Unravelling hazards of nanoparticles to earthworms, from gene to population

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Merel van der Ploeg

Thesis

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Chapter 1

General introduction

Nanotechnology

Nanotechnology is a novel technology, which is said to herald a new era or even the next industrial revolution (Hansen et al. 2008). This technology involves research and technological development at the atomic, molecular and macromolecular levels, creating and using structures, devices and systems at the scale of nanometers (Mason 2009; Miyazaki and Islam 2007; the Woodrow Wilson institute; Wijnhoven et al. 2010). Properties of the engineered nanoscale materials may be modified towards defined technological requirements. Nanomaterials tend to be more chemically reactive, have different strengths or electrical properties and are generally more mobile than similar materials at larger scales. In some cases, simply making things smaller changes the properties of materials, such as for example, exhibiting a different colour or starting to conduct electricity at the nanoscale (Mason 2009; the Woodrow Wilson institute). The definition of nanomaterials has been under debate, due to the criteria for defining a substance as a nanomaterial (Maynard 2011). On 18 October 2011, the European Commission recommended the following definition for a nanomaterial: "a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm – 100 nm" (European Union 2011). The term nanomaterials includes nano-objects (two dimensions less than 100 nanometer) and nanoparticles (three dimensions of 100 nanometer or smaller), but also fullerenes, graphene flakes and single wall carbon nanotubes with one or more dimensions below 1 nm (the Woodrow Wilson institute; Wijnhoven et al. 2010).

Although nanotechnology is said to have enormous potential to change society and daily life as we know it, it is just beginning to deliver on its promises. The number of products containing engineered nanomaterials is steadily growing and for the near future a further increase is expected (Gottschalk and Nowack 2011). From 2006 to 2011, the number of listed consumer products containing engineered nanomaterials increased worldwide from 212 to 1317 (the Woodrow Wilson institute). For the European market, the number of consumer products with a 'nanoclaim' (the claim that the product contains engineered nanomaterials), increased from 143 in 2007 to 858 in 2010 (Wijnhoven et al. 2010). The

type of products listed are diverse and include sunscreens, shampoos, toothpastes, vitamin sprays, sport-rackets, golf bats, clothes, food packaging and even food products, such as coffee creamer and instant noodles (Dekkers et al. 2011; the Woodrow Wilson institute; Wijnhoven et al. 2010). Apart from consumer products, engineered nanomaterials are also being used and studied for medical applications, such as drug delivery, bioseparation, wound dressing, contraceptive devices, coating of surgical instruments and orthopedic prostheses, nanorobots for surgery, imaging with nanoparticle contrast materials and nanobiosensors (Chen and Schluesener 2008; Ito et al. 2005; Jain 2008). Other fields where nanotechnology is used and tested are soil remediation (Andreescu et al. 2009; Tratnyek and Johnson 2006), environmental monitoring (Rogers 2006; Andreescu et al. 2009), energy generation and storage (Khodadadi and Hosseinizadeh 2007; Arico et al. 2005; Vaseashta and Mihailescu 2008) and drinking- and waste water-treatment (Savage and Diallo 2005; Vaseashta and Mihailescu 2008). By 2014, it is expected that 15% of the total global output of manufactured goods will have incorporated nanotechnology (Pilkington et al. 2009).

Hazard considerations

However, the same characteristics which make engineered nanomaterials useful in many products, such as chemical reactivity and persistence, cause concern about their potential adverse effects on humans (Rushton et al. 2010; Warheit et al. 2008). To examine the probability that engineered nanomaterials cause adverse effects to human health, proper risk assessments are essential (Hagens et al. 2007; Klaine et al. 2008; Navarro et al. 2008a; Tiede et al. 2009). The risk of a compound depends on the exposure and the potential to cause harm (the hazard) to an organism (Klaassen and Watkins 2003). The expected exposure routes associated with engineered nanomaterial production and use, which arise as a consequence of their diverse applications, include inhalation, ingestion injection and dermal routes (Casals et al. 2008; Oberdörster et al. 2005; Stone et al. 2007; Stebounova et al. 2011). Environmental exposure of humans through inhalation of nanoparticles (previously called ultrafine particles) associated with health effects has been studied most and started already more than twenty years ago (Oberdörster et al. 2007). Higher levels of

ultrafine particles (10 to 100 nm) were noted in urban areas compared with rural areas and exposure to these ultrafine particles was positively associated with symptoms of respiratory and cardiovascular diseases and even increased mortality (Brand et al. 1991; Clancy et al. 2002; Dockery et al. 1993; Donaldson and Stone 2003; Peters et al. 1997; Pope et al. 1991; Renwick et al. 2004; Stone et al. 2007).

These air pollution studies have contributed to the understanding of nanoparticle exposure and hazards (Oberdörster et al. 2005). In the last few years, research has resulted in a considerable amount of information on the hazards of nanoparticles to mammals (including humans), proceeding from toxicity studies using mammalian cells *in vitro* and as well as *in vivo* studies with different mammals (Ariano et al. 2011; Bhattacharjee et al. 2010; Fujita et al. 2009; Hansen et al. 2008; Hussain et al. 2005; Shvedova et al. 2005). Overall, the studies demonstrated that nanoparticles can enter cells by diffusing through cell membranes, but also by active uptake, such as endocytosis (Barillet et al. 2010; Bhattacharjee et al. 2011; Johnston et al. 2010). Within cells, nanoparticles may accumulate and cause toxicity (Bullard-Dillard et al. 1996). Many studies have demonstrated the formation of reactive oxygen species (ROS) (Barillet et al. 2010; Bhattacharjee et al. 2010; Foldbjerg et al. 2009; Hsin et al. 2008; Li et al. 2008; Park et al. 2011). Intracellular accumulation of ROS may either come from direct ROS production on the surface of nanoparticles, from indirect generation of ROS in cells due to interruption of the mitochondrial electron transport chain, or from destabilization of cellular ROS elimination pathways (AshaRani et al. 2008; Bhattacharjee et al. 2011; Hsin et al. 2008; Li et al. 2008; Ma et al. 2011; Pan et al. 2009). Other toxic effects associated with nanoparticle exposure and often associated with the ROS formation, are damage to membrane integrity, inflammation, DNA damage, perturbation of cellular calcium homeostasis and apoptosis (Ariano et al. 2011; Arora et al. 2008; Green and Howman 2005; Park et al. 2011; Xia et al. 2008; Zhao et al. 2011).

Nanoparticles in the environment

As the toxicity data obtained in mammalian test systems suggest that nanoparticles may affect human health, the question also arises if engineered nanoparticles have the potential

to become hazardous pollutants affecting the environment (Farré et al. 2011; Klaine et al. 2008). Due to the steady increase of production and use of engineered nanoparticles, discharge into the environment (intentionally or unintentionally) during production, transport, use and disposal is inevitable (Gottschalk and Nowack 2011; Lin et al. 2010; Oberdörster et al. 2005), Therefore the environmental discharge, exposure and effects are becoming an increasing concern and need to be addressed (Klaine et al. 2008). Ecotoxicology of engineered nanoparticles (nanoecotoxicology) is not in its infancy anymore, but still has an explorative character (Nowack 2009; Kahru and Dubourguier 2010). The risk of a substance depends both on exposure and hazards. For engineered nanoparticles there is little knowledge about exposure, as the real concentrations of these particles present in the environment are hardly characterized (Gottschalk and Nowack 2011; Handy et al. 2008a; Paterson et al. 2011; Vonk et al. 2009). It is not yet possible to monitor engineered nanoparticle concentrations in the environment, due to the lack of standard analytical methods for the analysis of environmentally relevant concentrations (Handy et al. 2008a; Nowack 2009; Paterson et al. 2011; Tiede et al. 2008). At the moment only few studies report on detecting engineered nanoparticle discharge into the environment (Farré et al. 2010; Hsu and Chein 2007; Kaegi et al. 2008; Kiser et al. 2009), investigating engineered nanoparticle release from paints used on exterior surfaces into waste waters (Kaegi et al. 2008) or engineered nanoparticle concentrations and fate in waste water treatment plants (Farré et al. 2010; Hsu and Chein 2007; Kiser et al. 2009;). The environmental samples in these studies were analysed using electron microscopy (transmission electron microscopy (TEM) and scanning electron microscopy (SEM)) mostly in combination with electron dispersive X-ray microanalysis (EDX), inductively coupled plasma optical emission spectroscopy (ICP-OES) and mass spectrometry (ICP-MS), and liquid chromatography–quadrupole linear ion trap–mass spectrometry (LC–QqLIT–MS). Limitations of these studies are that they only represent a small part of the potential environmental release situations and products, and are often not repeated enough to get a good statistical evaluation. Furthermore, a combination of methods is needed, which makes analysis expensive and the interpretation difficult. Therefore, these experimental methods used are often not suitable for large scale measurements of

environmental concentrations of engineered nanoparticles (Gottschalk and Nowack 2011). Even though interpretation of these studies on engineered nanoparticles discharge and other studies on exposure modelling should be treated cautiously, these studies indicate that increasing concentrations of engineered nanoparticles are present in the environment (Gottschalk and Nowack 2011; Mueller and Nowack 2008). Predicted environmental concentrations of engineered nanoparticles approach the milligram per kilogram level (0.5 mg/kg soil for TiO_2 nanoparticles in soil treated with sludge from wastewater treatment plants; Gottschalk et al. 2009).

Once in the environment, engineered nanoparticles can go through different behavioural changes, including aggregation, adsorption and dissolution (Farré et al. 2011; Klaine et al. 2008; Lin et al. 2010; Paterson et al. 2011). Behaviour of engineered nanoparticles in the environment depends on their characteristics, among which are size (distribution), composition, possible contaminants, shape, zeta potential and photoactivation (Paterson et al. 2011; Vonk et al. 2009). Once released into the environment, the characteristics and thus the behaviour of engineered nanoparticles will be modified by parameters such as pH, ionic strength and natural organic matter (Handy et al. 2008b; Lin et al. 2010). Depending on their behaviour, engineered nanoparticles may interact with chemicals, macromolecules, other nanoparticles and organisms in the environment in different ways. Animals may be exposed to engineered nanoparticles via skin contact, oral uptake through the gastrointestinal tract or through inhalation (Fujita et al. 2009; Handy et al. 2008b; Klaassen and Watkins 2003; Klaine et al. 2008; Scott-Fordsmand et al. 2008a; Shvedova et al. 2008; Smith et al. 2007; van Ravenzwaay et al. 2009).

So far, nanoecotoxicological research has focussed mostly on aquatic test organisms, including water fleas (*Daphnia magna*) and various species of algae and fish (Fabrega et al. 2011; Kahru and Dubourguier 2010; Navarro et al. 2008b). Compared with studies on the possible adverse effects of engineered nanoparticles in aquatic test organisms, effects on soil organisms were less frequently included in environmental hazard identification studies (Crane et al. 2008; Handy et al. 2008c; Shoults-Wilson et al. 2011a). However, engineered

nanoparticles may accumulate in soils through the application of sewage sludge, accidental spills, deposition from the air, agrochemicals or soil remediation (Cornelis et al. 2010). Furthermore, soil has been indicated as a sink for many (conventional) pollutants and therefore, long-term exposure of soil organisms is plausible (Rodriguez-Castellanos and Sanchez-Hernandez 2007). These perceptions make soil organisms a target to be taken into account. And more recently, attention for the possible adverse effects of engineered nanoparticles on soil organisms is increasing.

Aim of the thesis study

Given the need for better characterization of hazards of engineered nanoparticles to the environment and soil organisms in particular, the aim of the present thesis was to investigate effects of nanoparticle exposure on the earthworm *Lumbricus rubellus*, as a model organism for soil ecotoxicology, and to contribute to the development of effect markers for engineered nanoparticle exposure in this model.

The following sections present in some more detail the model system selected, endpoints of interest and the nanoparticles chosen for the studies described in the subsequent chapters of the present thesis.

Model organism: the earthworm

Earthworms (from the Lumbricidae family) were selected as one of the key indicator organisms for ecotoxicological testing of industrial chemicals by organisations dealing with environmental pollution and pesticides, including the European Community, the global Organization for Economic Co-operation and Development (OECD) and the Food and Agriculture Organization of the United Nations (FAO) (Edwards and Bater 1992). At present, earthworms are one of the most common organisms used in soil toxicity testing (Spurgeon et al. 2003; Rodriguez-Castellanos and Sanchez-Hernandez 2007). Earthworms are excellent subjects for toxicological research, for several reasons. One reason is that working with these animals is relatively easy, because common expertise about how to handle them is increasing, standardized guidelines have been developed, identification keys

are available and there is a vast and growing body of knowledge on their biology and ecology (Spurgeon et al. 2003; Römbke et al. 2005). A second reason is that there are few ethical objections to the use of these non-controversial experimental organisms (Römbke et al. 2005; Stenersen et al. 1992). Earthworms are also excellent subjects for ecotoxicological research, because of their different ways of potential exposure. Earthworms are continuously exposed both via their exterior epidermis, but also via their intestinal tract, because they ingest soil (Sanchez-Hernandez et al. 2006; Vijver et al. 2003). In addition, in the soil, earthworms are in contact with both the aqueous and the solid phase of the substrate (Römbke et al. 2005). Their reactions to exposure are detectable at various levels of biological organisation varying from gene expression at the cellular level, to population dynamics (Spurgeon et al. 2004; Stenersen et al. 1992). Furthermore, although the complexity of earthworms is low compared with vertebrates, they have highly differentiated organs and tissues, and possess an immune system that is comparable to that of vertebrates (Chen et al. 1991; Stenersen et al. 1992). In addition, earthworms are the common prey of many vertebrates. Therefore, they play a key role in the biomagnification process of several soil pollutants and in the occurrence of indirect effects of soil pollution on terrestrial vertebrates (Rodriguez-Castellanos and Sanchez-Hernandez 2007; Roodbergen et al. 2008).

The earthworm species *Eisenia fetida* and *Eisenia andrei*, from the family Lumbricidae, are the model species used in many studies and the species for which the OECD guidelines are designed (OECD 207:1984; OECD 222: 2004). These species are recommended because of their rapid life cycle and ease of culturing in laboratories (Edwards and Bohlen 1996). However, these are not the ideal species for extrapolation of laboratory data to field conditions, because these species are limited to sites rich in organic matter, such as compost and manure (Ma 1984; Tiunov et al. 2006; Lapied et al. 2010) and generally less sensitive to chemical exposure through the soil, compared with other Lumbricidae species (Dean-Ross 1983; Frampton et al. 2006; Ma 1984; Ma and Bodt 1993). For the studies described in the present thesis, another earthworm species from the family Lumbricidae was selected, e.g. *L. rubellus*. This is an epi-endogeic and abundant earthworm species, found in

temperate regions all over the world (Frampton et al. 2006; Ma and Bodt 1993; Sims and Gerard 1985; Tiunov et al. 2006).

The endpoints of interest

In this thesis, endpoints at different levels of biological organization were studied. At the individual level, mortality, growth and reproduction were measured and these were used to model population growth rate and composition (Figure 1). This integration of individual effects to population level consequences makes it possible to predict population behaviour under field conditions, which is essential for environmental risk assessment (Klok and De Roos 1996; Klok et al. 2006). However, measuring stress responses at just one level of biological organization yields little insight into the mode of action of the effects (Bundy et al. 2008; Heckmann et al. 2008; Rodriguez-Castellanos and Sanchez-Hernandez 2007; Spurgeon et al. 2008). Therefore, effect markers at lower levels of biological organization, e.g. at tissue, cellular and molecular levels, were used to improve understanding of the hazards and risks of nanoparticle exposure (Figure 1). In the following sections, the endpoints studied at the different levels are discussed in more detail.

Endpoints at gene expression level

Profiles of proteins, metabolites and gene expression can demonstrate rapid and sensitive responses of an organism to contaminant exposure, which has been validated for earthworms (Bundy et al. 2008; Burgos et al. 2005; Kuperman et al. 2003; Spurgeon et al. 2004; Wang et al. 2010). For the research discussed in the present thesis, effects on gene expression were assessed. Gene expression research in ecotoxicology compares mRNA levels for selected genes or complete gene sequences and can be used to assess effects of exposure to environmental pollutants, including nanoparticles. Altered mRNA levels for certain genes may indicate specific mechanistic pathways of toxicity and possibly adaptation, and often precedes effects found in functional parameters at the cellular level, in tissues and on whole organisms (Burgos et al. 2005; Owen et al. 2008; Snell et al. 2003; Stürzenbaum et al. 1998a). Earthworms of the Lumbricidae family have been used for gene expression alteration investigations after exposure to toxicants, mostly to heavy metals, in

several studies (Burgos et al. 2005; Homa et al. 2005; Owen et al. 2008; Ricketts et al. 2004; Spurgeon et al. 2004; Stürzenbaum et al. 1998b). Within these studies, alterations were studied for effects on gene expression for the functioning of proteins for detoxification (such as metallothionein and lysosomal glycoprotein), general and oxidative stress (including heat shock protein 70 (HSP70) and glutathione S-transferase (GST)) and reproduction (annetocin).

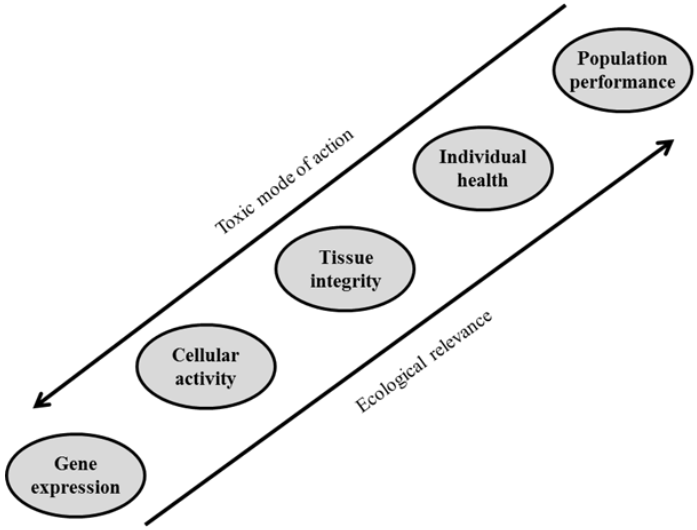

Figure 1. Schematic overview of the endpoints studied in the present thesis, at different levels of biological organization, which provides the possibility to study toxic mode of action at lower levels, as well as exemplify more about the ecological relevance of demonstrated effects, using the endpoints at higher levels.

Endpoints at cellular level

Using *in vitro* models, a wide variety of endpoints may be employed to study the cellular effects of nanoparticles. Endpoints studied and shown to be affected by nanoparticle exposure include intracellular production of ROS, disturbance of the mitochondrial membrane potential, reduction in cellular ATP levels, increased cytoplasmic calcium levels, induction of apoptosis, impairment of cell proliferation and reduced viability (Bhattacharjee et al. 2011; Pan et al. 2009; Park et al. 2011; Schaeublin et al 2011; Xia et al. 2008). Cellular models have also been used to study endpoints related to the immune system specifically, such as production of cytokines and changes in phagocytic activity

(Bhattacharjee et al. 2011; Nguyen et al. 2012). Since immune cells deal with invasive particles, including bacteria, viruses and nanoparticles, these cells may be a specific target for nanoparticle toxicity. For the immune system of earthworms, the free circulating immune cells present in the coelomic fluid, called coelomocytes, play an important role (Hayashi et al. 2012; Stein et al. 1977). The earthworm phagocytic assay, using such coelomocytes, may be considered equivalent to phagocytic assays using vertebrate cells, because phagocytosis is phylogenetically conserved within the animal kingdom as a first-line non-specific immune defence against microbial invaders (Burch et al. 1999; Fournier et al. 2000). Coelomocytes have already been used in several ecotoxicology studies to demonstrate effects of conventional toxic compounds, and these studies demonstrated that the model provides a simple, rapid and sensitive tool to assess immunotoxicological hazards of environmental exposure at the cellular level (Adamowicz 2005; Bilej et al. 1990; Bilej et al. 1992; Brousseau et al. 1997; Burch et al. 1999; Engelmann et al. 2005; Massicotte et al. 2004; Ville et al. 1995).

Endpoints at tissue level

Histology is the study of cells, tissues, and organs as seen with a microscope (Sharma and Satyanarayan 2011). Histological changes in the tissues can be used to determine modifications and damaging effects on tissues and cells, caused by prior or on-going exposure to contaminants (Muthukaruppan et al. 2005; Sharma and Satyanarayan 2011). The changes observed depend on the exposure and on the ability of the organisms to repair the injury (Muthukaruppan et al. 2005). Histological observations of tissues and cells are valuable tools to evaluate toxic effects of contaminants, which have been illustrated in several ecotoxicological studies with different species of Lumbricidae earthworms (Amaral et al. 2006; Amaral and Rodrigues 2005; Fischer and Molnar 1992; Giovanetti et al. 2010; Kiliç 2011; Morgan and Turner 2005; Muthukaruppan and Paramasamy 2010).

Endpoints at individual level

The most generally used effect markers in ecotoxicological studies with earthworms are survival, growth and reproduction. These endpoints are broadly accepted as

ecotoxicological endpoints, because they have been demonstrated as reliable and sensitive indicators of toxicity (Roh et al. 2007). There are two OECD guidelines for ecotoxicological studies with earthworms. The first and oldest is the acute toxicity test, OECD207 (OECD 1984), assessing survival only. The second is the four week reproduction test, OECD222 (OECD 2004), which also includes observations on growth, cocoon production and hatchability. Survival is a less sensitive endpoint and from an ecological point of view less relevant, compared to growth and reproduction (van Gestel et al. 1992). Therefore the four week reproduction test was used in this study. The offspring of the earthworms were also exposed and observed until adulthood, considering the potential prolonged availability of nanoparticles and the potentially higher sensitivity of juveniles compared with adults for exposure (Booth and O'Halloran 2001; Nielsen et al. 2008; Widarto et al. 2004).

Endpoints at population level

To assess the ecological impact of nanoparticle exposure, consequences at the population level may be more relevant than effects observed at the individual level and may thus be better predictors of hazards that nanoparticle exposure may pose to earthworm populations under field conditions (Klok et al. 2006; Widarto et al. 2004). For *L. rubellus* earthworms, observations made at the organismal level (on survival, growth and cocoon production) have been used to demonstrate effects of heavy metal and pesticide exposure on growth and development of earthworm populations in the field, with various population models (Baveco and de Roos 1996; Klok et al. 2006; Spurgeon et al. 2003). In the present study, a continuous-time life-history model (Baveco and De Roos, 1996; De Roos, 2008) was used to integrate effects of nanoparticle exposure on organismal endpoints into effects on population growth rate and stage distribution, i.e. the composition of the population in the different life stages of cocoon, juvenile, subadult and adult.

The nanoparticles tested

The nanoparticles used in the *in vivo* and most *in vitro* experiments of the present thesis are from the category of the carbon and silver nanoparticles. These two categories of

nanoparticles were selected because they are much employed in consumer products and investigated for various applications (Gelderman et al. 2008; Klaine et al. 2008; Stone et al. 2010; the Woodrow Wilson Institute).

Carbon nanoparticles

Carbon nanoparticles can originate from natural origin or (intentional and unintentional) anthropogenic sources. Exposure of organisms to carbon nanoparticles with a natural origin can occur due to volcano eruptions and forest fires, and it has taken place since organisms live on the earth (Bastús et al. 2008). Exposure to carbon nanoparticles has increased over the last century due to anthropogenic sources, including the large scale use of internal combustion engines and, later on, the intentional production of engineered carbon nanoparticles for consumer products and medical applications (Gelderman et al. 2008; the Woodrow Wilson institute). The carbon nanoparticle studied in the present thesis is the fullerene C_{60}, which has a size of approximately 0.7 nm (Goel et al. 2004). As pristine C_{60} is extremely insoluble in water and very stable, soils are likely to serve as a sink for C_{60} (Li and Alvarez 2011; Nielsen et al. 2008). In soil, these nanoparticles are expected to form clusters together (agglomerates and/or aggregates) and to bind to compounds present in the soil, including clay and organic matter, as shown in figure 2 (Ben-Moshe et al. 2010; Brant et al. 2005; Chen and Elimelech 2006).

At the time the present thesis study started, one study on earthworms exposed to nanoparticles was published, by Scott-Fordsmand et al. (2008a). This study demonstrated effects of C_{60}, applied through the food, on the reproduction of the earthworm *E. veneta*. During the last four years, only one other ecotoxicology study on effects of C_{60} exposure has been conducted with earthworms (see table 1). Apart from effect studies, a bioaccumulation study was performed by Li et al. (2010), which demonstrated the bioaccumulation of (radiolabelled) C_{60} added to the soil by *E. fetida* earthworms.

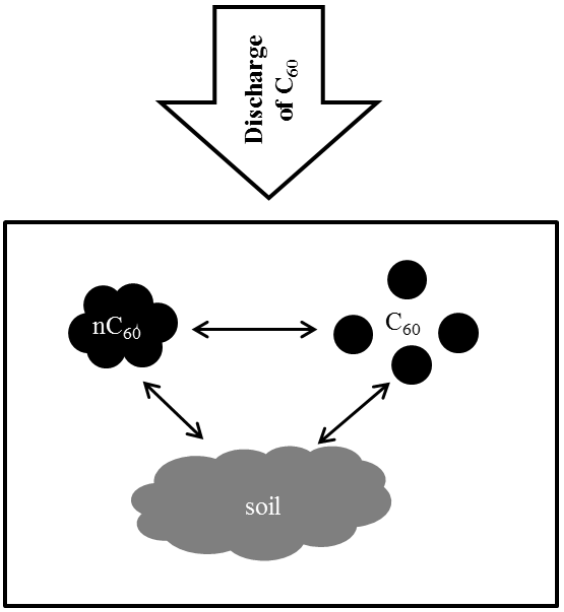

Figure 2. Schematic overview of the behaviour of C_{60} in the soil, with clustering of the single particles (nC_{60}) and the binding of single particles (C_{60}) and nC_{60} to compounds present in the soil.

Table 1. Studies demonstrating toxic effects of C_{60} nanoparticle exposure on earthworms. The exposure is presented as nominal concentrations, added to food or soil. Reproduction was quantified as the cocoon production, and the effects are calculated as compared with control (100%).

Species	Exposure	Effect	Reference
E. veneta	1 g/kg food	↓ reproduction (to 22%)	Scott-Fordsmand et al. 2008a
E. fetida	2 and 10 g/kg soil	No avoidance of the C_{60} amended soil	Li and Alvarez 2011
	5 to 50 g/kg soil	↓ reproduction (to 40%) at 50 g/kg soil	

Silver nanoparticles

The second type of nanoparticles investigated in this study are silver nanoparticles (AgNP). The AgNP used in this thesis had an average size of 15 nm (as stated by the manufacturer) and have been chosen by the European Commission's Joint Research Centre (JRC) as a

representative as-produced commercial nanomaterial. JRC is of the opinion that "representative nanomaterials are of utmost importance to be made available to the international scientific community to enable innovation and development of safe materials and products" (JRC 2011).

Silver (Ag) is a rare but naturally occurring element. Exposure of Ag to humans and the environment has already taken place for many centuries, because Ag has been used in many applications, including photography, jewellery and as an antimicrobial (Atkins and Jones 2000; Rai et al. 2009; Wijnhoven et al. 2009). These days Ag is applied to many products as AgNP, including personal care products and textiles. Several studies have investigated the release of AgNP from textiles during washing and demonstrated release of Ag (both ionic Ag and AgNP) up to 377 µg/g product per washing (Benn and Westerhoff 2008; Benn et al. 2010; Geranio et al. 2009). However, results depended on the type of AgNP, the product and the washing conditions (Gottschalk and Nowack 2011). Due to the use of AgNP in textiles and other consumer products, wastewater and eventually sewage sludge are likely places for AgNP to be found (Cornelis et al. 2010; Mitrano et al. 2012). Sewage sludge application may be one of the ways AgNP is discharged to the soil. AgNP may be present in the soil as single particles, dissolve to silver ions or cluster together, and all these forms may bind to compounds present in the soil (Figure 3; Stebounova et al. 2011; Tourinho et al. 2012). Conditions under which these nanoparticles dissolve or cluster and the extent to which these changes occur, depend on the characteristics of the nanoparticles, including size, coating, surface charge, possible contaminants and shape (Coutris et al. 2012; Park et al. 2011; Vonk et al. 2009). In addition, the type of soil is an important factor influencing nanoparticle behaviour and therewith toxicity (Cornelis et al. 2010; Shoults-Wilson et al. 2011a).

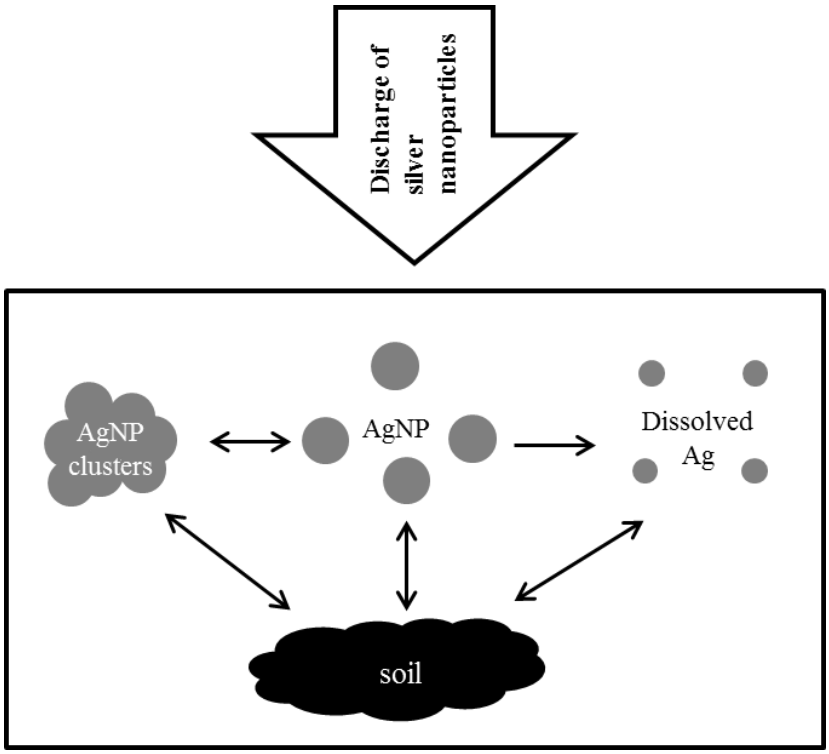

Figure 3. Schematic overview of the behaviour of silver nanoparticles (AgNP) in the soil, showing potential clustering and dissolution of AgNP, and the binding of these Ag forms to soil compounds (including organic matter, chloride and sulphide).

The mechanisms underlying the toxicity of AgNP have not been fully elucidated, but there are indications that the toxicity not only depends on ionic Ag, but also on nanoparticle specific effects (Demir et al. 2011; Hayashi et al. 2012; Kawata et al. 2009; Park et al. 2011). A few studies have indicated that bioaccumulation of AgNP is low in earthworms (Coutris et al. 2012; Shoults-Wilson et al. 2011a, 2011c). However, toxic effects have been observed in earthworms exposed to AgNP (Table 2).

Table 2. Toxic effects observed in earthworms (*Lumbricus terrestris* and *E. fetida*) after exposure to AgNP, which were uncoated, colloid or coated with polyvinyl pyrolidone (PVP) or oleic acid (OA). Reproduction was quantified as cocoon production of the earthworms. The exposure is given as nominal concentrations and the % effects are compared with the control (=100%).

Species	Exposure	Effect	Reference
L. terrestris	8.8 nm; colloid 0.4 to 8 mg/kg soil	↑ apoptotic activity (to 500%) from 4 mg/kg	Lapied et al. 2010
	20 nm; uncoated 1 to 100 mg/kg soil	↑ apoptotic activity (to 400%) at 100 mg/kg	
E. fetida	10 nm; PVP-coated 10 to 1000 mg/kg soil	↓ reproduction (to 55%) at 1000 mg/kg	Shoults-Wilson et al. 2011a
	30-50 nm; PVP-coated 10 to 1000 mg/kg soil	↓ reproduction (to 40%) from 1000 mg/kg	
E. fetida	10 and 30-50 nm; PVP-coated 0.3 to 54 mg/kg soil	Avoidance from 7 mg/kg	Shoults-Wilson et al. 2011b
E. fetida	30-50 nm; PVP-coated 10 to 1000 mg/kg soil	↓ reproduction (to 40%) from 1000 mg/kg	Shoults-Wilson et al. 2011c
	30-50 nm; OA-coated 10 to 1000 mg/kg soil	↓ reproduction (to 40%) from 1000 mg/kg	
E. fetida	30-50 nm; PVP-coated 1000 mg/kg soil	↓ growth (to 73%) Total reproductive failure	Heckmann et al. 2011a
E. fetida	10 and 80 nm; OA-coated 20 to 500 mg/kg soil	↓ enzymatic activities from 100 mg/kg	Hu et al. 2012

Outline of the thesis

Given the need for better characterization of hazards of engineered nanoparticles to soil organisms, the present thesis investigated effects of nanoparticle exposure on the earthworm *L. rubellus* and contributed to the development of effect markers for engineered nanoparticle exposure in this model soil organism for soil ecotoxicological studies. This work is presented in six chapters. The present **chapter 1** provides an introduction and background information on the work discussed in this thesis. In order to investigate effects of C_{60} exposure at the population level, **Chapter 2** presents results from *in vivo* experiments in which earthworms were exposed to C_{60}, spiked to the soil in different concentrations. From these experiments, individual endpoints were deployed to model population level effects. **Chapter 3** and **chapter 4** complement the observations made in **chapter 2**, studying cellular and molecular responses of earthworms to exposure to C_{60}. These responses may inform on the toxic mechanisms of C_{60} exposure and may also contribute to the development of additional effect markers for C_{60} exposure. In **chapter 3**, histopathology and gene expression analysis of earthworms exposed *in vivo* to C_{60} were investigated. **Chapter 4** presents results of experiments using immune cells (coelomocytes) extracted from earthworms and exposed *in vitro* to nanoparticles. This test system was used to study possible effects of C_{60} exposure on important cells for the immune response in earthworms. **Chapter 5** describes similar experiments as presented in **Chapter 2-4**, but performed for AgNP. *In vivo* and *in vitro* experiments are discussed, exposing earthworms and coelomocytes to AgNP. Finally, **chapter 6** discusses the findings described in the thesis and presents suggestions for future research.

