compared to the mean of the control (ISO, 2008). If for instance, the mean number of surviving worms in the test substrate was significantly lower than that of the control substrate, it indicates that there was an avoidance response to the test substrate or a preference to the control soil (ISO, 2008).

The equation that was used to calculate the avoidance behaviour:

$$A = (\frac{C-T}{N}) \times 100$$

Where: A: Avoidance in %

C: The number of worms in the control (either per vessel or all of the control replicates).

T: The number of worms in the test substrate (either per vessel or all of the test replicates).

N: The total number of worms (usually 10; either per vessel or all of the control replicates).

3.6. Biomarkers:

3.6.1. Sample preparation:

Frozen samples in homogenizing buffer (HSB) were thawed and the total mass of the organisms was determined and the appropriate homogenizing buffer was then adjusted according to the mass of the organisms. Eppendorph A, which was for PC, CAT, SOD, AChE and MDA had a total mass of 0.1 g and 1 mL General Homogenising Buffer (GHB) [1.15% KCl, 0.1 M potassium phosphate buffer (PPB), 0.1 nM phenyl methane sulphonyl fluoride (PMSF), 20% glycerol and 1 nM EDTA] was added. The final batch (Eppendorph B) consisted of 0.08 g worm tissue in 400 µL electron transport system (ETS) buffer [0.1 M tris-HCl; 0.2% triton X-100; 15% polyvinyl pyrrolidone and 153 µM $MgSO_4$]. For each biomarker response, the aliquots were taken from each batch. Biomarkers were expressed as activity per milligram protein and therefore the protein content of each sample batch was determined using the Bradford (1976) method. The method is based on the active ingredient in Bradford's reagent and then measuring the absorbance at 590 nm and compared against the curve of bovine serum albumin (BSA).

3.6.2. Biomarkers of exposure:

3.6.2.1. Catalase activity:

The Cohen *et al*. (1970) protocol was used for CAT activity and performed in the dark, because CAT assay is light sensitive. The supernatant (eppendorph A) was centrifuged at 9 500 g for 10 min at 4°C. Only 10 samples (in triplicate) per microtitre plate were analyzed at a time. H_2O_2 (93 µL) [6 mM] was added to the 10 µL sample in each well (samples and blank) and then left to incubate for 3 min. H_2SO_4 (19 µL) was added to stop the reaction and 2 mM $KMnO_4$ (130 µL) was added immediately after. Absorbance was measured at 490 nm for 30-60 s with a BioTek FLx800.

CAT activity was determined using the following equation:

$$k = \log\left(\frac{S_0}{S_3}\right) \times \frac{2.3}{t}$$

Where: S_0 = the mean of standard absorbance readings, S_3 is the standard – mean absorbance of sample and t equals time (3 min). After the calculation has been done, the k value is divided by protein and is then expressed as mg/l and then the K value is adjusted to µM by dividing by 1000. CAT activity is expressed as µM H_2O_2/min/mg protein.

3.6.2.2. Superoxide dismutase activity:

In brief, the SOD assay was adapted from Greenwald (1989). Fifty mM Tris buffer (pH adjusted to 8.2) containing 1 mM diethylenetriamine-pentacetic acid (DTPA) aerated vigorously at room temperature for 20 min was prepared on the same day of analysis. A 24 mM pyrogallol sollution was prepared in 10 mM HCl on the day of analysis. Homogenized samples and blank (Tris buffer) [4 µL] were added to the Tris buffer/DTPA [242 µL] in the 96 well microtitre plate in triplicates. By adding 4 µL pyrogallol solution, the reaction was initiated. Samples were read immediately in the dark for 10 min at 60 s intervals in the microplate reader (BioTek FLx800). SOD activity was expressed as ng SOD/mg protein.

3.6.2.3. Protein carbonyl induction:

The method described by Parves & Riasuddin (2005) was used to determine PC content. The method was originally described by Levine *et al*. (1990) and then modified by Floor & Wetzel (1998). Samples were prepared by homogenizing (10% w/v) the sample with the General homogenizing buffer (see *Sample preparations*) and the supernatant was then filtered through a cloth and centrifuged at 800 g for 5 min at 4°C. Samples (eppendorph A) were then centrifuged at 10 500 g at 4°C for 30 min. Sample supernatant (500 µL) was added to 500 µL 2,4-dinitrophenylhydrazine (2 M HCl; 10 mM) and left to incubate for an hour at room temperature while being vortexed every 10-15 min. Equal volumes (500 µL) of trichloroacetic acid (TCA)

[6%] were added to precipitate proteins out and centrifuged at 10 000 g for 3 min. The supernatant was discarded carefully by draining the TCA and washed three times with 1 mL ethanol:ethyl ether (1:1 v/v) and were left to stand for 10 min before centrifugation and discarding the supernatant each time. 400 µL of guanidine hydrochloride (6 M in 50% formic acid) was added to solubilise the proteins and then left for 15 min at room temperature. Samples were centrifuged at 16 000 g for 5 min. Supernatant was read in triplicate at 366 nm using an automated microplate reader (BioTek FLx800). Protein carbonyl content is expressed as nM carbonyl/mg protein.

3.6.2.4. Malondialdehyde content:

This assay was adapted from Ohkawa *et al*. (1979) and modified by Üner *et al*. (2005) to determine malondialdehyde (MDA) content. Sample homogenate of eppendorph A was centrifuged at 9 500 g (4°C) for 10 min and supernatant was used for MDA and protein analysis. To each supernatant (25 µL) sample, tris-sucrose buffer (same volume as supernatant) [25 mM tris-HCl, 250 nM sucrose] was added as blanks, sodium dodecyl sulphate (SDS) [50 µL, 8.1% in deionsed water], acetic acid (375 µL, 20%), thiobarbituric acid (375 µL, 0.8%) deionised water (175 µL) was added in an eppendorph tube (3 mL) and placed in a water bath (95°C) for 30 min to incubate and afterwards allowed to cool down to room temperature (20°C). Deionised water (250 µL) together with 1250 µL n-butanol:pyridine (15:1, v/v) was added and vortexed and centrifuged at 2 700 g for 10 min at room temperature. The supernatant (245 µL) was added into the microplate wells and the absorbance was read at 540 nm using an automated microplate reader. MDA content is expressed as nM/mg protein.

3.6.3. Biomarkers of Exposure:

3.6.3.1. Acetylcholinesterase activity assay:

In brief, this assay was adapted from Ellman *et al*. (1961). The samples prepared in eppendorph B, were centrifuged at 9 500 g for 10 min at 4°C. The procedure was performed on ice. PPB (0.09 M, adjusted to pH 7.4, 210 µL), s-acetylthiocholine iodide (30 mM, 10 µL) and Ellman's reagent (2,2'-dinitro-5,5'-dithio-dibenzoic acid, 10 µL) were added into the wells of a 96 microtitre plate and mixed carefully. Only 7 samples (in triplicate) per microtitre plate were analyzed at a time in order to ensure accurate readings. Samples were then left to incubate for 5 min at 37°C. After incubation, a 5 µL sample aliquot was added and mixed carefully and was then read immediately. Absorbance was read at 405 nm using an automated microplate reader (BioTek FLx800) and the kinetic reaction was read in 60 s intervals over 6 min (7 intervals) with reading 1 starting at 0 s. AChE activity is expressed as (Abs/min/mg protein).

3.6.4. Energy Allocation Biomarkers:

3.6.4.1. Protein (P) and Lipid allocation (L):

Muscle tissue was used to determine protein content and lipid reserves. The method for cellular energy allocation was adapted from De Coen & Janssen (1997a) and De Coen & Janssen (2003). CEA consists of four different assays namely; protein content, carbohydrate content, lipid content and electron transport system activity. Batch C was only for energy available (E_a). The 100 µL homogenate was further diluted by adding 400 µL deionized water and then used for the E_a assay.

Available energy reserves were divided into three separate assays: the protein content, carbohydrate levels, and lipid content. For protein determination, the Bradford (1976) [see sample preparations] method was followed. In brief, 5 µL deionized water (used as a blank) and 245 µL Bradford reagent was added to the wells in triplicate and left for 5 min to incubate. Absorbance was read at 595 nm with an automated microplate reader.

The Bligh & Dryer (1959) method was used to determine lipid content. A stock solution of tripalmitin (0–6 000 µg/mL) was prepared. Chloroform (500 µL) was added to 250 µL of the sample homogenate and mixed by vortexing. Methanol (500 µL) and 250 µL deionized water was added to the homogenate and again vortexed. After vortexing, the sample was centrifuged at 3 000 g for 5 min at 4ºC. Thereafter, 100 µL of the organic phase was transferred into glass test tubes. Choloroform (100 µL) was prepared as a blank. H_2SO_4 (500 µL) and was added to the blank, samples and standards and then covered with aluminium foil. These samples were left to incubate at 200ºC for 15 min and 1 mL deionized water was added afterwards. Samples were left to cool to room temperature and then 245 µL was taken out of each sample and added in triplicate to the glass microtiter plates. Absorbance was read at 360 nm using an automated microplate reader.

3.7. Nanomaterial uptake and distribution in *E. albidus*:

3.7.1. Metal bioaccumulation analysis:

Following exposures, organisms were pooled into at least three replicates per exposure group and accurately weighed to the nearest 4 decimals. Samples were placed in 9 mm x 21 mm tubes within a 150 mm x 150 mm x 24 mm Teflon digestion block and 1 mL suprapure 65% HNO_3 was added. Samples were placed in the fume hood and the Teflon digestion block was tightly covered and placed in an oven at 70°C and left overnight to digest. Thereafter, samples were left to cool to room temperature and 1 mL subsamples were removed and pipetted into 15 mL Falcon tubes. These samples were diluted, by adding 15 mL with milliQ water. Samples were analysed using ICP-MS techniques described in section 3.3.1.

3.7.2. Dark field microscopy using CytoViva imaging:

Triplicate samples for each exposure group were prepared on the final day of the exposure period (Day 21). Worms were gently collected with a small needle and placed in Cryopreserve gel (Tissue-Tek® OCT™ Compound) on a microscope slide and covered with a cover slip. The visualization was done through CytoViva® 150 Unit integrated onto an Olympus BX43 microscope.

3.8. Statistical Analysis:

For all toxicity analysis, ToxRat Software was used to calculate the NOEC, LOEC, EC_x and LC_x values. These tests are statistical analysis according to OECD, ISO protocols (ToxRat™, 2017).

3.8.1. LCx:

To determine any LC_x value, probit analysis or a logistic regression should be applied. When less than three concentrations with only limited mortality are available, this method is unsuitable and alternative methods can be used to calculate this value (OECD, 2015). These methods include the trimmed Spearman-Karber method, moving averages and simple interpolation (OECD, 2015).

3.8.2. ECx:

After the appropriate dose-response function has been obtained, any EC_x value can be calculated using the regression analysis (linear or non-linear). The EC_x value can be determined using a suitable regression analysis if looking at the growth of worms as a continuous response (OECD, 2015). For mortality/survival and the number of offspring, more suitable functions were used such as the normal sigmoid, logistic or Weibull functions [which contains two or four parameters, some of which can model hormetic response] (OECD, 2015).

3.8.3. NOEC:

The no observed effect concentration (NOEC) is the highest concentration where there was no significant statistical difference ($p < 0.05$) to that of the control (Newman, 2015).

3.8.4. LOEC:

The lowest observed effect concentration (LOEC) is the lowest concentration that had a significant statistical difference to that of the control (Newman, 2015).

3.8.5. Means testing:

One-way Analysis of Variance (ANOVA) was performed to calculate the statistical differences ($p<0.05$) in metal bioaccumulation and biomarkers responses between the different nanomaterial exposure groups, metal ion exposures and the control. Prior to analysis, the

distributions of the data were checked for homogeneity of variance (Levene's test) and normality (Kolmogorov–Smirnov test). Depending on the outcome, ANOVA with Tukey post hoc test (parametric data) or Kruskal-Wallis with Dunette T3 test (nonparametric data) were performed to calculate the statistical differences.

3.8.6. Multivariate analysis:

In this study a redundancy analysis (RDA) was performed to assess the relationship between metal bioaccumulation and biomarker responses in the different exposures of *E. albidus* to the metal ions, the QDs and their functionalised groups. The rationale behind using this analysis is to provide an indication of the degree of similarity in the responses to metal ions and their NM equivalents and whether the functionalised group elicits similar or different responses. Canoco statistical package were used to construct the RDA bi-plots. An angle close to 0° indicates positively correlated variables, whereas closer to 90°, the variables are uncorrelated and lastly when the angle was closer to 180°, the variables were negatively correlated (Ter Braak & Smilauer, 2004).

Results

4.1. Characterization of Cd/Te QDs:

4.1.1. Characterization in Milli-Q water:

The QD-NH_3 group had the lowest particle diameter compared to the other two functional groups (Table 4.1) with a mean diameter of 75 nm while PEG showed the highest with 159 nm which indicates higher agglomeration within Milli-Q water. The Cd/Te QDs showed a low dissolution rate, the QD-NH_3 group had the lowest of the three different functional groups in terms of the Cd released with 0.74% and Te 0,39%. The QD-COOH displayed the highest dissolution rate for Te with 23.7% released, but the QD-PEG group had the highest percentage Cd released from all three functional groups with 2.43% dissolution. Figure 4.1 displays the transmission electron microscope (TEM) images of the particles, which shows the agglomeration of the particles in Milli-Q water. Table 4.2 indicates the measured concentration (mg/kg) of the ionic metal controls in powder form, as well as the measured powder concentration of the QDs. The NMs had more than 100 mg Cd and the QD-NH_3 (366130 mg/kg) had the highest concentration compared to others. Table 4.3 indicates the amount of Cd and Te in a stock solution, which was a 100 mg powder mixed with a litre of Milli-Q water.