Chapter 2

Effects of C_{60} nanoparticle exposure on earthworms (*Lumbricus rubellus*) and implications for population dynamics

Based on:

MJC van der Ploeg, JM Baveco, A van der Hout, R Bakker, IMCM Rietjens, NW van den Brink. 2011. Effects of C_{60} nanoparticle exposure on earthworms (*Lumbricus rubellus*) and implications for population dynamics. Environmental Pollution 159: 198-203.

Abstract

Effects of C_{60} nanoparticles (nominal concentrations 0, 15.4 and 154 mg/kg soil) on mortality, growth and reproduction of *Lumbricus rubellus* earthworms were assessed. C_{60} exposure had a significant effect on cocoon production, juvenile growth rate and mortality. These endpoints were used to model effects on the population level. This demonstrated reduced population growth rate with increasing C_{60} concentrations. Furthermore, a shift in stage structure was shown for C_{60} exposed populations, i.e. a larger proportion of juveniles. This result implies that the lower juvenile growth rate due to exposure to C_{60} resulted in a larger proportion of juveniles, despite increased mortality among juveniles. Overall, this study indicates that C_{60} exposure may seriously affect earthworm populations. Furthermore, it was demonstrated that juveniles were more sensitive to C_{60} exposure than adults.

Introduction

The production and use of nanoparticles, such as fullerenes and quantum dots, have increased for several decades and are expected to increase dramatically in the near future (Navarro et al. 2008a; Nowack and Bucheli 2007). Therefore, exposure of humans and the environment to these particles seems inevitable (Handy et al. 2008c) and evaluation of potential impact of engineered nanoparticles on human and environmental health requires attention (Baun et al. 2008; Nel et al. 2006; Nowack and Bucheli 2007; Scott-Fordsmand et al. 2008a; Usenko et al. 2008).

In spite of their increased use and development for several decades (Navarro et al. 2008a), interest in the possible negative effects of nanoparticles was only instigated a decade ago (Kamat et al. 1998; Li et al. 1996; Sera et al. 1996; Stone et al. 1998). At present, possible hazards and risks are still mostly unknown (Baun et al. 2008; Handy et al. 2008b). So far, environmental hazards of nanoparticles have mainly been studied in aquatic organisms (Baun et al. 2008; Handy et al. 2008b; Isaacson et al. 2007; Moore 2006; Oberdörster 2004; Oberdörster et al. 2006; Smith et al. 2007; Usenko et al. 2008; Zhu et al. 2006) and relatively little is known about possible effects on soil organisms and ecosystems (Handy et al. 2008a; Navarro et al. 2008a; Scott-Fordsmand et al. 2008a). However, well-functioning soils are the basis of terrestrial ecosystems and essential for the society. Since soil may serve as a sink for many pollutants (Rodriguez-Castellanos and Sanchez-Hernandez 2007), including nanoparticles, long-term exposure to nanoparticles is plausible. This demands detailed ecotoxicological studies on hazards and risks that nanoparticles may pose to soil organisms. Earthworms, abundant soil organisms, have been used in many conventional soil ecotoxicology studies, because they are in close contact with the soil and thus good indicators for risks of soil contaminants (Rodriguez-Castellanos and Sanchez-Hernandez 2007; Spurgeon et al. 2003).

The objective of the present study was to investigate the potential impacts of fullerene nanoparticles (C_{60}) on earthworms. Effects of C_{60} exposure on growth, mortality and reproduction were studied during different life stages of *Lumbricus rubellus* (cocoon, juvenile, subadult and adult). These important individual endpoints for population dynamics (Baveco and De Roos 1996) were used in a continuous-time life-history model

(De Roos 2008). In this way, effects of C_{60} exposure on the individual endpoints could be integrated into effects on population growth rate and stage distribution, i.e. the development of the population in terms of composition and number of individuals. Consequences at the population level may be more relevant for ecological impact of C_{60} than endpoints at the individual level (Klok et al. 2006; Widarto et al. 2004) and may thus be better predictors of hazards that C_{60} exposure may pose to earthworm populations under field conditions.

Materials and Methods

Earthworms

Adult (clitellated) individuals of *L. rubellus* were obtained from an uncontaminated location in the Netherlands (Nijkerkerveen). The earthworms ranged in weight from 1415 to 1950 mg. Prior to the experiment, they were maintained for two weeks under constant conditions (24 hrs light, 15°C, 61% relative humidity) in uncontaminated soil, similar to the experimental soil.

Soil preparation

Experiments were performed using clean soil with 4.3% organic matter and soil pH was 5.0 (Proefboerderij Kooijenburg, Marwijksoord, the Netherlands). A week before the start of the experiment, the soil was sifted through a 5 mm sieve and transferred to glass containers (650 gram per container). Containers were kept under the same conditions as during the acclimatisation period.

C_{60} was obtained from SES Research (99.5+%, Houston USA). Metal impurities in the C_{60} were measured by ICP-AES (Table 1), after destruction with aqua regia (NEN 6465, 1992). As the maximum additional concentrations of metals from C_{60} when added to the soil, were far below threshold levels (Abdul Rida 1996; Klok et al. 2006; Scott-Fordsmand et al. 2008a), potential side effects of metals were not considered in further evaluation of the data.

Table 1. Measured concentrations of different metals detected in C_{60} powder, and concentrations added to the soil at an exposure level of 154 mg C_{60}/kg soil.

Compound	Fe	Ni	Zn	Cd	Co	Cu	Pb
mg/kg C_{60}	14.3	0.02	1.5	0.0	0.0	1.2	0.05
mg/kg soil	2.2	0.002	0.2	0.0	0.0	0.2	0.008

To obtain a stable C_{60} stock solution, C_{60} was dissolved in an aqueous soil extract. This extract was obtained by stirring control soil in ultrapure water (0.4 g/mL) for one hour and then filtering it (Whatman filters type 597, Fisher Scientific). After filtering, C_{60} was added to a part of the extract, to a final nominal concentration of 2 g/L. The suspension thus obtained was stirred for three days, in order to acquire an even suspension. Nominal soil concentrations were set at 0, 15.4 and 154 mg C_{60}/kg soil (dry weight) and a moisture content of 17.2% (dry weight). Concentrations were selected based on Scott-Fordsmand et al. (2008a). For the control (0 mg/kg) and the high exposure (154 mg/kg), 50 mL of soil suspension without and with C_{60}, respectively, was added to every container with 650 g soil. For the 15.4 mg/kg soil exposure, 5 mL suspension with and 45 mL suspension without C_{60} was added to the soil. Subsequently, the soil was mixed thoroughly. Worms were added immediately after preparation of the soil.

Characterisation of actual exposure is important in ecotoxicology (Spurgeon et al. 2003; Scott-Fordsmand et al. 2008b). Therefore, in the present study, appearance and aggregation of C_{60} particles in the soil extract were characterised using transmission electron microscopy (TEM), and concentrations of C_{60} in the soil were determined by spectrophotometry. C_{60} nanoparticles were visualized by a TEM (JEOL JEM 1011) as well as a high resolution TEM (HRTEM; JEOL JEM 2100), operated at 60 kV and 200 kV, respectively. Samples were taken from freshly made stocks of ultrapure water with C_{60} and soil extract with and without C_{60}. These samples were dropped on a 400 mesh copper Formvar/carbon grid and left to dry, before examination with the TEM or HRTEM. Spectrophotometry (Aquamate from Thermo Electron Corporation) was used to determine actual concentrations of C_{60}, for the control and 154 mg C_{60}/kg exposure soil. Per treatment five soil samples were collected. C_{60} was extracted from the soil using toluene and

concentrations were quantified spectrophotometrically (wavelength: 335 nm and extinction coefficient: 0.072 L/cm/mg; Bensasson et al. 1994; Çiçek et al. 2001). In earthworms it was not possible to quantify C_{60} concentrations due to interference of the matrix with the measurements.

Experimental design

Experiments were conducted conform ISO guidelines 11268-2 :1998, with minor modifications in exposure scenarios (see above) and use of another species (*L. rubellus*). Two experiments were carried out. The first experiment was conducted with adult earthworms and the second experiment was performed with offspring from the first experiment. To assess effects on growth rate at different life stages, experimental data on adult and juvenile growth were analyzed separately.

Adult growth, mortality and reproduction

This experiment consisted of three exposure levels, with nominal concentrations of 0 mg/kg (n = 8), 15.4 mg/kg (n = 6) and 154 mg/kg (n = 6). Each container housed five earthworms and the containers were considered to be the experimental units. At the start of the experiment the worms were weighted and randomly assigned to a container and treatment (weights did not differ significantly between treatments, $\alpha = 0.05$). Every week the worms were fed dried alder leaves (*Alnus glutinosa*), from an uncontaminated location (Vossemeerdijk, Dronten, the Netherlands), ad libitum. Before placing the leaves on top of the soil, they were moistened with demineralised water for at least an hour. After four weeks, the experiment was terminated and the worms were counted and weighted again. The number of produced cocoons was determined per container by wet-sieving and hand-sorting.

Offspring growth and mortality

Ten cocoons (if possible) were taken from each container and were incubated in a large Petri dish with soil of the corresponding treatment of their parent earthworms. After

hatching, juveniles were weighted and kept in a glass container with soil under the same exposure as their parents. Earthworms were sorted monthly from the soil over a period of 326 days, to determine weight and life stage. They were considered subadult when the tubercula pubertatis was present, but before the clitellum had reached its full development. Clitellated worms were indicated as adults (Sims and Gerard 1985).

Population model

The population model is based on a simple Dynamic Energy Budget (DEB) model (Kooijman and Metz 1984). This DEB model assumes that a fixed fraction of the incoming energy is spend on respiration, for maintenance and growth, and the remainder is invested in reproduction. Under constant environmental conditions the age-dependent individual growth with age is defined by the DEB model as

$$l_{(a)} = l_{\mathrm{m}} - (l_{\mathrm{m}} - l_{\mathrm{b}})e^{-\gamma a} \qquad \text{equation 1}$$

where size $l_{(a)}$ equals the weight to the power of one-third ($mg^{1/3}$) of an individual of age a (in days), l_{m} the maximum attainable size ($mg^{1/3}$), l_{b} the size ($mg^{1/3}$) at hatching and γ the individual growth rate constant ($mg^{1/3}/mg^{1/3}$/day) (Klok and Roos 1996).

Following the DEB model, reproduction is proportional to surface area ($mg^{2/3}$)

$$m_{(a)} = r_{\mathrm{m}}\left[l_{\mathrm{m}} - (l_{\mathrm{m}} - l_{\mathrm{b}})e^{-\gamma a}\right]^2 \text{ for } l_{(a)} \geq l_{\mathrm{ad}} \qquad \text{equation 2}$$

with $m_{(a)}$ as the reproduction rate of an individual of age a (cocoons/day), r_{m} the maximum reproduction rate (calculated from the number of cocoons/worm/day divided by $(l_{(a)})^2$). Maturation and reaching the subadult stage are assumed to be determined by size. Thus, l_{ad} is the size ($mg^{1/3}$) at which individuals become mature.

In contrast to the experimental data, in which growth rate was analyzed for the different stages separately, for modelling purposes growth rate γ was calculated for the entire life of the earthworms, integrating the growth rates of the different life stages. For each container, a growth curve was obtained (more details are discussed in the supplementary data) and all curves were highly significant ($p < 0.05$). Growth rate γ was adjusted for the number of earthworms per container, because Faber et al. (2004) demonstrated under similar experimental conditions, that growth rate of earthworms correlates negatively with worm density. The reduction in growth rate due to worm density as determined by Faber et al. (2004), was used to adjust γ, with a density of 5 worms per container set as the reference.

For the population parameters, averages and standard deviations were calculated per treatment group. These parameters were used in a continuous-time life-history model (De Roos 2008). Population growth rate was determined by solving Lotka's integral equation:

$$1 = \int_0^{A_m} e^{-ra} m(a) F(a) da \qquad \text{equation 3}$$

in which *r* represents population growth rate, *m(a)* is the individual fecundity at age *a* (see above) and *F(a)* the survival probability up to age *a*.

Survival function *F(a)* under natural conditions was derived from field data for a related species, *Lumbricus terrestris* (Lakhani and Satchell 1970), and scaled to the shorter lifespan of *L. rubellus*. Following the same procedure as Klok and De Roos (1996), we estimated stage-specific constant mortality rates (μ_c, μ_j, μ_s, μ_a, for cocoons, juveniles, subadults and adults, respectively) separately from the continuous survival curve. This enabled us to include additional toxicant-induced stage-specific mortality into the model, as derived from the experiments. This additional mortality was only included for the juvenile stage, because this was the only stage in which we found a significantly increased mortality in the high exposure group.

Under constant conditions, the population will grow with fixed rate $\bar{r}$. The associated stable stage distribution is given by:

$$S(a) = e^{-\bar{r}} F(a) \qquad \text{equation 4}$$

in which S(a) represents the density of individuals with age *a* in the exponentially growing population relative to the density of newborn individuals (De Roos 2008).
For the stage distribution this implies that fractions of cocoons, juveniles, subadults and adults are given by:

equation 5

$$C = \frac{\int_0^{\tau_0} e^{-\bar{r}} F(a)da}{\int_0^{A_m} e^{-\bar{r}} F(a)da} \quad J = \frac{\int_{\tau_0}^{A_s} e^{-\bar{r}} F(a)da}{\int_0^{A_m} e^{-\bar{r}} F(a)da} \quad S = \frac{\int_{A_s}^{A_{ad}} e^{-\bar{r}} F(a)da}{\int_0^{A_m} e^{-\bar{r}} F(a)da} \quad A = \frac{\int_{A_{ad}}^{A_m} e^{-\bar{r}} F(a)da}{\int_0^{A_m} e^{-\bar{r}} F(a)da}$$

Here τ_0, A_s, A_{ad} and A_m represent the duration of the cocoon stage, the age at becoming subadult and adult, and the maximum age, respectively. Note that these thresholds, except for the cocoon stage duration, are defined by size and can be determined by solving equation 1 for age at a specified size.

All the parameters used for the different treatments are given in table 2.

Table 2. Input parameters for the population model are presented as mean values, with the individual growth rate constant γ in $mg^{1/3}/mg^{1/3}/day$, the maximum reproduction rate (r_m) in $cocoons/mg^{1/3}/day$ and the duration of the cocoon stage (τ_0) in days. The mean length at birth (L_b), the mean lengths at reaching subadulthood (L_s) and adulthood (L_{ad}), and the estimated maximum length (L_m) are presented in $mg^{1/3}$. The mortality chance per life stage (μ_c, μ_j, μ_s, μ_a) is given per day. The standard deviation is shown when this was required for the model.

Parameter	$[C_{60}]$ (mg/kg soil)		
	0	15.4	154
γ	0.011 ± 0.002	0.008 ± 0.002	0.011 ± 0.003
L_m	12.8 ± 0.9	15.0 ± 1.4	13.3 ± 1.2
L_b	3.2 ± 0.26	3.1 ± 0.28	2.8 ± 0.27
r_m	0.0016 ± 0.0008	0.00097 ± 0.0001	0.0011 ± 0.0004
τ_0	42	42	42
L_s	8.3	8.8	8.7
L_a	9.9	10.2	9.9
μ_c	0.001	0.001	0.001
μ_j	0.005	0.005	0.0074
μ_s	0.003	0.003	0.003
μ_a	0.002	0.002	0.002

To estimate variances for population growth rate and stage distribution, we applied a Monte Carlo approach. For four of the estimated coefficients (γ, r_m, l_m and l_b) we used the mean and standard deviation observed in the experiments to define probability density functions, assuming for each a Gaussian distribution. Coefficients were assumed to vary independently. For 1000 random values drawn for each coefficient, population growth rate and stable stage distribution were calculated. All calculations were performed using MathCad 14 (Parametric Technology Corporation 2007).

Statistical analysis

Differences in growth rate and reproduction between the three treatments were tested using ANOVA ($\alpha = 0.05$), with Least Significant Differences (LSD) as post-hoc test (Burgers and Oude Voshaar 2010). Binomial testing was used to analyze differences between treatments

for juvenile mortality and for population stable stage distribution (Burgers and Oude Voshaar 2010). Since juvenile earthworms are relatively fast growing, an exponential growth curve was assumed for this life stage, while for adult earthworms the growth rate was assumed to be linear (Burgers and Oude Voshaar 2010). All statistics were conducted using GENstat (12th Edition, VSN International Ltd.).

Results

Characterisation of exposure media

The TEM images shown in figure 1 demonstrate that soilextract only does not contain C_{60} aggregates (Fig. 1A), but these aggregates are present in the soilextract with C_{60} (Fig. 1B). C_{60} appears to form aggregates of 10-15 nm in size, in soilextract (Fig. 1B) as well as in demineralised water (Fig. 1C), and these aggregates form loose clusters with each other. The HRTEM image of soilextract with C_{60} (Fig. 1D) shows the crystal structure of the aggregated C_{60} nanoparticles, demonstrated for these particles in other studies as well (Fujita et al. 2009; Goel et al. 2004). This indicates that the structures visualized in figure 1B and 1C indeed consist of C_{60}.

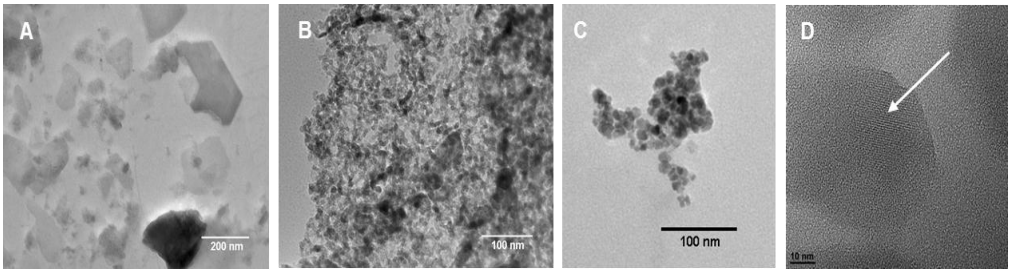

Figure 1. TEM images of soilextract without C_{60} (A) and with C_{60} (2 mg/mL) (B), and demineralised water with C_{60} (1 mg/mL freshly made stocks) (C). HRTEM image of the soilextract with C_{60} (D), showing the crystal structure of the C_{60} nanoparticles in the aggregates.

Spectrophotometry was used to estimate concentrations of C_{60} in the soil. In the control soil a concentration of 6.6 ± 0.9 mg/kg soil (mean ± S.E.) was measured and in the high exposure soil (with nominal concentration of 154 mg C_{60}/kg soil) the measured C_{60}

concentration was 125.2 ± 3.3 mg/kg soil. We assume that the 6.6 mg C_{60}/kg soil in the control soil (nominal C_{60} concentration of 0 mg/kg) is a natural background of unknown carbon particles. This could consist of C_{60} particles, but also of other carbon particles, because the method is not size selective. The measured concentration at the high exposure could then be adjusted to (125.2–6.6=) 118.6 mg/kg soil. Measured concentrations are in the range of the expected/nominal levels (with ~75% of nominal concentration for the measured high concentration), so we assume that nominal levels approach actual levels of C_{60}. Furthermore, the small standard errors indicate that the C_{60} is well mixed through the soil.

Adult mortality, growth rate and cocoon production

No significant differences were observed between treatments for survival and growth rate of adult earthworms (Table 3), but cocoon production was significantly affected by C_{60} exposure (p=0.011). Earthworms exposed to nominal C_{60} concentrations of 154 mg/kg soil produced significantly fewer cocoons than the control (~60% of control). Cocoon production of the earthworms in the group exposed to nominal C_{60} concentrations of 15.4 mg/kg soil did not differ significantly from either the control or the high exposure group.

Table 3. Average mortality (%/day), growth rate (mg/worm/day) and cocoon production (number of cocoons/worm/day) for adult *L. rubellus* earthworms in the different treatment groups, exposed for four weeks. Capitals A and B indicate significant differences within a column (α = 0.05). Mean values ± S.E.

[C_{60}] mg/kg soil	Mortality	Growth rate	Cocoon production	N
0	0.1 ± 0.09 ^{A}	12.8 ± 1.9 ^{A}	0.25 ± 0.01 ^{A}	8
15.4	0.2 ± 0.24 ^{A}	11.4 ± 2.7 ^{A}	0.21 ± 0.02 ^{AB}	6
154	0.1 ± 0.12 ^{A}	8.5 ± 3.8 ^{A}	0.15 ± 0.03 ^{B}	6

Juvenile growth rate and mortality

Mortality among juveniles exposed to nominal C_{60} concentrations of 154 mg/kg soil was significantly higher than the control group (p=0.002), while exposure to nominal C_{60}

concentrations of 15.4 mg/kg soil did not significantly affect juvenile mortality (figure 2; p=0.363).

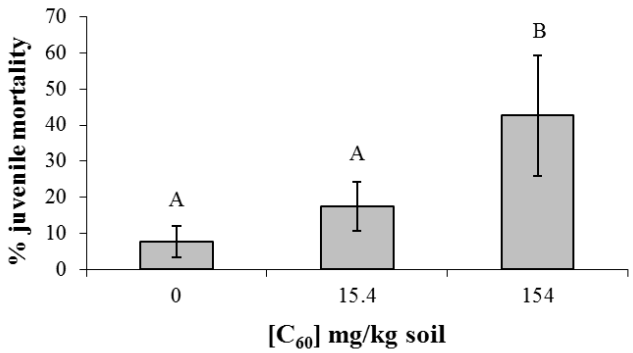

Figure 2. Juvenile mortality (percentage dead juveniles of the total number of hatched juveniles) for *L. rubellus* earthworms per treatment. Capital A and B indicate significant differences (α = 0.05). Mean values ± S.E.

Table 4 shows that exposure to nominal C_{60} concentrations of 15.4 mg/kg soil resulted in a significantly reduced growth rate for juveniles, compared to the control group. No significant difference was demonstrated between the growth rates of the juveniles exposed to nominal C_{60} concentrations of 154 mg/kg soil and the control.

The size at which individuals developed from juvenile to subadult tended to increase with higher exposure level, but this effect was not significant (0 mg/kg: 8.5 ± 0.46 cm; 15.4 mg/kg: 8.9 ± 0.66 cm; 154mg/kg: 9.0 ± 0.46 cm; mean ± S.E.).

Table 4. Growth rate (R; within regression used: A+B*(RX)) for juvenile *L. rubellus* earthworms per treatment. Capitals (A or B) describe significant differences (α = 0.05). Mean values ± S.E.

[C_{60}] mg/kg soil	Growth rate	N
0	1.007 ± 0.002 ^{C}	8
5.4	0.998 ± 0.003 ^{D}	6
154	1.007 ± 0.004 ^{C}	5

Population model

The estimated coefficients for individual growth and development (Table 2), were used in the population model to calculate population growth rate and stage distribution per treatment. The modelled growth rate decreased significantly with increasing C_{60} concentration (Figure 3).

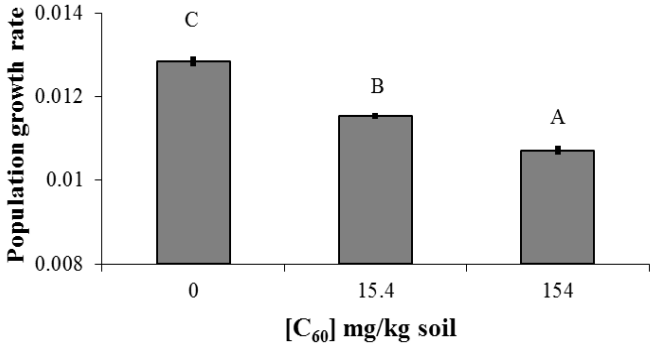

Figure 3. Modelled population growth rate (number of individuals/individual/week) per treatment for *L. rubellus* earthworms. Significant differences are shown as capital A, B and C (α = 0.05). Mean values ± S.E.

For stage distribution of C_{60} exposed populations, the modelling results showed significantly higher percentages of individuals in the juvenile stage and lower percentages of subadults, compared to the control group (Table 5).

Table 5. Modelled population stage distribution: percentages of individual *L. rubellus* earthworms per treatment group and life stage (cocoon stage not included). Significant differences within a column are presented as capitals A, B and C (α = 0.05). Mean values ± S.E.

[C_{60}] mg/kg soil	Juvenile %	Subadult %	Adult %
0	66 ± 0.3^{A}	15 ± 0.1^{C}	19 ± 0.3^{A}
15.4	71 ± 0.1^{C}	10 ± 0.0^{B}	19 ± 0.1^{A}
154	69 ± 0.3^{B}	9.7 ± 0.1^{A}	22 ± 0.3^{B}

Discussion

Exposure scenario

An important issue when performing ecotoxicity experiments is the exposure scenario (Scott-Fordsmand et al. 2008b). For this scenario, the addition of the potential toxic compound to the soil is a significant aspect. Nanoparticles are generally applied to the soil in a suspension. Such a suspension can be produced by prolonged stirring of the nanoparticles in water, sonication of the nanoparticle solution or dissolving the nanoparticles in a carrier solvent, such as THF (Oberdörster 2004; Scott-Fordsmand et al. 2008a; Zhu et al. 2006). However, these dissolving methods can have an effect on the toxicity of nanoparticles (Henry et al. 2007; Oberdörster et al. 2006; Spohn et al. 2009; Zhu et al. 2006). Furthermore, such exposure may not mimic ecologically relevant exposure scenarios. To avoid this possible drawback, we dissolved C_{60} in an aqueous solution containing extracted organic material from the experimental soil, before adding it to the soil. In this way, no additional compounds or co-solvents were required.

Another aspect of the exposure scenario is characterization of the potential toxic compound in the soil. We characterized aggregation status and estimated particle size distribution of C_{60} in the soil using (HR-)TEM. As demonstrated in figure 1, the crystal structure characteristic for nanoparticles was only visible in the soil extract with C_{60} and not in the soil extract only. Also, soilextract with C_{60} nanoparticles appeared to contain similar nanoparticle aggregates as the solution of C_{60} in demineralised water (which contains no other particles than the C_{60} nanoparticles). These samples demonstrated C_{60} particles in aggregates within the size range of 10-15 nm and these tight aggregates appeared to form loose clusters with other C_{60} aggregates. When total C_{60} concentrations were measured in the soil, using spectrophotometry, this demonstrated that measured C_{60} concentrations in the high treatment group were in the same range as the nominal concentrations (with an extraction efficiency of ~75%). This indicates that nominal concentrations of C_{60} appeared to be similar to actual concentrations.

Experimental endpoints

Adult cocoon production

Cocoon production of adult earthworms decreased with increasing C_{60} concentrations (Table 3), which is consistent with a study by Scott-Fordsmand et al. (2008a). Although there were differences between the experimental methods of Scott-Fordsmand et al. (2008a) and our study, for example the use of a different earthworm species (*Eisenia veneta*) and a different exposure route of C_{60} (through food), effects on cocoon production were similar between the studies.

Juvenile growth rate and mortality

The severity of the effects of C_{60} on the juvenile earthworms increased with the exposure level. At the low exposure level non-lethal effects were visible on growth rates (Table 4), but at the high exposure level lethality increased significantly (Figure 2). Lower juvenile growth rate combined with an increased size needed to develop to subadult (demonstrated as a trend in the current study), leads to a higher fraction of the population remaining in the juvenile stage. At a nominal C_{60} exposure level of 154 mg/kg soil, juveniles demonstrated a similar growth rate as the control group, which was unexpected. However, this treatment group showed a high mortality level (~40%). Two hypotheses can be postulated for the mechanism that could overshadow possible effects on growth rate in the 154 mg/kg exposure group. The first hypothesis is that C_{60} exposure caused slow-growing juveniles to die, resulting in a selection towards individuals with a normal growth rate. A second hypothesis may be related to a density-dependent growth rate under the experimental conditions, which was demonstrated by Faber et al. (2004). Due to the induced mortality, density decreased for the 154 mg/kg soil treatment. Hence, earthworms that survived C_{60} exposure could have had a density advantage. This latter hypothesis was assumed to be the most important mechanism and therefore the growth rates used in the population modelling were adjusted for the earthworm densities (see supplementary data).

Population model

When applying the experimental data in the population model (with correction for density), the population growth rate was found to be reduced significantly with increasing C_{60} concentrations (figure 3). The model also demonstrated effects of C_{60} exposure on the stage distribution of the populations, with increased percentages of C_{60} exposed individuals in the juvenile stage (Table 5). Experimental data on reduced juvenile growth rate (Table 4), combined with a longer length needed to reach subadulthood, confirm that exposed earthworms may stay longer in the juvenile stage. However, this effect is not detectable at the adult stage, likely due to a faster development through the subadult stage, as demonstrated by the smaller fraction of earthworms in this stage (Table 5).

Juvenile sensitivity

Both the individual and the modelled data demonstrate effects of C_{60} on the juvenile stage. Similar to these results, other studies using annelids have also demonstrated the juvenile stage as most sensitive to toxic compounds (Booth and O'Halloran 2001; Levin et al. 1996; Widarto et al. 2004). The studies of Booth and O'Halloran (2001) and Widarto et al. (2004) found that juvenile growth rate was more reduced than adult growth rate, upon exposure to pesticides and nonylphenol, respectively. These two studies and the study by Levin et al. (1996) also showed that time to maturity was affected by exposure to the toxic compounds.

Responses of juveniles may predict long-term impact of exposure to toxic compounds on populations better than responses in adults (Booth and O'Halloran 2001), especially when effects demonstrated under laboratory conditions are extrapolated to the field situation. In the field, the lifespan of earthworms is generally shorter than under laboratory conditions (Edwards and Bohlen 1996), because earthworms are not only exposed to the studied toxic compound but also to other stress factors, including seasonal variation, predation, competition, parasites, water motion and other pollutants (Klok et al. 2006; Levin et al. 2006). When the juvenile growth rate is reduced, this results in a prolonged juvenile stage and thus in shorter subadult and adult stages under field conditions. Some juveniles might not even reach adulthood within their lifespan (Klok and De Roos 1996). Lower number of

earthworms growing to adulthood in combination with reduced fertility, would then aggravate the negative effects of C_{60} on cocoon production.

Conclusions

This study shows that C_{60} exposure affects *L. rubellus* in a dose-dependent way, both at individual and at population level. Data at the individual level demonstrated reduced cocoon production and juvenile growth rate, and a higher juvenile mortality for the exposed earthworms. When these observed effects were extrapolated to the population level, this resulted in a lower growth rate and a stage distribution shift towards a larger proportion of juveniles, for C_{60} exposed populations. Hence, we conclude that exposure to C_{60} may pose a hazard to earthworm populations. The present study also suggests that earthworms in the juvenile stage represent a more sensitive target for long-term effects on populations than adult earthworms.

Acknowledgements

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Supplementary data

To obtain averages of the parameters for the population model per treatment, we used a Gompertz curve to describe the growth of the earthworms. This curve provided us with information about the rate constant (γ), maximum length (L_m) and weight at hatching (L_b)

for every container (Table S1). All regressions were (highly) significant, as is demonstrated by the F_{prob} in table S1 and is illustrated for two examples in figures S1A and S1B.

Table S1: Per container γ, L_m, L_b and F_{prob} were obtained by fitting the data on a growth curve.

pot #	[C_{60}] (mg/kg soil)	γ estimated ($mg^{1/3}/mg^{1/3}$/day)	γ adjusted ($mg^{1/3}/mg^{1/3}$/day)	L_m ($mg^{1/3}$)	L_b ($mg^{1/3}$)	F_{prob}
1	0	0.01	0.014	9.79	3.25	< 0.001
2	0	0.01	0.012	13.48	3.57	< 0.001
3	0	0.013	0.012	12.04	2.78	< 0.001
4	0	0.011	0.011	13.85	3.22	< 0.001
7	0	0.009	0.007	13.22	3.32	< 0.001
8	0	0.013	0.012	14.5	3.2	0.031
9	15	0.012	0.009	14.85	2.73	< 0.001
11	15	0.007	0.006	16.86	3.4	< 0.001
13	15	0.009	0.008	14.96	3.01	< 0.001
14	15	0.009	0.01	13.48	3.14	< 0.001
15	154	0.009	0.007	14.12	3.06	< 0.001
16	154	0.017	0.015	11.77	2.8	< 0.001
17	154	0.013	0.012	13	2.88	< 0.001
19	154	0.015	0.011	14.33	2.41	0.008

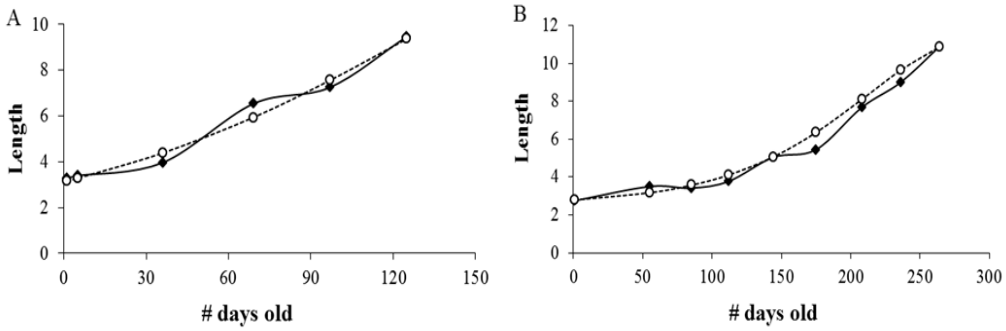

Figure S1. Individual *L. rubellus* earthworms with measured data (black line) and modelled data (dotted line) from container 8 (A) and 16 (B), with length in $mg^{1/3}$ plotted against age (# days after hatching).

Chapter 3

C_{60} exposure induced tissue damage and gene expression alterations in the earthworm *Lumbricus rubellus*

Based on:

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Abstract

Effects of C_{60} exposure (0, 15.4 or 154 mg/kg soil) on the earthworm *Lumbricus rubellus* were assessed at the tissue and molecular level, in two experiments. In the first experiment earthworms were exposed for four weeks, and in the second lifelong. In both experiments gene expression of heat shock protein 70 (HSP70) decreased. For catalase and glutathione-S-transferase (GST) no significant trends in gene expression or enzyme activity were observed. Gene expression of coelomic cytolytic factor-1 (CCF-1) did not alter in earthworms exposed for four weeks, but was significantly down-regulated in the lifelong exposure. Histology of earthworms exposed to C_{60} in both experiments showed a damaged cuticle, with underlying pathologies of epidermis and muscles, as well as effects on the gut barrier. However, tissue repair was also observed in these earthworms. Overall, these data show that sub-lethal C_{60} exposure to earthworms via the soil affects gene expression and causes tissue pathologies.

Introduction

The field of nanotechnology is rapidly expanding and carbon-based nanomaterials represent the second largest category of nanomaterials (second only to silver; as discussed in the Nanotechnology Project of the Woodrow Wilson International Centre for Scholars). Carbon-based nanomaterials, which include fullerenes, are used in health and fitness products, electronic goods, medical technology, and to encapsulate active ingredients for bioremediation (Cheng et al. 2004; Aitken et al. 2006). It is therefore inevitable that fullerenes, such as C_{60}, will be released into the environment (Gottschalk et al. 2009). C_{60} exposure can be toxic to animals (Handy et al. 2008a; Klaine et al. 2008; Kahru and Dubourguier 2010), including earthworms (Scott-Fordsmand et al. 2008a; **Chapter 2**).

Environmental risk assessments typically use survival, growth and reproductive success to predict population level responses to chemicals (Roh et al. 2007). For earthworms, reduced survival, growth and reproduction have been described for C_{60} exposure in the soil (Scott-Fordsmand et al. 2008a; **Chapter 2**), including their consequences for the population growth rate. However, effects of C_{60} exposure on earthworms below the whole organism level are much less clear and studies on the possible target tissues, biological processes as well as modes of action underlying the toxicity of these nanoparticles are needed.

Research on the sub-lethal effects of C_{60} has been driven partly by the chemistry of C_{60}, with a focus on the ability of C_{60} to generate reactive oxygen species (ROS) (Taylor and Walton 1993; Kamat et al. 2000). Evidence for the oxidative stress hypothesis arises from studies on mammalian cells exposed to C_{60}, where antioxidants (such as ascorbic acid and alpha-tocopherol) protected against membrane damage and the activity of antioxidant enzymes (e.g., catalase and superoxide dismutase) was altered (Kamat et al. 2000; Usenko et al. 2008). Moreover, other mammalian studies demonstrate evidence of inflammatory responses (Fujita et al. 2009; Mühlfeld et al. 2008) and alterations of macrophage immune function (Hamilton et al. 2007), due to C_{60} exposure. However, toxic mechanisms and stress responses of earthworms to C_{60} exposure remain to be investigated.

The overall aim of the present study was to investigate cellular and molecular responses of earthworms to C_{60} exposure, to complement the observations already made on survival, growth and reproduction (**Chapter 2**). The secondary objective was to contribute to the

development of effect markers for C_{60} exposure, by using a set of established effect markers which reflect the different levels of biological organisation in the earthworm and may also inform on the toxic mechanism of C_{60} exposure (Handy et al. 2002a; Heckmann et al. 2008). At the molecular level, four specific effect markers were selected; heat shock protein 70 (HSP70) as a well-known effect marker of generic stress (van Straalen and Roelofs 2006), catalase and glutathione-S-transferase (GST) as effect markers for oxidative stress (Kohen and Nyska 2002), and coelomic cytolytic factor-1 (CCF-1) as an immune response effect marker (Olivares Fontt et al. 2002). At the cellular and tissue level, histological analyses were used to identify pathology and evidence of inflammation in the tissue. Finally, the molecular and tissue level effects were interpreted in the context of effects on mortality, growth and reproduction observed in the same experiments, as reported in **chapter 2**.

Materials and Methods

Soil preparations and C_{60} characterisation

The earthworms investigated in this study are from the same experiments as described in **chapter 2**, where details about soil preparations and C_{60} characterisation are described. In short, the experiments were carried out with clean soil, consisting of 4.3% organic matter and the pH was 5.0 (Proefboerderij Kooijenburg, Marwijksoord, the Netherlands). The containers, with soil and earthworms, were maintained under constant conditions (24 hours light, 15°C, 61% relative humidity) throughout the experiments. C_{60} was obtained as a dry powder from SES Research (99.5+%, Houston USA). To be able to get an indication of metal impurities in the C_{60}, a chemical analysis on the C_{60} powder was performed, by inductively coupled plasma-atomic emission spectroscopy (ICP-AES). As shown in table 1, metal levels were well below threshold levels for toxic effects, even with maximum addition of C_{60} to the soil (Neuhauser et al. 1984; Abdul Rida 1996; Scott-Fordsmand et al. 1998; Klok et al. 2006).

Table 1. Overview of measured concentrations of different metals detected in C_{60} (mg/kg C_{60}) and nominal concentrations of the metals (mg/kg soil) for the highest exposure level of C_{60} (154 mg C_{60}/kg soil). Threshold levels, or no effect levels, for earthworms (at mg/kg soil) from the literature are also given (Neuhauser et al. 1984; Abdul Rida, 1996; Scott-Fordsmand et al. 1998; Klok et al. 2006).

Metal (mg/kg)	Cd	Co	Cu	Fe	Ni	Pb	Zn
In C_{60}	>0.0001	>0.0001	1.2	14	0.016	0.05	1.48
In soil with C_{60}	>0.0001	>0.0001	0.18	2.2	0.002	0.008	0.23
Threshold level	1.6	26	22	28495	80	52	85

To obtain a stable C_{60} stock solution, C_{60} was dissolved in an aqueous soil extract (as also described in **Chapter 2**). This extract was obtained by stirring control soil in ultrapure water (0.4 g soil/mL) for one hour at 180 rpm, after which it was filtered (Whatman filters type 597, Fisher Scientific). After filtering, C_{60} was added to a part of the soil extract, to a final nominal concentration of 2 g/L. The C_{60} suspension thus obtained was stirred for three days. Nominal soil concentrations were set at 0 (control), 15.4 and 154 mg C_{60}/kg soil (dry weight), with a moisture content of 17.2% (dry weight). To obtain these concentrations in the soil, 650 gram soil was mixed with soil extract for two minutes (Hobart mixer, speed 2), for every container separately. For the control and the 154 mg/kg exposure 50 mL of soil extract, without and with C_{60} respectively, was added to every container. For the 15.4 mg/kg soil exposure, the soil extract added to each container consisted of 5 mL soil extract with C_{60} and 45 mL soil extract without C_{60}. Earthworms were added to the containers immediately after preparation of the soil.

Size and aggregation of C_{60} particles in the soil extract were characterised using both transmission electron microscopy (TEM) (JEOL JEM 1011) and high resolution TEM (HRTEM; JEOL JEM 2100), operated at 60 kV and 200 kV, respectively. Samples of C_{60} in the soil extracts demonstrated that C_{60} appeared to form aggregates of 10-15 nm in size, which clustered together loosely (as also discussed in **chapter 2**). The HRTEM image of the soil extract with C_{60} showed the normal crystal structure of the aggregated C_{60}, as observed in other studies also (Fujita et al. 2009; Goel et al. 2004).

Concentrations of C_{60} in the soil were determined with a toluene extraction method and followed by spectrophotometry (Aquamate from Thermo Electron Corporation), for five

samples per treatment (as described in **chapter 2**). Measured concentrations of C_{60} were in the range of the nominal levels (125.2 ± 3.3 mg/kg soil (n=5) for the high nominal concentration of 154 mg/kg soil), suggesting that nominal levels approached actual levels of C_{60}. Furthermore, the small standard errors between replicate soil samples indicated that the C_{60} was well mixed throughout the soil. For earthworms, it was not technically possible to quantify C_{60} concentrations in the tissues, due to interference from the organic matrix of the tissues.

Experimental designs

Two experiments were carried out. In the first experiment healthy adult earthworms (*Lumbricus rubellus*), ranging in weight from 1241 to 1663 mg, were exposed to soil with nominal concentrations of 0, 15.4 or 154 mg C_{60}/kg soil for four weeks. Eight containers were used for each treatment and each container housed five earthworms. The containers were considered to be the experimental units for replicates, so there were eight replicates and 40 earthworms per treatment. Every week the earthworms were fed alder leaves (Alnus glutinosa), from an uncontaminated location (Vossemeerdijk, Dronten, the Netherlands), ad libitum. In the second experiment offspring, from parent earthworms exposed to the same C_{60} treatment as above, were exposed from hatching up to adulthood (approximately 350 days; 'lifelong'). The number of replicates was eight for the control treatment and six for both 15.4 and 154 mg C_{60}/kg soil. Earthworms were fed alder leaves as discussed above and the containers were checked every four weeks, to monitor the survival and body weight of the earthworms. At the end of each of the experiments, earthworms were washed in cold demineralized water, weighed and then randomly allocated for histological examination (two earthworms per container) or for gene expression and enzyme activity analysis (one earthworm per container).

Gene expression analysis

Earthworms used for gene expression analysis were washed in cold demineralized water, weighed and then immediately snap frozen in liquid nitrogen and stored at -80°C. Earthworms were transported on dry ice to the National Environmental Research Institute

(NERI) in Silkeborg Denmark, where gene expression measurements were performed. For these measurements, earthworms from four replicates were used per exposure group (n = 4 containers per treatment), but for the earthworms exposed lifelong to 154 mg C_{60}/kg soil only earthworms from three containers were available due to high mortality at this treatment level (n = 3). Individual whole earthworms were taken from the -80°C freezer and immediately ground to a fine powder, under liquid nitrogen, using a pestal and mortar. The resulting powder was mixed thoroughly to ensure that a representative sub-sample (approximately 20 mg tissue) could be used for gene expression analysis. Total RNA was extracted using the NucleoSpin RNA II kit from Macherey-Nagel (Germany). RNA concentrations were determined by spectrophotometry using an Implen Nanophotometer (AH Diagnostics, Denmark) and the integrity of the RNA was verified by 1% agarose gel electrophoresis. For each sample 2 µg of DNAse-treated total RNA was reverse transcribed to cDNA using anchored oligo$(dT)_{20}$ primers (Invitrogen, Denmark) and the Omniscript Reverse Transcription kit (Qiagen, Germany). cDNA samples were diluted in RNAse free water to a concentration of 4 ng/µL. Expressed Sequence Tags (ESTs) for the seven investigated genes were obtained from Lumbribase (Lumbribase homepage) and NCBI websites (NCBI homepage), and verified by BLASTX analysis (NCBI homepage). Forward and backward primers for these ESTs were designed using Primer3 (Rozen and Skaletsky 1999) and synthesized by Eurofins (Ebersberg, Germany). EST number, gene ontology and primer sequences of the investigated genes are shown in table 2.

Table 2. EST number, gene ontology and primer sequences of the investigated genes.

Gene name	Lumbribase EST no.	Gene ontology	Forward primer (5'-3')	Reverse primer (5'-3')	Amplicon size (bp)
HSP70	LRC01283	General stress protein	TTGCATATGGAGCTGCTGTC	GAGGAGTCACGTCGAGAAGC	106
Catalase	LRC01429	Anti-oxidant enzyme	ACATGAACGGATACGGAAGC	ATTCTTGATGCCTTGGTTCG	120
GST-pi	LRC06119	Anti-oxidant enzyme	CACTTGGCAACACTGGAGAA	CTGACCTTGTCACCAACGAA	93
SOD	LRC09106	Anti-oxidant enzyme	CCACGTTCACACATCTGGAG	GCGATTATGCGGATTGAAGT	92
CCF-1	LRC00385	Cytokine	GTACGTGACAGCCTTGCAGA	GGCATTGTTGTCTCCCTCAT	101
GAPDH	LRC07381	Glycolytic enzyme	TGTCTCGACCGACTTCAACA	GTGATCATTGAGGGCGATTC	95
PFK-1	LRC00993	Glycolytic enzyme	TACGTCGCTGAATGAGATCG	CTTGGGTCTCGATGACGAAT	111

Real-time quantitative polymerase chain reaction (qPCR) was conducted using Brilliant II SYBR Green qPCR Master. Each sample was run in duplicate and each well contained a 5 µL sample (equivalent to 20 ng total RNA) along with 900 nM of each primer, in a total volume of 25 µL. The plate was shaken and centrifuged for one minute (at 4600 *g*), to ensure a homogenous mixture on the bottom of the plate without bubbles. The cycling program was started at 95°C for ten minutes to activate the DNA polymerase, then 40 cycles of 95°C for ten seconds and 60°C for sixty seconds. Subsequently, melting curves were performed by Stratagene MxPro Software 4.1 (Agilent Technologies, Denmark), to validate that only one target had been amplified. Threshold cycles and amplification efficiencies were calculated for each sample using Data Analysis for Real-Time PCR (DART-PCR) version 1.0 (Peirson et al. 2003). Outliers identified by DART-PCR and samples diverging from the dissociation curve were omitted from further analysis. NORMA-Gene was used to normalize the relative expression of the samples calculated by DART-PCR (Heckmann et al. 2011b).

Enzyme activity assays

Total protein concentrations were estimated using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, US). The activity of two antioxidant enzymes, catalase and GST, was determined using commercially available assay kits from Cayman Chemical (Ann Arbor, Michigan, US), with item numbers 707002 and 703302, respectively. Samples were taken from the same earthworms used for gene expression quantification. For the catalase assay, a 50 mg sample was homogenized in 40 mL of a catalase assay buffer (50 mM potassium phosphate buffer, prepared with 0.07 g/mL KH_2PO_4 and 0.11 g/mL K_2HPO_4*$3H_2O$; 0.3 mg/mL EDTA; pH 7.0; (Sambrook and Russell 2001)). The homogenate was centrifuged (10,000 g for 15 minutes) and the supernatant was used for further analysis. CAT activity was determined using H_2O_2 as the substrate and the formaldehyde produced was measured at 540 nm. For the GST, 50 mg sample was homogenized in 400 µL of GST assay buffer (100 mM potassium phosphate buffer, prepared with 0.14 g/mL KH_2PO_4 and 0.23 g/mL K_2HPO_4*$3H_2O$; 0.6 mg/mL EDTA; pH 7.0; (Sambrook and Russell 2001)) and centrifuged for 15 minutes at 10,000 g. The

supernatant was used for the GST activity determination. Activities of total GST were determined using 1-chloro-2,4-dinitrobenzene (CDNB) as substrate and absorbance was measured at 340 nm. Enzyme activities were related to the protein content (determined at 562 nm) of the sample. All samples were analysed at least as duplicates and as described by the assay kit supplier. C_{60} interference with the kits for gene expression and biochemistry is not expected, because the high ionic strengths of the salines used to make the tissue homogenates would rapidly precipitate the C_{60}, which is then lost in the pellet at the centrifugation steps, leaving a clean supernatant for biochemistry (Hou et al. 2009).

Histological observations

Earthworms were carefully collected, washed in cold demineralized water and weighed. Subsequently, segments were carefully cut from the clitellum (covering the region approximately one centimetre anterior and posterior to the clitellum), using a sharp scalpel blade, and were placed immediately into buffered 4 % formal saline to fix the specimens (Handy et al. 2002b). Fixed samples were sent to the University of Plymouth for processing of routine wax histology (Handy et al. 2002b). For the first experiment two earthworms from every replicate (sixteen earthworms per treatment) were processed. For the second lifelong experiment five adult earthworms per treatment, all from different containers, were processed for histology. Earthworms from both experiments were processed into wax blocks and 8 µm sections were cut from each earthworm. Sections were stained with haematoxylin and eosin for general structural observations or with Mallory's trichrome to reveal additional details of connective tissues. All tissues were prepared simultaneously in batches containing tissues from control earthworms and the C_{60} treatments at the same time point, in order to eliminate differences in fixation or staining artefacts between treatments. Tissues were examined under an Olympus Vanox-T microscope and photographs were produced using an Olympus digital camera (C-2020 Z). The thickness of each layer of tissue from the ectoderm (cuticle, epidermis, circular muscle and longitudinal muscle when intact, and clitellum parenchyma when present) were measured manually for each slide to analyse morphometrics.

Statistics

Differences in gene expression and enzyme activity between the three treatments were analyzed using analysis of variance (ANOVA, with $\alpha = 0.05$), with Least Significant Differences (LSD) as the post-hoc test (Burgers and Oude Voshaar 2010). Histological observations on cuticle loss were statistically tested for differences between exposure levels and between exposure periods, using a binomial regression analysis. These statistical analysis were carried out with GENstat (13th Edition, VSN International Ltd.). For other histological observations, data were similarly analysed by StatGraphics Plus version 5.1. No container effects were observed, so data was pooled by treatment for ANOVA (after checking for kurtosis, skewedness, and unequal variance with Bartlett's test). In cases where data transformation was not effective, the Kruskal–Wallis test was used and differences were located by notched box and whisker plots. Results are presented as mean ± standard error of the mean (SEM) unless otherwise specified.

Results

Gene expression analysis

For the adult earthworms exposed to C_{60} for four weeks, only one of the genes examined (HSP70) showed a clear concentration-dependent change of expression in whole tissue homogenates (Figure 1). HSP70 gene expression in both the 15.4 and 154 mg C_{60}/kg soil treatments decreased significantly (ANOVA; p=0.018) compared with the control (Figure 1A). In the 154 mg C_{60}/kg treatment group, the HSP70 expression had decreased down to 34% of the control value. For catalase, GST and CCF-1 gene expressions (Figures 1B-1D) there were no statistical differences noted due to C_{60} exposure. The gene expression of glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an enzyme involved in the citric acid cycle, did not differ between the three treatments as well (as shown in Supplementary Table S1).

In the lifelong experiment the HSP70 gene expression was also down-regulated with increasing C_{60} concentration, to 60% of the control level (Figure 1A), but this trend was only statistically significant at the 10% rejection level (ANOVA; p = 0.1). The gene

expressions of the antioxidant enzymes catalase and GST did not change significantly due to C_{60} exposure (Figures 1B and 1C). However, unlike the four weeks experiment, the CCF-1 gene expression of earthworms exposed lifelong to both C_{60} concentrations showed a statistically significant decrease (ANOVA; p=0.04) compared to the control treatment (Figure 1D). In the 154 mg C_{60}/kg soil treatment group the CCF-1 expression had decreased down to 12% of the control value. Gene expressions of superoxide dismutase (SOD), GAPDH and phosphofructokinase-1 (PFK-1) did not demonstrate differences between the treatments (see Supplementary Table S1).

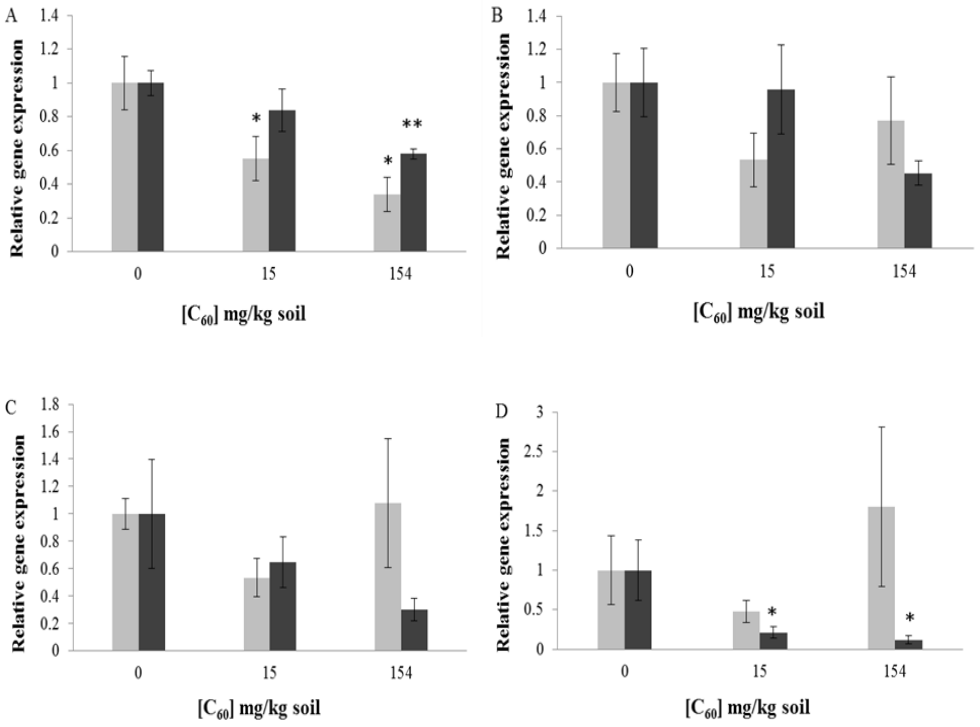

Figure 1. Relative gene expression levels (compared with average of the control) of (A) heat shock protein 70 (HSP70), (B) catalase, (C) glutathione S-transferase (GST) and (D) coelomic cytolytic factor 1 (CCF-1), for the four weeks (light grey) and for the lifelong (dark grey) exposed *L. rubellus* earthworms. Data are shown per exposure group (0, 15.4 and 154 mg C_{60}/kg soil), as mean values ± SEM. Significant differences demonstrated are compared to control, *p<0.05 and **p<0.01.

Enzyme activity assays

Table 3 shows that the enzyme activity for catalase and GST did not differ significantly between treatments for the four weeks experiment. In the lifelong exposed earthworms also no treatment-dependent changes in catalase and GST activity were noted (Table 3).

Table 3. Measured levels of catalase and glutathione-S-transferase for *L rubellus* earthworms in the different treatment groups, exposed to C_{60} for four weeks or lifelong. Mean values ± SEM. No significant differences were demonstrated between treatments, within the assays ($\alpha = 0.05$).

Exposure period	[C_{60}] (mg/kg soil)	Catalase (nmol/min/mg protein)	GST (nmol/min/mg protein)
Four weeks	0	295 ± 32	184 ± 53
	15.4	318 ± 48	154 ± 62
	154	327 ± 67	158 ± 49
Lifelong	0	394 ± 38	212 ± 89
	15.4	464 ± 51	133 ± 56
	154	298 ± 48	148 ± 40

Histological observations

In the four week experiment quantitative histological analysis of the thickness of cuticle, epidermis and the circular muscles of the ectoderm showed no significant differences between treatments, for any of the segments (see Supplementary Table S2). Further histological examination demonstrated changes in the tissue of adult earthworms after four weeks of exposure to C_{60} (Figure 2). Control earthworms showed normal structure of the ectoderm, without epithelial lifting or necrosis (Figure 2A). The underlying circular and longitudinal muscles were normal for all control earthworms, and pathologies such as fibrosis, hydropic change or atrophy were absent. There was also no evidence of coelomocyte infiltration in any of the tissues, which renders the absence of an inflammatory response of the cells.

In the tissues of earthworms exposed to 15.4 mg C_{60}/kg soil for four weeks, some histological changes were observed. Eleven out of the sixteen earthworms showed an intact

cuticle, but the staining was more basophilic than for the controls. Three out of the five earthworms with damaged cuticle also showed enlarged mucocytes and a fine basophilic granular deposit in some epithelial cells. This indicates an increase in negatively charged material in the epidermis. Earthworms with a damaged cuticle also demonstrated some hyperplasia of the epidermal and basal cells, as well as granular lipofuscin-like deposits in the circular muscle layer (Figure 2B) in combination with mild fibrosis. Some loss of architecture and oedema in the longitudinal muscle was also observed (Figure 2B). The gut epithelium of all the earthworms in this group showed hyperplasia of the sub-mucosa and epithelium, accompanied by necrosis. In six out of the sixteen earthworms, the changes to the gut were so severe that the normal architecture was no longer evident. The clitellum segments of the earthworms exposed to 15.4 mg C_{60}/kg soil were comparable to the controls, except for one earthworm with damaged cuticle and eosinophilic epidermal cells. This earthworm also had eosinophilic granular deposits in the damaged parenchyma of the clitellum.

In earthworms exposed for four weeks to 154 mg C_{60}/kg soil, cuticle damage was similar to that observed at the lower C_{60} concentration (observed in five of the sixteen earthworms), but three earthworms also showed areas of thickened and very basophilic cuticles. For all sixteen earthworms, mild hydropic changes in the epidermis were noted, which was not observed for the 0 and 15.4 mg C_{60}/kg soil treatment groups. Three earthworms with a damaged cuticle also showed some erosion of the epidermal cells, lipofuscin-like deposits and fibrosis in the circular muscle (Figure 2C), as well as oedema in the longitudinal muscle. Two earthworms with a damaged cuticle, demonstrated severe erosion of the longitudinal muscle with loss of architecture (Figure 2C). In the gut sections of the earthworms in this exposure group, injuries to the intestinal cells were similar to those in the 15.4 mg C_{60}/kg soil exposure group. All sixteen earthworms showed hyperplasia and necrosis and in nine earthworms this resulted in complete loss of the normal architecture of the gut epithelium. The clitellum segment from earthworms exposed to 154 mg C_{60}/kg soil were normal, except for one earthworm with diffuse parenchyma.

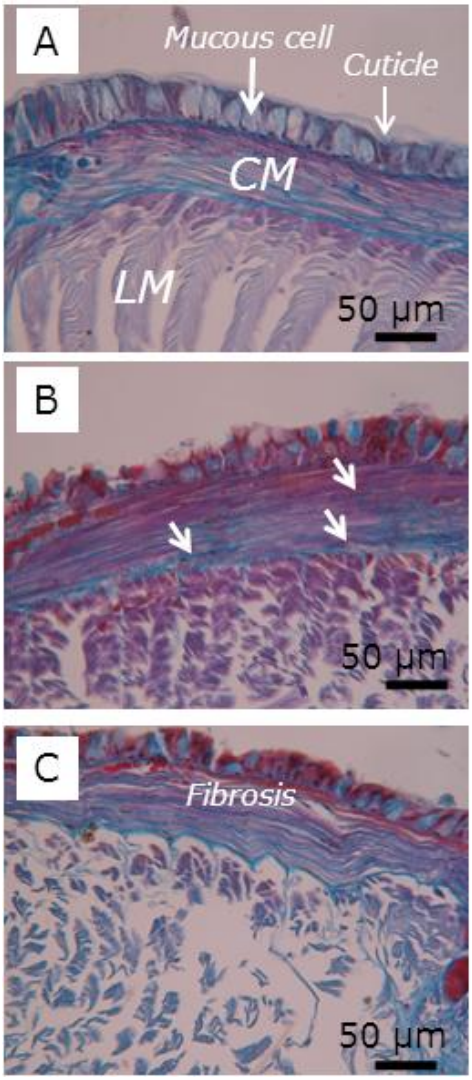

Figure 2. Transverse sections of segments from the anterior region of earthworms exposed to (A) control, (B) 15.4 and (C) 154 mg/kg of C_{60} nanoparticles. Controls show normal morphology of the epidermis with an intact cuticle on the surface and undischarged mucous cells. The circular (CM) and longitudinal muscle (LM) layers are normal. Panel B and C demonstrate the erosion of the epithelium, with loss of the cuticle and discharge of mucous cells for both C_{60} concentrations. For these earthworms granulation in the circular muscle was observed (panel B, white arrows), as well as fibrosis of the circular muscle and some loss of integrity of the longitudinal muscle (panel C). The magnification was X400. Sections were cut 8 µm thick and stained with Mallory's trichrome.

Lifelong exposure also caused no notable changes in the thicknesses of the tissue layers in the ectoderm (see Supplementary Table S2). Further examination of the tissues of the control earthworms demonstrated normal histology, as also described above for the four weeks exposed control group.

Tissues of earthworms exposed to 15.4 mg C_{60}/kg soil showed some loss of the cuticle, which was accompanied by enlarged mucocytes and some hyperplasia of the epidermal cells. In four earthworms the circular muscle also demonstrated mild atrophy, and for two earthworms mild fibrosis and granular lipofuscin-like deposits was noted. The gut epithelial cells of these earthworms were thinner and showed more elongated nuclei than the cells of the control earthworms. This demonstrates that these cells have increased their metabolic activity.

Segments from earthworms after lifelong exposure to 154 mg C_{60}/kg soil showed loss of the cuticle in three out of the five earthworms examined. In the earthworms with damaged cuticles effects were similar to the four week experiment, except for one lifelong exposed earthworm which also showed atrophy of the longitudinal muscles. Changes in the gut epithelium were similar to the changes of the earthworms exposed lifelong to 15.4 mg C_{60}/kg soil. However, the earthworms in the highest exposure group also showed areas of necrotic epithelium, where all cells showed extensive vacuoles. The clitellum of three out of the five earthworms exposed to 154 mg C_{60}/kg soil was different from the controls. For one earthworm depletion of the parenchyma of the clitellum was observed and in two other earthworms severe erosion of the parenchyma had occurred.

Statistical tests of the above described effects on the cuticle showed that exposed earthworms experienced significantly more cuticle loss (binomial regression analysis; $p < 0.001$). Also, the cuticle damage was more severe in the earthworms exposed to C_{60} lifelong than in the adults exposed for four weeks (binomial regression analysis; $p = 0.039$).

Discussion

This study shows that earthworms exposed to C_{60} in soil can suffer adverse sub-lethal effects, which include loss of integrity of the protective cuticle, pathology of the ectoderm, intestinal erosion and altered gene expression in the whole organism. These changes occur against a background of increased juvenile mortality and decreases in reproductive success and juvenile growth, reported in a previous ecotoxicological study on earthworms exposed to C_{60} under the same conditions (discussed in **Chapter 2**). In that study it was demonstrated that reproductive success decreased with increasing C_{60} concentration, down to 60% of the control level at the highest concentration of 154 mg C_{60}/kg soil. Effects on juvenile earthworms also increased with the C_{60} exposure level. At the sub-lethal exposure level of 15.4 mg C_{60}/kg soil, growth rate was significantly reduced. At the highest exposure level mortality was the dominant effect, with 40% of the juvenile earthworms passing away after hatching.

Gene expression of HSP70 was down-regulated with increasing C_{60} concentration, for both exposure periods. Such down-regulation is not unique to C_{60} exposed earthworms and has been demonstrated for other compounds and organisms as well, such as for cadmium exposed mussels (Brown et al. 1995), for amphipods exposed to dieldrin and fluoranthene (Werner and Nagel 1997), and for isopoda species exposed to several toxic metals (Eckwert et al. 1997). The down-regulation of HSP70 expression could be explained by emerging tolerance for C_{60} exposure (Eckwert and Köhler 1997; Croute et al. 2000) and by the up-regulation of another member of the HSP family. HSP70 is part of a family of heat shock proteins, which respond in a dynamic way to exposure, and therefore other members of the HSP family may have been up-regulated to protect the earthworms instead (Werner and Nagel 1997; Bierkens 2000). Lower HSP70 expression levels could also be explained by tissue repair, which is indicated by the histology, e.g. hyperplasia and the presence of thinner cells with elongated nuclei in the gut epithelium, and also observed in fish epithelia due to carbon nanotube exposure (Smith et al. 2007). HSP70 is known to inhibit global protein synthesis (van Straalen and Roelofs 2006). During tissue repair, protein synthesis

will be increased in the tissue and this is usually accompanied by low HSP70 expression (Feder 1999; Bierkens 2000).