Table 4.1: Characterization of QDs in terms of particle size and dissolution rate:

QDs	Particle size		Dissolution (% Metal released)
	Max (nm)	Mean (nm)	
COOH	370	84±58	Cd 2.1%
			Te 23.7%
PEG	510	159±72	Cd 2.43%
			Te 3.23%
NH_3	290	75±50	Cd 0.74%
			Te 0.39%

Table 4.2: Concentrations of Cd and Te (mg/kg) in ionic metal exposures and QD exposures and ± standard error (±SE):

Exposure	Nominal Concentration (mg/kg)		Measured Concentration (mg/kg)	
	Cd	Te	Cd	Te
100 mg/kg Cd as $CdCl_2$	100	-	163.079	-
500 mg/kg Cd as $CdCl_2$	500	-	815.397	-
500 mg/kg Te as $TeCl_4$		500	-	1.056
100 mg/kg QD-COOH	-	-	261.26±701	49.11±124.2
100 mg/kg QD-PEG	-	-	277.1±7246	58.83±1846
100 mg/kg QD-NH_3	-	-	366.1±6382	185.62±4685

Table 4.3: Cd and Te concentrations of QD stock solution (100mg/L) and ± standard error (±SE):

QD stock solution (100 mg/L)	Nominal Concentration (mg/kg)		Measured Concentration (mg/kg)	
	Cd	Te	Cd	Te
QD-COOH	-	-	30.45±0.354	5.67±0.139
QD-PEG	-	-	33.03±0.301	6.91±0.183
QD-NH_3	-	-	18.87±0.355	7.3±0.059

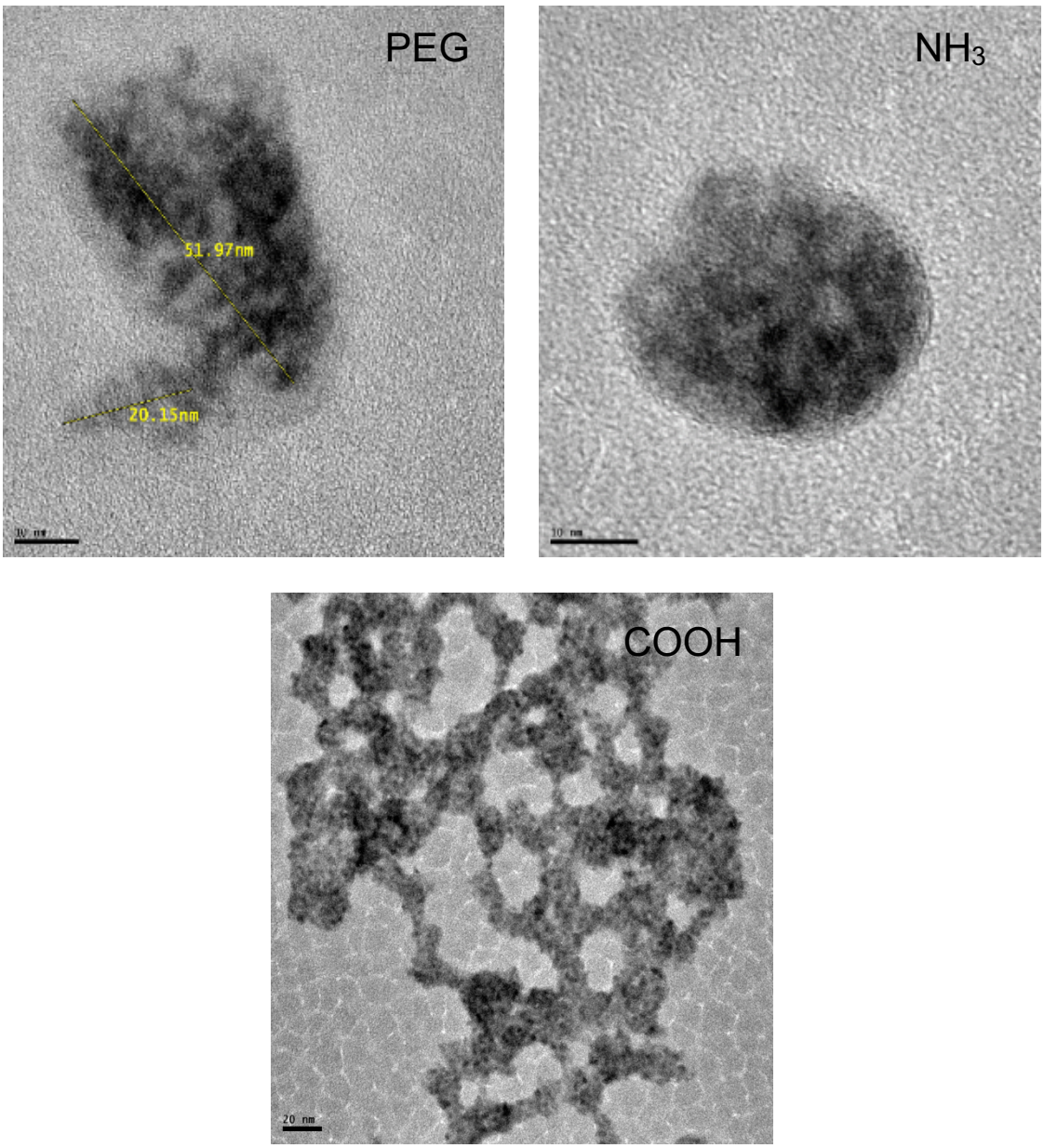

Figure 4.1: Transmission electron microscope images of QDs (PEG, NH_3 and COOH) prepared in Milli-Q water.

4.1.2. Chemical characterization of the exposure soils:

The cadmium content in the soil exposed to three functional groups of QDs (Figure 4.2) was not significantly different from the control, but the Cd ionic control differed significantly from all of the groups. The Cd differed significantly ($p<0.05$) from the control and the three functional groups of the same concentration (100 mg/kg). Figure 4.3 illustrates the significance between all of the groups tested for Te content. The 500 mg/kg QD-PEG, QD-NH_3 and the Te ionic control differed significantly ($p<0.05$) from the control. The Te ionic control differed from all three functional groups of the same concentration. The only significant difference ($p<0.05$) between QD-PEG, QD-COOH and QD-NH_3 was at the 500 mg/kg concentration.

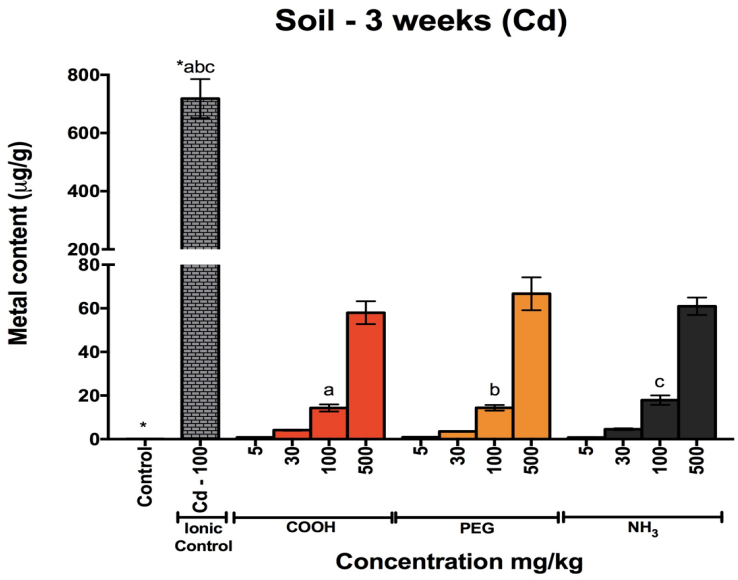

*Figure 4.2: Cadmium metal content of soil (µg/g dry mass) of exposed (3 weeks) soil to QDs (PEG, COOH & NH_3) with standard error (SE). The groups with the * differ from the control and the different superscript alphabetical letter displays the significant difference between the same concentrations.*

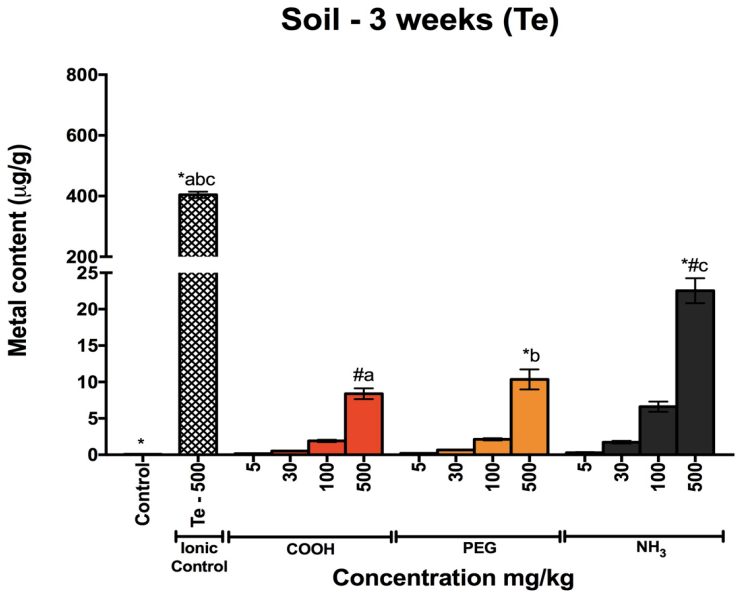

*Figure 4.3: Tellurium metal content of soil (µg/g dry mass) after exposure of 21 days exposed to QDs (PEG, COOH & NH_3) with SE. The * indicate statistical differences from the control and the rest of the groups. Different alphabetical superscripts indicate statistical differences between the same concentrations with the # indicating significance between different functional groups of the same concentration.*

4.1.3. Transport of nanomaterials utilizing a flow-through system:

Figures 4.4 and 4.5 illustrate heat maps of the total Cd and Te metal concentrations measured in the sediment column, eluted water and clay after 24 h following exposure to the different functionalized QDs. These heat maps provide an indication of the fate and transport of the metals following the addition of the QDs as an aqueous solution to the surface of the soil column or mixing the QDs in the sediment. For the purposes of the heat maps high is defined as a concentration range of (250-1500 µg/L or µg/kg), while medium is (50-250 µg/L or µg/kg) and low is (0-50 µg/L or µg/kg).

From Figure 4.4, there is a clear indication that applying the stock solution of NM on surface of the soil column resulted in elution of the majority of the particles through the soil into the beaker irrespective of the different functionalization of QDs. The highest Cd concentrations remaining in the soil column were measured in the top region (1) and decreased towards the bottom of the column. The eluted water had the highest Cd content with the clay having the second highest. Figure 4.4B displays the results following application of Milli-Q water to soils with QDs mixed homogenously in the soil column. The opposite trend was observed with the Cd concentrations decreasing from the top of the column with low concentrations to low-medium concentrations at the bottom of the column. Similarly to A, the highest Cd concentrations were measured in the eluted water and the second highest in the clay fraction. For QD-NH_3 (Figure 4.4B), the Cd content is slightly different than the other two experiments, where the third highest was found in the middle region of the tube and became lower towards the bottom. The water and clay remained to be the highest and second highest respectively.

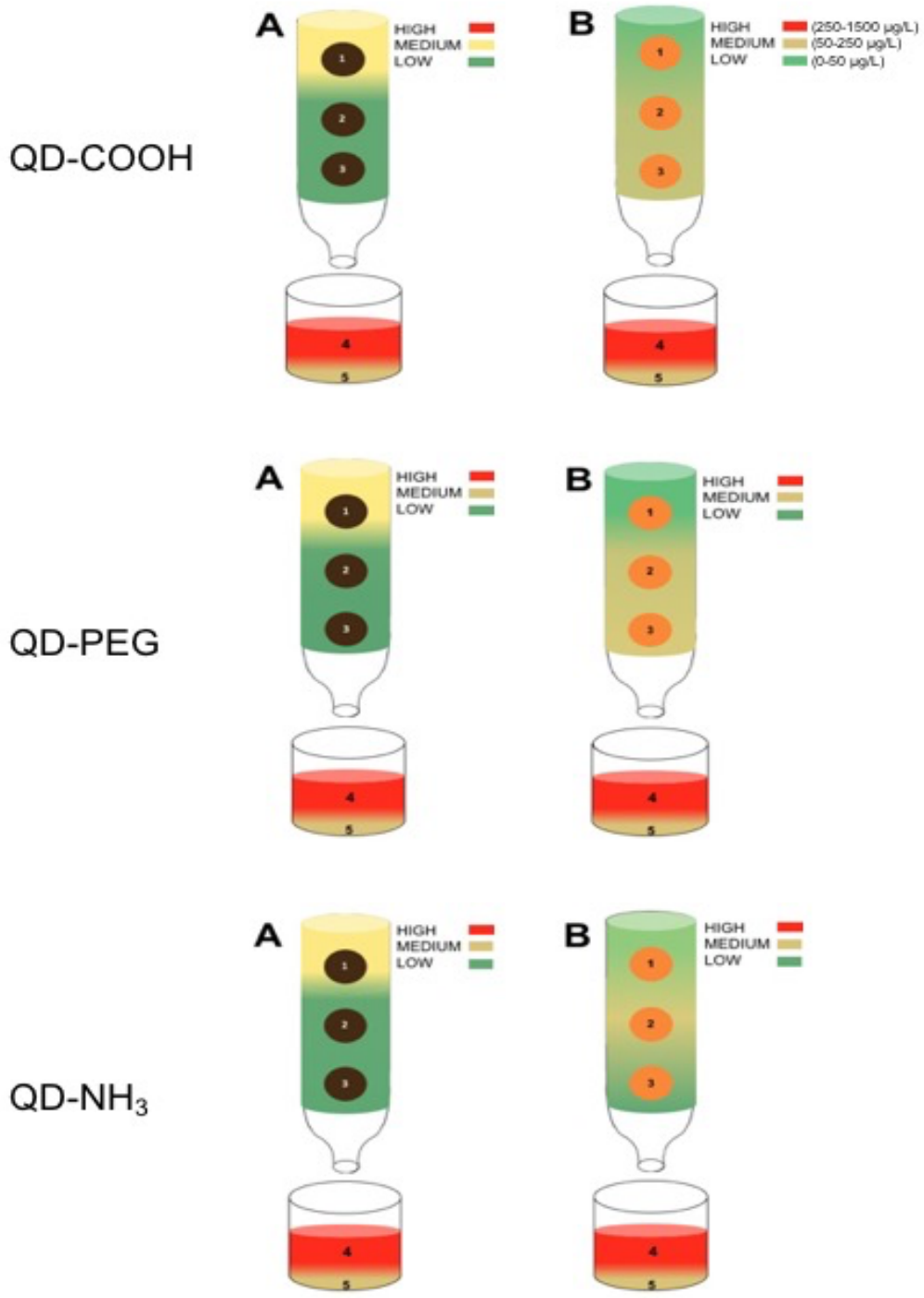

Figure 4.4: A heat map of the Cd concentrations in the soil column, eluted water and clay fractions following application of Cd/Te-quantum dots - A) as a dispersion mixture applied to the top of the soil and B) where the nanomaterials were homogenously mixed in the soil. The 5-sampling areas are represented by the surface of the soil column (1), the middle of the soil column (2), bottom of the soil column (3), eluted water (4) and eluted clay fraction (5).

Tellurium released in the soil by the QDs had a much lower concentration (Figure 4.5) in comparison with the cadmium. High is defined as a concentration range of (30-100 µg/L or µg/kg), while medium is (10-30 µg/L or µg/kg) and low is (0-10 µg/L or µg/kg). For the tellurium released all QDs (PEG, COOH and NH_3), the same trend can be seen as with the cadmium released in the soil. The middle and bottom part of the tube had less Te content than at the top of the tube. The Te content that was found in the clay and the water fractions were within the same concentration range (sampling areas 4 & 5). The Te content found in the clay and water

with QD-NH_3 group differed from the other two groups, where less Te was found in the clay compared to the water. When the NMs were homogenously mixed into the soil, the clay fraction had the highest Te concentration (Figure 4.5B). When mixing the NMs with the soil, the Te concentration became higher as it moved towards the bottom of the tube (Figure 4.5B). The clay had the highest Te concentration for all three functional groups when the NMs were mixed in the soil. The QD-PEG had less Te content in the water than the other two groups. The Cd and Te concentrations that were measured in the five areas for the different exposures reported in Figures 4.4 and 4.5 are reported in Annexure A.