In the present study, no statistically significant effects of C_{60} exposure on antioxidant enzyme expression and activity were observed, in either the four week or the lifelong experiment. Numerous other nanotoxicology studies suggest oxidative stress as a likely mechanism of C_{60} toxicity (Kamat et al. 1998; Sayes et al. 2005; Usenko et al. 2008; Klaper et al. 2009; Barillet et al. 2010). However, these studies have used short, acute exposures and/or *in vitro* studies on cells. The lack of clear dose-response relationships between C_{60} exposure and oxidative stress may be explained by different phenomena. Earthworms may adapt to oxidative stress associated with sub-lethal pollutant exposure quickly (Maity et al. 2008) and effects may have only been detectable a couple of days after the start of the C_{60} exposure, rather than 4 weeks later. Furthermore, the oxidative stress response usually involves a cascade, including various chemical antioxidants and antioxidant enzymes, and it may not be necessary to induce all of the antioxidant enzymes. In addition, the concentrations of antioxidant enzymes are known to vary across different tissues, and a whole body measurement may not detect fine changes in individual tissues (Korsloot et al. 2004; Kohen and Nyska 2002; Smith et al. 2007; Barillet et al. 2010). The tissue repair demonstrated with the histological observations could also affect antioxidant enzyme activity.

There are some concerns that C_{60} could produce inflammation and may be immunotoxic (Fujita et al. 2009; Hamilton et al. 2007). However, in the histological analysis of this study no inflammation response in the tissues was observed (no coelomocyte infiltration into the tissues) and also gene expression of the cytokine-like protein CCF-1 did not increase. Tissue injury in the absence of an immune response can be explained by (i) death of the coelomocytes or (ii) immunosuppression (Stebbing 1981; van Straalen and Roelofs 2006). The latter seems most likely, as the lifelong exposed earthworms demonstrated a statistically significant down-regulation of CCF-1 expression (to < 20% compared to the control). Immunosuppression due to chronic exposure has also been demonstrated for

Lumbricus terrestris earthworms exposed to the polychlorinated biphenyl (PCB) Aroclor 1254 (Goven et al. 1993).

Reliable measurements or visualization of C_{60} in the internal tissues of earthworms are not feasible without fluorescent or radio-active labelling of the nanoparticle. So, in the absence of routine analytical methods to measure C_{60} in the tissues of earthworms, target organs for C_{60} can only be inferred from biological effects and the integrity of the external barriers on the animal at the moment. In both the four weeks and lifelong C_{60} exposed earthworms damaged cuticles were observed, accompanied by underlying pathology in the rest of the ectoderm. This suggests that C_{60} would have direct access to the internal tissues of the earthworms. For the life time exposure study, the incidence of cuticle pathology was higher (> 60%) than for the four weeks exposure (around 30%), suggesting a cumulative effect over time. In both experiments the gut barrier was also compromised by C_{60} exposure, indicating that any C_{60} in ingested soil would also be able to access the body. Unlike the cuticle, the gut showed an adaptive response with evidence of tissue repair. The four week exposed earthworms demonstrated loss of gut epithelial architecture in both the 15.4 and 154 mg C_{60}/kg soil exposure groups. However, the situation was less severe for the lifelong exposed earthworms, which showed only patches of necrotic gut epithelial cells mainly in the 154 mg C_{60}/kg soil exposure group. This indicates that the repair mechanisms of the lifelong exposed earthworms may have been active, but nonetheless the gut barrier was not fully intact. Overall, the incidence of tissue injuries was broadly the same at the two C_{60} concentrations, suggesting that even the lowest C_{60} concentration tested was well above the exposure threshold.

Tissue injury has consequences for the bioenergetics of earthworms. The tissue injury and repair observed in the present study would inevitably increase the body maintenance component of the overall energy budget, leaving less energy for other processes like growth and reproduction (Kooijman 2000). The earthworms examined in this study were from the same experiments as discussed in **chapter 2**, which demonstrated that C_{60} exposure can indeed reduce reproduction (in a concentration-dependent way) and juvenile growth (as a

sub-lethal effect, at the exposure level of 15.4 mg C_{60}/kg soil). The decrease in reproductive success may also be explained by a direct effect of C_{60} exposure on the reproductive organs. Histological observations of the clitellum, an important part of the earthworm reproductive system, showed a damaged parenchyma of the clitellum in C_{60} exposed earthworms.

The mg/kg concentrations used in the present study demonstrate toxic effects due to four weeks up to lifelong exposure. A no observable adverse effect level (NOAEL) could be established. Verified measurements of actual soil C_{60} concentrations present in the environment are lacking. However, modelled environmental concentrations of C_{60} predict an annual increase of C_{60} concentrations in the soil at the ng/kg level (Gottschalk et al. 2009). C_{60} is very stable, so persistence of these nanoparticles can be expected (Nielsen et al. 2008). Furthermore, the predicted values for C_{60} in sewage sludge are approaching the mg/kg range (Gottschalk et al. 2009) and this waste is often applied to agricultural soils. The commercial production of C_{60} is also expected to increase in the coming decades (Nielsen et al. 2008). Taken together, these observations suggest that the exposures in the present study are 1000 fold higher than modelled environmental C_{60} concentrations in soils at the moment, but only a lowest observed adverse effect level (LOAEL) was established in the present study and environmental concentration are likely to increase with a growing production and the environmental persistence of C_{60}.

In conclusion, this study demonstrates sub-lethal effects of C_{60} exposure at the level of gene expression and tissue integrity on earthworms. The gene expression data demonstrated down-regulation of some stress and immune-related genes, but no response of antioxidant enzyme expression or activity was observed. Histological observations confirmed tissue injuries and showed that the external barriers (cuticle and gut epithelium) of the C_{60} exposed earthworms were partly damaged. The surviving earthworms appeared to cope with the injuries during their life time and even showed evidence of tissue repair. However, these sub-lethal changes resulted in effects on growth and reproduction, as shown in an earlier study on the same experiments (**Chapter 2**). This indicates that repair of effects at a

lower level of biological integrity may result in effects at higher levels of biological organisation. Further research is needed to identify the precise mode of action of (chronic) C_{60} exposure to earthworms, with emphasis on the time-sequence of expression of genomic effect markers and the appearance of (tissue) pathology, reproductive failure, growth retardation and finally death of the earthworms. Also, a range of concentrations below 15.4 mg C_{60}/kg soil needs to be studied to assess the NOAEL for C_{60} exposure to earthworms.

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Supplementary data

Table S1. Relative gene expression levels (compared to the average of the control) of superoxide dismutase (SOD), glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and phosphofructokinase-1 (PFK-1) for the four weeks and for the lifelong treated *L rubellus* earthworms, exposed to different concentrations of C_{60} in the soil. No significant differences were demonstrated between treatments, within the assays ($\alpha = 0.05$). Data are shown per exposure group (0, 15.4 and 154 mg C_{60}/kg soil), as mean values ± S.E.

Exposure period	[C_{60}] (mg/kg soil)	SOD	GAPDH	PFK1
Four weeks	0	-	1.0 ± 0.35	-
Four weeks	15.4	-	0.8 ± 0.44	-
Four weeks	154	-	0.8 ± 0.37	-
Lifelong	0	1.0 ± 0.27	1.0 ± 0.26	1.0 ± 0.10
Lifelong	15.4	1.1 ± 0.07	1.8 ± 0.39	0.8 ± 0.11
Lifelong	154	0.54 ± 0.29	0.7 ± 0.05	0.8 ± 0.18

Table S2. Quantitative histology of the ectodermal layers (cuticle, epidermis and the circular and longitudinal muscles) of *L. rubellus* earthworms, showing either the thickness of each tissue layer in the ectoderm (µm, ectoderm thickness) for the four weeks exposure or the percentage thickness of each tissue layer (% of ectoderm thickness) for the lifelong exposed earthworms. Data are means ± S.E. and data are only given where tissue structure enabled accurate measurements to be made. *p<0.05 compared to the control. **Tissue injury prevented measurements on more than one earthworm.

[C_{60}] (mg/kg soil)	Exposure period	Cuticle	Epidermis	Parenchyma	Circular muscle	Longitudinal muscle
		Anterior segments				
0	4 weeks	0.32 ± 0.10	7.89 ± 2.43	-	5.53 ± 0.73	-
15	4 weeks	0.77 ± 0.41	4.36 ± 1.51	-	6.04 ± 0.54	-
154	4 weeks	0.28 ± 0.04	2.93 ± 0.38	-	5.42 ± 0.76	-
		Anterior segments				
0	Lifelong	0.7 ± 0.3	15.5 ± 3.2	-	13.3 ± 2.5	58.4 ± 18.6
15	Lifelong	0.1 ± 0.1	17.9 ± 2.9	-	14.7 ± 2.8	64.2± 1.3 *
154	Lifelong	0.4 ± 0.26	9.7 ± 3.0	-	11.4 ± 7.4	73.6 ± 1.5
		Clitellum segments				
0	Lifelong	-	10.41**	43.1 ± 16.5	3.1 ± 1.0	37.5 ± 20.0
15	Lifelong	0.1 ± 0.1	16.0 ± 1.0	39.5 ± 15.9	9.2 ± 2.8	45.0 ± 9.4
154	Lifelong	0.5 ± 0.26	14.8 ± 7.8	18.0 ± 9.0	7.5 ± 1.5	43.2 ± 18.0
		Posterior segments				
0	Lifelong	0.4 ± 0.2	20.2 ± 6.7	-	14.3 ± 4.4	59.6 ± 9.1
15	Lifelong	1.0 ± 0.2	11.0 ± 2.1	-	11.5 ± 1.7	74.9 ± 2.4
154	Lifelong	0.5 ± 0.5	20.7 ± 6.7	-	7.69**	63.6 ± 5.9

Chapter 4

In vitro nanoparticle toxicity to rat alveolar cells and coelomocytes from the earthworm *Lumbricus rubellus*

Based on:

MJC van der Ploeg, JHJ van den Berg, S Bhattacharjee, LHJ de Haan, DS Ershov, RG Fokkink, H Zuilhof, IMCM Rietjens, NW van den Brink. *In vitro* nanoparticle toxicity to rat alveolar cells and coelomocytes from the earthworm *Lumbricus rubellus*. Nanotoxicicology, in press, DOI: 10.3109/17435390.2012.744857.

Abstract

Sensitivity of immune cells (coelomocytes) of *Lumbricus rubellus* earthworms was investigated for exposure to selected nanoparticles, in order to obtain further insight in mechanisms of effects observed after *in vivo* C_{60} exposure. In the *in vivo* study, tissue damage appeared to occur without accompanying increased immune responses. Coelomocytes exposed *in vitro* to C_{60} showed no decrease of their cellular viability, but demonstrated a decrease in gene expression of the cytokine-like protein CCF-1, indicating immunosuppression. Experiments with NR8383 rat macrophage cells and tri-block copolymer nanoparticles were used to compare sensitivity and to demonstrate the usefulness of coelomocytes as a test system for nano-immunotoxicity, respectively. Overall, the results imply that sensitivity towards nanoparticles differs between cell types and nanoparticles. Moreover, this study indicates that injuries in absence of an immune response, observed after *in vivo* C_{60} exposure in **chapter 3**, are caused by immunosuppression rather than coelomocyte mortality.

Introduction

Due to the rapid increase of nanoparticle production, these particles are expected to end up in the environment (Kahru and Dubourguier 2010; Nowack 2009). The possible environmental hazards of nanoparticles are not well known and nanoecotoxicological data should be obtained with high priority (Handy et al. 2008a; Stone et al. 2010). In our previous work (**Chapter 2 and 3**) we have shown that fullerene (C_{60}) exposure can affect the earthworm *Lumbricus rubellus*. In **chapter 2** it was demonstrated that *in vivo* exposure of these earthworms to C_{60} in the soil caused a reduction of cocoon production and juvenile survival and growth. Investigation at the molecular level (**Chapter 3**) demonstrated a concentration-dependent down-regulation of the gene expression of heat shock protein 70 (HSP70), a well-known effect marker for generic stress (van Straalen and Roelofs 2006), and of coelomic cytolytic factor-1 (CCF-1), a cytokine-like protein in earthworms with similar functions as the mammalian tumor necrosis factor-alpha (TNF-α) (Bilej et al. 1995; Olivares Fontt et al. 2002). This down-regulation instead of up-regulation of CCF-1 indicated a reduced instead of an increased immune response in earthworms upon C_{60} exposure. The exact mode of action underlying this effect, immunosuppression or mortality of immune cells, remained to be elucidated. Additionally, histological observations of the C_{60} exposed earthworms demonstrated damage to the external barriers (cuticle and gut epithelium), in combination with strong effects on the structure of muscle tissues (**Chapter 3**). In general, tissue injury is associated with an inflammatory response of the coelomocytes, as part of tissue repair (Cikutovic et al. 1999; Goven et al. 1994). However, no inflammatory response was observed in our previous study described in **Chapter 3**. Perceiving such injuries in the absence of an expected immune response may be explained by either immunosuppression or mortality of the coelomocytes, important cells in the earthworm immune system (Stebbing 1981; van Straalen and Roelofs 2006).

Coelomocytes are free flowing cells, present in the coelomic cavity of earthworms. These cells are responsible for cellular immune functions, including phagocytosis and release of cytokine-like proteins to stimulate immune responses (Adamowicz and Wojtaszek 2001; Adamowicz 2005; Bilej et al. 1990; Diogéne et al. 1997; Hrženjak et al. 1992; Engelmann et al. 2005; Kauschke et al. 2007; Ville et al., 1995; Weeks and Svendsen 1996).

Coelomocytes have already been used in several ecotoxicology studies to demonstrate effects of conventional toxic compounds, and these studies demonstrated that the model provides a simple, rapid and sensitive tool to assess immunotoxicological hazards (Burch et al. 1999; Calisi et al. 2009; Massicotte et al. 2004; Sauvé et al. 2002; Sauvé and Fournier 2005; Scott-Fordsmand et al. 1998; Ville et al. 1995).

In order to address the impact of nanoparticle exposure on the immune cells of earthworms in more detail, coelomocytes were isolated from the earthworm *L. rubellus* and exposed to C_{60} *in vitro*. These coelomocytes were analyzed for their viability, phagocytic activity and CCF-1 gene expression levels. Coelomocytes were also exposed to tri-block copolymer nanoparticles (Bhattacharjee et al. 2011), in order to validate the use of coelomocytes as a test system for immunotoxicity of nanoparticles. Finally, for comparison, mammalian rat lung macrophage NR8383 cells were also exposed to the same nanoparticles, characterizing their effects on cell viability, phagocytosis and production of TNF-α.

Materials and methods

Nanoparticles and characterization

Positively (with an amine terminal group; PNP-NH_2) and negatively (with a carboxylic acid terminal group; PNP-COOH) charged tri-block copolymer nanoparticles, with and without fluorescent probe, were prepared as described by Bhattacharjee et al. (2011). The stock consisted of polymer nanoparticles suspended in nanopure water. For the experiments, serial dilutions of exposure medium were made in ultrapure water, and for all concentrations the same amount (20%) of Ham's F-12K (Kaighn's) medium (culture medium; from Invitrogen/Life Technologies Ltd, UK) containing 10% heat-inactivated fetal calf serum (FCS; from PAA Laboratories GmbH, Germany) was added. C_{60} nanoparticle powder was obtained from SES Research (99.5+%, Houston USA). As discussed in **chapter 2**, an analysis with inductively coupled plasma-atomic emission spectroscopy (ICP-AES) on the C_{60} powder demonstrated that metal impurities in the C_{60} did not pose any risk. For the experiments, a stock of 8 mg/ml C_{60} powder in culture medium supplemented with 10% FCS was made, which was stirred overnight to obtain an

even suspension. Serial dilutions of exposure medium were made with the culture medium containing 10% FCS. The size distribution of the nanoparticles was determined by dynamic light scattering (DLS), using a Cobolt Samba 300mW DPSS laser at a wavelength of 532 nm. For each sample, the mean diameter of the peaks with the associated polydispersity index (PDI), an indication of the width of the diameter peak, was determined for twenty measurements. Due to the presence of more than one peak for the C_{60} samples, the PDI was established by determining the width of each peak at half-height and using that width in the equation PDI = [width/radius]^2. The polymer nanoparticles were characterized in ultrapure water as well as in culture medium containing 10% FCS, at a concentration of 0.1 µg/ml. C_{60} characterization was only performed in culture medium supplemented with 10% FCS. Two C_{60} concentrations (10 and 100 µg/ml) were tested, to study possible changes in aggregation with an increase in C_{60} concentration. The zeta potential was analyzed using a Malvern ZetaSizer 2000. Suspensions were similar to the ones used for the DLS analysis. Data were processed with Zeta mode V1.51 software (Chen et al. 2004). Five measurements were used to establish average and SEM for each sample. Aqueous dispersions of the polymer nanoparticles were also examined using a field emission scanning electron microscope, as reported by Bhattacharjee et al. (2011). C_{60} was viewed by transmission electron microscopy (TEM), at a concentration of 10 µg C_{60}/ml culture medium. A drop of sample was deposited on a copper grid coated with formvar and carbon. After an incubation of two minutes, excess suspension was carefully removed with a filter paper and the grid was allowed to dry at room temperature. The grids were observed in a JEOL 2100 TEM (Jeol, Japan), operating at 200 kV. Micrographs were taken with a GATAN US4000 4K digital camera.

Cell types

Coelomocytes

Primary immune cells (coelomocytes) in the coelomic fluid were extruded from unexposed adult *L. rubellus* earthworms. Coelomocyte extrusion was performed using a modification of the extrusion method as described by Eyambe et al. (1991). 24 Hours before extrusion, earthworms were rinsed in demineralized water, placed in a petri dish on a moist filter

paper (Whatman filters type 597, Fisher Scientific) and incubated in a climate room at 15°C. After 24 hours, the earthworms were rinsed again, placed on paper towel and massaged to expel the contents from the gut. 24 hour starvation and the massage were performed in order to prevent fecal contamination of the extrusion medium upon collection of the coelomocytes in a subsequent step. Subsequently, individual earthworms were placed in 10 ml tubes and 3 ml extrusion medium was added. The extrusion medium consisted of 95% phosphate-buffered saline (PBS), supplemented with 2 mg/ml ethylene-diaminetetraacetic acid (EDTA, 99% purified grade; from Sigma-Aldrich, St Louis, USA) and 4 mg/ml guaiacol glycerol ether (GGE, >98%; from Sigma-Aldrich, St Louis, USA), adjusted to pH 7.3 with sodium hydroxide. Just before extrusion, 5% (of final volume) of 96%-ethanol was added to the medium. The earthworms were incubated in the extrusion medium for three minutes, during which they excreted coelomocytes into the medium. After three minutes the earthworms were removed from the medium, rinsed in demineralized water and released in soil. The tubes with the coelomocyte suspensions were put on ice and 5 ml of culture medium containing 10% FCS was added to each tube. The coelomocytes were centrifuged (at 200 g for 10 minutes) and washed with culture medium to remove the extrusion medium. Subsequently, the coelomocytes were resuspended in 200 µl culture medium containing 10% FCS and the coelomocyte suspensions from the different earthworms were combined. Cells were counted using a Bürker bright line counting chamber and cell number was adjusted to approximately 4 x 10^6 cells/ml. Thereafter, 50 µl or 150 µl of the adjusted cell suspension was added to each well of a 96-well or a 48-well plate (with a flat-bottom; from Greiner Bio-One, Germany), respectively, and directly used in the experiments.

NR8383 cells

NR8383 rat macrophage cells (ATCC, Manassas, USA) were cultured and obtained according to the procedure described by Bhattacharjee et al. (2011). For exposure, 50 µl of a cell suspension (of approximately 4 x 10^6 cells/ml) was added to each well of a 96-well plate and directly used in the experiments.

Cellular uptake of nanoparticles by coelomocytes

For studying effects of inhibition of clathrin-mediated and caveolin-mediated endocytosis, sucrose and MβCD pre-incubations were carried out. The procedure has been described in detail by Bhattacharjee et al. (2011), who studied the same nanoparticles in NR8383 cells. After pre-incubation with the inhibitors, the cells were plated, exposed to the fluorescent polymer nanoparticles (at 1 μg/ml) and incubated at 15°C overnight. After the incubation, coelomocytes were studied with a confocal laser scanning microscope (CLSM; Zeiss 200M Axiovert). Details on the CLSM procedure can be found in Bhattacharjee et al. (2011). Z-stack imaging of coelomocytes was performed, to ascertain that the particles were inside the coelomocytes and not on the surface (Cartiera et al. 2009). Samples with coelomocytes exposed to PNP-NH_2 or PNP-COOH in the absence of inhibitors were also analyzed, for comparison.

Cell survival and phagocytic activity

To quantify cell survival and phagocytic activity, serial dilutions of exposure medium were made as discussed before. Subsequently, 50 μl of exposure medium was added to 50 μl of cell suspension, to obtain the acquired final concentrations and expose the cells. The polymer nanoparticles used in these experiments were non-fluorescent, to avoid interference with the fluorescent beads used in the phagocytosis assay. Culture medium supplemented with 10% FCS only and culture medium containing 10% FCS as well as $CuSO_4$ (100 μM for the NR8383 cells and 300 μM for the coelomocytes) were used as negative and positive control, respectively. Fluorescent beads (size 1 um, L-1030 latex beads; from Sigma-Aldrich, St Louis, USA) were added to the wells at a cell to bead ratio of 1:50. For the coelomocytes, the beads were added before overnight incubation and for the NR8383 cells the beads were added only four hours before scoring the phagocytosis, because coelomocytes have a lower uptake rate than NR8383 cells (Brousseau et al. 1997). The cells were incubated overnight at 37°C (NR8383 cells) or 15°C (coelomocytes). To test for viability of the cells, a trypan blue solution (in PBS) was added to each well (final concentration 0.05%). From each well, a sample was taken for quantification of the cell viability and the phagocytic activity, using a light microscope (Olympus CK2) and a

fluorescent microscope (Olympus T2) with fluorescent light (Olympus BH2-RFL-T2), respectively. For each sample the number of blue cells per 100 cells was counted randomly to assess viability of the cells. Quantification of the phagocytic activity was achieved using the procedure discussed by Bhattacharjee et al. (2011).

Cytokine (-like) assays

Measurement of CCF-1 gene expression in coelomocytes

Coelomocytes (150 µl/well) were seeded in a 48-well plate and 150 µl of serial dilutions of the C_{60} exposure medium were added to each well, to obtain the acquired final exposure concentrations. Exposing cells to culture medium containing 10% FCS only was tested as a negative control. The coelomocytes were exposed overnight and then RNA was extracted, using a Qiashreddar and a RNeasy kit (Qiagen GmbH, Germany). RNA concentrations were determined by a Nanodrop spectrophotometer (ND-1000 Spectrophotometer, from Thermo Scientific, USA). For each sample, 300 ng total RNA (15 ng/µl as final concentration) was reverse transcribed to cDNA using iScript Reverse Transcription Supermix (Bio-rad Laboratories, USA). Expressed Sequence Tags (ESTs), and forward and backward primers for the target gene CCF-1 and the reference gene (glyceraldehyde-3-phosphate dehydrogenase (GAPDH)) were verified and designed as discussed in **chapter 3**. The primers were obtained from Biolegio (the Netherlands). Using these primers, real-time quantitative polymerase chain reaction (qPCR) was conducted with iQ Sybr Green Supermix (Bio-rad Laboratories, USA). All samples were diluted 40 times and a dilution series of a mix of all the samples was also tested. This dilution series was used to make a dissociation curve afterwards, to be able to eliminate outliers. Each well of a 96-well plate contained 5 µl of a sample (equivalent to 15 ng cDNA) along with 400 nM of each primer, in a total volume of 25 µl. The plate was centrifuged for one minute (at 120 *g*), to ensure a homogenous mixture on the bottom of the plate without bubbles. The cycling program was started at 95°C for fifteen minutes to activate the DNA polymerase, followed by 45 cycles of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The program was completed with 72°C for five minutes and 95°C for one minute. Thereafter melting curves were performed by iCycle (Bio-rad Laboratories, USA) to validate that only one target had

been amplified. With this program, threshold cycles and amplification efficiencies were also calculated for each sample. Samples with distinct divergence from the dissociation curve were omitted from further analysis (Heckmann et al. 2006). The induction factor was calculated for each sample, using the reference gene to normalize for reverse transcription efficiency (Pfaffl 2001).

Measurement of TNF-α produced by NR8383 cells.

The NR8383 cells were seeded in a 96-well plate (final concentration of 2 x 10^5 cells/well) and serial dilutions of the exposure medium were added to obtain the acquired final C_{60} concentrations. Exposure of the cells to culture medium containing 10% FCS only was used as a negative control. After 24 hours, 50 µl was taken from each sample and added to the anti-rat TNF-α antibody pre-coated 96-well plate of a commercially available rat TNF-α ELISA kit (Thermo Fisher Scientific, Rockford, USA). TNF-α levels were analysed according to the protocol described by the ELISA kit manufacturer.

Statistics

All experiments were performed in triplicate. The results were calculated as % of the respective negative control and are presented as mean of the replicates ± standard error of the mean (SEM). Data were analyzed in GENstat (14^{th} Edition, VSN International Ltd.). Differences between treatments were analyzed using analysis of variance (ANOVA, with $\alpha = 0.05$), with Least Significant Differences (LSD) as the post-hoc test (Burgers and Oude Voshaar 2010). $\alpha = 0.05$ is considered to be the level of significance and marked with an asterisk sign (*). In specific cases a trend at the level of $\alpha = 0.10$ was determined and noted by a hashtag (#). When possible, concentration response curves were generated, by applying logistic nonlinear regression analysis. EC_{50} was defined as the concentration of the nanoparticle exposure that led to an effect of 50%, compared with the negative control, of the recorded response.

Results

Characterization of the nanoparticles

In the present study two types of nanoparticles were applied: C_{60} and tri-block copolymer nanoparticles. C_{60} was already studied previously in an *in vivo* toxicity study with *L. rubellus* earthworms (**Chapter 2 and 3**). The polymer nanoparticles were used because they enabled uptake studies in our *in vitro* cellular system, given the fact that they can be prepared containing a fluorescent label.

The synthesis and characterization of the polymer nanoparticles was described in detail before by Bhattacharjee et al. (2011). In short, the size distribution of both PNP-NH_2 and PNP-COOH as analyzed by DLS, was 45 ± 5 nm when dispersed in ultrapure water. Scanning electron microscope imaging confirmed the presence of particles of this size in the dispersions. In culture medium supplemented with 10% FCS, however, PNP-NH_2 showed a size distribution of 106 ± 5 nm and PNP-COOH of 67 ± 5 nm. The zeta potential in water was +20 mV for PNP-NH_2 and -22 mV for PNP-COOH. In culture medium containing 10% FCS though, the zeta potential was -12 mV and -35 mV for PNP-NH_2 and PNP-COOH, respectively.

For C_{60} in culture medium supplemented with 10% FCS, analysis by DLS demonstrated that a prominent component was approximately 200 nm in size for both examined concentrations. Lower peaks were also observed, indicating the presence of much smaller (26 and 5 nm) and larger (~10 μm) particles, in addition to the prominent 200 nm particles. The zeta potential of C_{60} in culture medium supplemented with 10% FCS was -14 mV at 10 μg/ml and -13 mV at 100 μg/ml. In the TEM images, C_{60} clusters (nC_{60}) with a size between 100 and 200 nm were observed (Figure 1A), which contained the crystalline structure of C_{60} (Figure 1B). Furthermore, the electron diffraction demonstrated discrete spots (not shown), suggesting a crystalline structure pattern (Bokare and Patnaik 2005; Deguchi et al. 2001).

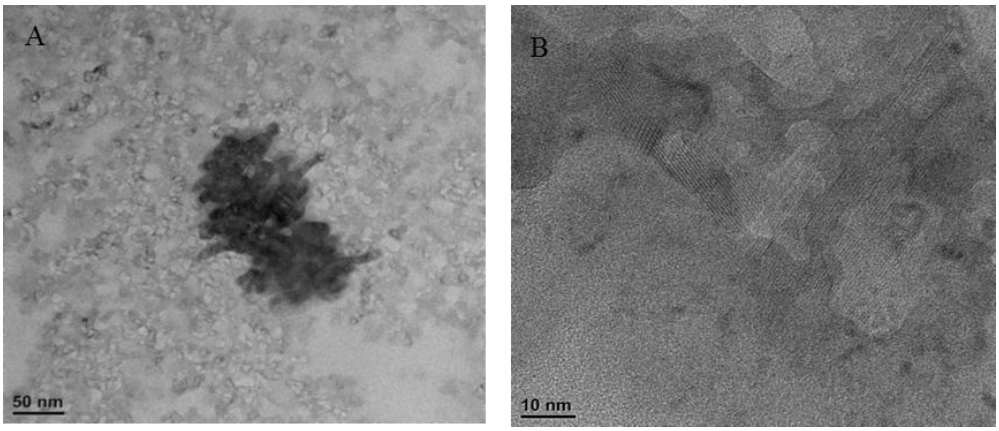

Figure 1. TEM images of (A) a C_{60} cluster (nC_{60}) in culture medium and (B) a close up of the C_{60} cluster showing the crystalline structure known for C_{60} nanoparticles (Bokare and Patnaik 2005; Deguchi et al. 2001).

Cellular uptake of nanoparticles

Significant cellular uptake of the polymer nanoparticles by coelomocytes could be detected, after incubation with the fluorescent polymer nanoparticles, using Z-stack imaging of coelomocytes (Figure 2). These images indicated that PNP-COOH were more clustered and that the PNP-NH_2 nanoparticles were spread more diffusely in the cytoplasm of the coelomocytes. The overall uptake did not differ significantly between PNP-NH_2 and PNP-COOH.

After inhibition of the specific endocytosis pathways, cellular uptake of PNP-COOH was affected for both the caveolin- and clathrin-mediated pathways, to 56% and 65% compared to the control, respectively (Figure 3A). For PNP-NH_2, cellular uptake was especially reduced after inhibition of the clathrin-mediated endocytosis pathway, to 59% of the control (Figure 3B). Together these data reveal that the coelomocytes, in analogy with rat macrophage NR8383 cells studied before, are able to internalize nanoparticles of different charges by several active uptake mechanisms.

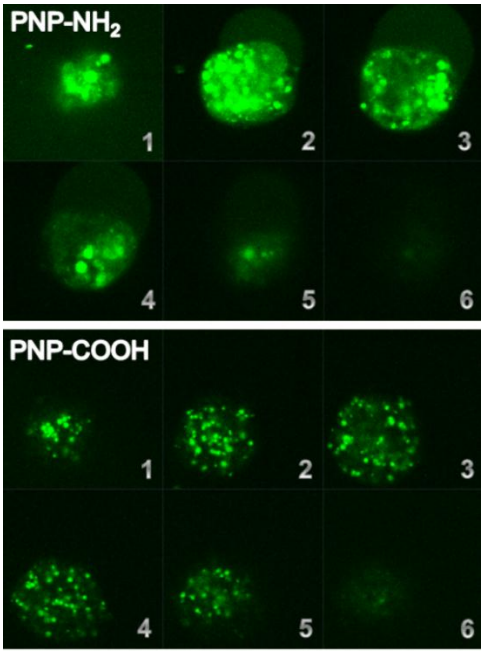

Figure 2. CLSM images of cross sections of a coelomocyte after overnight exposure to PNP-NH_2 (top) or PNP-COOH (bottom). The results are shown as z-stack images, where slice 1 demonstrates the bottom and slice 6 the top part of the cell.

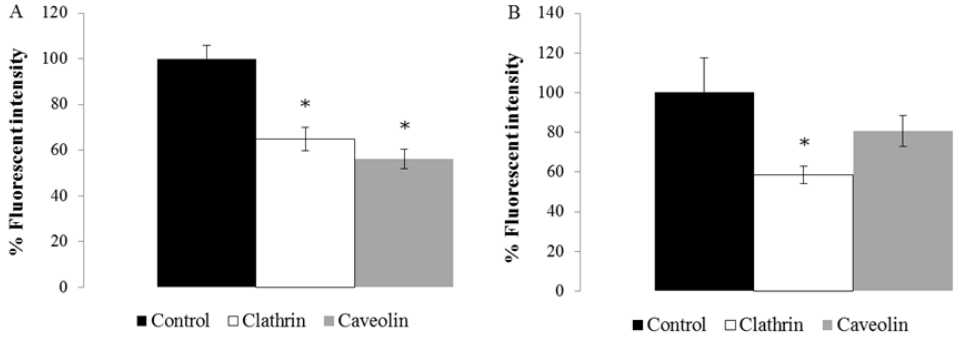

Figure 3. Fluorescent intensity of (A) PNP-COOH and (B) PNP-NH_2 internalized by coelomocytes, for control (black) and for specific inhibition of the clathrin- (white) or caveolin-mediated (grey) endocytosis pathways. Values are shown as mean % of the respective control ± SEM and statistical different values from the respective control are marked with an asterisk (*).