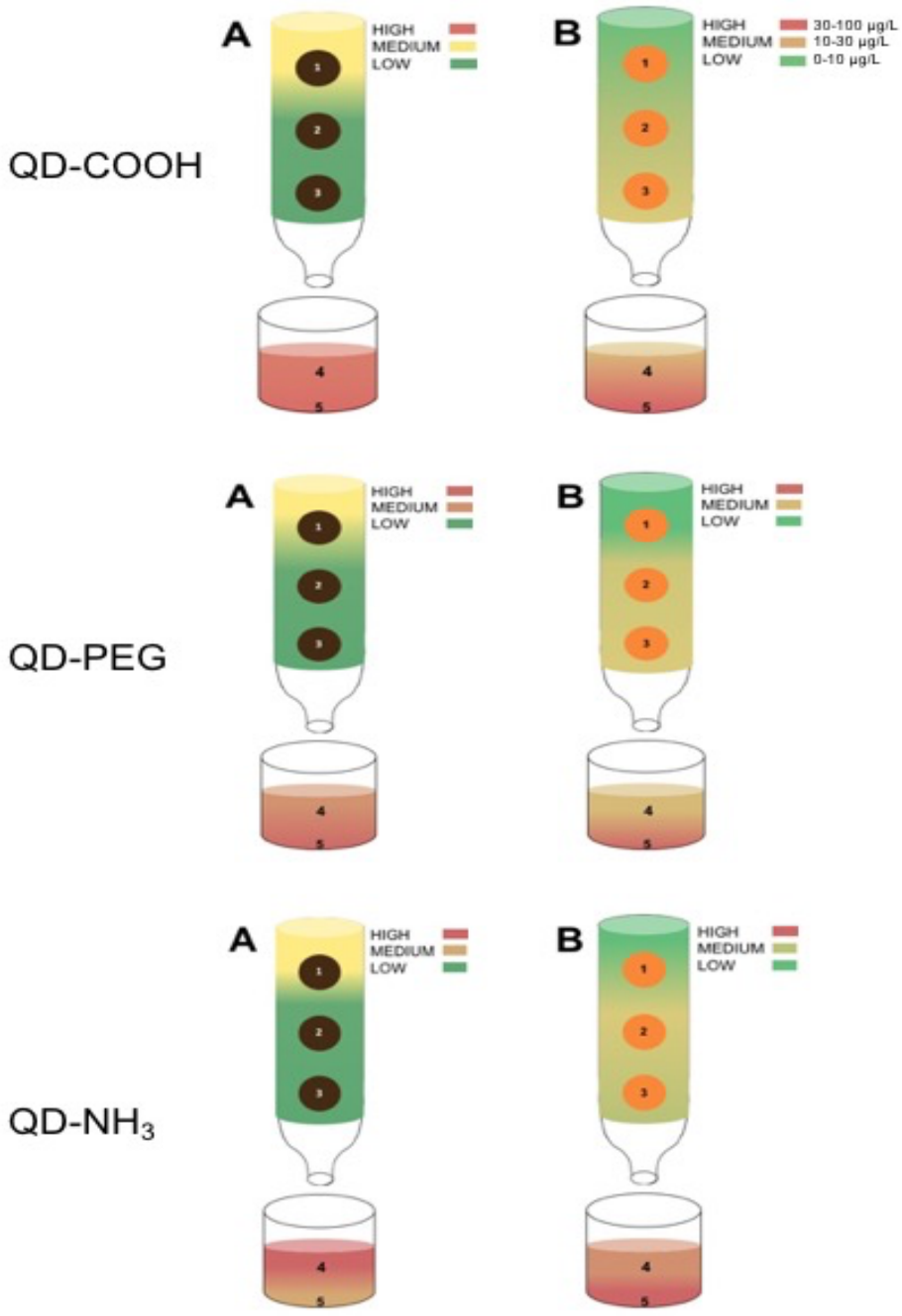

Figure 4.5: A heat map of the Te concentrations in the soil column, eluted water and clay fractions following application of Cd/Te-quantum dots - A) as a dispersion mixture applied to the top of the soil and B) where the nanomaterials were homogenously mixed in the soil. The 5-sampling areas are represented by the surface of the soil column (1), the middle of the soil column (2), bottom of the soil column (3), eluted water (4) and eluted clay fraction (5).

Exposure assessment:

4.2.1. Tissue distribution of quantum dots using CytoViva imaging:

Dark field hyperspectral imaging (CytoViva) revealed that in *E. albidus* the Cd/Te QDs were present within the worms in comparison to the control where QDs were not present (Figure 4.6). The worms were depurated overnight and placed on a microscope slide. The depuration revealed that once soil was removed from the gut of the worms QD's were still present within the intestine (Figure 4.10B).

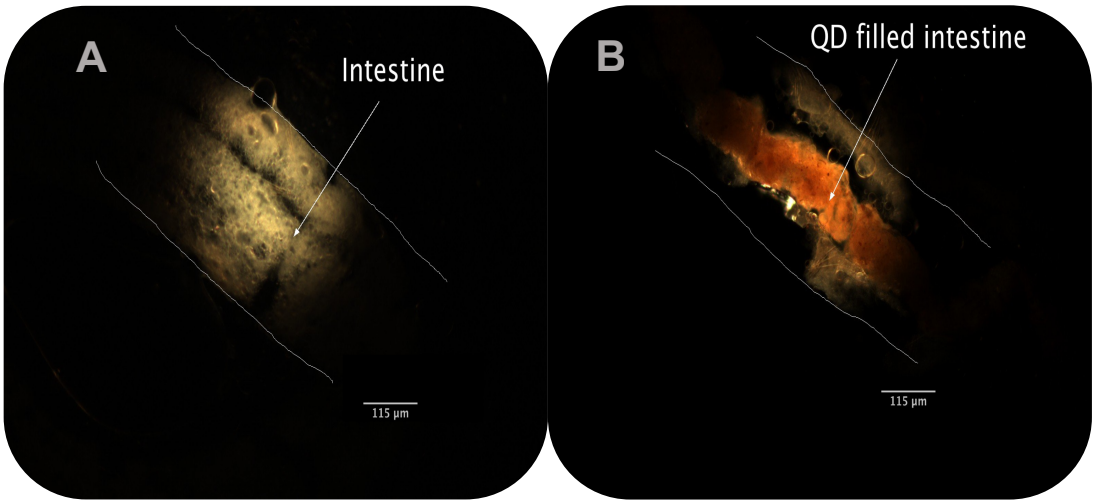

Figure 4.6: CytoViva® dark field hyperspectral imaging of Enchytraeus albidus in A) control and B) in exposed 1.0 mg/kg QD-PEG.

4.2.2. Bioaccumulation of Cd and Te:

Figure 4.7 indicates clear differences in Cd content in tissue of the adult test organisms between the different groups exposed to QDs. There was a significant difference between the control and the Cd control. In terms of the control and the three functional groups, there was a significant difference between the control and the 500 mg/kg QD-COOH exposed individuals. The remaining concentrations of the three functional groups were not significantly different. There is significantly lower difference between the Cd control and the 100 mg/kg concentration of all groups. Within the exposure groups, there was no significant difference between the same concentrations.

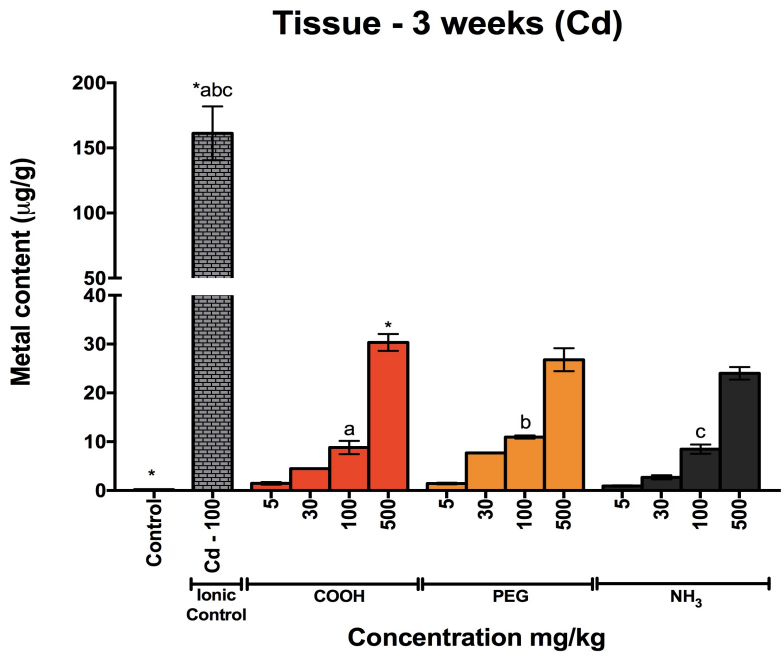

*Figure 4.7: Cadmium metal content of tissue and exposed to QDs. The groups with the * differ from the control and the different superscript alphabetical letters displays the significant difference between the same concentrations.*

There was a clear significant difference in tellurium found in the tissue between the ionic Te control and the control exposure groups as shown in Figure 4.8. The Te concentration is lower in the particle of QDs hence why tellurium content in Te are significantly different from the three functional groups with the same concentrations. Also, the control shows no significance between the three functional groups. There was no significant difference in uptake of Te from the QD particle between the 500 mg/kg QD-COOH, 500 mg/kg QD-PEG and 500 mg/kg QD-NH_3 groups.

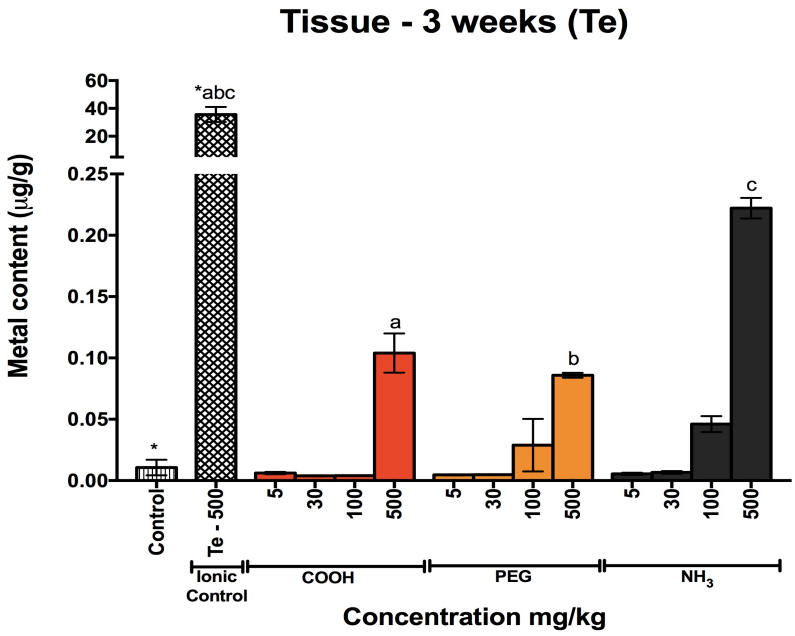

*Figure 4.8: Tellurium tissue metal content after exposure of 21 days exposed to QDs. The * indicate statistical differences from the control and the rest of the groups. Different alphabetical superscripts indicate statistical differences between the same concentrations.*

4.2. Effects assessment:

4.3.1. Survival

There were mortalities in any of the exposures except in the Cd 500 mg/kg ionic control which had a mortality rate of 100% As a result of this LC_x values could not be calculated (Table 4.2). No significant mortalities in any of the exposures except Cd 500 mg/kg.

Table 4.4: Mean survival percentage and ± standard deviation (± SD) of Enchytraeus albidus after 21 days:

Controls

Negative control	**Positive control**			
Control	**Cd 100 mg/kg**	**Cd 500 mg/kg**	**Te 500 mg/kg**	**Te 1 000 mg/kg**
100	98 ± 4.47	0	94 ± 8.94	90 ± 7.07

QDs

	0.5 mg/kg	**1 mg/kg**	**5 mg/kg**	**15 mg/kg**	**30 mg/kg**	**100 mg/kg**	**500 mg/kg**
COOH	100	98 ± 0.45	100	100	100	96 ± 0.54	98 ± 0.45
PEG	100	98 ± 0.45	96 ± 0.89	100	100	100	96 ± 0.89
NH_3	100	100	100	96 ±0.89	96 ± 0.89	96 ± 0.54	96 ± 0.54

4.3.2. Reproduction:

The reproductive success of *E. albidus* following exposure to different concentrations of QD-COOH is presented in Figure 4.9. There were no significant differences ($p>0.05$) between the different exposures compared to the control.

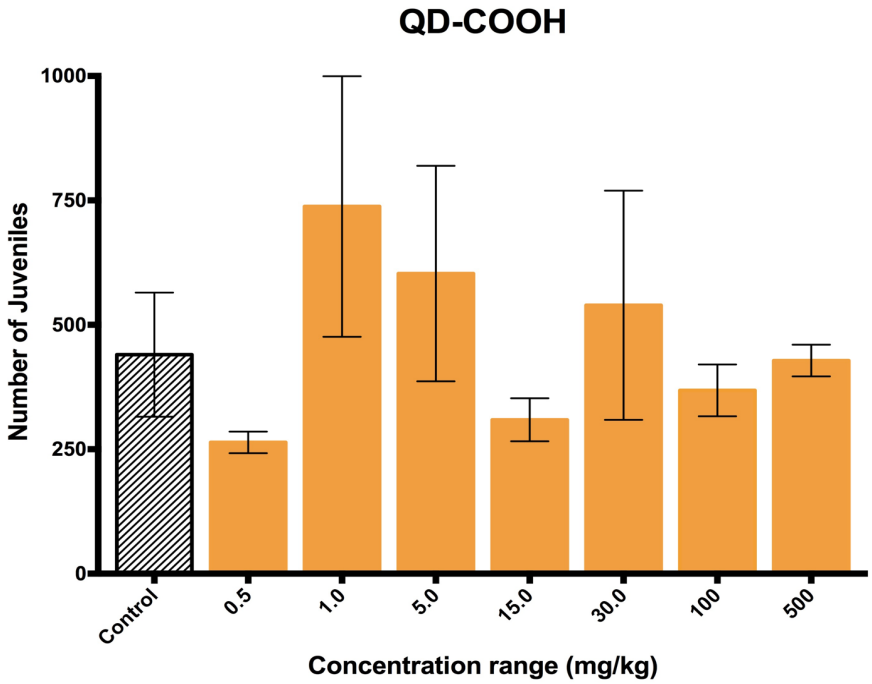

Figure 4.9: Reproductive success of Enchytraeus albidus exposed to QD-COOH for 42 days.

The number of offspring produced following exposure to QD-NH_3 is presented in Figure 4.10. All the exposure concentrations resulted in lower offspring production compared to the control with only the 5 mg/kg being significantly lower in offspring production and the 5 mg/kg differs significantly form the 100 mg/kg. EC_x values are provided in Annexure B.

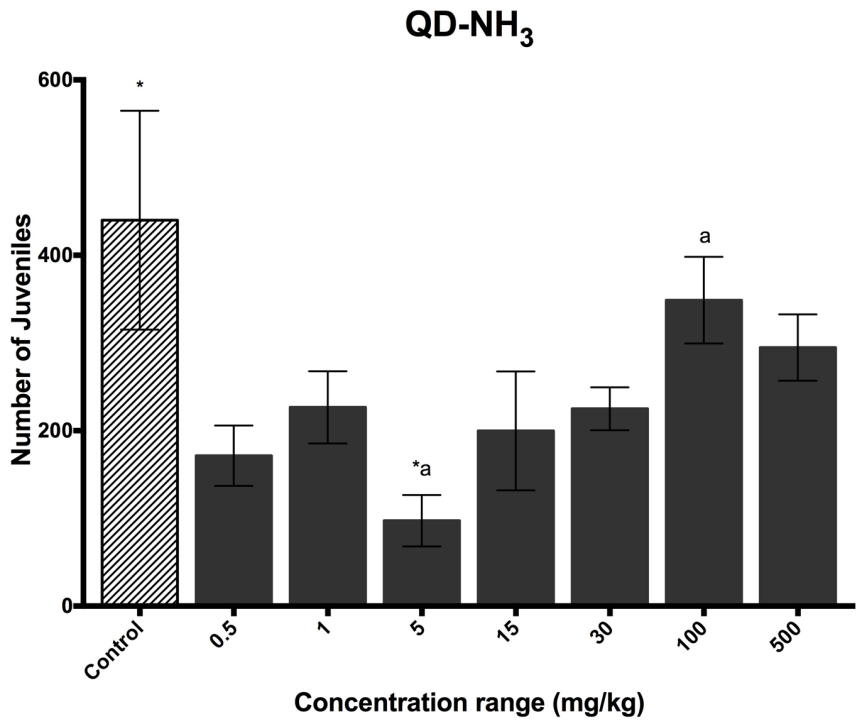

*Figure 4.10: Number of offspring exposed to QD-NH_3 for 42 days. * indicate statistical differences to the control and alphabetical letters indicate statistical differences between concentrations.*

Figure 4.11 displays an approximate bimodal trend with the number of juveniles hatched after 42 days. With a Kruskal-Wallis and one-way ANOVA analysis, no significant difference in

number of offspring was found between the control and the concentrations tested. Although not significant, several groups showed higher offspring numbers than those produced in the control group. EC_x values are provided in Annexure B.

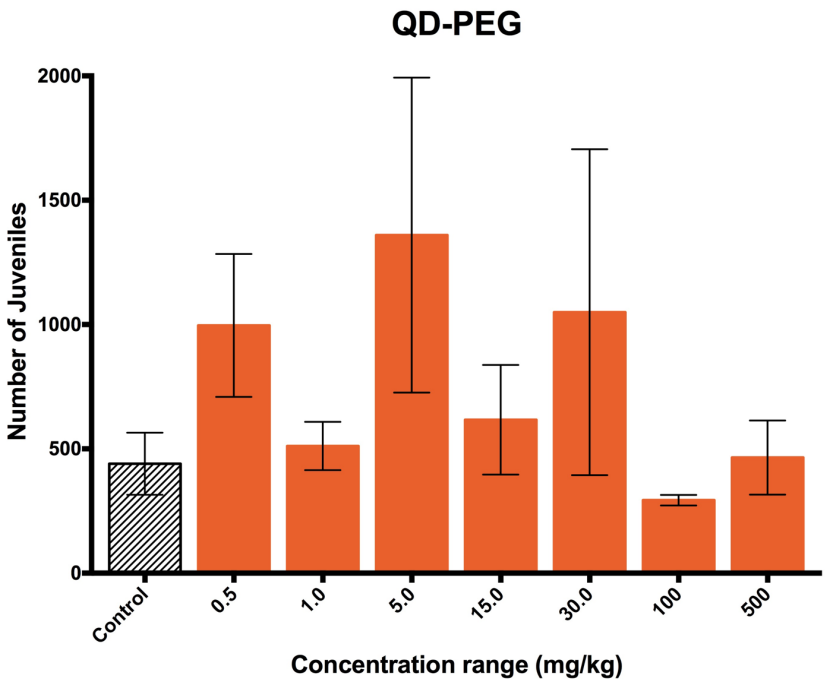

Figure 4.11: Total number of juveniles produced after 42 days exposed to QD-PEG.

Significant differences in the number of offspring between the control, 100 mg/kg Cd, 500 mg/kg Cd and the 500 mg/kg Te were found (Figure 4.12). The control, Cd 100 and Cd 500 mg/kg differ significantly from each other. Te of the same concentration is also significantly different from the 500 mg/kg Cd.

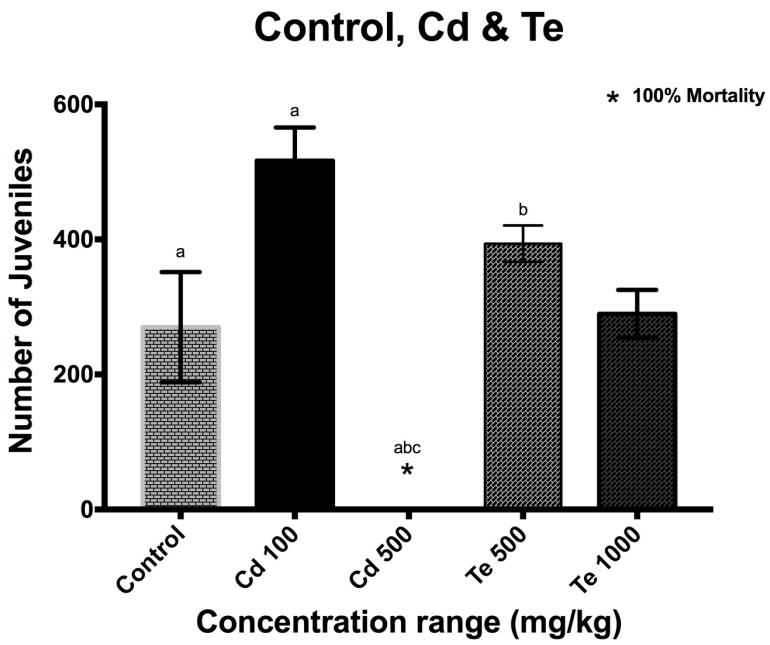

Figure 4.12: Offspring number of Enchytraeus albidus exposed to the bulk metals Cd and Te for 42 days. The same alphabet letters indicate statistical differences between the groups.

4.3.3. Avoidance

There was no significant ($p>0.05$) avoidance response of adult potworms following exposure to QD-COOH (Figure 4.13). The greatest avoidance to QD-COOH exposure was observed at the 100 mg/kg concentration. EC_x values are provided in Annexure B.

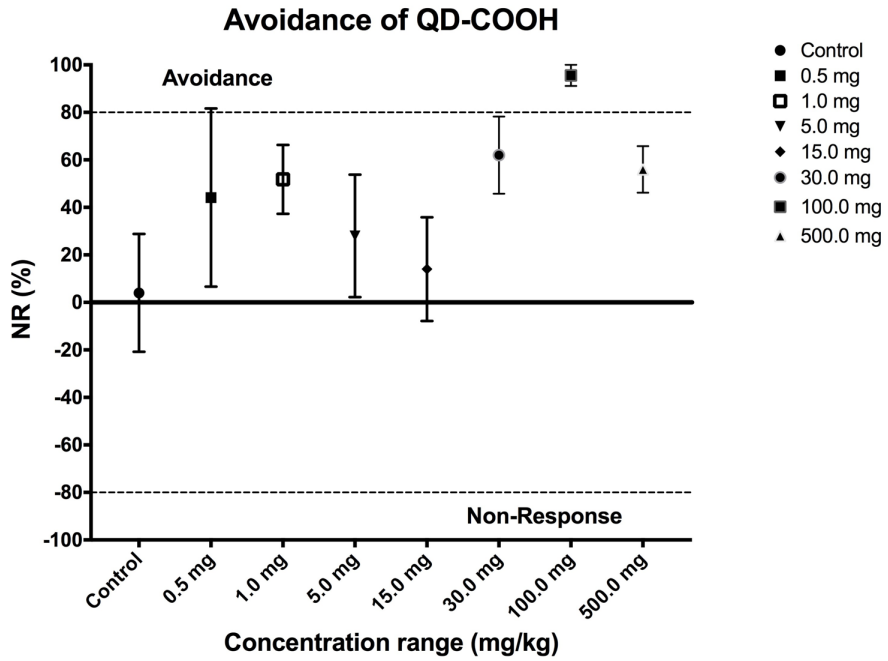

Figure 4.13: Behavioural response of Enchytraeus albidus following exposure to QD-COOH for 48 h. The NR (%) indicate the Net Response in percentage to the QDs

A sinoid response to QD-NH_3 exposure was observed but it was not significant different ($p>0.05$) from the control (Figure 4.14). EC_x values are provided in Annexure B.

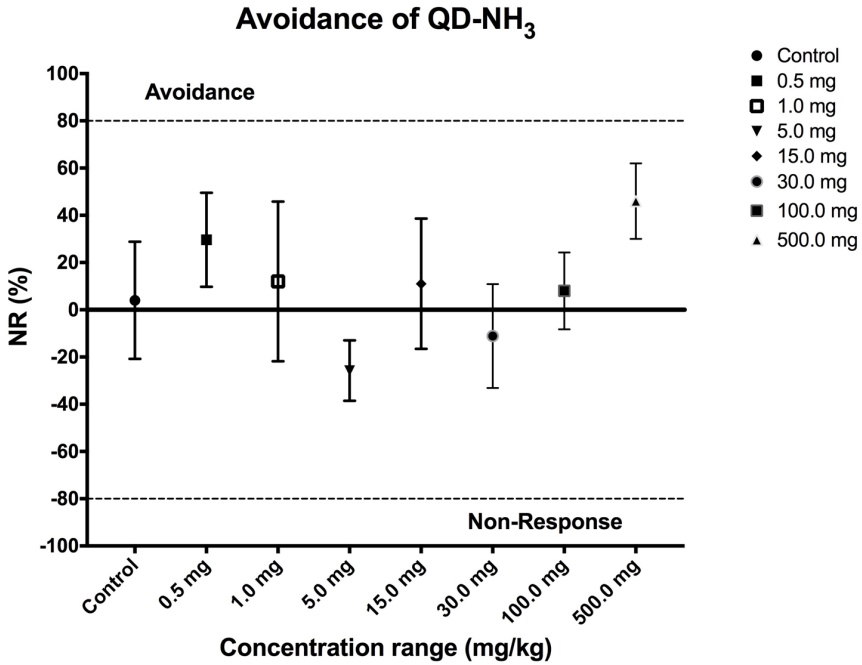

Figure 4.14: Avoidance behaviour of Enchytraeus albidus to QD-NH_3.

Figure 4.15 shows an increase in avoidance behaviour for the enchytraeids from concentrations 0.5 mg/kg to 5 mg/kg. There was statistical significance between the 1 mg/kg concentration and 15 mg/kg as well as between the 5 mg/kg and the 15 mg/kg. The 15 mg/kg concentration is also significantly different from the 100 mg/kg and the 500 mg/kg. EC_x values are provided in Annexure B.

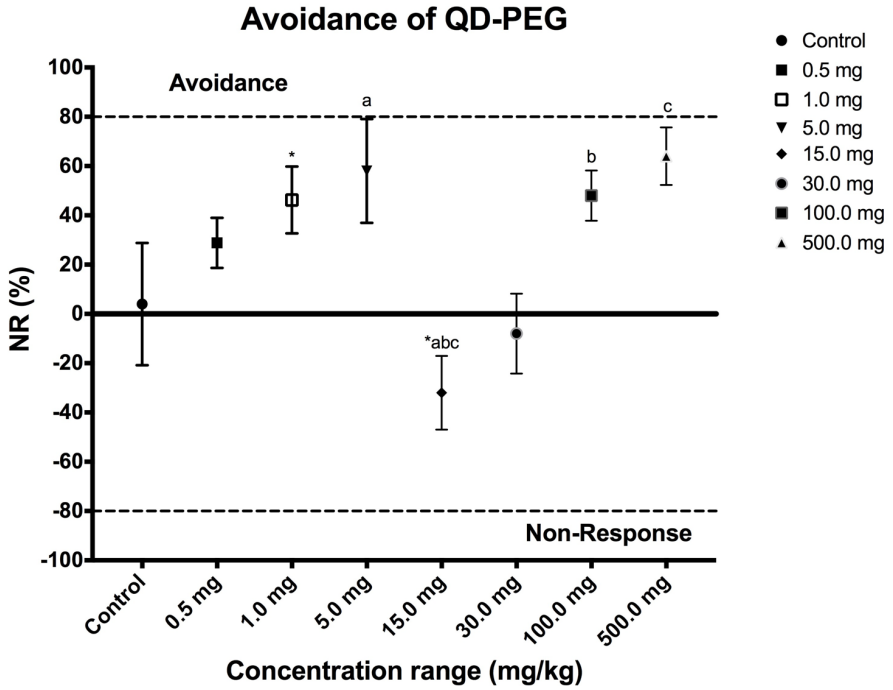

*Figure 4.15: Enchytraeus albidus illustrating their behavioural response to QD-PEG. The * indicate statistical differences between 1 mg/kg and 15 mg/kg, the same alphabetical letter indicate significance between 5 mg/kg and 15 mg/kg (a), 15 mg/kg with 100 and 500 mg/kg (b).*

Figure 4.16 indicates the avoidance behaviour of *E. albidus* to heavy metals such as Cd and Te. There is statistically significant difference ($p<0.05$) between the control and the heavy metals tested. Between the two concentrations of Cd there is statistical differences, but no significance between the Cd and Te of the same concentration (500 mg/kg). Both 500 mg/kg heavy metal concentrations exhibit a clear behavioural response as the response is above the 80% threshold.

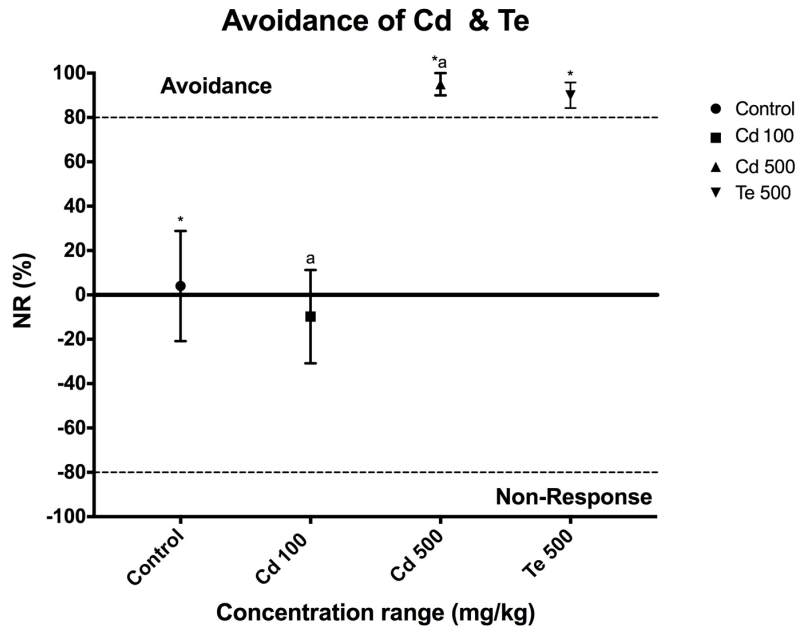

*Figure 4.16: The avoidance response of Enchytraeus albidus to the bulk metals Cd and Te. The * indicate significance between the control and the rest of the groups tested. Similar alphabetical superscripts indicate statistical significance between exposure groups.*

4.3.4. Biomarkers:

The catalase activity displayed in Figure 4.17, illustrates statistical decreases ($p<0.05$) between the control and the rest of the functional groups and positive controls. For all three functional groups a bimodal trend in CAT activity was observed. The bulk metal exposures resulted in greatest inhibition of CAT activity with the Cd the greatest, whereas the QD-NH_3 exposures had the greatest effect on CAT inhibition when comparing the functionalized QD to each other.