Cell survival and phagocytic activity

For the coelomocytes exposed to PNP-COOH, no clear concentration-dependent decrease for viability as well as phagocytic activity was observed (Figure 4A and 4C). The results presented in figures 4B and 4D show that PNP-NH_2 did induce a concentration-dependent reduction of the viability and phagocytic activity of the coelomocytes. EC_{50} values of 45 μg/ml for the effects of PNP-NH_2 exposure on cell viability (Figure 4B), and of 7.8 μg/ml for the effects on phagocytic activity (Figure 4D) were derived. For comparison, the effects of PNP-NH_2 on viability and phagocytosis of NR8383 are also displayed (Figure 4) and these data reveal that these cells also only demonstrated adverse effects on cell viability and phagocytic activity after exposure to PNP-NH_2, with EC_{50} values of 31 μg/ml and 64 μg/ml, respectively.

As shown in figure 5A, C_{60} exposed coelomocytes demonstrated no decrease in cell viability. However, a concentration-dependent decrease of the phagocytic activity was observed for these cells, with an EC_{50} value of 2000 μg/ml (Figure 5B). For comparison, the effect of increasing C_{60} concentrations on viability and phagocytic activity of NR8383 macrophage cells was also tested (Figure 5). For these cells, exposure to C_{60} resulted in a concentration-dependent decrease of cell viability as well as of the phagocytic activity. The EC_{50} values for these effects of C_{60} exposure were 1.04 μg/ml and 1.87 μg/ml, respectively.

Cytokine (-like) assays

CCF-1 gene expression in coelomocytes (Figure 6A) decreased at lower concentrations, but an increase was observed from 4000 μg C_{60}/ml culture medium onwards, which is just above the EC_{50} of phagocytic activity. An EC_{50} for the effect of C_{60} on CCF-1 activity could not be derived, but the lowest observable effect concentration (LOEC) was 100 μg/ml.

In addition, a considerable increase of TNF-α levels produced by the NR8383 cells was observed with increasing C_{60} concentrations, starting from a concentration around the EC_{50} for phagocytic activity (Figure 6B). At the highest C_{60} concentration of 400 μg/ml, the TNF-α level was seven times higher compared to the negative control. As the effect curve

was still in the exponential stage at the highest concentration tested, no EC_{50} could be established. The LOEC could be derived, which was 1 µg C_{60}/ml culture medium.

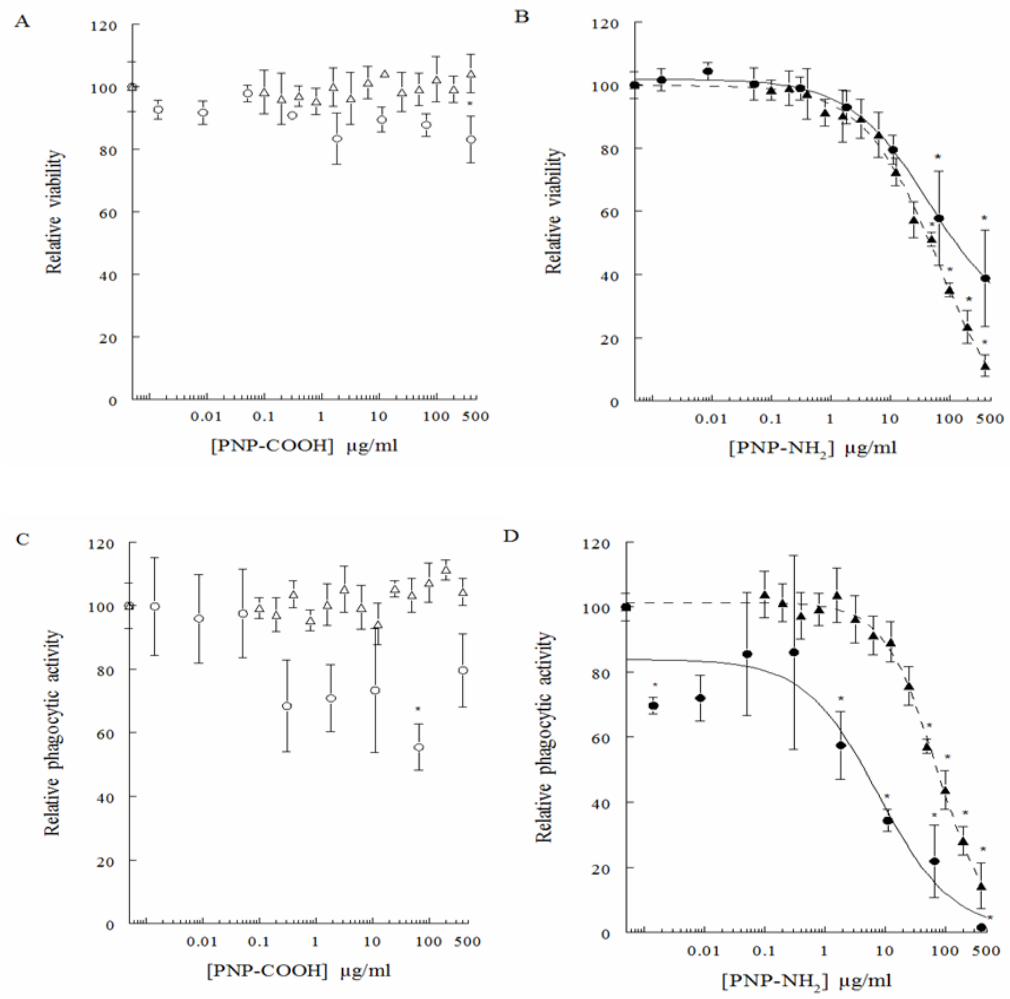

Figure 4. Viability (A and B) and phagocytic activity (C and D) of NR8383 cells (triangles) and coelomocytes (circles), compared to the respective unexposed control (= 100%), after exposure to increasing PNP-COOH (open, A and C) and PNP-NH_2 (filled, B and D). Values are presented in mean ± SEM, with an asterisk (*) indicating statistical difference from the control. Only significant regression curves are displayed (coelomocytes normal line and NR8383 cells dashed line).

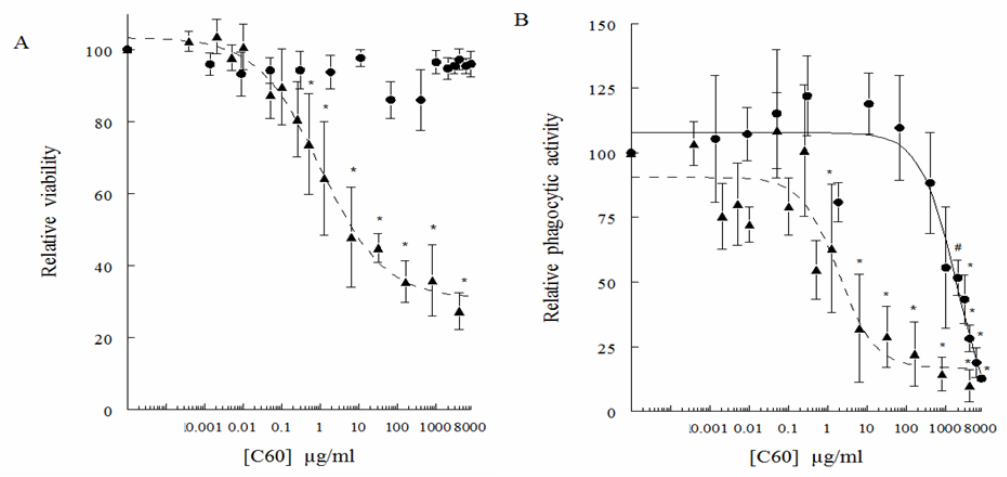

Figure 5. Viability (A) and phagocytic activity (B) of NR8383 cells (triangle) and coelomocytes (round) after exposure to increasing C_{60} concentrations. Values are expressed compared to the control (= 100%) and are shown in mean ± SEM. An asterisk (*) indicates a statistically significant difference compared with the respective control at the level of α=0.05 and hashtag (#) indicates a significant difference with α=0.10. Only significant regression curves for the coelomocytes (normal line) and the NR8383 cells (dashed line) are displayed.

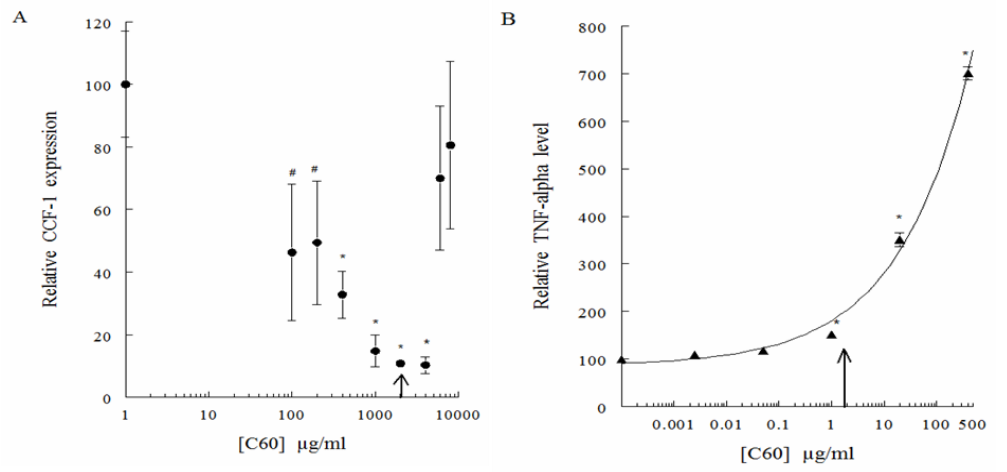

Figure 6. CCF-1 gene expression levels of coelomocytes (A) and TNF-α levels in NR8383 cells (B) exposed to increasing C_{60} concentrations. Values are shown in mean ± SEM. Statistical differences compared to the respective control are marked with an asterisk (*) for α=0.05 and with a hashtag (#) at the level of α=0.10. The arrow indicates the EC_{50} value of the phagocytic activity.

Discussion and conclusions

Our previous work (**Chapter 2 and 3**) has demonstrated toxic effects of *in vivo* C_{60} exposure on *L. rubellus* earthworms, as reflected by adverse effects on reproduction, juvenile survival and growth, tissue integrity and by altered gene expression of HSP70 and CCF-1. In the present study we investigated the impact of nanoparticle exposure, including C_{60}, towards isolated coelomocytes of the earthworm *L. rubellus*. These investigations were performed in order to obtain insight into the sensitivity of these important cells of the earthworm's immune system to nanoparticle exposure.

For the polymer nanoparticles, it is reported that they do not have a strong tendency to aggregate (Bhattacharjee et al. 2011). The hydrodynamic diameter of the polymer nanoparticles, analyzed by DLS, was larger in culture medium containing 10% FCS compared with water. This difference was especially observed for PNP-NH_2 and may be due to negatively charged proteins present in the medium, which have a preference for adsorption on positive nanoparticles (Cherng et al. 1996; Montes-Burgos et al. 2010). Adsorption of (negatively charged) proteins on the polymer nanoparticles was illustrated by the zeta potential data of the polymer nanoparticles measured in culture medium containing 10% FCS. These data demonstrate a weak negative charge for PNP-NH_2 and a stronger negative charge for PNP-COOH, as compared with the zeta potential in water. Even though the PNP-NH_2 were coated with a protein corona and their zeta potential had changed, they were readily taken up by the coelomocytes, which was also observed in other studies investigating the uptake of positively charged nanoparticles (Asati et al. 2010; Bhattacharjee et al. 2011; Chen et al. 2011). Studies by Lesniak et al. (2010) and Maiorano et al. (2010) have demonstrated that the stability of the protein corona may vary and does not always oppose the uptake of nanoparticles. Therefore, the protein corona of the PNP-NH_2 may have been quite unstable, not hindering cellular uptake and subsequent adverse effects on the cells.

C_{60} size distribution and aggregation in culture medium supplemented with 10% FCS was assessed using DLS. The prominent size distribution was about 200 nm, for both

concentrations analyzed. TEM imaging also demonstrated clusters within this size range and moreover, the observed crystalline structure pattern indicates that they contained C_{60}. The large clusters observed, may be due to aggregates of C_{60} (Gelderman et al. 2008). Measurements of C_{60} in culture medium containing 10% FCS showed low negative zeta potential values, which is in line with observations reported for C_{60} solutions in other studies (Chen and Elimelech 2006; Spohn et al. 2009; Han and Karim 2008). These slightly negative zeta potentials were not strong enough to cause electrostatic repulsion of the particles, suggesting that the system was not stable and agglomeration of particles was to be expected (Berg et al. 2009; Jiang et al. 2009; Xu et al. 2007).

The CLSM observations for cellular uptake of the fluorescent polymer nanoparticles, demonstrated that both PNP-NH_2 and PNP-COOH were internalized. However, these observations indicated that PNP-COOH were clustered, most likely in lysosomes, but PNP-NH_2 were distributed more diffusely throughout the coelomocytes. This difference was also noted for NR8383 cells, by Bhattacharjee et al. (2011). When observing the inhibition of the specific pathways, the CLSM data indicate that for the positively charged nanoparticles only inhibition of the clathrin-mediated endocytosis had a significant effect on the uptake. Bhattacharjee et al. (2011), using NR8383 cells, also observed the clathrin-dependent endocytosis to be of importance for the cellular uptake of especially PNP-NH_2. This finding indicates that endocytosis through the clathrin-mediated pathway is a possible mechanism of internalization for PNP-NH_2, for both NR8383 cells and coelomocytes. Other studies (Harush-Frenkel et al. 2007; Ma and Lim 2003), using various cultured cells, also demonstrated that clathrin-mediated endocytosis was mainly involved in the uptake of positively charged nanoparticles. Uptake of PNP-COOH was significantly reduced after inhibition of both the caveolin- and clathrin-mediated endocytosis pathways. This differs from the expectations, because Bhattacharjee et al. (2011) found that the caveolin-mediated endocytosis was a more important pathway for uptake of PNP-COOH by the NR8383 cells, than the clathrin-mediated pathway. Overall, the data on the cellular uptake of the polymer nanoparticles demonstrate that both nanoparticles were internalized by the coelomocytes, and that the caveolin- and clathrin-mediated pathways were involved. The CLSM data also

indicate that distribution within the coelomocytes differed between PNP-NH_2 and PNP-COOH, e.g. PNP-NH_2 were more present in the cytosol of the cells.

Even though no evident difference was noted between the total uptake levels of the differently charged polymer nanoparticles, they induced different toxicity to the coelomocytes. PNP-COOH induced no clear cytotoxicity, but exposure of the coelomocytes to PNP-NH_2 caused significantly lower cell viability and phagocytic activity. Bhattacharjee et al. (2011) observed similar effects for NR8383 cells, showing cytotoxicity for PNP-NH_2 but not for PNP-COOH. PNP-NH_2 may be more cytotoxic due to their more diffuse distribution. These nanoparticles may instigate production of reactive oxygen species (ROS) and interact with mitochondria, thereby hampering normal mitochondrial functioning and causing depletion of the high energy substrate adenosine-5'-triphosphate (ATP) (AshaRani et al. 2008; Bhattacharjee et al. 2011; Karatas et al. 2009; Pan et al. 2009; Xia et al. 2008).

Several *in vitro* studies have demonstrated toxicity due to C_{60} exposure in mammalian cells (Gelderman et al. 2008; Jacobsen et al. 2008; Jia et al. 2005). In these studies, DNA damage, loss of phagocytic ability, cell cycle arrest and increased influx of calcium were detected. To the knowledge of the authors, this is the first study discussing *in vitro* C_{60} exposure of earthworm coelomocytes. The results demonstrate hardly any effect of C_{60} exposure on coelomocyte survival. The phagocytic activity was affected at high concentrations, with significantly decreased activity from 2000 µg C_{60}/ml culture medium onwards, down to 13% phagocytic activity compared with the control at 8000 µg C_{60}/ml culture medium. CCF-1 gene expression of coelomocytes appeared to be a more sensitive parameter, showing a statistically significant decrease already at the lowest tested C_{60} concentration of 100 µg/ml. Decreased CCF-1 expression was also observed in the earthworms exposed *in vivo* in our previous study (**Chapter 3**). This demonstrates that, at relatively low concentrations, C_{60} exposure may suppress an inflammatory reaction of the coelomocytes. Other studies have also demonstrated decreases in phagocytic activity in combination with a reduction in cytokine release, after exposure to magnetic and cadmium

quantum dot (CdTe-QD) nanoparticles (Hsiao et al. 2009; Nguyen et al. 2012). Moreover, Nguyen et al. (2012) demonstrated that CdTe-QD nanoparticles, by lowering cytokine levels, could make the cells more sensitive to a bacterial infection. Such reduced resistance to opportunistic infections, in combination with the inability of the coelomocytes to induce adequate tissue repair (Goven et al. 1994; Napolitano et al. 1996), may explain the tissue injuries previously observed (**Chapter 3**).

At very high C_{60} concentrations, above the EC_{50} for phagocytosis, the measured CCF-1 expression increased again. For the NR8383 cells, the cytokine (TNF-α) level also showed an increase after phagocytic activity had decreased below 50%. However, NR8383 cells demonstrated a clear concentration-dependent cytotoxicity and inflammatory response (TNF-α increase), already at 1 μg/ml (close to the EC_{50} for phagocytosis which is 1.87 μg/ml). These differences between the cell types demonstrate that for viability and phagocytic activity, the coelomocytes appear to be less sensitive to C_{60} exposure than the NR8383 cells. However, the CCF-1 gene expression data and the data on the polymer nanoparticle exposure demonstrate that coelomocytes are a sensitive test system for determining immunotoxicity of nanoparticles.

Differences in sensitivity and intracellular processing between cell types after exposure to the same type of nanoparticle, has been demonstrated in other studies as well. Xia et al. (2008) observed that mouse macrophage (RAW 264.7) and human lung epithelial (BEAS-2B) cultured cells were more sensitive to cationic polystyrene nanosphere exposure, than human microvascular endothelial (HMEC), mouse hepatoma (HEPA-1), rat pheochromocytoma (PC-12) cells. However, only the BEAS-2B cells were found to internalize more nanoparticles than the other cell types. Nguyen et al. (2012) found that mouse macrophage cells (J774A.1) were more sensitive to CdTe-QD exposure than human colonic epithelial cells (HT29), in terms of metabolism loss, changes in morphology and rate of QD internalization. Differences in sensitivity and uptake of nanoparticles were also found between coelomocytes and a cultured cell line, in a study by Hayashi et al. (2012). This study demonstrated that coelomocytes from the earthworm *Eisenia fetida* were more

sensitive to silver nanoparticle exposure than the cultured human leukemia (THP-1) cell line and also accumulated more silver nanoparticles.

In conclusion, the results of this study demonstrate that earthworm immune cells can be a target for nanoparticles. The present study also shows the usefulness of primary immune cells, from *L. rubellus* earthworms, as a test system to determine immunotoxicity of nanoparticles on these earthworms.

For C_{60} nanoparticles, tissue injuries were demonstrated in the absence of an immune response, and this was ascribed to immunosuppression or mortality of immune cells (**Chapter 3**). The present *in vitro* study indicates that C_{60} does not affect the viability of the coelomocytes. However, C_{60} exposure reduced CCF-1 gene expression levels in *in vitro* exposed coelomocytes, similar to the effect after exposure to earthworms *in vivo*. Although *in vivo* observations are difficult to compare with *in vitro* effects and a relationship between coelomic fluid concentrations and body burden of C_{60} could not be established, the results of the present study suggest that similar mechanisms of action may apply to *in vitro* exposed cells and *in vivo* exposed organisms. In both studies, levels of CCF-1 were reduced due to C_{60} exposure, indicating that the tissue injuries at the organismal level may be associated with immunosuppression rather than mortality of the coelomocytes.

Acknowledgements

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Chapter 5

Effects of silver nanoparticles on *Lumbricus rubellus* earthworms and particle characterisation in relevant test matrices, including soil

Submitted for publication

Abstract

The impact of silver nanoparticles (AgNP) and silver nitrate ($AgNO_3$) on *Lumbricus rubellus* earthworms was assessed. Four week exposure to the highest AgNP treatment (154 mg Ag/kg) reduced growth and reproduction, compared with the control. $AgNO_3$ exposure also caused lowered reproduction, but not as much as the equivalent AgNP treatment. Lifelong exposure to the highest AgNP treatment caused complete juvenile mortality. All AgNP treatments induced tissue pathology. Population modelling demonstrated reduced population growth rates for the AgNP and $AgNO_3$ treatments, and no growth at the highest AgNP treatment, due to juvenile mortality. Analysis of AgNP treated soil samples revealed that single AgNP and AgNP clusters were present in the soil, and that total Ag in soil pore water remained high throughout the lifelong experiment. Overall, this study indicates that AgNP exposure may affect earthworm populations and that the exposure may be prolonged, due to continuous release of Ag to the soil pore water.

Introduction

Nanotechnology has an increasing societal impact, with great expectations for the use of nanoparticles in a wide range of applications including food additives, medical devices and soil remediation (Andreescu et al. 2009; Calzolai et al. 2012; Dekkers et al. 2011). Silver nanoparticles (AgNP) are among the most widely used nanoparticles in consumer products and inevitably, the unintended exposure of humans and the environment is of concern (Calzolai et al. 2012; Wijnhoven et al. 2010). Consequently, there have been efforts to collect data on the environmental hazards and behaviour of AgNP (Arnaout and Gunsch 2012; Stone et al. 2010; Wijnhoven et al. 2010). Kahru and Dubourguier (2010) evaluated the toxicity of nanoparticles on species from different levels in the aquatic ecosystem (including bacteria, algae, yeast, ciliates, crustaceans, nematodes and fish) from 77 studies, and classified AgNP as "extremely toxic". Several studies suggest that the toxicity of AgNP to the environment may be derived from the release of silver (Ag) ions, because ionic Ag is known as one of the most toxic heavy metal species, but the hazards of AgNP may not be completely explained by ionic Ag toxicity (Arnaout and Gunsch 2012; Colman et al. 2012; Griffitt et al. 2009; Mitrano et al. 2012; Ratte 1999).

The risks of AgNP exposure to terrestrial ecosystems and especially soil organisms is still poorly understood (Shoults-Wilson et al. 2011c). However, the antimicrobial properties of AgNP have raised concerns that soil microbial communities may be disturbed, with deleterious consequences for important ecosystem functions, including decomposition and nutrient cycling (Colman et al. 2012; Rai et al. 2009). In addition, direct AgNP toxicity to other soil organisms is also of concern (Tourinho et al. 2012).

A limited number of studies have investigated the impact of AgNP exposure to earthworms, quantifying effects on survival, growth, avoidance, inhibition of the Na+, K+ ATPase and apoptotic activity in tissues, for *Eisenia fetida* and *Lumbricus terrestris* earthworms (Heckmann et al. 2011a; Hu et al. 2012; Lapied et al. 2010; Shoults-Wilson et al. 2011a, 2011b and 2011c). The toxicity of metal nanoparticles to soil organisms has been tentatively associated with ionic metal fractions appearing in the soil pore water (Kool et al. 2011; Shoults-Wilson et al. 2011a). Furthermore, the behaviour of AgNP may be modified by interactions of these nanoparticles with soil components, such as organic matter and

clay, and by soil characteristics, including ionic strength and pH (Cornelis et al. 2010; Coutris et al. 2012; Lapied et al. 2010; Shoults-Wilson et al. 2011a; Stebounova et al. 2011; Tourinho et al. 2012). Therefore, for good interpretation of soil toxicity experiments, characterisation of the nanoparticles in different soil compartments (e.g. soil and soil pore water) is required (Cornelis et al. 2010; Jiang et al. 2009; Montes-Burgos et al. 2010; Stone et al. 2010).

Our previous work on C_{60} exposure to earthworms highlighted some important timescale issues in nanoparticle toxicity. *In vivo* experiments demonstrated lower reproduction after four week C_{60} exposure, and reduced survival and growth after lifelong C_{60} exposure, which are all critical endpoints for population dynamics (**Chapter 2**). For these C_{60} exposed earthworms, tissue pathology and genomic responses were observed as well, as discussed in **Chapter 3**. Additional *in vitro* experiments, using earthworm immune cells (coelomocytes), demonstrated effects of exposure to C_{60} and polymer nanoparticles, which implicated the immunotoxic potential of nanoparticles (**Chapter 4**). In the present study a similar approach was employed, using *Lumbricus rubellus* earthworms exposed to AgNP *in vivo* for four weeks with a subsequent lifelong exposure of the offspring and the assessment of a range of endpoints (survival, growth, reproduction and histopathology). A population model was used to quantify the potential impact of AgNP induced changes of these individual endpoints on *L. rubellus* populations. In an attempt to link exposure to effects, Ag concentrations were characterized in different test matrices, using several independent analytical techniques. In addition, given our previous observations of immunotoxicity of nanoparticles, an *in vitro* experiment with coelomocytes was performed to give insight in possible modes of action of AgNP toxicity.

Materials and Methods

Three experiments were conducted, two *in vivo* experiments and one *in vitro* experiment. The first *in vivo* experiment encompassed the four week exposure of adult earthworms, and the assessment of their survival, growth and reproduction. In the second *in vivo* experiment, offspring from the first experiment were monitored lifelong for survival and growth. In addition, an *in vitro* experiment was conducted, to assess the possible immunotoxic effects

of AgNP. In the latter experiment, earthworm coelomocytes were exposed overnight and cell survival was determined.

Test compounds

Commercially available NM-300K silver nanoparticles (AgNP) were obtained from Mercator GmbH (Germany), with a mean reported particle diameter of 15 nm (90% < 20 nm). These nanoparticles have been selected as a representative nanomaterial by the OECD Working Party on Manufactured Nanomaterials (WPMN) international testing program (OECD 2010). The stock suspension of AgNP contained 10.16% Ag (w/w) dispersed in a stabilizing vehicle material, consisting of polyoxyethylene glycerol trioleate (4%; w/w) and polyoxyethylene (20) sorbitan mono-laurat (Tween 20) (4%; w/w) (manufacturer's information). The vehicle material without AgNP was also purchased from Mercator as NM-300K DIS Ag-dispersant, and used as a control. Silver nitrate ($AgNO_3$) was obtained from Merck KGaA (Germany), as a powder with a purity of 99.8% (w/w), and used to benchmark the AgNP effects against Ag salt.

Metal impurities in the AgNP stock suspension and the $AgNO_3$ powder were measured by an Element 2 high resolution inductively coupled plasma-mass spectrometry (HR-ICP-MS; Element 2; Thermo Scientific, Germany), after dissolving in 0.14M HNO_3 (1/1000). The levels of metal impurities were very low (as shown in Table S1 of the Supplementary Data) and therefore, no additional risks were expected from these impurities.

***In vivo* experiments**

Experimental design

The *in vivo* experiments were conducted following ISO guidelines 11268-2: 1998, with minor adjustments in exposure scenarios and using the earthworm species *L. rubellus*. For the four week exposure experiment, adult (clitellated) earthworms were acquired (Nijkerkerveen, the Netherlands). The average weight of the earthworms was 1373 ± 33 mg (mean ± SEM; n = 180) and the weights did not differ significantly between exposure groups ($p > 0.05$). Prior to the experiment, the earthworms were acclimatized for two weeks

under constant conditions (24 hours light, 15°C and 61% relative humidity) in uncontaminated sandy test soil, with 1.6% clay, 4.3% organic matter and a pH-H_2O of 5.96.

For the *in vivo* experiments, the soil was prepared as described in **chapter 2**, using a wet-spiking procedure with a soil extract. For each treatment, six glass containers were used (n = 6), each housing five earthworms. Different soil exposure concentrations of Ag in the form of AgNP and $AgNO_3$ were used. Three nominal exposure concentrations of AgNP were tested: 1.5 (low), 15.4 (medium) and 154 (high) mg Ag/kg soil. Due to limitations in the experimental logistics of the *in vivo* experiments, only one concentration of $AgNO_3$ was tested, at 15.4 mg Ag/kg soil, to compare the Ag salt with the highest AgNP treatment. In addition, soil without Ag and soil with vehicle material were used as controls. The soil extract used to spike the soil, was obtained by addition of soil to ultrapure water, after which the suspension was shaken and filtrated. Subsequently, the soil extract was spiked with AgNP (2.0 Ag/l), $AgNO_3$ (0.2 g Ag/l), vehicle material (20 g/l; as a control for AgNP) or without any addition (control for $AgNO_3$) and added to the soil. The soil was mixed thoroughly and added to the container, after which the earthworms were placed in the container immediately. In the materials and methods section of the Supplementary Data, further details about the soil and the spiking procedure are provided.

For the four week experiment the endpoints included survival, growth (weight gain compared between beginning and end of experiment) and reproduction (the cocoon production per container at the end of the experiment, converted to the average number of cocoons produced per earthworm per day). Furthermore, at the end of the experiment, histological observations of different tissues were made and total Ag concentrations were analysed in tissues. In addition, imaging of Ag particle uptake (only for the high AgNP treatment) was conducted at the end of the experiment, to underpin the analytical results. In the lifelong experiment earthworms were checked monthly, over a period of ten months, for survival and growth. Furthermore, histological examinations were made at the end of the lifelong experiment (after ten months).

Table 1. Overview of the different analytical techniques used to characterize *in vivo* exposure. The exposure was assessed in soil extract (SE) and ultrapure water (UPW) by asymmetric flow field flow fractionation system (AF4) to determine the size of AgNP, and in soil (S) and earthworms (E) to quantify total Ag and Ag particle diameter and concentrations using inductively coupled plasma mass spectrometry (ICP-MS) and single particle ICP-MS (SP-ICP-MS). Furthermore, soil pore water was analysed by flame atomic absorption spectrometry (F-AAS) to measure total Ag concentratis present in the soil pore water samples. Samples for the analyses were freshly made (t = 0) or taken at the end of the four week (t = 1 month) or the lifelong experiment (t = 10 or 11 months). In addition, freshly made samples of soil extract (SE), as well as samples from soil (S) and earthworms (E) taken at the end of the four week experiment were analysed by field emission gun scanning electron microscopy in combination with energy dispersive analysis of X-rays (SEM/EDX). The abbreviation n.a. means not applicable.

Matrix	**SE and UPW**	**Soil**	**Soil pore water**	**Earthworm**	**SE, Soil, Earthworms**
Endpoint	Size	Total Ag and Ag particle (>30 nm) conc.	Total Ag conc.	Total Ag and Ag particle (>30 nm) conc.	Imaging of Ag particles
Method	AF4	ICP-MS, SP-ICP-MS	F-AAS	ICP-MS, SP-ICP-MS	SEM/EDX
Time point	t=0	t=1 and t=10	t=1 and t=11	t=1	t=0 (SE), t=1 (S, E)
Treatment unit analysed	n.a.	All AgNP and $AgNO_3$ treatments	All AgNP and $AgNO_3$ treatments	All AgNP and $AgNO_3$ treatments	High AgNP and $AgNO_3$ (S), high AgNP (E)

Exposure characterisation

In this section, an overview of the methods used to characterise the *in vivo* exposure will be presented. Due to the extent of the methods, details are provided in the materials and methods section of the Supplementary Data.

Characterisation of the AgNP and total Ag concentrations was performed in the soil extract (used for addition of AgNP, $AgNO_3$ and vehicle material to the soil), the soil, the soil pore water and the earthworms, where technically possible. An overview of the *in vivo* exposure characterisation is presented in table 1 and the results are discussed in the results section.

For analysis of the soil extract, which was used for spiking the soil, a freshly made stock of AgNP in the soil extract was diluted with clean soil extract before determination of the particle diameter of AgNP with asymmetric flow field flow fractionation (AF4). An accompanying experiment was performed with the soil extract, during which the ionic strength of the soil extract was increased. This experiment was performed because of the idea that the ionic strength of the soil pore water during the *in vivo* experiments was probably (much) higher than of the soil extract (Houba et al. 2000) and therefore AgNP aggregation is expected when adding the soil extract to the soil (Cornelis et al. 2010; Tourinho et al. 2012).

For analysis of the soil, soil samples were taken at the end of the four week and lifelong experiments (after ten months). After acid digestion of the soil, inductively coupled plasma mass spectrometry (ICP-MS) was used to measure total Ag concentrations. In addition, a novel method for quantification of relative particle size distribution and particle number concentrations of Ag particles was applied, using single particle ICP-MS (SP-ICP-MS). Similar soil samples as used for the ICP-MS measurements were analysed after water extraction, in order to preserve the structural integrity of the Ag particles.