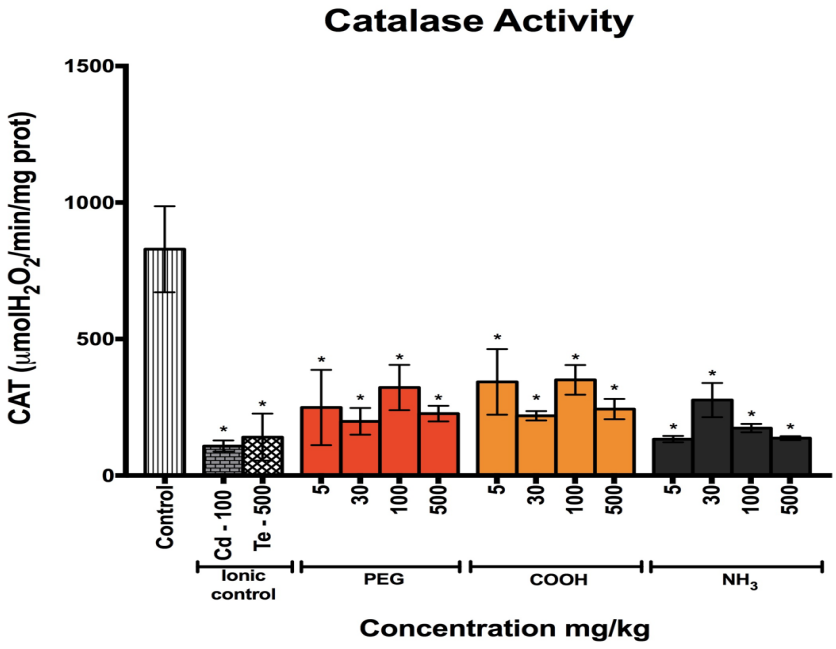

*Figure 4.17: Catalase activity in 21-day exposed Enchytraeus albidus. The * indicate statistical differences between the control, bulk metals and the three functional groups.*

Figure 4.18 presents the superoxide dismutase results obtained from exposing *E. albidus* to a series of concentrations of QDs (PEG, COOH, NH_3). The content ranged from the highest (26.21 ng SOD/mg protein – Control) to the lowest (3.5 ng SOD/mg protein – 500 mg/kg PEG). QD-NH_3 indicates the most superoxide dismutase activity with the lowest at the 5 mg/kg concentration (12.66 ng SOD/mg protein) and the highest at the 30 mg/kg concentration (18.44 ng SOD/mg protein). The superoxide dismutase activity at 100 mg/kg QD-PEG is much higher than the other concentrations of the same group which is lower than the two ionic controls. The three functional groups displayed no significance ($p>0.05$) from the control and the two ionic controls.

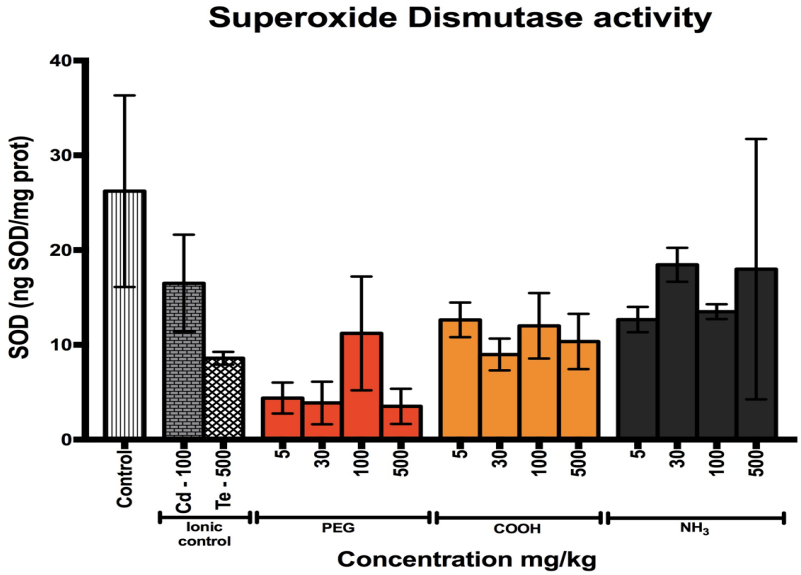

Figure 4.18: Superoxide dismutase activity from Enchytraeus albidus after an exposure of 21 days.

Figures 4.19 (PC) and 4.20 (MDA) showed no significant differences. For all exposures for the functional group QD-NH_3, the protein carbonyl (PC) increases with the increase in concentration, whereas the QD-COOH group decreases with the increase in concentration. All three functional groups had higher response in PC in comparison towards the control and ionic controls. In Figure 4.24, The PEG functional group shows an increase in MDA. Carboxylic acid demonstrates a bimodal response, different from the QD-PEG and the QD-NH_3. Malondialdehyde content for QD-NH_3 group decreased as the concentration increased, which has the lowest MDA content of all the functional groups (0.39 nmol/mg protein). The Te 500 mg/kg indicate more MDA content (1,13 nmol/mg protein) has occurred than the 500 mg/kg QD-COOH and QD-NH_3, but less than the QD-PEG group and much more than the control (0.26 nmol/mg protein).

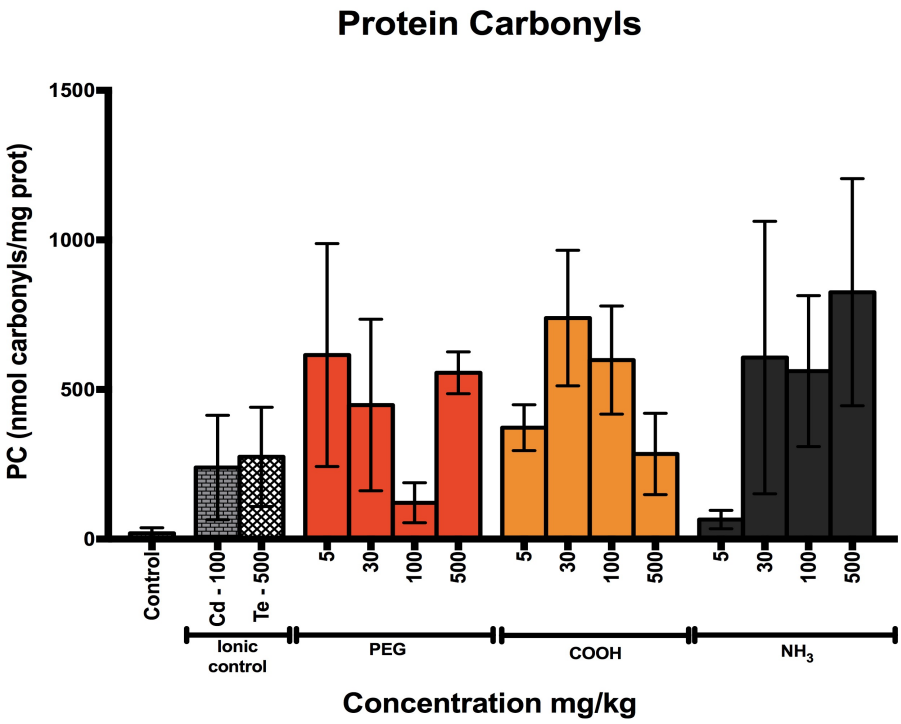

Figure 4.19: Protein carbonyl content of Enchytraeus albidus after exposed to QDs for 21 days.

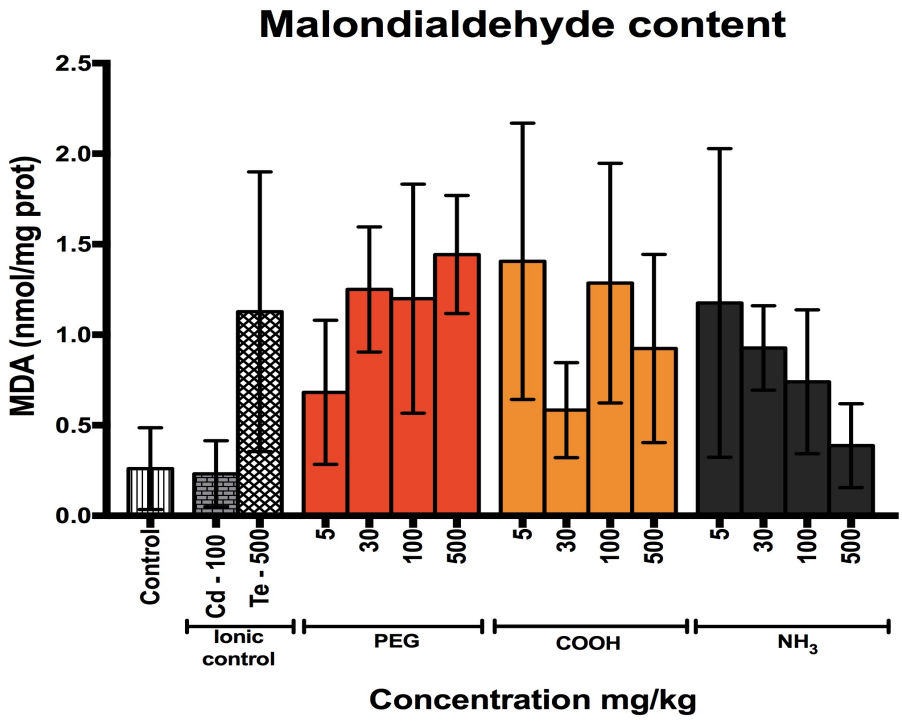

Figure 4.20: Lipid peroxidation measured as malondialdehyde (MDA) of Enchytraeus albidus displayed after 21-days exposed to QDs.

Figure 4.21 displays a significant difference in AChE activity from QD-PEG 30 mg/kg and 100 mg.kg to the control activity, where the control was the lowest (0.001 Abs/min/mg protein) and the 100 mg/kg PEG group was the highest (0.006 Abs/min/mg protein). There is no significant difference (one-way ANOVA) between the rest of the concentrations and the control. From the

ionic controls, there is no significant difference between them and all three functional groups. QD-COOH 500 mg/kg displayed the lowest AChE activity of the three functional groups.

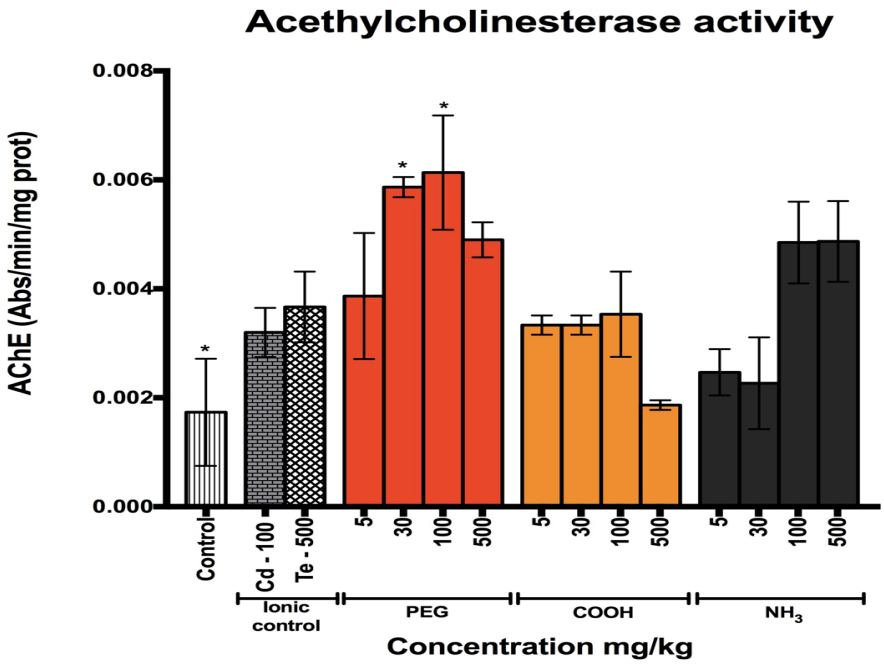

*Figure 4.21: Acethyelcholinesterase activity after a 21-day exposure of QDs. The * indicate statistical differences from the control and the 30 and 100 mg/kg PEG group.*

The lipid content of *E. albidus* after exposure to QDs for 21 days is displayed in Figure 4.22. After analysing the data with a one-way ANOVA analysis, significant differences ($p<0.05$) was found between the control and the ionic control - Cd (*), the Cd 100 mg/kg and 100 mg/kg QD-PEG, QD-COOH and QD-NH_3 (a) and $TeCl_4$ 500 mg/kg and the 500 mg/kg QD-PEG, QD-COOH and QD-NH_3 (b). No significance was observed between the Cd/Te QD groups. These results can be compared to figure 4.20, the lipid peroxidation damage as these results in figure 4.22 indicate low lipid content after 21 days for the QDs and there is high lipid peroxidation for the QDs. In comparison with the ionic controls, the Cd lipid content is high as no lipid peroxidation has occurred and with the Te is lower than the Cd but it more or less the same as the for the lipid peroxidation.

The protein content displayed in Figure 4.23 shows a significant decrease in protein content in comparison with the control and ionic controls except for the QD-PEG group (5, 30 and 100 mg/kg). The significance ($p<0.05$) was calculated by using a one-way ANOVA analysis. No significance was found between the three groups tested as well as the QDs and the ionic controls.

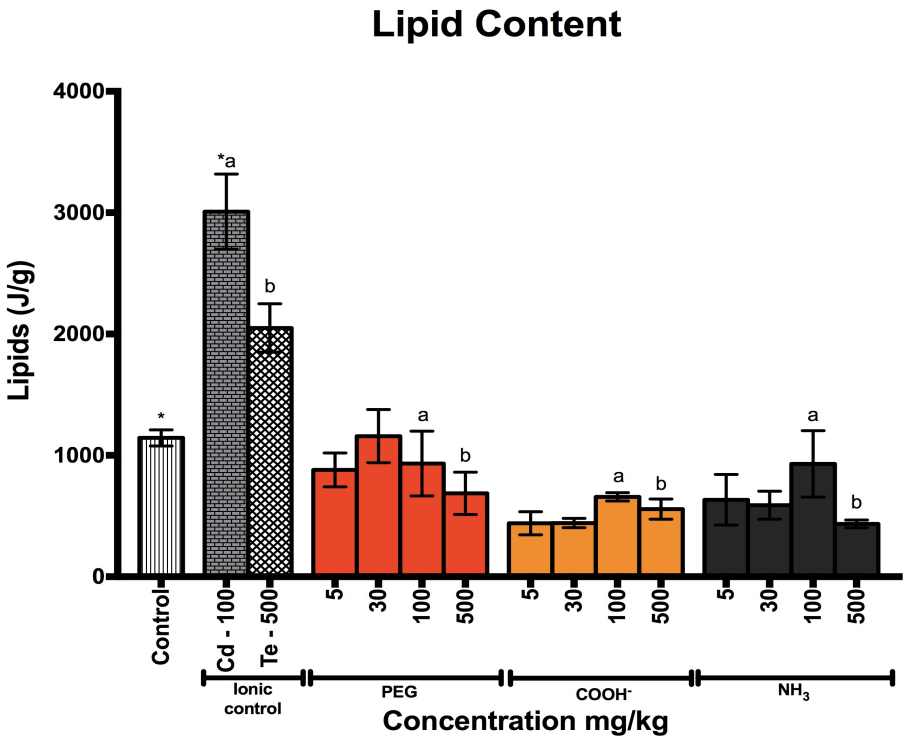

Figure 4.22: The lipid content after exposing Enchytraeus albidus to QDs for 21 days.