For analysis of total Ag in the soil pore water, samples were obtained after saturation and centrifugation of soil samples taken at the end of the four week and lifelong experiments (after eleven months). Subsequently, total Ag concentrations in the soil pore water samples were directly measured with flame atomic absorption spectrometry (F-AAS).

For the earthworm analysis, whole earthworm samples were collected at the end of the four week experiment and analysed to assess the uptake of Ag. Measurements by ICP-MS were performed to determine total Ag concentrations in the earthworm samples, after acid digestion of whole earthworms. In addition, particle size distribution and particle number concentration of Ag particles were measured by SP-ICP-MS, in water extracts of the earthworms.

Furthermore, in almost all matrices of the *in vivo* experiment (soil extract, soil and earthworm) the presence of Ag particles was qualitatively assessed by field emission gun scanning electron microscopy in combination with energy dispersive analysis of X-rays (SEM/EDX). SEM/EDX provides information about the Ag particle size and composition, and the elements associated with these particles.

Effect assessment: Individual and population endpoints

At the start of the four week experiment, the earthworms were weighted and added to the containers. Earthworms were fed alder leaves (*Alnus glutinosa*) from an uncontaminated location (Vossemeerdijk, Dronten, the Netherlands), ad libitum. After four weeks, the experiment was terminated and the earthworms were counted and weighted again. One earthworm per container was stored in 4% buffered formal saline for histological examination (Handy et al. 2002b), after segments were carefully cut with a sharp scalpel blade, covering the region approximately one centimetre anterior and posterior to the clitellum. The other earthworms were put in liquid nitrogen and stored at -80°C, until further analysis. Subsequently, the number of cocoons present per container was determined by wet-sieving and hand-sorting.

For the consecutive lifelong experiment, the cocoons from the first experiment were incubated in petri dishes with soil of the corresponding treatment of their parent earthworms. After hatching, juveniles were transferred to containers with soil of the same exposure as their parents, again with six containers per treatment (n = 6) and five earthworms per container. Earthworms were checked monthly over a period of ten months, to determine weight and life stage: juvenile, subadult (showing a tubercula pubertatis, but before the clitellum has fully developed) or adult (with a fully developed clitellum) (Sims

and Gerard 1985). At the end of the experiment, earthworms were counted and weighted. For histological examination, one earthworm was randomly chosen from each container, carefully cut and then immediately fixed in 4% buffered formal saline. The other earthworms were put in liquid nitrogen and stored at -80°C, until further analysis.

The individual effect markers on adult reproduction and offspring survival and growth, were integrated to assess consequences at the population level, using a continuous-time life-history model as described in **chapter 2**. Within this model, several parameters were taken from the literature (Klok and De Roos 1996), including the duration of the cocoon stage (τ_0; 42 days), and the mortality chance per day (with 1 = 100% mortality at day 1) for the cocoon stage ($\mu_{c;}$ 0.001) and the adult life stage (μ_{ad}; 0.0027).

Effect assessment: Histological observations

For the histological examinations, the fixed segments were processed into wax blocks and transverse sections of 7 µm were cut from each segment. The staining of these sections was performed using Mallory's trichrome. All sections were prepared simultaneously, in batches containing both samples from earthworms of the control, AgNP and $AgNO_3$ treatments, in order to eliminate differences in fixation or staining artefacts between treatments. Sections were examined with an Olympus Vanox-T microscope and photographs were obtained using an Olympus digital camera (C-2020 Z). Eventually, not all earthworms were examined, due to some preservation artefacts, which made the histological observations semi-quantitative.

***In vitro* experiment**

Exposure characterization

Dynamic light scattering (DLS) and Zetasizer analyses were applied on freshly made stocks of AgNP and $AgNO_3$ (10 µg/ml) in ultrapure water and Ham's F-12K (Kaighn's) medium (cell culture medium; from Invitrogen/Life Technologies Ltd, UK) containing 10% fetal calf serum (FCS), to measure particle size distribution and zeta potential for AgNP and

$AgNO_3$ (results are presented in the Results section). Furthermore, the presence of Ag particles was qualitatively established using SEM/EDX.

Effect assessment: Coelomocyte viability

Primary immune cells from the coelomic fluid (coelomocytes) were exposed to AgNP and $AgNO_3$ *in vitro*. Coelomocytes were extruded from unexposed adult *L. rubellus* earthworms as described in **chapter 4**. Serial dilutions of AgNP and $AgNO_3$ in cell culture medium containing 10% FCS were made, to obtain a concentration range of exposure medium from 0 to 2000 µg Ag/ml cell culture medium, for AgNP as well as $AgNO_3$. In addition, serial dilutions of vehicle material without AgNP were prepared and used as negative control for AgNP exposure. Subsequently, 50 µl of exposure medium was added to 50 µl of coelomocytes suspension (with approximately 5 x 10^6 coelomocytes/ml) in a 96 well-plate, to obtain the acquired final concentrations of AgNP and $AgNO_3$ from 0 to 1000 µg Ag/ml cell culture medium. The coelomocytes were incubated overnight, at 15°C. Hereafter, cell viability was assessed using 0.05% trypan blue (final concentration), as described in **chapter 4**.

Statistical analyses

Differences between treatments were analysed using analysis of variance (ANOVA, with $\alpha = 0.05$), with least significant differences (LSD) as the post-hoc test (Burgers and Oude Voshaar 2010). The ANOVA tests were carried out using GENstat (14th Edition, VSN International Ltd.). Results are presented relative to the corresponding control (= 100%), as mean ± standard error of the mean (SEM) per treatment, unless otherwise specified.

Results

In vivo experiments

Exposure characterisation

The particle diameter of AgNP in the soil extract (used for spiking the soil) was determined by AF4, which demonstrated a clear peak with a maximum absorption at a particle diameter of 16 nm (Supplementary Data Figure S1). Integration of the fractograms with total Ag measurements in the eluent fractions demonstrated that more than 90% of the total Ag amount in the soil extract had a particle diameter < 32 nm (Figure S2). The addition of calcium to the soil extract with AgNP at a concentration of 5 mM and higher, caused complete destabilization of the colloidal AgNP suspension (Figure S3), suggesting clustering and settling of the AgNP.

SEM/EDX analysis of the soil extract containing AgNP demonstrated the presence of single Ag particles, with a particle diameter of approximately 20 nm. Some larger structures (50 to 250 nm) of Ag were also present, consisting of Ag only or Ag in combination with carbon, chloride and sulfide. In the soil extract with $AgNO_3$, large Ag flake-like structures ($\geq$ 1 μm) were formed, which also contained chloride and carbon.

Soil samples were taken at the end of the four week and the lifelong (ten months) *in vivo* experiments. For the four week experiment, the total Ag concentrations were 1.2 ± 0.03, 10.5 ± 0.2 and 118 ± 4 mg Ag/kg for the low, medium and high AgNP treatments, respectively (Table 2). Hence, 68 to 80% of the nominal AgNP soil exposure concentration was extracted from the soil samples, with the acid digestion method. After ten months the low, medium and high AgNP treatments demonstrated total Ag concentrations of 1.3 ± 0.3, 8.3 ± 0.3 and 104 ± 4 mg Ag/kg soil, respectively. The $AgNO_3$ treatment showed a total Ag concentration of 11.5 ± 0.1 mg Ag/kg after four weeks (Table 2) and a concentration of 6.3 ± 0.01 mg Ag/kg soil after ten months. A novel method for SP-ICP-MS analysis of soil was applied, with which a water extractable fraction of 10 to 27% was detected. Furthermore, due to the instrumental parameters used for the SP-ICP-MS, it was only feasible to detect particles larger than 30-40 nm. Consequently, the present results are semi-quantitative and

provide insight into the occurrence of particles larger than 30 nm, excluding the single AgNP used in this study. Ag particle concentrations for the low AgNP and the $AgNO_3$ treatments were below the detection limit of the SP-ICP-MS (< 0.10 mg Ag/kg soil) for all samples. Soil samples taken after four weeks from the medium AgNP treatment showed an Ag particle concentration of 0.13 ± 0.02 mg Ag/kg and for the high AgNP exposure soil this concentration was 5.5 ± 0.9 mg Ag/kg soil (Figure S4), with average Ag particle sizes of 42 nm and 46 nm, respectively. After ten months, the Ag particle concentration for medium AgNP soil was below the detection limit. The high AgNP treatment showed a concentration of 1.9 ± 0.3 mg Ag/kg soil, with Ag particles of 48 nm, at this time point.
SEM/EDX imaging of the soil samples taken at the end of the four week experiment indicated the presence of AgNP in the soil in three different forms: (1) single AgNP of approximately 20 nm in size, which were most abundant, (2) larger spherical structures with a size of 50 to 250 nm, containing only Ag (Figure S6A), and (3) structures of approximately 500 nm in the shape of a star (Figure S6B). These star shaped structures consisted of Ag particles of 50 to 100 nm, in combination with carbon and chloride. In the soil samples treated with $AgNO_3$ no Ag structures were visible.

Analyses of the soil pore water samples were carried out at the end of the four week and the lifelong *in vivo* experiments, in order to assess whether any changes of the total Ag levels in the soil pore water occurred over time. Total Ag concentrations in the soil pore water samples from the low and medium AgNP treatments were below the detection limit of the F-AAS (< 3 µg Ag/l). Soil pore water samples from the high AgNP treatment showed a total Ag concentration of 96 ± 6 µg Ag/l after four weeks (Table 2) and after eleven months the total Ag concentration had increased to 260 ± 21 µg Ag/l. For the $AgNO_3$ treatment, total Ag could only be detected in soil pore water samples after four weeks, with an Ag concentration of 16 ± 7 µg Ag/l (Table 2).

Using ICP-MS, total Ag concentrations were measured in earthworms exposed *in vivo* for four weeks and the uptake ratio of the total Ag concentration in the earthworm to the total Ag concentration in the soil could be established. Ag concentrations of the earthworms

sampled from the low AgNP treatment did not exceed the detection limit for the ICP-MS (< 1 mg Ag/kg earthworm) and the SP-ICP-MS (< 0.2 µg Ag/g earthworm). Earthworms from the medium AgNP treatment group showed a total Ag concentration of 4.5 ± 0.5 µg Ag/g earthworm (Table 2), which indicates a corresponding uptake ratio of 0.43. In earthworms from the high AgNP treatment, the total Ag concentration was 2.7 ± 0.3 µg Ag/g earthworm (Table 2), suggesting an uptake ratio of 0.02. For $AgNO_3$ exposed earthworms, total Ag concentration and the corresponding uptake ratio were 2.3 ± 0.7 µg Ag/g earthworm (Table 2) and 0.2, respectively. The SP-ICP-MS analysis of earthworms from the medium AgNP, high AgNP and $AgNO_3$ treatments showed Ag particle concentrations of 0.3 ± 0.16 µg Ag/g earthworm (average size 47 nm), 1.2 ± 0.97 µg Ag/g earthworms, with an average size of 51 nm (Figure S5), and particles of 49 nm at a concentration of 2.2 ± 0.8 µg Ag/g earthworm, respectively.

Table 2. Exposure characterisation of the four week experiment presented with the nominal concentrations (mg Ag/kg soil) for the AgNP and $AgNO_3$ treatments, as well as the total Ag concentrations measured in soil (mg Ag/kg soil). Furthermore, total Ag concentration in the soil pore water (µg Ag/l soil pore water) and the earthworms (µg Ag/g earthworm) are demonstrated. In addition, effects of four week exposure to AgNP and $AgNO_3$ on relative weight gain and cocoon production of the *L. rubellus* earthworms (n = 6 containers) are shown. Values are presented as mean ± SEM. For the effects, values are compared with the corresponding control (= 100%), between vehicle material control (V. control) and AgNP treatments or control and $AgNO_3$ treatments, and for these data an asterisk sign (*) shows an assessed significant difference.

Treatment	Nominal [Ag]	Total Ag in soil	Total Ag in soil pore water	Total Ag in earthworms	Relative weight gain	Relative # cocoons
V. control	0	< 1	< 3	< 1	100 ± 12	100 ± 4
AgNP	1.5	1.2 ± 0.03	< 3	< 1	121 ± 17	103 ± 5
	15.4	10.5 ± 0.2	< 3	4.5 ± 0.5	136 ± 4	91 ± 11
	154	118 ± 4	96 ± 6	2.7 ± 0.3	44 ± 12 *	18 ± 3 *
Control	0	< 1	< 3	< 1	100 ± 17	100 ± 8
$AgNO_3$	15.4	11.5 ± 0.1	16 ± 7	2.3 ± 0.7	118 ± 14	60 ± 10 *

SEM/EDX analysis of the earthworm slices from the high AgNP treatment of the four week experiment demonstrated the presence of Ag particles in the intestine, consisting of Ag only

(Figure 1A) or along with sulfide and chloride. When focussing on the dermis of the earthworms, Ag particles were found within the layer of epithelial cells. These were single AgNP with a size of approximately 20 nm (Figure 1B), but also larger particles (≤ 100 nm) consisting mainly of Ag.

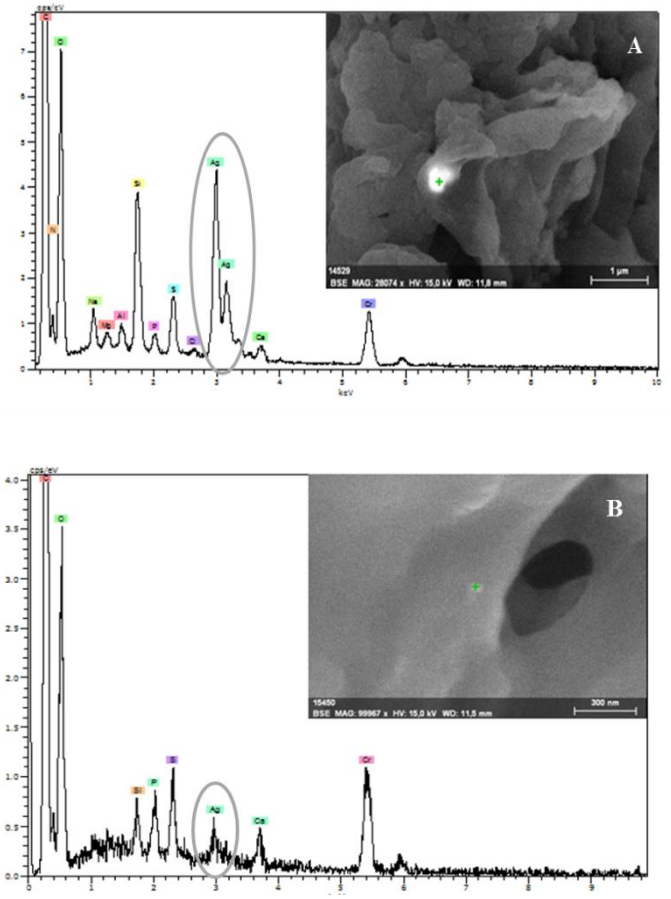

Figure 1. SEM/EDX images and EDX spectrum analyses obtained from a *L. rubellus* earthworm exposed to the high AgNP treatment for four weeks, indicating the presence of an Ag only structure in the intestine (A) and a single AgNP in the dermis (B). The grey oval line in the EDX spectrum encircles the Ag element peak.

Effect assessment: Individual and population endpoints

All adult earthworms survived the four week exposure. Table 2 shows that the adult weight gain in the high AgNP treatment group was significantly reduced (down to 44%), compared

with the vehicle material control group, as was the reproduction (down to 18%). $AgNO_3$ exposure did not reduce weight gain, but a significant reduction in cocoon production down to 60% was observed, as compared with the unexposed control treatment (Table 2).

In the lifelong experiment the hatchability of the offspring was only significantly affected in the high AgNP treatment (ANOVA, p=0.001), where only two cocoons hatched and the hatched juveniles died shortly afterwards.

Table 3 shows the parameters used in the continuous-time life-history model for the different treatments, which were estimated from the individual endpoints, e.g. adult reproduction, and survival and growth of the offspring. The low and medium AgNP treatments caused significantly reduced population growth rates (ANOVA; $p < 0.001$), decreasing down to 95% and 89%, respectively, compared with the vehicle material control. For the high AgNP, no population growth calculation was possible, due to the 100% mortality shortly after hatching. The population growth rate of the $AgNO_3$ treatment was significantly reduced compared with the corresponding unexposed control (87%; ANOVA; $p < 0.001$) and slightly, but significantly, lower than the medium AgNP treatment. The life stage distribution of the populations did not show significant differences between treatments, aside from the high AgNP treatment with the 100% juvenile mortality.

Table 3. Input parameters for the population model, estimated from the four week and lifelong *in vivo* experiments, are presented per treatment group. The growth rate constant for the individual earthworms (γ) is given in $mg^{1/3}/mg^{1/3}$/day and the maximum reproduction rate (R_m) per treatment group is presented as cocoons/$mg^{1/3}$/day. Earthworm length at birth (L_b), the lengths at reaching subadulthood (L_s) and adulthood (L_{ad}), and the estimated maximum length of the earthworms (L_m) are displayed as $mg^{1/3}$. In addition, the mortality chance for the juveniles (μ_j) and subadults (μ_s) are given per day (with 1 = 100% mortality at day 1). For all data, the mean values are presented and the standard deviation (SD) is displayed when this was required for the model. Significant differences between vehicle material control and AgNP treatments or control and $AgNO_3$ treatments are displayed with an asterisk sign (*).

Treatment	Input parameters							
	γ	R_m	L_b	L_s	L_{ad}	L_m	μ_j	μ_s
Vehicle control	0.022 ± 0.005	0.0033 ± 0.0005	2.49 ± 0.3	10.5	11.2	13.3 ± 0.8	0.005	0.0043
Low AgNP	0.023 ± 0.005	0.0039 ± 0.0003	2.426 ± 0.1	9.93	11.2	12.5 ± 0.6	0.0051	0.0043
Medium AgNP	0.021 ± 0.006	0.0027 ± 0.0004*	2.46 ± 0.2	10.4	11.8	14.0 ± 0.1	0.0061	0.0059
Control	0.021 ± 0.004	0.0032 ± 0.0005	2.68 ± 0.1	9.9	11.4	13.4 ± 1.0	0.0047	0.0043
$AgNO_3$	0.021 ± 0.005	0.0018 ± 0.0002*	2.69 ± 0.2	10.2	11.5	13.9 ± 0.9	0.0053	0.0043

Effect assessment: Histological observations

For the four week experiment, observations of the tissue segments anterior to the clitellum and of the clitellum region from the control and the vehicle material control earthworms showed a normal histology. The tissue segments anterior to the clitellum of these earthworms showed an epithelium consisting of columnar epithelial cells, some mucous and basal cells, and normal looking underlying circular and longitudinal muscles (Figure 2A and 2B). One of the four vehicle material control earthworms, showed some slight eosinophilic granular material in the epithelium of these segments. The clitellum region of the control and vehicle control earthworms showed a pseudostratified epithelium with soft parenchyma underneath and normal circular and longitudinal muscle layers.

The AgNP treatments caused mild or moderate erosion in parts of the epithelium of the tissue anterior to the clitellum (Figure 2C, 2D and 2E), for three of the four low AgNP treated earthworms and two of the four medium AgNP and high AgNP examined earthworms, respectively. Mild fibrosis was noted in the circular muscles of these earthworms as well. The tissues of the clitellum region were also affected by four week exposure to AgNP. Exposure to the low and medium AgNP treatment caused granular lipofuscin-like deposits in the clitellum tissue and epidermis, and mild fibrosis of the circular muscle, in two of six (low) and two of three (medium) examined earthworms. For the high AgNP treatment, only tissue from one earthworm, with normal histology, was examined. Earthworms from the $AgNO_3$ treatment showed some hyperplasia in the epidermis and some granular lipofuscin-like deposits in the circular muscle (2 of 3 earthworms), in the tissue anterior to the clitellum (Figure 2F). In addition, in the clitellum region of one of the three $AgNO_3$ exposed earthworms some hyperplasia of the pseudostratisfied epithelium was noted.

For the lifelong experiment, examination of the segments of the clitellum region and the tissue anterior to the clitellum of the control and the vehicle material control earthworms showed normal histology.

The tissue anterior to the clitellum of earthworms from the low and medium AgNP treatments showed some erosion of the epithelium. In addition, one earthworm from the

medium AgNP treatment showed excessive hyperplasia of mucus cells in the epidermis, which suggests increased mucus production (a defence mechanism). When looking at the clitellum region, low AgNP treatment resulted in hyperplasia of mucus cells in the epidermis and some loss of the clitellum tissue thickness in one of the five earthworms. All four earthworms from the medium AgNP treatment demonstrated slight or moderate loss of architecture for the epithelial cells of the clitellum region, and one earthworm showed additional fibrosis and lipofuscin-like deposits in the circular muscle, as well as some damage to the longitudinal muscle. For the $AgNO_3$ treatment, slight erosion of epithelial cells (with otherwise intact epithelium) and some thickening of the circular muscle were noted in tissue anterior to the clitellum of two of the three earthworms. In the clitellum region, $AgNO_3$ treatment had less effect than the AgNP treatments, causing some erosion of the epithelium, and some thickening of the circular muscle in two of the three earthworms.

The *in vitro* experiment

Exposure characterisation

For the *in vitro* experiment, Ag exposure was analysed using SEM/EDX, DLS and zeta potential. SEM/EDX analysis of cell culture medium with AgNP demonstrated single Ag particles of approximately 20 nm (most abundant) and some larger Ag structures (50-250 nm), which appeared to include chloride and sometimes sulfide. DLS analysis of AgNP in the ultrapure water demonstrated particles with a hydrodynamic diameter of 50 nm (Figure 3) and a zeta potential of -25 ± 1. When AgNP was analysed in the cell culture medium, the particle hydrodynamic diameter became slightly larger (58 nm; Figure 3) and the zeta potential became less negative (-11 ± 1 mV). SEM/EDX imaging of $AgNO_3$ in the cell culture medium showed large structures of Ag ($\geq$ 1 μm), which were associated with chloride. No consistent results were obtained for DLS measurements of $AgNO_3$ in ultrapure water and the cell culture medium. Zeta potential measurements of $AgNO_3$ showed a slightly positive zeta potential in ultrapure water (1.6 ± 0.4 mV) and a zeta potential of -12 ± 0.3 mV in the cell culture medium.

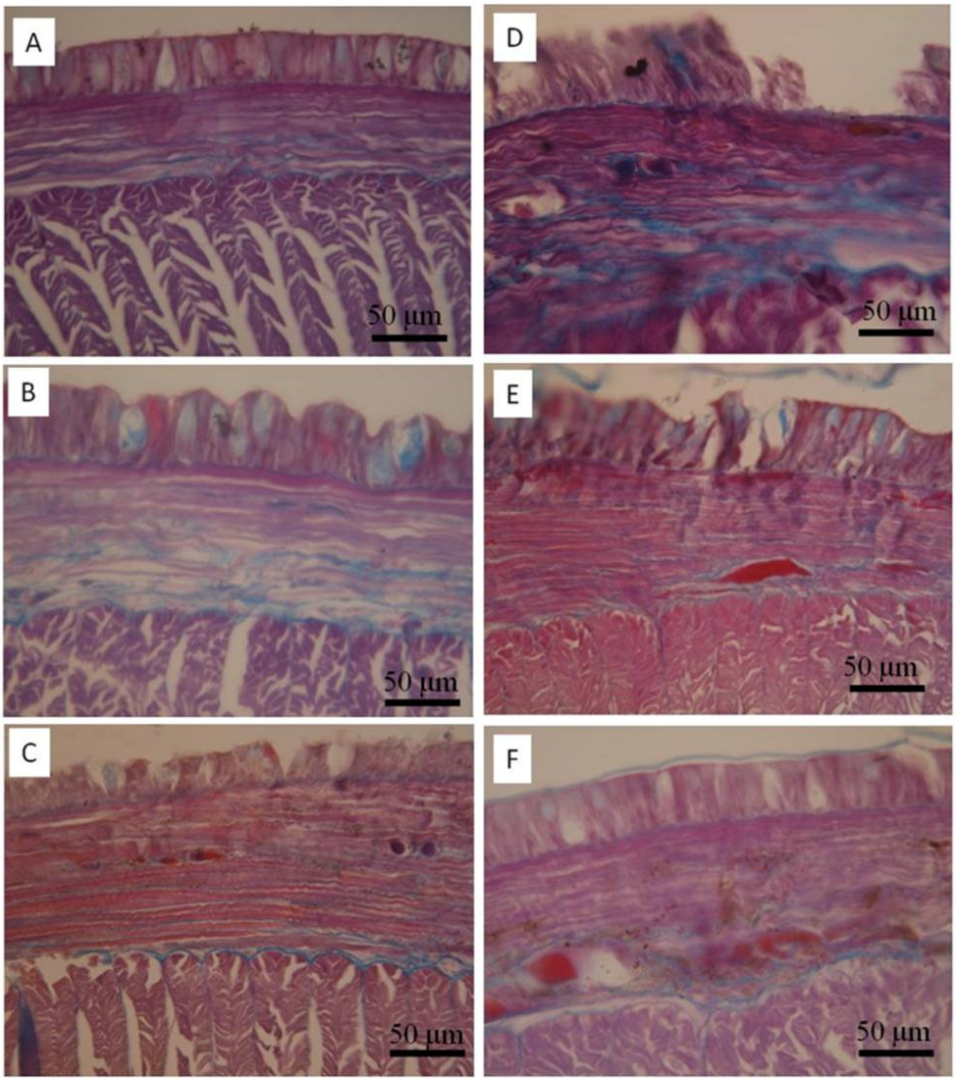

Figure 2. Transverse sections of segments from the anterior region of *L.rubellus* earthworms exposed for four weeks to control (A), vehicle material control (B), low (C), medium (D), high (E) Ag NP, or $AgNO_3$ (F) soil. Controls and vehicle material controls showed normal morphology of the epidermis and underlying muscles. Note the erosion of the epithelium and fibrosis of the circular muscle in panel C, D and E due to Ag NP exposure, with the medium treatment usually worse than the others. $AgNO_3$ treatment (F) had only minor impact with some granular lipofuscin-like deposits, mainly in the circular muscle. Magnification X400. Sections cut 7 µm stained with Mallorys trichrome.

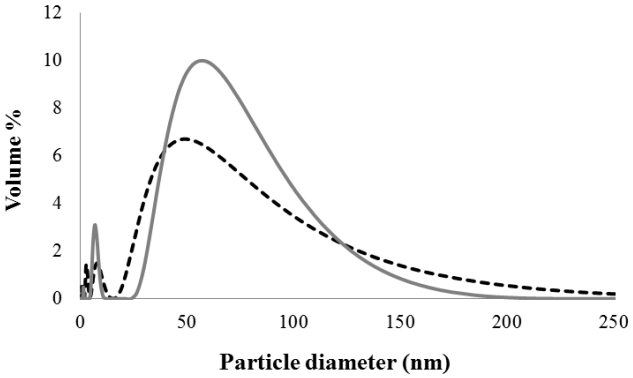

Figure 3. Hydrodynamic diameters of AgNP in ultrapure water (black dotted line) and cell culture medium with 10% FCS (grey line), measured with DLS at t = 0.

Effect assessment: Coelomocyte viability

Figure 4 shows that viability of the coelomocytes decreased with increasing AgNP concentrations, demonstrating an EC_{50} of 290 µg Ag/ml cell culture medium. Viability of the coelomocytes was affected more by $AgNO_3$ exposure, with an EC_{50} of 21 µg Ag/ml cell culture medium (Figure 4).

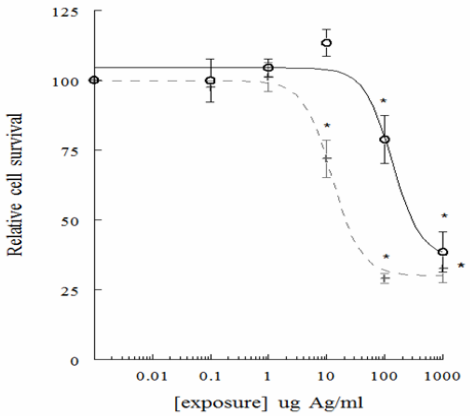

Figure 4. Viability of the coelomocytes exposed to increasing concentrations of AgNP (black open circle) or $AgNO_3$ (grey cross). Values are compared with the control (=100%) and are shown as mean ± SEM (N=3). An asterisk sign (*) indicates a statistically significant difference compared with the control. The regression curves for AgNP (continuous black line) and $AgNO_3$ (dashed grey line) exposure are displayed.

Discussion

The present study has investigated the effects of *in vivo* exposure of earthworms to AgNP through the soil. Effects of AgNP exposure were not only observed in a four week exposure experiment on growth, reproduction and histopathology, but also after lifelong exposure on survival, growth and histopathology. The modelling approach made it possible to observe effects of AgNP exposure on *L. rubellus* population growth rate, even at the lowest AgNP concentration (1.5 mg Ag/kg soil) tested. Furthermore, the *in vitro* assay showed the potential immunotoxicity of AgNP exposure to coelomocytes, which may have implications on whole body functioning. Exposure characterization indicated that the soil extract (used for spiking the soil) contained many single AgNP, but addition of the soil extract with AgNP to the soil led to formation of larger Ag structures, in addition to the single AgNP. Furthermore, total Ag levels in the soil pore water of the AgNP exposure soil remained high during the lifelong experiment, which was not observed for soil spiked with $AgNO_3$. This suggests differences in the behaviour of the nano form compared with the metal salt in the soil.

Exposure characterisation

In this study, several analytical techniques were used to characterize the exposure of earthworms *in vivo*. With these different techniques a thorough characterization of AgNP was established, before addition of AgNP to the soils (using the soil extract), and during exposure of the earthworms in the soil and in the soil pore water. In addition, uptake of AgNP by the earthworms was analysed.

Analysis of AgNP in the soil extract by AF4 showed a peak diameter at 16 nm, which is in good agreement with Klein et al. (2011) for the same NM-300K AgNP, who reported a particle diameter varying from 14 to 17 nm using transmission electron microscopy. The ionic strength of the soil extract was rather low (2.3 mM), while the ionic strength in the soil pore water during the *in vivo* experiment may have been much higher (Houba et al. 2000). This higher ionic strength may have led to clustering of AgNP after the soil extract was added to the soil (Cumberland and Lead 2009; Delay et al. 2011; Stebounova et al. 2011). This phenomenon may explain the presence of larger Ag structures within the solid

phase of the soil, as indicated by SEM/EDX and SP-ICP-MS analyses, in addition to the single AgNP also observed in the soil by SEM/EDX. These larger Ag structures consisted of AgNP clusters (Figure S6A) or of AgCl and Ag_2S heteroaggregates (Figure S6B), possibly with ionic silver adsorbed to the AgNP surfaces and 'nanobridges' of AgCl or Ag_2S heteroaggregates between the AgNP (Levard et al. 2011; Li et al. 2010; Liu and Hurt 2010; Walczak et al. 2012). Furthermore, the results of the soil pore water from the high AgNP exposure soil suggest that Ag particles and structures provided continuous release of Ag to the soil pore water. This suggestion is in line with Coutris et al. (2012), who also observed a long-term release of (dissolved) Ag in their study using uncoated AgNP of 20 nm. In the SEM/EDX imaging and SP-ICP-MS analysis of soil with $AgNO_3$, no Ag particles and structures were detected. This indicates that $AgNO_3$ remained dissolved in the soil, as is also known and demonstrated for this metal salt (Atkins and Jones 2000; Coutris et al. 2012; Ratte 1999). The Ag from $AgNO_3$ may have undergone rapid fixation in the soil, binding to chloride, sulfide and soil organic matter, without forming large and detectable amounts of insoluble Ag heteroaggregates (Alberts and Filip 1998; Coutris et al. 2012; Kerndorff and Schnitzer 1980; Murray and Linder 1984). Hence, these results suggest that exposure of organisms to AgNP in soils has a more dynamic character than exposure to $AgNO_3$, because AgNP continuously release Ag to the soil pore water, unlike $AgNO_3$.

The uptake of Ag by four week exposed earthworms demonstrated that, even though the total Ag and nominal AgNP concentrations in the soil increased, the total Ag concentration in the earthworms was not affected. This was also observed by Shoults-Wilson et al. (2011c) for *E. fetida* earthworms exposed to polyvinylpyrrolidone (PVP)-coated or oleic acid (OA)-coated AgNP through the soil, and this indicates that accumulation of Ag by earthworms may not be directly related to the total Ag and nominal AgNP concentrations in the soil. In this study, only total Ag concentrations in the soil pore water were measured, but other studies on nanometals (Kool et al. 2011; Shoults-Wilson et al. 2011a) suggest that the bioavailability and uptake of Ag may be more directly linked to the ionic fraction present in the soil pore water.