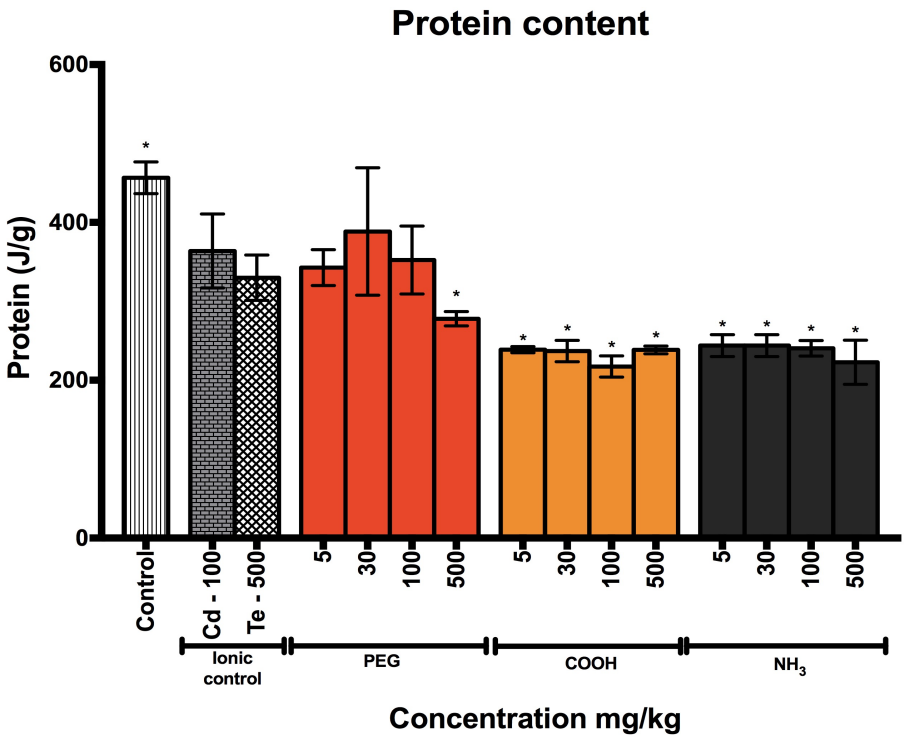

Figure 4.23: The Protein content of Enchytraeus albidus exposed to QDs for 21 days.

A principal component analysis (PCA) of the biomarker responses in relation to exposure groups described 79.6% of the variation in the data (Figure 4.24). The biomarker data were subjected to an RDA with soil Cd and Te exposure in soil and bioaccumulation of Cd and Te in *E. albidus* as the descriptive environmental variables. The redundancy analysis (RDA) in Figure 4.28 describes 20.3% of the variability in the data with axis 1 describing 19.1% and axis 2

1.15%. The three controls and lower exposure concentrations were negatively correlated to the Cd and not so much the Te levels in soil and potworms. In the organisms, the SOD and CAT levels were also high, indicating the protective role that these anti-oxidant enzymes play. The highest concentrations of the QDs (100 and 500 mg/kg) are associated with an increase in PC and a decrease in CAT and SOD activity. The QD-PEG and QD-COOH exposures are also associated with increases in lipid damage in the form of increased lipid reserves and lipid peroxidation (MDA). The QD-NH_3 exposure group did not display any lipid damage. The biomarker responses in the high concentrations of QD-PEG and QD-COOH were associated with higher soil and tissue Cd levels, while the QD-NH_3 exposure was associated with Te exposure. However, exposure to the metals only accounts for 20.3% of the variation in the data.

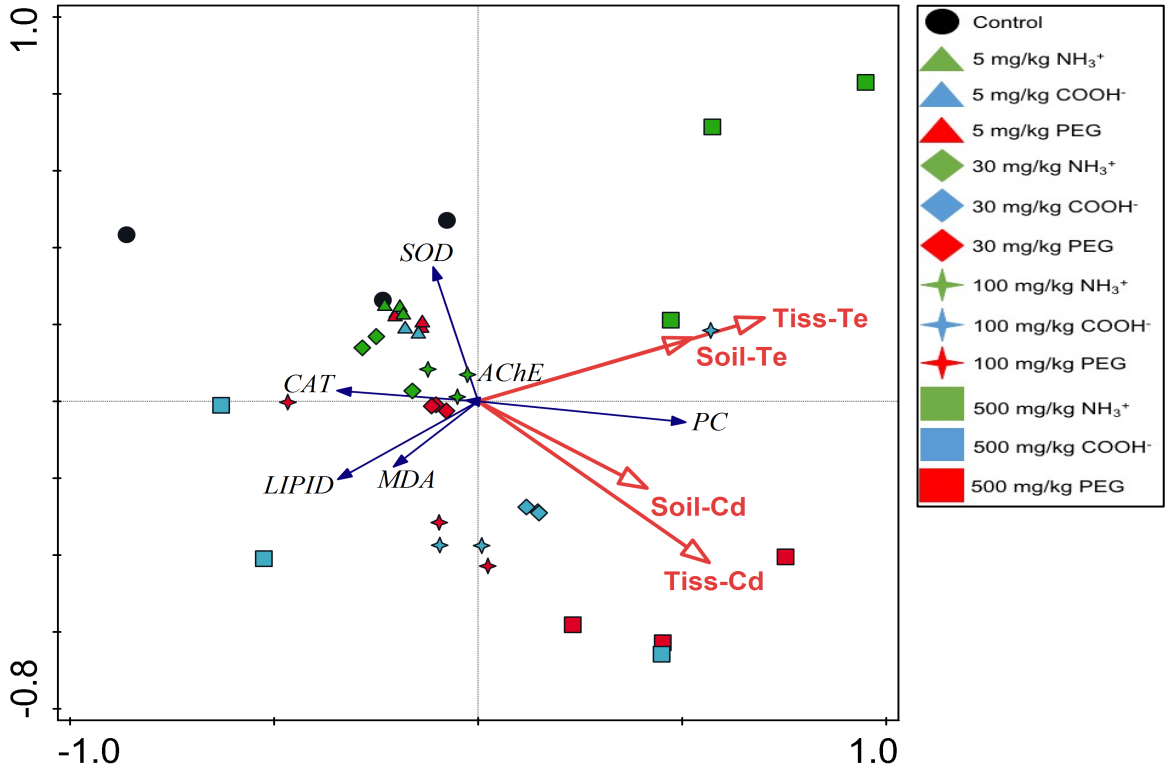

Figure 4.24: A Redundancy Analysis of the biomarkers in comparison with the metal accumulation of QDs in E. albidus after 21 days. Both axes explain 20,27% of the variability in the data.

Discussion:

5.1. Characterization of Cd/Te QDs (PEG, COOH & NH_3):

Characterization is important because the physico-chemical properties of different NMs cause different effects, hence, the characterization of the QDs used in our study. Dissolution rate is an important aspect as it will determine how fast molecules or individual ions will be released from the particle (Nowack *et al*., 2012; Tourinho *et al*., 2012). In the case of metal-based NMs, the release of the ions could result in metal associated toxicity as metals are known to be toxic in their ionic form (Tourinho *et al*., 2012; Schultz *et al*., 2015). The dissolution of Cd and Te from the QDs was very low (< 23.7 % for Te which was found in low concentrations in the QDs) and consequently it is extremely unlikely that the little freed metal ions would exert a negative influence. Key factors that drive dissolution are: surface area, particle size, chemistry and their shape (Schultz *et al*., 2015). Thus, the dissolution of NMs in both the nanoparticulate and in the dissolved forms needs to be considered in order to better understand the fate and toxicity of NMs on organisms and the environment (Tourinho *et al*., 2012). According to literature (Tourinho *et al*. (2012) and Nowack *et al*. (2012)) the characterization of NMs will affect their behaviour and toxicity in the environment and ultimately the accumulation in soil and organisms.

Physical forces (Brownian motion, fluid motion and gravity) and NM characteristics (particle size and surface properties) will have an effect on the aggregation and agglomeration of NMs (Tourinho *et al*., 2012). The QD-PEG group had the highest agglomeration due to the maximum particle size (510 nm) of the three functional groups and could be because of the chain length of the functional group, QD-COOH (370 nm) the second and the QD-NH_3 (290 nm) group agglomerated the least because of smaller particle diameter.

Surface properties affect the ability of NMs to bind to organic material that comes in contact with them. In this study, the positive (QD-NH3) and negative (QD-COOH) charged QDs had a higher binding affinity to the soil in the flow through (Figures 4.4 to 4.5) than the neutral group. As explained by Schultz *et al*. (2015) surface properties will on their own affect the fate and behaviour. Thus, the surface charge will affect the potential of exposure, which will have a critical influence on the bioavailability of the NMs. Previous studies have concluded that positively charged Ag-NMs have higher toxicity compared to negative ones (El Badawy *et al*., 2011). When adding NMs to soil, their properties might change due to the presence of soil clay particles and humic materials, having their own surface charge. The charged surfaces of these components might influence their association with NMs and ultimately control the transport of NMs in the soil (Tourinho *et al*., 2012). Heckmann *et al*. (2011) illustrated that the negative zeta potential for Ag-NMs causes repulsion to the soil therefore leading to higher bioavailability. The type of soil matrix will have an influence on the fate, behaviour and bioavailability of the test material. In natural soils, the NMs will filter through the soil matrix if the clay content or ionic strength is elevated (Handy *et al*., 2012).

In terms of particle size, the mobility of particles decreases with larger particles, because these particles will be retained by pore straining (Tourinho *et al*., 2012). In this study, the mean particle size of the negative (QD-COOH) and positive (QD-NH_3) charged QDs was smaller than the neutral PEG functionalized group. The flow through transport revealed that the third highest Cd concentration was found in the top region of the soil column (Figure 4.4A – Chapter 4) with the highest and second highest in the eluted water and eluted clay fraction respectively. One possible explanation for this is that the particles are not equally sized because of agglomeration, and the smaller particles could have better mobility through the soil transporting to the elution while the larger particles agglomerated on the surface when being added to the soil.

When the QDs were mixed with the soil (Figure 4.4B – Chapter 4), the total metal content in the glass tube was found to be different compared to the NMs poured on top. The metal accumulated at the bottom of the tube and most were found in the eluted clay fraction and in the eluted water. Using stock concentrations to elute the QDs, the NMs passed through the soil more rapidly, but when already mixed and eluted with water the NMs had time to settle and bind to soil particulates. This might explain why the second highest concentration was found in the eluted clay fraction. The other particles could mobilize within water as the water flows through. Since NMs in medium always agglomerate, it can be assumed that agglomeration and aggregation also influence the mobility of the NMs in soil. As agglomeration increases gravitational forces increase which will lead to more particle interaction with soil surfaces (Tourinho *et al*., 2012).

5.2. Exposure Assessment:

5.2.1. Uptake and distribution of Cd/Te QDs in *E. albidus*:

5.2.1.1. CytoViva dark field imaging:

From the CytoViva dark field imaging, a conclusion can be made that the enchytraeids do ingest the QDs which bind to the surface of the gut. Even after 24 h of depuration the QDs are seen to be present within the gut however more research is required to determine whether ionic uptake occurs because of the changes in pH or whether NMs themselves are able to cross into the tissue of the organism. Since whole worm digestion was used the metal uptake data could also be a reflection of the QDs present inside the gut and can be related to the metal analysis. No acute toxicity was observed but chronic toxicity showed a biomarker response which can be related to uptake. Unrine *et al*. (2010) observed that Cu-NMs remains as particles in the soil and the earthworms did internalize the particles and that this internalization might be connected with enchytraeids as their uptake mechanisms might be connected to that of earthworms even though their gut components are different (Amorim *et al*., 2012).

5.2.1.2. Metal bioaccumulation:

A dose-response relationship occurred with the bioaccumulation of QDs in the tissue from the present study. Reinecke *et al*. (1999) explained that earthworms have the ability to develop increased resistance, but at the expense of reproduction and reduced growth rate. As in the case of the present study of Cd/Te QDs, the QDs accumulation in the tissue was relatively high, which is a known effect for Cd exposures in earthworms (*E. fetida*) (Reinecke *et al*. 1999). The Cd uptake is associated with Cd-binding proteins which play an important role in the detoxification of metals. Another study (Spurgeon *et al*., 1994) exposing Cd to *E. fetida*, could not determine the LC_{50} values due to no significant mortality observed, but 14-day LC_{50} values were determined by van Gestel & Dis (1988) and Neuhauser *et al*. (1985) as >1000 mg/kg and 1843 mg/kg respectively. The earthworms exhibited high tolerance to being exposed to Cd as a result of the detoxification of metallothionein proteins.

Toxicity data for Te on soil invertebrates are limited. In the present study, there was a dose-response relationship in terms of the accumulation of Te in the tissue of *E. albidus*. Although, as a result of the low dissolution rate of the QDs, it can be assumed that the metal content in the tissue of the worms is in nanoparticulate form. Therefore, both metals (Cd and Te) should be compared with each other in the context of accumulation. The Cd was found to be higher in the tissue than the Te which was also found in the QD exposures. Bioaccumulation of metals in the tissue of *E. albidus* suggests that it is the QD particle itself that gets taken up, however, the conclusion can be made that because of the low dissolution rate and toxicity, it is not the free ion of Cd or Te. Whether the uptake is through adsorption or ingestion remains unknown.

Particle size of NMs is an important aspect, as the size play a role in the uptake and internalisation mechanism (Shang *et al*., 2014). Kettiger *et al*. (2013) argued that NMs with a particle size of 50 nm get internalized more efficiently by the cells than smaller (15-30 nm) or larger (70-240) particles. Nanomaterials smaller than 6 nm get eliminated much quicker from the body, because they get excreted, whereas the larger materials (> 200 nm) have higher accumulation in the body (Albanese *et al*., 2012). Nanomaterials that are larger than 50 nm have a higher binding affinity to a great number of receptors (Albanese *et al*., 2012). Fröhlich (2012) explained that when NMs are smaller than 100 nm, the still-smaller NMs is acting more toxic than the larger NMs such as QDs and TiO_2. Shang *et al*. (2014) explained that smaller NMs are more toxic than larger NMs because they have a high surface area available for interaction with the environment. This in affect increases the chance to interact with the surrounding biomolecules, triggering unfavourable responses.

An article by Verma & Stellacci (2010) stated that the functional groups of NMs play an important role in the interaction with cells. They argued that neutral functional groups are excellent at preventing nanomaterial-biological interactions, whereas charged functional groups are more responsible for active NM interaction. This could explain the higher toxicity observed with the charged functional groups (QD-NH_3 and QD-COOH). The charged groups had less agglomeration and could be internalised by the worms, thereby leading to higher toxicity. Charged NMs have a higher toxicity than the neutral groups and positively charged NMs are more toxic than the negatively charged NMs of the same size (Fröhlich, 2012). Kettiger *et al*., (2013) stated that positively charged NMs get taken up more readily and they can disrupt the integrity of the plasma membrane due to strong interactions with the anionic plasma membrane.

When linking the metal content available in the worms to the reproductive output and the survival rate, to uptake in particulate form, the QDs possibly take longer to have an effect on the worms and longer exposures are needed (Gomes *et al*. 2013). The low dissolution rate could also have an effect; dissolution of the ions takes longer within the gut of the enchytraeids. As Ma *et al*. (2013) explained in terms of the dissolution rate for ZnO-NMs, the toxicity of this specific NM is related to the high dissolution of the particle causing more zinc ions to be released and made available for uptake causing higher toxicity because zinc was found to be one of the more toxic metals than other metal ions. The total concentration of the metals which make up the QDs in the present study was lower in the tissue than in the worms exposed to the ion itself. More bioaccumulation occurred with the Cd than for the QDs. Lower accumulation of the QDs in the gut could mean that the QDs are first degraded and deposited in the soil before being taken up by the organisms or the acidic environment within the gut is not high enough to be able to completely release the ions present within the stable NM (Stewart *et al*. 2013).

5.3. Effect Assessment:

5.3.1. Survival:

Numerous studies have been conducted using enchytraeids and exposure to different metal NMs. Amorim *et al*. (2012) and Bicho *et al*. (2016) exposed *E. albidus* to copper salt, copper-NM and silver-NM (Ag NM300K) together with silver nitrate respectively and found a decrease in the survival number of adults. A study by Gomes *et al*. (2013) showed similar results when exposing *E. albidus* to Ag-NM and $AgNO_3$ and found that the $AgNO_3$ were more toxic. A limit test using *Eisenia fetida* (earthworm) exposed to 1000 mg/kg Ag-NMs caused no mortality where the 1000 mg/kg $AgNO_3$ caused almost a mortality of a 100%. Gomes *et al*. (2013) explained that this difference in toxicity could be due to a release of Ag^+ ions from the particles or it could be because of slower uptake of the Ag-NM – which could be identified through a longer exposure period. Shoults-Wilson *et al*. (2011) explained that the size of the particles does play a role in influencing toxicity, but the soil type also plays an important role, with sandy loam soils bioaccumulating more Ag from Ag-NMs than in artificial soil. The sandy loam soils have lower pH and organic content which results in higher bioavailability of Ag ions (Shoults-Wilson *et al*., 2011). The present study was the first to be conducted on Cd/Te QD exposure in *E. albidus*. The survival of *E. albidus* was not affected following a 21-day exposure. Survival at all the exposure concentrations ranged between 90 – 100% after 21 days of exposure (Chapter 4 – Table 4.4). These results of the present study support the very low Cd and Te dissolution from the QDs (Table 4.2 & 4.3) with the consequence that no toxicity due to metal ions was observed, as opposed to the ionic control (500 mg/kg Cd) which showed 100% mortality.

Cadmium has been reported as being toxic by Spurgeon *et al*. (1992), Lock & Janssen (2002), Zhu *et al*. (2008), Novais *et al*. (2011), Castro-Ferreira *et al*. (2012), Novais *et al*. (2013), Gomes *et al*. (2016) & González-Alcaraz & Van Gestel (2016). Novais *et al*. (2011) showed that a significant decrease in adult survival occurred at concentrations greater than 3.2 mg/kg, and no survival occurred at 320 mg/kg. The results of the present study showed that no mortality was observed at the 100 mg/kg but at the 500 mg/kg Cd there was 100% mortality. The present study confirmed that Cd is more toxic than the Cd/Te QDs, when applied at similar nominal exposure concentrations which further confirm the low dissolution rates observed within this study. The functional groups did not have an effect of the toxicity of the QDs. In the case of the Te salt, no significant mortality was observed with the 500 mg/kg and 1000 mg/kg concentrations meaning that in the present study the associated toxicity after exposure to QDs, would be caused by Cd ions rather than Te ions.

5.3.2. Reproduction:

In the present study, reproductive output was not affected after an exposure of six weeks. Even at high concentrations, there was no clear observed effect on the number of juveniles across all functional groups. In comparison with the 500 mg/kg Cd exposure (100% mortality) and the three functional groups (PEG, COOH & NH_3) of the same concentration (mortality not affected), it is clear that the QDs have no effect on *E. albidus* reproduction. Therefore, when a great decline in reproduction is caused, the toxicity could be ion related and not NM specific because of the low dissolution rate. Uptake of QDs therefore causes low or no toxicity because of the low uptake through adsorption on the body surface, ingestion of soil particles and low dissolution rate of these particles (Gomes *et al*., 2015). Thus, the QDs toxicity becomes more pronounced over a longer exposure duration (Gomes *et al*., 2015).

Copper-NMs are believed to be more toxic than its corresponding metal-salt as found by Amorim *et al*. (2012), when the Cu-NMs caused a decrease in reproduction compared to the $CuCl_2$-salt. Gomes *et al*. (2015) proved that by exposing *Enchytraeus crypticus* (*E. crypticus*) to titanium dioxide (TiO_2) and zirconium dioxide (ZrO_2) some NMs are less toxic to its corresponding metal dioxides. The study showed that both NMs (TiO_2 and ZrO_2) displayed no severe effect on reproduction with no toxicity observed in the TiO_2 up to 1000 mg/kg, but at 502 mg/kg (EC_{50}) ZrO_2 toxicity was observed. In this study, no EC_x value could be determined because of a lack of effects except for, QD-COOH where the EC_{10} was 124 mg/kg and the EC_{20} was 720.6 mg/kg, indicating that the QDs are not as toxic as its corresponding salt with a higher concentration could possibly have yielded an EC_{50} result. A study by Wang *et al*. (2009), showed that exposing *C. elegans* to a series of toxicants (ZnO-NM, ZnO, $ZnCl_2$, TiO_2-NMs and TiO_2) can reduce the growth, number of eggs inside the worm and the offspring per worm, but no significance was found when exposed to Zn-NMs and the corresponding metal or metal-salts. Thus, different species will therefore be affected differently. The offspring was significantly affected at only two concentrations at 0.8 and 1.6 mg/L. Voua Otomo *et al*. (2014) found that Au-NMs did not have an effect on the reproduction of *Enchytraeus bucholzi*.

Contradicting results were found in literature, for example Roh *et al*. (2009) observed that the *C. elegans* reproductive success are more affected with the Ag-NMs than the $AgNO_3$, whereas Kim *et al*. (2012) found that Ag-NMs are less toxic. Thus, the release of ions is not providing a clear answer explaining the difference in the toxicity between metal salts and corresponding NMs. A study done on *E. fetida* explained the toxicity to the reproduction of the earthworms is likely linked to ionization (10-15%) of the Ag-NMs (Shoults-Wilson *et al*., 2011). When earthworms (*E. fetida*) were exposed to 1000 mg/kg Cu Heckmann *et al*. (2011) observed that the reproductive output was decreased to almost a 100% when exposed to Cu-salt and Cu-NMs. Bicho *et al*. (2016) showed that *Enchytraeus crypticus* were able to respond to ionic exposures by activating an antioxidant defense mechanism which assisted the worms in detoxifying and allowing them

to survive at high concentrations. This mechanism was not activated when exposed to Ag-NMs and directly affected reproduction.

It can therefore be concluded that in the present study the reproduction of *E. albidus* exposed to QD-NH_3 was lower than the control, but the survival was not affected. A possible increased resistance against the QDs could have developed to enhance their chance of survival.

5.3.3. Avoidance:

Avoidance behaviour of *E. albidus* to Cd/Te-QD exposure has not been investigated and this study is the first of its kind. Using enchytraeids as a test species to determine avoidance behaviour needs further standardization to experimental procedures, soil properties and duration of exposure (Amorim *et al*., 2008). The results obtained from the present study, displayed a variety of different results and no clear pattern could be identified from the three different groups functional.

Avoidance may be a good indicator for nanotoxicity. Coleman *et al*. (2010) found that Ag-NMs have an effect has on *E. fetida* in terms of avoidance behaviour. Amorim & Scott-Fordsmand (2012) found that Cu-NMs (EC_{50} = 242 mg/kg) caused increased avoidance by *E. albidus* compared to Cu-salt (475 mg/kg). Compared to the present study, only the highest concentrations of the bulk materials caused an avoidance response, with the QDs the 30, 100 and 500 mg/kg QD-COOH showing a response. In the QD-NH_3 no avoidance was observed and with the QD-PEG the 1, 5, 100 and 500 mg/kg displayed a response. Exposing the enchytraeids to Cu-NMs and $CuCl_2$, Amorim & Scott-Fordsmand (2012) concluded that toxicity could be nano-specific with the avoidance being more affected than the reproductive output for Cu-NMs which was the case in our study of QDs. It is evident that the avoidance results for the three different groups were contrasting one another. The enchytraeids displays avoidance behaviour with the QD-COOH groups especially with the 100 mg/kg concentration exhibiting a 95% avoidance result which is above the 80% threshold. The QD-NH_3 group exhibited no avoidance in comparison with the control and with the QD-PEG group the avoidance increased with the lowest concentration (0.5 mg/kg) up until the 5 mg/kg with the 15 mg/kg displaying no avoidance and then increased again as the concentration increased with 500 mg/kg illustrating high avoidance. In comparison to the ionic controls, the 500 mg/kg of both heavy metals displayed response in the high 90% with 100 mg/kg Cd showing no response to the metal. A possible explanation for this kind of fluctuating results is that the time of exposure (48 h) was not long enough for the worms to experience any stress i.e. exposure of NMs not triggering the chemoreceptors of the worms, thus causing no avoidance.

5.3.4. Biomarkers:

The exposure and the effects of pollutants can be estimated in terrestrial invertebrates through the use of biomarkers (Gao *et al*., 2015). Biomarkers are effective to determine the toxicity of chemicals which exhibit low toxicity and concentrations (Gao *et al*., 2015). Biomarkers have been utilized to profile toxicological effects such as lethal concentrations, reproduction, lysosomal membrane stability, enzyme activity, apoptosis and DNA damage (Gao *et al*., 2015).

5.3.4.1. Catalase and Superoxide Dismutase activity:

Superoxide dismutase, an antioxidant enzyme that protects the tissue against superoxide anion radical by catalyzing conversion into H_2O_2., while CAT aids the detoxification process through breaking down the H_2O_2 into H_2 and H_2O (Gomes *et al*., 2012).

The CAT activity in the present study revealed a bimodal response to the concentration of QDs, but in comparison with the control, the activity decreased after an exposure of 21 days. Following exposure of *Mytilus galloprovincialis* to Cd (Vlahogianni & Valavanidis, 2007) a decrease in the levels of CAT activity was observed. This is in agreement with findings of the present study where the Cd 100 mg/kg produced the lowest CAT activity of all treatments. In the present study, the SOD activity seems to be impaired in comparison with the control and the two ionic controls. The QD-PEG and QD-COOH exhibits lower activity than Cd 100 mg/kg and the control, whereas the QD-NH_3 group of 5 and a 100 mg/kg illustrates less activity than Cd. Redundancy Analysis showed that with the SOD and CAT levels being high in the control, the protective role that these anti-oxidant enzymes play is evident. This means that QDs have the ability to cause ROS as was found by Lovric *et al*. (2005) following exposure of MCF-7 to QDs. They attributed the formation of ROS toplasma membrane, mitochondria and nucleus damage in MCF-7 cells and secondly induced cellular damage with a resultant antioxidant response. Gomes *et al*. (2012) found that exposing *E. albidus* to Cu-salt and Cu-NMs, the Cu-salt caused lower CAT activity than the Cu-NMs. This study also showed that Cd exposures resulted in lower CAT activity compared to the QD exposures. This can possibly be attributed to Cd binding directly to the CAT enzyme –SH groups (Atli *et al*. 2006), indicating that the Cu binding is higher with the salt than for the NMs. In a study done by Gomes *et al*. (2015) the Ag-NMs caused a decrease in the CAT activity, which was also seen in this study.

Increased SOD levels, because of exposure to metals have already been observed such as *E. albidus*, following exposure to 320 mg/kg Cu metal (Howcroft *et al*., 2009). Gomes *et al*. (2012) found that SOD levels decreased (did not change) following exposure of *E. albidus* to Cu. They attributed this to decreased bioavailability of Cu in the exposure medium. The lower SOD measured in this study can therefore be attributed to the exposure medium used.

Nanomaterials have the ability to cause reactive oxygen species (ROS) because NMs have intoxicating redox properties (Gomes *et al*. 2012). Because of inhibition, ROS is being

overproduced which in effects leads to degradation of enzymes (Gomes *et al*., 2011). The ROS is available to react to the Cu^{2+} ions from the dissolution of CuO-NMs that will lead to the forming of hydroxyl radical that is created from hydrogenperoxide under Cu^{+} because of Fenton and Haber Weiss reactions, leading to probable CAT and SOD inactivation (Gomes *et al*., 2011). When CAT activity is activated, the formation of ROS could be avoided, protecting the organism of undergoing oxidative stress (Vlahogianni & Valavanidis, 2007). Thus, lower levels of CAT and SOD activity will affect the organisms' protective mechanisms against oxidative stress causing more ROS.

5.3.4.2. Malondialdehyde:

In the present study, the QDs resulted in increased lipid peroxidation (LPO) levels in comparison with the control. This was explained by the fact that the antioxidant mechanisms (SOD and CAT) in *E. albidus* were not activated, as Cd does cause lipid damage in tissues (Vlahogianni & Valavanidis 2007). Metals have the potential for redox reactions that contribute to the production of ROS and consequently oxidative damage to membrane lipids. They also found that the increase in LPO levels were correlated with a decrease in CAT activity, which was also found in the present study. In the current study the fluctuations in LPO levels at different exposure concentrations (i.e. QD-COOH 5 mg/kg and 30 mg/kg) could be attributed to the NM agglomeration state as was also observed by Ribeiro *et al*. (2015).