The ICP-MS and SEM/EDX data of the earthworms exposed to AgNP and $AgNO_3$ for four weeks provide evidence that Ag particles were taken up by the earthworms (Figure 1) and that these particles were in close contact with the earthworm tissues, especially the external barriers. As we were not able to distinguish between adsorption on the outside and true uptake (on the inside), it is not known to what extent the earthworms were accumulating Ag. For AgNP exposed earthworms, the Ag particles which were taken up were single AgNP or AgNP clusters, but also consisted of AgCl or Ag_2S heteroaggregates. AgCl and Ag_2S heteroaggregate formation has been observed and discussed in studies for *in vivo* oral exposure to AgNP and Ag ions, added as $AgNO_3$ or Ag acetate, and may also explain the presence of Ag particles in earthworms exposed to $AgNO_3$ (Danscher and Stoltenberg 2006; Loeschner et al. 2011; Walczak et al. 2012).

***In vivo* effect assessment**

The four week experiment demonstrated effects of exposure to the high AgNP (154 mg Ag/kg soil) and the $AgNO_3$ (15 mg Ag/kg soil) treatments. Other four week reproduction studies exposing earthworms to AgNP also demonstrated effects of $AgNO_3$ at lower Ag concentrations than AgNP (Heckmann et al. 2011a; Shoults-Wilson 2011c). This indicates that the effects of ionic Ag are important for the toxicity effects observed in four week exposure experiments. Within the lifelong experiment with the offspring, the effects for AgNP were more striking than for $AgNO_3$, because medium AgNP caused a reduction in juvenile and subadult survival to a higher extent than the $AgNO_3$ treatment, and high AgNP treatment resulted in complete mortality during the cocoon and juvenile stages. These results also indicate that juveniles may be more sensitive to (chronic) AgNP exposure than adults, as was also observed for C_{60} (**chapter 2**).

The effects on growth and reproduction after four week exposure to AgNP in this study (Table 2), were observed at lower concentrations that in other studies. Shoults-Wilson et al. (2011c) noted a decrease in reproduction for *E. fetida* earthworms of 40% at 1000 mg Ag/kg soil after four week exposure to PVP-coated or OA-coated AgNP, with a size of 30 to 50 nm. Heckmann et al. (2011) tested the effect of PVP-coated AgNP (30 - 50 nm) on *E. fetida* earthworms only at 1000 mg Ag/kg soil for four weeks, and the exposed earthworms

responded with a reduced growth (down to 73% compared with the control) and did not reproduce. The earthworms of the present study demonstrated a reduction in growth down to 44% and a very low reproduction (down to 18%) already after four week exposure to the 'high AgNP treatment' of 154 mg Ag/kg (Table 2). These differences may be explained by the use of a different AgNP. For example, toxicity of citrate-coated AgNP of 10 nm and PVP-coated AgNP of 20 nm to the bacteria *Nitrosomonas europaea* depended on coating and/or size of the AgNP (Arnaout and Gunsch 2012). Other factors explaining differences between toxicity studies are the characteristics of the soil. This was demonstrated by Shoults-Wilson et al. (2011a), who observed more effects of AgNP exposure to *E. fetida* earthworms when using sandy loam soil compared with artificial soil, with the first type of soil comprising lower levels of clay and organic matter, and a lower pH. In addition, sensitivity to exposure may differ between earthworm species, as was discussed by Frampton et al (2006) for different pesticides. In that study, the sensitivity varied between earthworm species and types of pesticide.

Adult reproduction and offspring survival and growth were integrated in the population model, to assess population level consequences. The resulting population growth rates were slightly higher than the data demonstrated in **chapter 2** for C_{60} exposure, which indicates that differences may be apparent between experiments and that caution should be taken when comparing between different experiments. Furthermore, all AgNP and $AgNO_3$ treatments in the present study showed a reduction in population growth rate. This indicates that the lowest adverse effect level (LOAEL) for AgNP exposure is 1.5 mg/kg and that even without observing significant effects for the individual endpoints of survival, growth and reproduction, population growth rate may be affected.

The histological examinations and SEM/EDX analysis of AgNP exposed earthworms indicate that AgNP may affect the external barriers, but are not expected to penetrate far into the earthworm body. Lapied et al. (2010) also demonstrated that the external barriers of the earthworm *L. terrestris* were affected most by AgNP exposure. Damage to the outer skin and the intestine may seriously affect the health of earthworms, because this damage

interferes with the correct functioning of these tissues and potentially the homeostasis of the earthworms (Lapied et al. 2010). In addition, when looking at the lifelong experiment, tissue injuries were more severe for the medium AgNP treatment, compared with the $AgNO_3$ treatment. This complements the idea indicated by the juvenile mortality data, that chronic AgNP treatment was more harmful for the earthworms than chronic $AgNO_3$ treatment. This difference may be explained by the exposure characterisation data, which suggest that AgNP may prolong the presence of a bioavailable fraction of Ag.

***In vitro* experiment**

Analysis of AgNP in the cell culture medium by DLS showed particles of 58 nm (Figure 3), which is in good agreement with DLS results presented by Klein et al. (2011) for the same NM-300K AgNP. Furthermore, characterisation of the *in vitro* exposure by SEM/EDX and DLS suggests that the coelomocytes were exposed *in vitro* mainly to single AgNP, or two clustered AgNP. The AgNP were presumably kept stable in this state due to a protein corona (Hayashi et al. 2012; Lundqvist et al. 2008; Murdock et al. 2008), which caused the zeta potential to become less negative in cell culture medium when compared with ultrapure water.

When comparing the AgNP EC_{50} with the EC_{50} for $AgNO_3$, within the *in vitro* experiment, a 14-fold difference is observed. The soluble Ag fraction was not measured in the present study, but Hayashi et al. (2012) demonstrated an ionic Ag fraction between 2 and 8% for the AgNP in cell culture medium after incubation for 24 hours. If this is similar for the AgNP used in the present study, the toxicity of ionic Ag may explain a large portion of the effects of AgNP exposure. Overall, this *in vitro* experiment indicates that AgNP exposure may impair functioning of the coelomocytes, including immune responses against pathogens and tissue repair (as also discussed for C_{60} in **chapter 4**), possibly mainly through the ionic Ag fraction. These effects on immune functioning and tissue repair may have had consequences for the bioenergetics of the exposed earthworms, increasing the body maintenance and leaving less energy for other processes (Kooijman 2000), as is demonstrated by the reduced growth and reproduction of the earthworms exposed to AgNP *in vivo* (Table 2).

In conclusion, AgNP exposure may impact earthworm populations, by affecting growth, reproduction, juvenile survival, tissue integrity and immune cell viability of the earthworms. Chronic effects of AgNP exposure are readily observed at 1.5 mg Ag/kg soil, hence the no observed adverse effect level (NOAEL) is below that concentration. The expected environmental concentrations of AgNP in soil are modelled at the ng Ag/kg level, although sewage sludge is expected to contain concentrations of milligrams Ag/kg (Gottschalk et al. 2009) and the deposited AgNP may accumulate in the top layer of the soil, as is also demonstrated for conventional pollutants (Hou et al. 2005; Mikkelsen et al. 1996). In this way, organisms living in the upper soil layer, including *L. rubellus* earthworms, may be at risk of chronic exposure to Ag.

Acknowledgements

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Supplementary data

Details on materials and methods

Soil preparation for the in vivo experiments

Sandy, moderately humic, loamy soil (1.6% clay, 4.3% organic matter, pH-H_2O 5.96) was collected from an experimental organic farm in the Netherlands (Kooijenburg, Marwijksoord, the Netherlands). This location has a long history of organic farming and the soil contains only background levels of heavy metals (Lahr et al. 2008). After sampling, the soil was air-dried at room temperature. A week before the start of the experiment, the soil was sifted through a 5 mm sieve and glass containers (with a lid) were filled with 650 gram of the sieved soil. Moisture content of the soil was determined by overnight drying at 105°C and the soil in the containers was amended with the amount of water needed to obtain a final moisture content of 15% (based on dry weight) during the experiments. These containers were kept under the same constant conditions as during the acclimatisation period of the earthworms.

The earthworms were exposed to three different AgNP treatments of 1.5 (low AgNP treatment), 15.4 (medium AgNP treatment) and 154 mg Ag/kg soil (high AgNP treatment) and compared with the vehicle material. As reference, Ag salt was added as $AgNO_3$ at 15.4 mg Ag/kg soil, with an appropriate control where no Ag salt was added. All these soil exposure concentrations are expressed as Ag content/kg dry weight of soil. The test substances and the vehicle material were added to the soil containers using an aqueous soil extract, to ensure uniform additions to the soil. This soil extract was prepared by gently shaking soil in ultrapure water (Millipore; resistivity = 18.2 mΩ cm^{-1}), at a soil to water ratio of 1/2.5 (g/v) for one hour, followed by filtration over a 4-7 μm filter (Whatmann filters type 597, Fisher Scientific). The pH of the soil extract was 6.2 and the ionic strength was equivalent to 2.3 mM. After filtration of the soil extract, AgNP and $AgNO_3$ were added at nominal concentrations of 2 g Ag/l and 0.2 g Ag/l, respectively. In addition, a soil extract was prepared with only the vehicle material, at a concentration of 20 g NM-300K DIS/l. After addition of the AgNP, $AgNO_3$ or vehicle material, the soil extracts were gently shaken for one hour, immediately followed by addition of the soil extracts to the soil in the

containers. Soils were dosed carefully with 0.5, 5.0 and 50 ml of the soil extract spiked with AgNP (2.0 Ag/l), using glass pipettes, to realize nominal soil exposure concentrations of 1.5, 15.4 and 154 mg Ag/kg soil, respectively. The 15.4 mg Ag/kg soil for the $AgNO_3$ treatment was prepared by spiking the soil with 50 ml of soil extract with $AgNO_3$ (0.2 g Ag/l). For the controls, soil was amended with 50 ml soil extract without any addition as a control for $AgNO_3$ or 50 ml soil extract with the vehicle material (20 g/l) as a control for AgNP, to obtain a vehicle concentration similar to the vehicle concentration in the high AgNP treatment. After application of the soil extracts, the soil was mixed thoroughly for two minutes (Hobart mixer; speed 2), followed by the immediate addition of the earthworms.

Soil extract analysis with AF4

The hydrodynamic diameter of the AgNP present in the soil extract was determined within 48 hours after preparation, by an Asymmetric Flow Field Flow Fractionation system (AF4; Postnova Analytics, Munich Germany). For this purpose, the soil extract with AgNP, at a concentration of 2 g/l, was further diluted in clean soil extract to reach an actual Ag concentration of 7.8 ± 0.2 mg Ag/l. To verify if any changes in particle diameter had occurred after diluting the AgNP in the soil extract, an aliquot of the AgNP stock was diluted in ultrapure water (8.6 ± 0.3 mg Ag/l) to reach a similar Ag concentration as in the diluted soil extract with AgNP. The runs with the AF4 were performed in duplicate, with an injection volume of 100 µl. The accumulation wall of the AF4 consisted of a 1 kDa polyether sulfone membrane (Postnova Analytics, Munich, Germany) and the spacer thickness of the AF4 channel was 350 µm. The carrier consisted of a 0.01% (w/v) sodium dodecyl sulphate (SDS) solution at pH 8 in ultrapure water (Bolea et al. 2011). During the focusing step of twelve minutes, the channel flow was 0.02 ml/min, while the cross flow and detector flow were 0.4 and 1.0 ml/min, respectively. A transition time of one minute was used to separate the elution step from the focusing step. During the elution step, the channel flow was 1.4 ml/min, while the cross flow was 0.4 ml/min for 25 minutes. Afterwards, the cross flow was decreased in a linear fashion to 0 ml/min in one minute. Subsequently, the elution step was maintained for an additional ten minutes, with the cross

flow field turned off. The detector flow was interfaced online to an ultraviolet diode-array-detection detector (Postnova Analytics, Germany), which measured the UV-VIS spectrum each 0.6 seconds during the elution step. Hereafter, AF4 theory was used to calculate the hydrodynamic diameter of the AgNP eluted during the first 25 minutes of the elution step (Litzén 1993). This theory is based on ideal behaviour of spherical particles, which is the case for the majority of the Ag present in the NM-300K AgNP (Klein et al. 2011).

For the off-line collection of the AF4 eluate, the detector outflow of the AF4 system was connected to a fraction collector (Retriever IV, Teledyne Isco, USA) to take samples over an interval of two minutes (2 ml) during the elution step of the AF4 runs. These samples correspond to an interval in particle diameter size of about 7 nm. After sampling, 0.2 ml of 65% (v/v) HNO_3 was added to each sample for 24 hours, for digestion of AgNP, after which the Ag concentration was measured using a high resolution-inductively coupled plasma-mass spectrometer (Thermo Element 2, Thermo Scientific). The recovery of AgNP after AF4 separation was calculated by dividing the total amount of Ag measured in all fractions collected during the elution step of an AF4 run, by the amount of Ag injected with the sample into the AF4 system, multiplied by 100%.

In addition, an accompanying experiment was performed, to investigate the stability and possible aggregation of AgNP in the soil, as compared with the soil extract. For this experiment, the soil extract containing AgNP was spiked with calcium nitrate ($Ca(NO_3)_2$), which was prepared in clean soil extract, to reach a final nominal Ca concentration of 5, 10, 25, or 50 mM. Calcium was selected, because this is generally the most important cation in soil pore water (Koopmans et al. 2006). Also, treatments consisting of the soil extract with and without AgNP, to which no calcium was added, were included in the experiment. The suspensions were shaken for 24 hours. Subsequently, the UV-vis absorption spectra of the suspensions were measured, using a Genesys 10S UV-VIS spectrophotometer (Thermo Scientific). The instrument was referenced with ultrapure water.

ICP-MS methods for soil and earthworm characterization

Total Ag concentrations were determined for soil and earthworms, from the four week and the lifelong (ten months) *in vivo* experiments, using ICP-MS. For this purpose, 0.5 gram per

soil sample or whole earthworms were taken (triplicates per treatment) and put into a microwave digestion vessel. Samples were digested with 6 ml of 37% (v/v) HCl and 2 ml of 70% (v/v) HNO_3. The vessels were microwaved at 1600 W, to 140°C with 20°/min and to 180°C with 15°/min, which was held for 60 minutes. Following digestion, the digests were transferred to 50 ml polypropylene tubes and 42 ml of ultrapure water was added to each tube. An aliquot of 1 ml was taken from each tube and diluted 6 times. Hereafter the samples were measured with an ICP-MS (Thermo Scientific X-series 2), equipped with a Burgener PEEK Mira Mist type nebulizer and a quartz impact bead spray chamber. The forward RF-power was 1400 W and the gas flows were 13 l/min for the plasma, 1.0 l/min for the nebulizer and 0.7 l/min for the auxiliary. The sample flow rate to the nebulizer was set at 0.5 ml/min, using an integrated peristaltic pump. The ICP-MS instrument was operated in the continuous analysis mode.

For the characterisation of Ag particles in soil, 1 gram of soil (from the four week and the lifelong *in vivo* experiments) was extracted with ultrapure water, at a soil to solution ratio of 1/25 (w/v). The soil was not pre-treated prior to this soil extraction. The extracts were shaken in a head-over-head apparatus for 16 hours. After settling for 15 minutes, an aliquot of 2 ml was collected and filtered through a 5 µm filter. The filtrate was diluted with ultrapure water at a ratio of 1/1000 and analysed with single particle inductively coupled plasma mass spectrometry (SP-ICP-MS). Triplicates were measured for soil from each treatment. In case of the earthworms, 5 ml of ultrapure water was added to the whole earthworm (stored at -80°C beforehand) and the vial was placed in an ultrasonic bath for 30 minutes. Hereafter, the entire sample was transferred into a 50 ml polypropylene tube. Subsequently, 10 ml ultrapure water was added to the tube and the sample was sonicated for 3 minutes. Following sonication, another 10 ml of ultrapure water was added and the sample was extracted in a head-over-head apparatus for 16 hours. An aliquot of 2 mL was collected from the tube and filtered through a 5 µm filter. The filtrate was diluted 1000 times and analysed by SP-ICP-MS. Three earthworms per treatments were analysed.

The SP-ICP-MS measurements were performed using the ICP-MS instrument and instrumental settings similar as for the ICP-MS, however, in this case the ICP-MS was operated in the time resolved mode (TRA) with a dwell time of 3 milliseconds and a typical

run time of 60 seconds per measurement. During SP-ICP-MS analysis, an Ag particle generates a pulse of ions, which results in a signal which is greater than the background. The intensity readings can be collected as a function of time, where pulses above the background represent the measurement of an individual nanoparticle (Mitrano et al. 2012). The Ag particle diameter size is determined from the intensity of the pulses, while the Ag particle size distribution is determined from the normalized frequency of all pulses. With the SP-ICP-MS analysis used, only particles > 30-40 nm could be detected.

Soil pore water analysis using F-AAS

Flame atomic absorption spectrometry (F-AAS) was used to determine the total Ag concentrations in the soil pore water samples. Soil samples (50 gram per sample; triplicates per treatment soil) were collected for the soil pore water measurements at two time points, e.g. at the end of the four week experiment and after the lifelong experiment (eleven months). Soil pore water samples were obtained after equilibration of the soil samples, which were saturated with ultrapure water (at a moisture content of 100% instead of 15%), for one week. Subsequently, the samples were centrifuged for 45 minutes (centrifuge Falcon 6/300 series, CFC Free) at 2000 *g* over two round filters with a pore size of 11 μm (Schleicher &Schuell) and a 0.45 μm membrane filter (Schleicher &Schuell), placed inside the centrifuge tubes (Hobbelen et al. 2004). All filters were pre-treated and saturated with a $Cu(NO_3)_2$ solution to prevent Ag loss, resulting from Ag binding to the filter material. Approximately 5 ml of soil pore water was collected for each soil, to perform analysis of the total Ag levels in the soil pore water by F-AAS (Perkin Elmer 1100B).

In vitro characterization by DLS and ZetaSizer

The particle size distribution of particles present in cell culture medium spiked with AgNP or $AgNO_3$ was performed by dynamic light scattering (DLS), using a Cobolt Samba 300mW DPSS laser at a wavelength of 532 nm as a light source. In addition, suspensions in ultrapure water were made, to verify if any changes in particle diameter had occurred after dilution in cell culture medium. The (nominal) concentration of AgNP stock and $AgNO_3$ powder in ultrapure water or cell culture medium containing 10% FCS was 10 μg Ag/ml.

For each sample, twenty measurements were made and the detector intensity data (measured with an ALV/SO SIPD Single Photon Detector with ALV Static and Dynamic Fiber optics) were processed with an ALV5000/60X0 external correlator and ALV-5000/E software (all from ALV-GmbH, Germany). The average autocorrelation data (g2) were analysed using AfterALV software (Dullware, the Netherlands), which uses the CONTIN algorithm as developed by Stephen Provencher (Provencher 1982). For each sample, the mean diameter of the peaks with the associated polydispersity index (PDI), an indication of the width of the diameter peak, was determined.

In addition, the zeta potential for the same samples as used for DLS measurements was analysed, using a Malvern ZetaSizer 2000 (Malvern Instruments, UK). Data were processed with Zeta mode V1.51 software (Chen et al. 2004). Five measurements were used to establish average and SEM for each sample.

SEM/EDX methods for Ag particle analysis

The presence of Ag particles in different sample types was visualized and qualitatively determined, shortly after preparation of the media, by field emission gun scanning electron microscopy (SEM). This technique was applied in combination with energy dispersive analysis of X-rays (EDX), which provides information about the elements associated with the Ag particles present in the samples. In case of soil, samples were dried at 40°C, placed on aluminium specimen holders with double sided adhesive carbon tape, and coated with a 5-10 nm layer of chromium, using an Emitech K575X turbo sputter coater. In case of the earthworms, earthworms (from -80°C) were fixated with glutardialdehyde in a solution of phosphate buffered saline (PBS). Subsequently, the earthworms were dehydrated with ethanol and xylene, using a vacuum infiltrating processor (VIP). These dehydrated samples were soaked in molten paraffin and then 4 micrometre-thick sections of the paraffin embedded material were produced, using a microtome. These sections were mounted on glass microscope slides and washed with xylene and ethanol for deparaffinization. Thereafter, the sections were dried at room temperature and coated with the chromium. In case of the soil extract and cell culture medium, samples (10 µg Ag/ml) of 1 to 2 ml were filtered over an Anopore aluminium oxide filter with a pore size of 20 nm (type Anodisc,

Whatman). The filters were rinsed with 5 ml of ultrapure water and mounted on the aluminium specimen holders. After drying at room temperature, the filters were coated with the chromium. All the prepared samples were analysed with a SEM/EDX (MIRA-LMH, from Tescan, Czech Republic). The microscope was operated at an accelerating voltage of 15 kV, with a working distance of 10 mm and a spot size 5 nm. The EDX spectrometer (Bruker AXS) contained a Quantax 800 workstation, a XFlash 4010 detector with an active area of 10 mm^2 and a super light element window (SLEW), which allows X-ray detection of elements higher than boron (Z > 5). The spectral resolution of the detector was 123 eV (Mn (10kcps) mean full width at half maximum (FWHM)). The samples were screened systematically at three different magnifications: 10.000x, 25.000x and 50.000x. These magnifications were chosen to be able to detect clustered particles as well as individual particles. After the identification of Ag particles, EDX was used to determine the chemical composition of the Ag particles.

Supplementary results

AF4 results for the soil extract

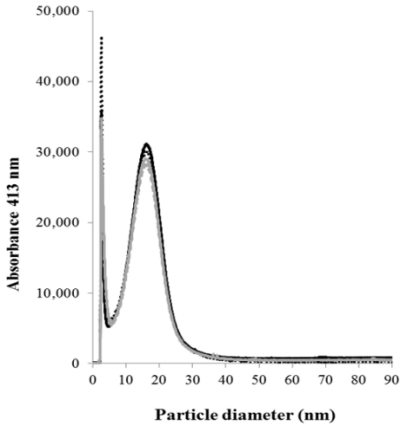

Figure S1. *Determination of AgNP diameter in ultrapure water and soil extract*
Fractograms of the AgNP diluted in ultrapure water (black straight and dotted lines) and in the soil extract (grey straight and dotted lines), with detection of AgNP by UV-VIS absorbance measurements at 413 nm, showing clear and very good overlapping peaks, with a maximum absorption at a particle diameter of 16 nm.

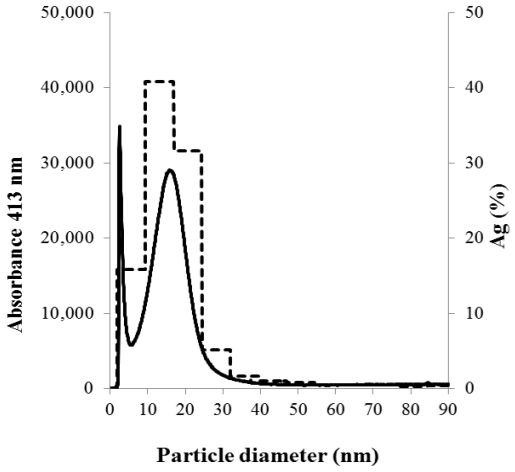

Figure S2. *Analysis of the fraction of Ag for each particle diameter interval*

The AF4 fractogram of one of the duplicate runs of soil extract with AgNP (straight line), with detection by UV-vis absorbance measurements, in combination with the results of the Ag concentration measurements in the eluent fractions collected during the AF4 run (dotted line), presented as a percentage of the total amount of Ag injected with the sample into the AF4 system.

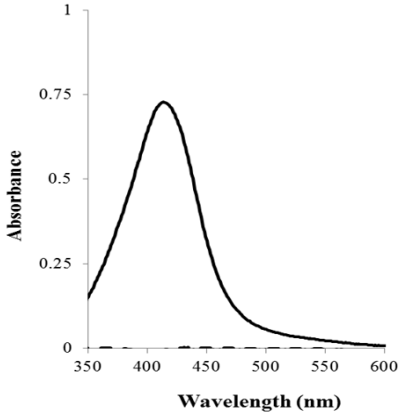

Figure S3. *The impact of ionic strength on AgNP clustering*

The UV-vis absorption spectra of the AgNP in the soil extract at different Ca concentrations. The particle diameter of AgNP was unaffected while diluted in the soil extract (black line). After addition of Ca (at 5mM Ca (black not-extended line), 10 mM Ca (black dotted line), 25 mM Ca (grey line) and 50 mM Ca (grey dotted line)), the absorption peaks were lost from the UV-vis absorption spectra, shown by the lines near and on the X-axes. This indicates that the colloidal AgNP suspension was destabilized.

SP-ICP-MS data

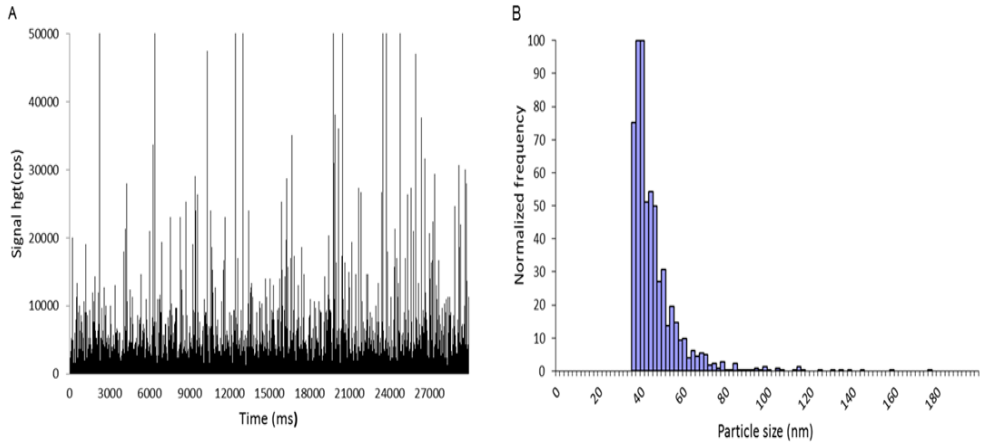

Figure S4. *Ag particles in soil*

SP-ICP-MS analysis for soil from the four weeks high AgNP treatment, showing the pulses (or signals) generated in time (A) and the particle size (nm) distribution calculated from the SP-ICP-MS data (B).

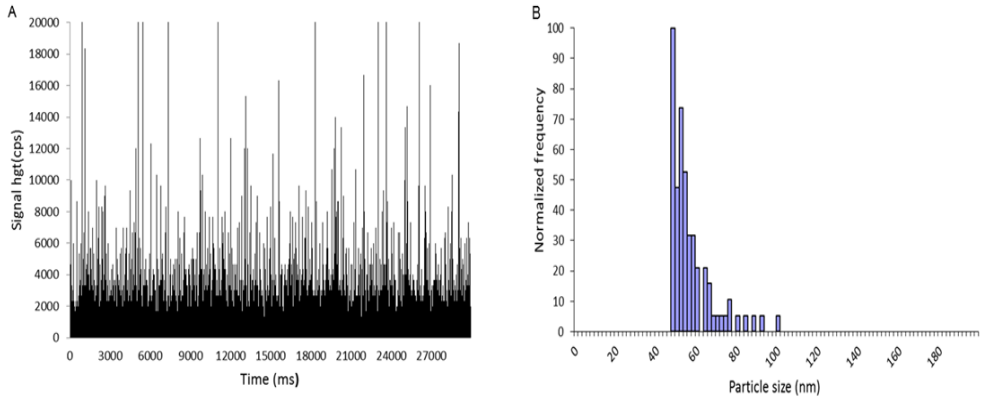

Figure S5. *Ag particles in earthworm*

The pulses generated in time (in ms) during the SP-ICP-MS analysis (A) and the size (nm) distribution of the Ag particles as calculated from the SP-ICP-MS data (B), for an *L. rubellus* earthworm sample from the four weeks exposure to the high AgNP treatment.

SEM/EDX imaging of Ag in soil

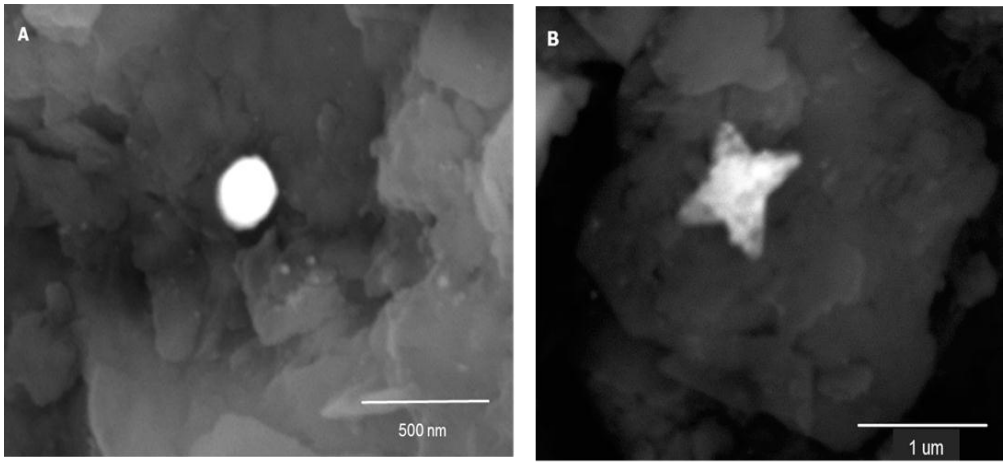

Figure S6. SEM images of Ag particles present in soil from the high AgNP treatment of the four week experiment, with an Ag only aggregate (A) and a star structure (B), which seemed to consist of Ag aggregates of 50 to 100 nm in combination with carbon and chloride.

Metal impurities in stocks

Table S1. Concentrations of different metals measured in the AgNP-stock suspension and for the AgNO3 powder, analysed by HR-ICP-MS and converted to mg Ag/kg soil, to present the data as possible metal impurity concentrations for the soil at the highest exposure treatments tested (154 mgAg/kg or 15.4 mg Ag/kg, respectively).

Material	Cd	Cr	Cu	Ni	Pb	Zn
AgNP	0.11	0.11	0.01	0.12	0.01	0.0
$AgNO_3$	0.03	0.004	0.0	0.07	0.04	0.0

Chapter 6

General discussion and future perspectives

General discussion

The production and use of nanoparticles have increased for several decades and the number of products with engineered nanoparticles incorporated is steadily growing (Gottschalk and Nowack 2011; Wijnhoven et al. 2010; Woodrow Wilson institute). The fields of application of nanoparticles are diverse and wide, including food, personal care, sports gear, medical applications, as well as devices for environmental monitoring and waste water treatment (Andreescu et al. 2009; Dekkers et al. 2011; Jain 2008; Savage and Diallo 2005). Due to this dramatic increase in production and applications of engineered nanoparticles, exposure of humans and the environment to these materials seems inevitable (Handy et al. 2008a; Oberdörster et al. 2005). And unfortunately, the same characteristics which make nanoparticles useful in many products, such as chemical reactivity and persistence, cause concern about their potential adverse health effects (Warheit et al. 2008; Farré et al. 2011).

A considerable number of studies has investigated the hazards of nanoparticles on mammals (including humans) and has demonstrated uptake, bioaccumulation and toxicity of different types of nanoparticles (Ariano et al. 2011; Bhattacharjee et al. 2010; Fujita et al. 2009; Hsin et al. 2008; Hussain et al. 2005; Park et al. 2011; Schaeublin et al. 2011; Shvedova et al. 2005; Xia et al. 2008). Discharge of nanoparticles into the environment, during production, transport, use and disposal, is expected (Gottschalk and Nowack 2011; Lin et al. 2010) and possible environmental exposure, hazards and risks need to be addressed (Baun et al. 2008a; Handy et al. 2008c; Nel et al. 2006; Nowack and Bucheli 2007; Stone et al. 2010). Research on the impact of nanoparticles on the environment has mainly focussed on aquatic organisms (Handy et al. 2008c; Kahru and Dubourguier 2010; Navarro et al. 2008; Shoults-Wilson et al. 2011c) and information on effects of nanoparticle exposure on soil organisms and ecosystems is scarce.

Given the need for better characterization of hazards of engineered nanoparticles to the environment and soil organisms in particular, the aim of the present thesis was to investigate effects of nanoparticle exposure on the earthworm *Lumbricus rubellus*, as a model organism for soil ecotoxicology, and to contribute to the development of effect markers for engineered nanoparticle exposure in this model.

The nanoparticles investigated in the studies described in the present thesis were fullerene (C_{60}) and silver nanoparticles (AgNP). These nanoparticles were selected because they are among the most widely used types of nanoparticles in consumer products and are deemed suitable as reference material (Gelderman et al. 2008; Klaine et al. 2008; Stone et al. 2010; Woodrow Wilson Institute). C_{60} was also chosen because of its known stability and very low solubility, which indicate that soil may serve as a sink for these nanoparticles (Li and Alvarez 2011; Nielsen et al. 2008). The type of AgNP selected for the experiments discussed in the present thesis were designated as a representative nanomaterial by the European Commission's Joint Research Centre (JRC), which means that this specific type of nanomaterial will be tested by different laboratories within Europe and the results can be compared more easily than for other types of AgNP. In the present thesis, effects of the selected nanoparticles on gene expression, tissue integrity, individual health and population growth were investigated after *in vivo* exposure. In addition, *in vitro* experiments were carried out using earthworm immune cells, to evaluate possible immunotoxic effects of the nanoparticles. By studying effects at different levels of biological integration further insight in the mode of action was obtained and a contribution to the development of effect markers for nanoparticle exposure was made. Furthermore, effects of nanoparticle exposure at the individual level were translated to the population level, in order to predict effects possibly occurring for earthworm populations in the field upon nanoparticle exposure.