The exposure of *E. albidus* to Cu-salt and Cu-NMs (Gomes *et al*., 2012) for eight days resulted in lipid peroxidation in the Cu-salt exposures. For the Cu-NMs an increase was observed at the lowest exposure concentration (450 mg/kg) and not at the highest concentration (750 mg/kg). Similar results were found by Park *et al*. (2009) who found an induction of ROS in bacteria exposed to silver at lower concentrations (0.01-0.1 mg/L), but nothing at higher concentrations (0.05 mg/L). This clearly indicates the influence that agglomeration at higher concentrations has an influence on toxicity. Howcroft *et al*. (2009) illustrated that *E. albidus* has the ability to prevent LPO with their antioxidant defence mechanisms during short-term exposures (2 days) but not after longer exposures (3 weeks). Gomes *et al*. (2015) found that when exposing *Eisenia fetida* to $AgNO_3$ for long exposure periods there was no significant change in the antioxidant enzymes. They attributed this to prolonged stress, which inhibited the antioxidant defence mechanisms. In general decreases in CAT and SOD activity leads to more LPO damage (Gomes *et al*., 2012).

The bimodal toxicity response that was observed in this study following exposure to QDs is attributed to the different agglomeration patterns of the NMs at different concentrations. The "Trojan horse" mechanism can explain this type of toxicity when toxicity is not concentration dependent and is due to changes in the amount of toxicant being able to enter cells based on their ability to move across the cell membrane (Ribeiro *et*., 2015). This study showed that QDs

cause ROS and with the depletion in CAT and SOD activity and as a result varying degrees of LPO was observed in all the exposure groups.

5.3.4.3. Protein Carbonyls (PC):

Protein Carbonyls provide an indication of protein damage as a result of oxidative stress (Levine *et al*., 1990). According to Parvez & Raisuddin (2005) oxidative variations in proteins may occur in a variety of physiological and pathological processes, which could be due to primary or secondary causes. In a study done by Tsyusko *et al*. (2012), PCs displayed a significant increase following exposure of *Eisenia fetida* to ionic Ag and Ag-NMs when compared to the control. The authors suggested that a decrease in protein levels is related to the activation of detoxification and repair mechanisms in their response to oxidative stress caused by the Ag ion and Ag-NM exposure. It therefore appears that oxidative stress is not NM specific as generally believed (Nel *et al*., 2009; Yang *et al*., 2011) but that this action applies for many toxicants such as metal ions as well (Valko *et al*., 2005).

The results from this study indicated that not only did PCs increases in all the exposure treatments but that the functional group also played a role in protein damage. This is the only biomarker where the functional group appears to have an effect. As with the other oxidative stress biomarkers, PCs also displayed a bimodal response with the QD-NH_3 group causing the highest level of PC formation. This can be attributed to oxidative stress resulting in the formation of hydroxyl radicals (OH^-) (Parvez & Raisuddin, 2005). The loss of acute sulfhydryl groups because due to protein oxidation may also lead to the formation of PCs (Parvez & Raisuddin, 2005).

Therefore the LPO levels, as well as the lipid fractions, found in the present study, validated the observation of oxidative stress has occurred when exposing *E. albidus* to QDs.

5.3.4.4. Acethylcholinesterase activity (AChE):

In the present study, there was inhibition of AChEs in the QDs as well as the two ionic control exposure groups. In particular there were significant changes in AChE from the control and the 30 and 100 mg/kg QD-PEG. Heavy metals are well-known anti-ChE substances in vertebrates and invertebrates (Howcroft *et al*., 2011). An inhibition of AChE activity occured when *E. albidus* was exposed to metals (Frasco *et al*., 2005; Amorim *et al*., 2008; Novais *et al*., 2011). The decreases in AChE activity compared to control can be attributed to the catalization of the hydrolysis of neurotransmitter acethylcholine (Lionetto *et al*., 2012). This would have resulted in the accumulation of acethylcholine, which will result in inhibition/damage to the neural and muscular system (Lionetto *et al*., 2012).

The studies by Frasco *et al*. (2005), Amorim *et al*. (2008) and Novais *et al*. (2011) also found that when the worms were able to avoid high metal concentrations the choline esterase

activities were not significantly affected. Thus the AChE inhibition assay could be used as a good sub-lethal stress indicator of avoidance reactions (Howcroft *et al*., 2011). Behavioural changes associated with AChE inhibition has been well documented in aquatic biota (Amiard-Triquet, 2009) and in this study the QD-PEG and QD-COOH exposure groups displayed greater avoidance when compared to the QD-NH_3 group. This was also reflected in the AChE activity where the AChE inhibition was higher in the QD-PEG and QD-COOH groups than the QD-NH_3 group.

5.3.4.5. Protein (P) and Lipid allocation (L):

Energy allocation in enchytraeids have been well documented following exposures to Cd (Novais *et al*., 2015), Zn (Novais *et al*., 2015), Cu (Amorim *et al*., 2012), Cu-NMs and Ag-NMs (Gomes *et al*., 2015; Amorim *et al*., 2012). In the present study there was also a decrease in the protein content following exposure to the Cd and Te metal ion exposures. A decrease in protein across the different functional groups was observed in the present study. There were only significant decreases at the highest QD-PEG exposure concentration (500mg/kg) while the negative (QD-COOH) and positive (QD-NH_3) charged groups showed a significant decrease in protein content. According to Gomes *et al*. (2015) low levels of stress can result in higher levels of protein synthesis for the detoxification process if the carbohydrates and lipids are available as an energy source. A decrease in protein content could be associated with an increase in protein utilisation through the induction of gluconeogenesis in order to keep glucose levels constant (Boeck *et al*., 1997).

In this study a clear relationship between the decreases in lipid energy fraction and the LPO for the same exposure concentrations. Gomes *et al*. (2015) explained that this can be explained by one of two or even a combination of both mechanisms: the NMs could be internalized differently in the cells or the dissolution rates of the metals from the the NMs are different, which causes the enchytraeids to be exposed differently over time.

Lipids are used as a first energy source in invertebrates following exposure of *Daphnia magna* to Cd (De Coen and Janssen, 1997; Soetaert *et al*., 2007) and in *E. albidus* to Cu (Amorim *et al*., 2012). A decrease in lipids can be attributed to oxidative damage of the cell membranes as demonstrated following Cd and Zn exposure in *E. albidus* (Novais *et al*., 2015) These authors stated that the effects on the reduced energy levels in the enchytraeids go along with oxidative stress to the cell membranes, which will have an effect on the reproductive output. They further explained that although no mortality has been observed, the reduced reproductive success could be as a result of change in energy allocation.

Conclusion:

The toxicity of NMs can either be nano-specific through the particle itself or it could be caused by releasing the specific metal ion which causes the toxicity (Amorim *et al*., 2012). Factors such as particle size, dissolution rate and surface properties influence the toxicity of NMs (Nowack *et al*., 2012; Tourinho *et al*., 2012; Ma *et al*., 2013). Particle size of NMs will affect their mobility in the environment and with aggregation and agglomeration, larger particles move slower through the environment and smaller particles are likely to move down to the groundwater level (Tourinho *et al*., 2012). The low dissolution rate of QDs implies that particles remain intact when in contact with the soil, thus particle uptake would occur more likely than free ion uptake. The different surface properties (neutral, negative and positive charged) of the three functional groups exhibited different soil distribution results. The surface charge influences their movement ability in soil due to binding effects and soil particulate surface area (i.e. clay and organic matter). Higher metal content was found in the soil exposed to the QD-COOH and QD-NH_3 functional groups when compared to QD-PEG than in the tissue of *E. albidus*. Thus, sorption of QD-COOH and QD-NH_3 occurs, which causes the particles to attach to different constituents of the soil.

There was no acute toxicity in terms of mortality and reproductive success of *E. albidus* exposed to QDs (PEG, COOH & NH_3). The bulk metal salts also did not cause any mortality except at 500 mg/kg Cd where 100% mortality was seen. With this being the first study exposing *E. albidus* to QDs no direct comparison could be made with previous studies in the literature. The different avoidance behaviours observed in the study can be attributed to a variety of explanations. Possible explanations are that the duration of exposure with the specific NM was too short for the worms to exhibit a reaction or the worms were not able to detect the QDs in the substrate. There is a lag in particle uptake which still occurs over time since the worm is unable to detect and avoid the toxicant. Another explanation can be that with the low dissolution the QD particle is taken up from the soil and possible toxic reaction will only take place in the gut of the worms where the pH of the gut could cause a release of ions.

The results obtained from the chronic toxicity test, illustrated that although no toxicity was observed in survival and reproduction, the QDs do cause toxicity at sub-lethal level. Biomarker analysis indicates that oxidative damage did occur during the exposure period, with high MDA content, low lipid fraction, stimulation of PCs, inhibition of CAT and SOD activity. When CAT and SOD activity is reduced, the protective role that antioxidant enzymes provide are not enough to counter the lipid peroxidation causing more reactive oxygen species (ROS) to form, which leads to more oxidative stress. Nanomaterials have the ability to cause ROS, thus causing higher oxidative damage (Gomes *et al*., 2011). Acethylcholinesterase (AChE) is good indicator of sub-lethal effects as it involves the neuromuscular transmission, meaning that avoidance can be associated with the AChE activity. The behaviour and AChE can be

associated with each other as the QD-PEG and QD-COOH group displayed more avoidance response than the QD-NH_3 group and the AChE activity for the QD-PEG and QD-COOH displayed more AchE inhibition than QD-NH_3.

All of the objectives of this present study have been met, indicating that acute toxicity data indicate that QDs have no real threat to *E. albidus*. The reproductive success was also not significantly affected, but internalization of the QDs does take place with biomarker responses indicating that sub-lethal effects do occur. Lastly, the QDs bind differently to the soil causing differences in uptake effects and COOH could calculate an EC_x but none of the others could. In conclusion, different functional groups with different charges will result in different uptake of NMs as charged groups resulted in higher toxicity because internalisation was higher as a result of less agglomeration This study sets baseline data for the effects of QDs on *E. albidus*. To our knowledge exposure of Te to terrestrial invertebrates has not been done, thus Te results provides new information. Soil organisms would get exposed to the free ions of QDs in the environment because they get degraded over time which will then alter the associated toxicity of nanomaterials (Stewart *et al*., 2013).

Recommendations:

- ✓ Longer exposure periods are required for avoidance behaviour to determine whether potworms are able to detect nanomaterials using a specialized mechanism.
- ✓ A second generation Enchytraeid Reproduction Test (ERT) should be done to determine whether offspring develop reproductive defects.
- ✓ The use of different artificial soil types should be used to determine if the soil properties would influence the toxicity of QDs.
- ✓ Physical damage in the gut caused by the particles should be studied.
- ✓ Different techniques for applying the nanomaterial should be analyzed to observe different toxicity, for example, the nanomaterials could be applied with the food or soil could stand for 24 h to settle and then introduce worms.

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Annexure A:

Table 1: Cadmium metal content (μg/g) in glass tube of flow through during nanomaterials poured on top and standard error (±SE).

	Control	COOH	PEG	NH_3
Top (1)	0.06 ± 0.046	57.1 ± 3.26	60.3 ± 15.7	62.8 ± 20.8
Middle (2)	0.15 ± 0.039	11.9 ± 1.5	12.8 ± 0.71	6.10 ± 0.55
Bottom (3)	0.10 ± 0.081	10.4 ± 1.71	8.5 ± 1.16	3.71 ± 0.42
Eluted Water (4)	191.2 ± 20	1,054.3 ± 45.5	1,037.5 ± 39.2	1,050.5 ± 57.6
Eluted Clay fraction (5)	0.46 ± 0.12	145.5 ± 3.79	244.1 ± 28.3	62.4 ± 1.45

3.79 0.948 28.3 1.5 1.45 0.944

Table 2: Nanomaterial mixed soil Cadmium content (μg/g) after pouring MilliQ H_2O on top and ±SE.

	Control	COOH	PEG	NH_3
Top (1)	0.06 ± 0.046	9.00 ± 1.9	10.8 ± 1.82	9.72 ± 2.2
Middle (2)	0.15 ± 0.039	12.7 ± 0.79	16.5 ± 1.71	10.8 ± 0.95
Bottom (3)	0.10 ± 0.081	13.9 ± 1.32	17.6 ± 1.97	9.25 ± 0.59
Eluted Water (4)	191.2 ± 20	961.7 ± 39.6	728.7 ± 45.1	794.4 ± 34.1
Eluted Clay fraction (5)	0.46 ± 0.12	49.7 ± 0.95	64.3 ± 1.5	32.1 ± 0.94

Table 3: Tellurium content (μg/g) in glass tube from pouring nanomaterial on top together with the ±SE.

	Control	**COOH**	**PEG**	**NH_3**
Top (1)	0.06 ± 0.013	5.51 ± 0.14	8.06 ± 1.86	15.1 ± 4.79
Middle (2)	0.15 ± 0.042	1.68 ± 0.15	1.87 ± 0.11	2.24 ± 0.19
Bottom (3)	0.10 ± 0.025	1.49 ± 0.18	1.47 ± 0.11	1.60 ± 0.042
Eluted Water (4)	0.02 ± 0	24.0 ± 3.18	31.5 ± 5.02	57.4 ± 2.74
Eluted Clay fraction (5)	0.25 ± 0.052	28.2 ± 0.79	50.6 ± 6.42	29.2 ± 0.88

Table 4: Tellurium content (μg/g) in mixed nanomaterial soil after pouring MilliQ H_2O on top as well as the ±SE.

	Control	**COOH**	**PEG**	**NH_3**
Top (1)	0.06 ± 0.013	0.89 ± 0.18	1.12 ± 0.20	2.62 ± 0.53
Middle (2)	0.15 ± 0.042	1.64 ± 0.10	2.33 ± 0.15	3.74 ± 0.26
Bottom (3)	0.10 ± 0.025	1.97 ± 0.19	2.48 ± 0.20	3.42 ± 0.19
Eluted Water (4)	0.02 ± 0	2.26 ± 0.44	4.37 ± 0.94	11.6 ± 0.17
Eluted Clay fraction (5)	0.25 ± 0.052	9.72 ± 0.10	13.4 ± 0.32	16.9 ± 0.11

Annexure B:

Table 1: Calculated EC_x and LC_x values (mg/kg) of $COOH^-$. CL: 95% Confidence limits. n.d. = not determined.

		EC_{10}	EC_{20}	EC_{50}	LC_{10}	LC_{20}	LC_{50}
COOH		124.1	720.6	n.d.	n.d.	n.d.	n.d.
95% CL	**Upper**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Lower	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 2: Calculated ECx and LCx values (mg/kg) of PEG. CL: 95% Confidence limits. n.d. = not determined.

		EC_{10}	EC_{20}	EC_{50}	LC_{10}	LC_{20}	LC_{50}
PEG		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
95% CL	**Upper**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	Lower	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.

Table 3: Calculated ECx and LCx values (mg/kg) of NH_3. CL: 95% Confidence limits. n.d. = not determined.

		EC_{10}	EC_{20}	EC_{50}	LC_{10}	LC_{20}	LC_{50}
NH_3		n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
95% CL	**Upper**	n.d.	n.d.	0.00	n.d.	n.d.	n.d.
	Lower	n.d.	n.d.	0.00	n.d.	n.d.	n.d.

Table 4: Offspring and survival LOEC and NOEC values (mg/kg) of the different functional groups. N.d. = not determined.

	Offspring number		**Survival**	
	LOEC	**NOEC**	**LOEC**	**NOEC**
$COOH^-$	n.d.	n.d.	n.d.	n.d.
PEG	>500	>=500	n.d.	n.d.
NH_3	n.d.	n.d.	n.d.	n.d.