Taken together, the results of the present thesis reveal that C_{60} and AgNP exposure affect *L. rubellus* earthworms in a dose dependent way, at different levels of biological organization. The histopathological observations made in the studies discussed in the present thesis provided insight in possible causes of effects. The observations on tissues of exposed earthworms showed damage to the external barriers, e.g. cuticle and gut epithelium (**chapter 3 and 5**), an effect which may seriously affect the health of earthworms (Lapied et al. 2010). For C_{60} exposure, the effects on underlying tissues were more visible than for the AgNP exposure. This indicates that at the dose levels tested, C_{60} may have had more effect on the external barriers and/or that C_{60} infiltrated into the earthworms to a higher extent than AgNP. In this way, C_{60} interfered more with the functioning of the external

barriers and the homeostasis of the earthworms, and may have made the underlying tissues more vulnerable (Lapied et al. 2010).

The decrease in growth and cocoon production observed upon C_{60} and AgNP exposure (**chapter 2 and 5**) may have occurred due to the effects of tissue injury and repair on the bioenergetics of the exposed earthworms, leaving less energy for growth and reproduction (Kooijman 2000). However, the decrease in reproductive success may also be explained by a direct effect of nanoparticle exposure on the reproductive organs. This applies at least for C_{60} exposure, because damage to the parenchyma of the clitellum (an important part of the earthworm reproductive system) was observed in C_{60} exposed earthworms. For the AgNP exposed earthworms no clear effects on the parenchyma were observed and the bioenergetics explanation seems most likely for reduced reproductive output in AgNP exposed earthworms, however, definite conclusions on the effects of AgNP on the reproductive system requires further future histopathological examinations.

Oxidative stress is a mode of action which has been much discussed and demonstrated for nanoparticle toxicity (Barillet et al. 2010; Bhattacharjee et al. 2011; Johnston et al. 2010; Klaper et al. 2009; Li et al. 2008; Oberdörster et al. 2005). However, *L. rubellus* earthworms exposed to C_{60} *in vivo* did not demonstrate an increase in antioxidant enzyme expression and activity (**Chapter 3**). This lack of a significant effect on oxidative stress marker enzymes may be explained by the experimental set-up, which may have masked the occurrence of oxidative stress (Johnston et al. 2010). An important factor of the experimental set-up in this case is the *in vivo* and relatively long lasting exposure, which differs from most nanotoxicology studies reporting on nanoparticle induced oxidative stress that have used short, acute and/or *in vitro* exposures. As effects may only be noticeable shortly after the start of the C_{60} exposure rather than four weeks or even several months later, due to feedback processes (Maity et al. 2008; Oberdörster et al. 2004), they may not have been noticed in the experiments discussed in the present thesis. The use of only a small set of antioxidant enzymes may be an additional masking factor, because it may not be necessary for the earthworms to induce all of the antioxidant enzymes present in the

earthworm body (Kohen and Nyska 2002; Martindale and Holbrook 2002). Furthermore, the use of whole body measurements may have masked the occurrence of oxidative stress, because this may impede the detection of subtle changes in individual tissues as concentrations of antioxidant enzymes may vary between different tissues (Barillet et al. 2010; Korsloot et al. 2004; Oberdörster et al. 2004; Smith et al. 2007).

The results from the *in vitro* experiments on C_{60} toxicity were not unambiguous. *In vitro* exposure of rat macrophage NR8383 cells to C_{60} demonstrated a reduction in cell survival and phagocytic activity, already at low µg/ml concentrations with EC_{50} values of 1.04 and 1.87 µg/ml, respectively (**chapter 4**). The coelomocytes, on the other hand, were much less sensitive and the survival and phagocytic activity of these cells seemed unaffected by C_{60} exposure until the mg/ml level. In contrast to this, the coelomocytes and NR8383 cells demonstrated similar responses to tri-block polymer nanoparticle exposure, indicating the usefulness of the coelomocytes for nanotoxicity testing. The cell culture media used in the C_{60} experiments were the same for both cell types and could not explain the demonstrated difference in effects to C_{60} exposure between the cell types. The most plausible explanation is the difference in sensitivity between the cell types. This phenomenon has been demonstrated in other studies testing nanoparticle exposure *in vitro* as well (Hayashi et al. 2012; Nguyen et al. 2012; Xia et al. 2008). For rat NR8383 cells C_{60} exposure may cause damage to cells, affecting cell viability and phagocytic activity, and C_{60} exposure to earthworm coelomocytes may merely cause suppression of the inflammatory response (as demonstrated by lowered CCF-1 levels).

Reliable, extensive and repeated measurements of actual soil nanoparticle concentrations are lacking, but modelled environmental concentrations of nanoparticles in the soil are close to the µg/kg level (Gottschalk et al. 2009). The nanoparticle concentrations applied in the experiments discussed in the present thesis are more than three orders of magnitude above these currently expected environmental concentrations. However, nanoparticle exposure may pose a threat to soil ecosystems in the near future, due to the expected prolonged presence of some types of nanoparticles (Coutris et al. 2012; Li et al. 2010;

Nielsen et al. 2008). In addition, in the experiments discussed in the present thesis only lowest observed adverse effect levels (LOAELs) could be determined and the no observed adverse effect levels (NOAELs) may be at much lower concentrations. Furthermore, the earthworms are used as model organisms for soil ecotoxicology and the impact of nanoparticles on the earthworms need to be extrapolated to other soil organisms, which may be more sensitive to nanoparticle exposure.

Future perspectives

The introduction of nanotechnology offered tremendous opportunities for all sorts of nano-applications in different fields, including medicines, foods, electronics, alternative energy and water remediation. During the production, use and disposal of a nanoproduct, it is likely that nanoparticles will eventually enter the soil environment. Currently, effects of exposure of nanoparticles on soil ecosystems are still largely unknown, but efforts are being made, including the present thesis. Even though the present thesis has shed some light on possible effects and modes of action of nanoparticle toxicity towards earthworms, ample questions remain for future research. The two main topics for environmental risk assessment of engineered nanoparticles in the soil are (**1**) the hazard assessment of nanoparticles to soil organisms, including characterisation of (**1a**) bioavailability and (**1b**) adverse effects and sensitive endpoints, and (**2**) the assessment of the actual exposure and behaviour of nanoparticles under field conditions.

1a. Characterisation of bioavalibility

If nanoparticles are discharged to the soil, soil organisms may be exposed and may accumulate the nanoparticles. Several studies have investigated the bioaccumulation of nanoparticles in soil organisms, but many questions remain (Hu et al. 2010; Kim et al. 2011; Lapied et al. 2011; Li et al. 2010; Shoults-Wilson et al. 2011c). Therefore, bioaccumulation should be considered for future research, especially in combination with the impact on the organisms, because studies on bioaccumulation and effects do not demonstrate a clear correlation. For example, the research by Shoults-Wilson et al. (2011c) on bioaccumulation of polyvinylpyrrolidon (PVP) and/or oleic acid (OA) coated AgNP by *Eisenia fetida* earthworms as well as the

AgNP bioaccumulation by *L. rubellus* earthworms discussed in **chapter 5** of the present thesis do not indicate a clear dose-dependent bioaccumulation. However, both studies demonstrated (nominal) concentration dependent effects of AgNP. One explanation for this discrepancy of bioaccumulation and effects may be that AgNP affects earthworms also without being taken up. The histopathology data (**chapter 5**) may suggest this, because the effects are mostly observed on the external barriers. Another explanation may be that earthworms accumulate Ag particles as well as Ag ions (Unrine et al. 2010). As it has been suggested that the particles may not contribute substantially to toxicity and merely increase the tissue Ag concentrations, the toxicity of AgNP may be primarily related to the fraction of Ag ions present in the earthworms (Kool et al. 2011; Shoults-Wilson et al. 2011c). Therefore, future research should account for the oxidation and dissolution of metal nanoparticles in order to explain potential accumulation and toxicity of metal nanoparticles and their ions for earthworms.

Furthermore, if nanoparticles are accumulated by soil organisms, these materials may be transferred within food chains. Holbrook et al. (2008) demonstrated the transfer of quantum dots in a simplified aquatic food chain, from ciliated protozoans to rotifers. Nanoparticle transfer may also occur in terrestrial food chains. Earthworms are considered to be very important for biomagnification of conventional pollutants in terrestrial ecosystems (Rogival et al. 2007; Roodbergen et al. 2008; Rozema et al. 2008; VandeCasteele et al. 2004) and may have the same role for nanoparticle biomagnification. Hence, possible biomagnification is an issue future research should deal with.

In addition to bioaccumulation of nanoparticles themselves, the impact of nanoparticles on bioaccumulation and toxicity of other environmental pollutants requires future investigation. Nanoparticles may influence bioaccumulation and toxicity of other environmental pollutants when they affect the external barriers, as observed in **chapter 3 and 5** for C_{60} and AgNP, enabling other compounds to enter the body more easily. In addition, nanoparticles may enhance the bioaccumulation of other compounds through adsorption, as was observed for C_{60} and TiO_2

nanoparticles by Baun et al. (2008b) and Zhang et al. (2007), respectively. These nanoparticles demonstrated strong adsorption capacities for other toxic compounds, e.g. the polycyclic aromatic hydrocarbon phenanthrene and the heavy metal cadmium, respectively, which enhanced the availability and bioaccumulation of phenanthrene and cadmium in aquatic organisms (Baun et al. 2008b; Zhang et al. 2007). The same phenomenon may occur in soil ecosystems, and for other nanoparticles and other environmental pollutants as well.

1b. Characterisation of adverse effects and sensitive endpoints

As soil organisms are and will be exposed to nanoparticles in the field, reliable and sensitive test systems are important to establish the relevant endpoints. The currently used toxicity tests may need modifications. The standard OECD toxicity test systems for exposure of earthworms encompass acute (48 hours) or four week exposure of adult earthworms (OECD 207:1984 and OECD 222: 2004). Both the C_{60} and the AgNP experiments discussed in the present thesis indicate that juveniles are more sensitive to the nanoparticle exposure and this can have adverse effects on earthworm populations. Therefore, experiments with earthworms from different life stages or lifelong exposure experiments may be useful in future research to better predict the impact of nanoparticle exposure under field conditions. Furthermore, the standard OECD toxicity test systems investigate mortality, growth and reproduction, using adult earthworms. These individual endpoints are very relevant to assess possible effects at the population level. However, they are usually affected at relatively high concentrations and additional endpoints are useful to inform about the mode of action of the toxicity (Lapied et al. 2010). In the present thesis, gene expression analysis was employed (**chapter 3**), which proved its usefulness to demonstrate toxicity of the C_{60} already from the lowest concentration examined. Gene expression of a small set of genes was studied, but profiling based techniques (including genomics, proteomics and metabolomics) are advancing and have been proven of use for earthworm research as well (Bundy et al. 2008; Gong et al. 2007; Guo et al. 2009; Kuperman et al. 2003; Wang et al. 2010). Therefore, the use of

these techniques at larger scale in future research studies may be helpful in unravelling the mode of action of nanoparticle toxicity.

Instead of *in vivo* experiments, the use of simple *in vitro* models using endpoints which may reveal general mechanisms of toxicity, may be a basis for further assessing the potential hazards of nanoparticle exposure and possibly in the future replace, reduce and refine *in vivo* experiments. For the most useful translation of the *in vitro* results to the *in vivo* situation, preferably a suitable and comparable *in vitro* system should be used. For example, as the results presented in **chapter 3 and 5** demonstrate the effects of C_{60} and AgNP on the external barriers, e.g. cuticle and gut epithelium, epithelial cells may be selected as an *in vitro* system for studying the mechanisms of this toxicity. Furthermore, in future research cultured cell types (such as NR8383 cells) may be used for large scale screening and the search for the mechanisms underlying the effects. In addition, primary cells (including earthworm coelomocytes), which are not easy to obtain and culture, may be used to ascertain the obtained results and to facilitate the translation of the *in vitro* to the *in vivo* situation. However, before (large scale) screening of *in vitro* nanotoxicity is possible, *in vitro* assays will need to be further developed, validated and standardized. One matter which needs further investigation is the best general theory behind cellular toxicity of nanoparticles. Oxidative stress, with the formation of reactive oxygen species (ROS), is considered as an important mode of action for nanoparticle induced cytotoxicity (Bhattacharjee et al. 2010; Li et al. 2008; Marambio-Jones and Hoek 2010; Stone and Donaldson 2006). However, findings differ between experimental set-ups and several studies indicate that the generation of ROS may be a secondary effect rather than causing the onset of toxicity (Bhattacharjee et al. 2011; Johnston et al. 2010; Park et al. 2011). Another important matter for *in vitro* assays is the selection of *in vitro* methods with endpoints that are not affected themselves by the nanoparticles. During our *in vitro* investigations interference of C_{60} and AgNP with the MTT and resazurin assays was observed (data not shown). This phenomenon has been demonstrated and discussed in other studies as well (Klaine et al. 2008; Kroll et al. 2009).

2. Exposure and behaviour of nanoparticles under field conditions

Apart from information on hazards, actual exposure levels under field conditions are also required for risk assessment. At the moment, information on release of nanoparticles into the environment is largely unknown and only small scale studies have investigated actual nanoparticle release into the environment (Farré et al. 2010; Hsu and Chein 2007; Kaegi et al. 2008; Kiser et al. 2009). Furthermore, standard analytical methods for the analysis of environmental concentrations of nanoparticles are lacking and actual exposure levels are still very difficult to determine (Handy et al. 2008a; Nowack 2009; Paterson et al. 2011; Vonk et al. 2009). Measuring nanoparticles in the soil is especially complicated because of the complex matrix (Gimbert et al. 2007). The nanoparticles may be present in the soil as single nanoparticles or as aggregates, or they may be dissolved or bound to soil compounds (including organic matter). These aspects seem to depend on the properties of the nanoparticle itself, including size, shape, charge, concentration and surface and core chemistry, as well as on the composition of the soil, e.g. content of organic matter and clay (Ben-Moshe et al. 2010; Cornelis et al. 2010; Darlington et al. 2009; Lapied et al. 2011; Stone et al. 2010; Zook et al. 2011). One of the biggest challenges for detection and characterisation of nanoparticles in soil is their separation from solid compounds naturally present in the soil without modifying the nanoparticles (Klaine et al. 2008). Here, the distinction between bioavailable and inaccessible nanoparticles (and their derivatives) also provides an analytical challenge for future research.

Furthermore, research on the perseverance and bioavailability of nanoparticles over time is needed, for a better understanding of exposure under field conditions. For example, the experiments discussed in **chapter 5** demonstrated more striking deviation in effects between AgNP and $AgNO_3$ for the long-term than for the four week experiment, because AgNP seemed able to provide prolonged exposure to dissolved Ag. In addition to the longer duration of the experiment, the use of 'aged' forms of nanoparticles may also be useful in future research to provide information on bioactivity, stability and prolonged presence or persistence of nanoparticles. 'Aged' and weathered nanoparticles may be the form in which nanoparticles are present in the environment rather than as the freshly suspended or synthesized nanoparticles generally used in experiments. 'Aged' nanoparticles may contain

modified or lost surface coatings, affecting nanoparticle properties (such as size, charge and surface chemistry) and behaviour (including clustering and dissolution) (Bastús et al. 2008; Zook et al. 2011).

In conclusion, the present thesis demonstrated hazards of exposure of the earthworm *L. rubellus* to the nanoparticles C_{60} and AgNP at different levels of biological organisation, which provided insight into possible modes of action of the nanoparticle toxicity as well as implications for earthworm populations. A valid translation of these results to the situation of populations of earthworms and other soil organisms under field conditions is not yet possible, because methods for reliable determination of nanoparticle levels in soil are lacking. The expected concentrations in the field are more than three orders of magnitude below the concentrations used in the experiments discussed in this thesis. However, due to expected and observed prolonged bioavailability of some nanoparticle species and the high levels of nanoparticles in sewage sludge, concentrations in the field may develop towards the levels of the experiments discussed in the present thesis in the near future. Furthermore, when focusing on the risk assessment procedure, it should be pointed out that in the experiments discussed in the present thesis effects were already observed at the lowest concentration used, for both C_{60} and AgNP. This implies that the NOAELs are below those concentrations. In addition, the experiments were performed using a single soil (model) species and assessment factors are needed to extrapolate to other soil organisms with unknown sensitivity. When taking these matters in consideration, the assessed levels discussed in the present thesis may be relevant for nanoparticle exposure to soil organisms in the near future. Therefore this work has contributed to understanding the potential hazards of nanoparticle addition (accidental or intentional) to the soil environment. Even though, as the future perspectives demonstrate, a lot of questions and challenges remain for future research.

Summary

Summary

Nanotechnology is an expeditiously growing field, where engineered nanoparticles are being incorporated in many different applications, from food to waste water treatment (Dekkers et al. 2011; Gottschalk and Nowack 2011; Savage and Diallo 2005). Due to this large scale production and use of nanoparticles, their release into the environment seems inevitable (Crane et al. 2008; Handy et al. 2008a; Oberdörster et al. 2005). Actual exposure levels of nanoparticles under field conditions and the hazards of nanoparticle exposure to the environment are poorly understood, especially for the soil environment (Kahru and Dubourguier 2010; Navarro et al. 2008; Shoults-Wilson et al. 2011a).

Given the need for better characterization of hazards of engineered nanoparticles to the environment and soil organisms in particular, the aim of the present thesis was to investigate effects of nanoparticle exposure on the earthworm *Lumbricus rubellus*, as a model organism for soil ecotoxicology, and to contribute to the development of effect markers for engineered nanoparticle exposure in this model.

The present thesis was divided in different chapters. **Chapter 1** provides an introduction to the topic and discusses the importance of research on the hazards of exposure to engineered nanoparticles. Furthermore, the aim and outline of the thesis are presented, with background information on the model organism, effect markers and nanoparticles.

In **chapter 2** effects of exposure to the fullerene C_{60} (nominal concentrations 0, 15.4 and 154 mg C_{60}/kg soil) on survival and growth during the different life stages of *L. rubellus* (cocoon, juvenile, subadult and adult), as well as reproduction were quantified. These important individual endpoints for population dynamics were incorporated in a continuous-time life-history model (Baveco and De Roos 1996; De Roos 2008). In this way, effects of C_{60} exposure on the individual endpoints could be extrapolated to implications for population growth rate and life stage distribution, i.e. the development of the population in terms of number of individuals in the different life stages. These implications at the population level may be more relevant for the ecological impact of C_{60} than effects on

endpoints at the individual level (Klok et al. 2006; Widarto et al. 2004). At the individual level C_{60} exposure caused significant adverse effects on cocoon production, juvenile growth rate and survival. When these endpoints were used to model effects on the population level, reduced population growth rates with increasing C_{60} concentrations were observed. Furthermore, a shift in life stage structure was shown for C_{60} exposed populations, towards a larger proportion of juveniles. This result implies that the lower juvenile growth rate induced by C_{60} exposure resulted in a larger proportion of juveniles, despite increased mortality among juveniles. Overall, this study implied serious consequences of C_{60} exposure for *L. rubellus* earthworm populations, even at the lowest level of exposure tested. Furthermore, it showed that juveniles were more sensitive to C_{60} exposure than adults.

To complement the observations made on survival, growth and reproduction described in **chapter 2**, subsequent investigations on cellular and molecular responses of the earthworms to C_{60} exposure were performed (**chapter 3**). A set of established effect markers was used, which reflect different levels of biological organisation in the earthworm and may inform on the toxic mechanisms of adverse effects induced by C_{60} exposure (Handy et al. 2002; Heckmann et al. 2008). At the molecular level, four specific effect markers were selected, including markers for generic stress (heat shock protein 70 (HSP70) (van Straalen and Roelofs 2006), for oxidative stress (catalase and glutathione-S-transferase (GST) (Kohen and Nyska 2002) and for an immune response (coelomic cytolytic factor-1 (CCF-1) (Olivares Fontt et al. 2002). At the tissue level, histological analyses were used to identify damage to cells and tissues, and indications of inflammation in the tissues. In these investigations, exposure to C_{60} (0, 15 or 154 mg C_{60}/kg soil) affected gene expression of HSP70 significantly. Gene expression of CCF-1 did not alter in adult earthworms exposed for four weeks, but was significantly down-regulated after lifelong exposure (from cocoon stage to adulthood) of earthworms, already to the lowest C_{60} exposure level. No significant trends were noted for catalase and glutathione-S-transferase (GST) gene expression or enzyme activity. Tissue samples of the C_{60} exposed earthworms from both experiments and exposure levels, showed a damaged cuticle with underlying pathologies of epidermis and muscles. Additionally, the gut barrier was not fully intact. However, tissue repair was also

observed in these earthworms. In conclusion, this study demonstrated effects of sub-lethal C_{60} exposure on *L. rubellus* earthworms, at the level of gene expression and tissue integrity.

Although tissue injury is generally associated with an inflammatory response, as part of tissue repair (Cikutovic et al. 1999; Goven et al. 1994), the tissue damage observed for the *in vivo* C_{60} exposed earthworms in **chapter 3** appeareded to occur without accompanying induced immune responses. The CCF-1 gene expression level was reduced in the C_{60} exposed earthworms, and histological observations did not show infiltration of damaged tissues by immune cells. In order to obtain further insight in mechanisms of effects observed at the molecular and tissue level on immune related parameters, the sensitivity of immune cells (coelomocytes) of *L. rubellus* earthworms towards exposure to selected nanoparticles was investigated *in vitro* (**chapter 4**). To this end, coelomocytes were isolated from unexposed adult *L. rubellus* earthworms and exposed to C_{60} *in vitro*. After exposure, these coelomocytes were tested for cellular viability, phagocytic activity and CCF-1 gene expression levels. The gene expression of CCF-1 was most affected, demonstrating a strong reduction, which indicated immunosuppression. Experiments with NR8383 rat macrophage cells and tri-block copolymer nanoparticles were used to compare sensitivity of the cell types and showed the usefulness of coelomocytes as a test system for nano-immunotoxicity in general. Overall, this study indicated that the absence of an immune response, in case of tissue injuries observed after *in vivo* C_{60} exposure, is likely caused by immunosuppression rather than coelomocyte mortality.

In subsequent investigations, the experiments performed for C_{60} were also carried out with silver nanoparticles (AgNP), both *in vivo* and *in vitro* (**chapter 5**). Effects of AgNP were assessed *in vivo* at nominal concentrations of 0, 1.5 (low), 15.4 (medium) and 154 (high) mg Ag/kg soil and compared to effects of silver ions, added as $AgNO_3$ (nominal concentration 15.4 mg Ag/kg soil). In a four week reproduction assay, the high AgNP and $AgNO_3$ treatments had a significant effect on cocoon production and high AgNP exposure also caused a reduction in weight gain of the adult earthworms. No juveniles survived the high AgNP treatment, therefore only F1 earthworms from the other exposure treatments

were monitored for survival and growth, until adulthood. These individual endpoints were used to model effects on the population level. The low and medium AgNP as well as the $AgNO_3$ treatments significantly reduced the population growth rate. The high AgNP treatment caused complete failure of the population growth. Furthermore, histological examination of the earthworms from all AgNP exposure treatments demonstrated tissue damage, with injuries mainly at the external barriers, e.g. the cuticle and the gut epithelium. In addition, effects of AgNP exposure were assessed *in vitro* and a reduction of coelomocyte viability was observed in a concentration-dependent manner, although the EC_{50} was fourteen times higher compared with that for Ag ions, added as $AgNO_3$. Furthermore, characterisation of the *in vivo* exposure media implied that AgNP remained present in the soil in single and aggregated state, releasing Ag to the soil pore water up to at least eleven months. The ionic fraction of Ag in soils has been suggested to be bioavailable to organisms and (largely) responsible for the observed AgNP toxicity (Coutris et al. 2012; Koo, et al. 2011; Shoults-Wilson et al. 2011b). In comparison, the $AgNO_3$ seemed to dissolve rapidly, as is also known for this metal salt, and fixation of Ag ions by the soil presumably led to a quick reduction of Ag bioavailability (Atkins and Jones 2000; Coutris et al. 2012; Ratte 1999). This is in line with the observation that effects were more prolonged in the AgNP treatments in comparison with the $AgNO_3$ exposed animals. In conclusion, this study indicated that AgNP exposure may seriously affect earthworm populations, with the ability to cause immunotoxicity, injury to the external barriers of the earthworm body and a reduction in growth, reproduction and juvenile survival.

Finally, **chapter 6** presents a discussion on the findings of the present thesis and provides suggestions for future research.

Samenvatting

Samenvatting

Nanotechnologie is een snel groeiende technologie waarbij kunstmatige, door de mens ontworpen deeltjes, zogeheten nanodeeltjes, worden gebruikt voor veel verschillende soorten toepassingen (Gottschalk and Nowack 2011; Wijnhoven et al. 2010; Woodrow Wilson institute). De manieren waarop nanodeeltjes worden toegepast zijn divers en breed; van voedsel, sportartikelen, verzorgingsproducten, medische applicaties, tot apparatuur voor rioolwaterzuivering en monitoring van het milieu (Andreescu et al. 2009; Dekkers et al. 2011; Gottschalk and Nowack 2011; Jain 2008; Savage and Diallo 2005). Gezien de grootschalige productie en toepassing van nanodeeltjes, lijkt het onvermijdelijk dat deze deeltjes vrijkomen in het milieu en dat mensen en andere organismen eraan worden blootgesteld (Crane et al. 2008; Handy et al. 2008a; Oberdörster et al. 2005). Helaas geven sommige eigenschappen die nanodeeltjes nuttig maken voor veel toepassingen, zoals chemische reactiviteit en persistentie, ook reden tot zorgen over de mogelijke negatieve effecten voor mens en milieu (Warheit et al. 2008; Farré et al. 2011). Over de concentraties van nanodeeltjes in het milieu en de gevaren voor het milieu van blootstelling aan nanodeeltjes, is nog onvoldoende bekend. Dit geldt vooral voor de bodem (Kahru and Dubourguier 2010; Navarro et al. 2008; Shoults-Wilson et al. 2011a).

Inspelend op de behoefte om de gevaren van kunstmatige nanodeeltjes voor het milieu in het algemeen en voor bodemorganismen in het bijzonder beter in kaart te brengen, was het doel van de studies beschreven in dit proefschrift om de effecten van de blootstelling van nanodeeltjes op de regenworm *Lumbricus rubellus* te onderzoeken. Deze wormensoort diende als modelorganisme voor bodem ecotoxicologie. Daarnaast waren de studies ook opgezet om bij te dragen aan de ontwikkeling van effect-indicatoren voor de blootstelling aan kunstmatige nanodeeltjes in dit onderzoeksmodel.

Het proefschrift is opgedeeld in zes hoofdstukken. **Hoofdstuk 1** geeft een introductie in het onderwerp en beschrijft het nut van onderzoek naar de gevaren van blootstelling aan kunstmatige nanodeeltjes. Verder bespreekt het hoofdstuk het doel van de studies

beschreven in het proefschrift, met achtergrond-informatie over het modelorganisme *L. rubellus*, effect-indicatoren en nanodeeltjes.

In **hoofdstuk 2** werd onderzocht welke effecten de blootstelling aan het koolstofnanodeeltje C_{60} (met nominale concentraties van 0, 15.4, 154 mg C_{60}/kg grond) heeft op de voortplanting van *L. rubellus* regenwormen en op de overleving en groei van de regenwormen tijdens verschillende levensfasen (cocon, juveniel, subadult en volwassen). Deze kenmerken of parameters van de individuele regenwormen zijn belangrijk in de populatie dynamica en werden opgenomen in een 'continuous-time life-history' model (Baveco and De Roos 1996; De Roos 2008). Dit model maakte het mogelijk om de effecten van blootstelling aan C_{60} op de individuele parameters te vertalen naar gevolgen voor populatie-groeisnelheid en levensfase-verdeling. De levensfase-verdeling laat de ontwikkeling van de populatie zien voor het aantal individuen per levensfase. Deze gevolgen op populatie niveau kunnen relevanter zijn voor het ecologische effect van C_{60} dan effecten op de individuele parameters (Klok et al. 2006; Widarto et al. 2004).
Op het individuele niveau werden bij de regenwormen significant nadelige effecten van blootstelling aan C_{60} vastgesteld voor de cocon-productie, en de juveniele groei en overleving. Vertaling van deze parameters naar het populatieniveau liet zien dat de populatie-groeisnelheid afnam naarmate de C_{60} testconcentraties werden verhoogd. Daarnaast was de levensfase-verdeling voor de populaties van de aan C_{60} blootgestelde regenwormen verschoven, want er was een relatief grotere groep van regenwormen in de juveniele fase. De lagere juveniele groeisnelheid, veroorzaakt door C_{60} blootstelling, zorgde voor een grotere groep regenwormen in de juveniele fase, ondanks de grotere sterfte onder juvenielen. Uit dit experiment werd geconcludeerd dat blootstelling aan C_{60}, bij de concentraties die gebruikt zijn in dit experiment, ernstige gevolgen kan hebben voor populaties van regenwormen, en dat juveniele regenwormen gevoeliger zijn voor deze blootstelling dan volwassen regenwormen.

In aanvulling op het experiment in **hoofdstuk 2**, zijn er in **hoofdstuk 3** ook op cellulair en moleculair niveau testen uitgevoerd met de aan C_{60} blootgestelde regenwormen. Hiervoor is

een set van bestaande effect-indicatoren gebruikt. Deze indicatoren geven informatie over de effecten van blootstelling aan C_{60} op verschillende niveaus van de biologische organisatie van de regenworm en over de toxische mechanismen van deze effecten (Handy et al. 2002; Heckmann et al. 2008). Op moleculair niveau werden enkele effect indicatoren geselecteerd, waaronder indicatoren voor algemene stress (heat shock protein 70, HSP70) (van Straalen and Roelofs 2006), voor oxidatieve stress (catalase and glutathione-S-transferase, GST) (Kohen and Nyska 2002) en een indicator voor een immuun-reactie (coelomic cytolytic factor-1, CCF-1) (Olivares Fontt et al. 2002). Op het cellulaire weefsel-niveau zijn histologische analyses uitgevoerd, waarbij gekeken is naar beschadigingen aan cellen en weefsels, en naar ontstekingen in de weefsels.
De testen op moleculair niveau lieten zien dat blootstelling aan C_{60} (0, 15 of 154 mg C_{60}/kg grond) een significant effect had op de genexpressie van de algemene stress-indicator HSP70. Bij de genexpressie van CCF-1 was geen effect te zien voor de regenwormen die vier weken waren blootgesteld. De levenslang blootgestelde regenwormen hadden, bij beide C_{60} concentraties, echter een significant lagere genexpressie van deze immuun-reactie indicator ten opzichte van de controle-groep. Voor catalase en GST, indicatoren voor oxidatieve stress, werden geen significante effecten op de genexpressie vastgesteld. De histologische analyse van de weefselmonsters lieten een beschadigde cuticula (buitenste huidlaag) zien, met schade aan onderliggende epidermis en spieren, voor regenwormen van beide C_{60} concentraties en experimenten. Daarnaast was de darmwand van deze wormen beschadigd. Tegelijkertijd was er echter herstel van de darmwandweefsels te zien. Op basis van de resultaten uit dit hoofdstuk werd geconcludeerd dat effecten van niet-dodelijke blootstelling van regenwormen aan C_{60} ook op het niveau van genexpressie en weefselstructuur te bepalen is.

Over het algemeen gaat weefselschade gepaard met ontsteking als onderdeel van weefselherstel (Cikutovic et al. 1999; Goven et al. 1994). Echter, in de beschadigde weefsels van de regenwormen die *in vivo* waren blootgesteld aan C_{60} , zoals beschreven in **hoofdstuk 3**, werden geen ontstekingen waargenomen. Deze regenwormen lieten namelijk

een verlaagde CCF-1 genexpressie zien en bovendien werd tijdens de histologische analyse van de weefsels geen infiltratie geobserveerd van beschadigde weefsels met immuuncellen.
Om meer inzicht te krijgen in de mechanismen van de aan het immuunsysteem-gerelateerde effecten die zijn waargenomen op moleculair en weefsel-niveau, zijn in **hoofdstuk 4** immuuncellen (coelomocyten) van niet-blootgestelde *L. rubellus* regenwormen geïsoleerd en *in vitro* getest op gevoeligheid voor blootstelling aan C_{60}. De coelomocyten werden getest op cellulaire overleving, fagocytische activiteit en CCF-1 genexpressie. Uit deze experimenten bleek dat CCF-1 genexpressie de meest gevoelige parameter was voor C_{60} blootstelling, aangezien deze een sterke reductie liet zien bij toenemende C_{60} concentraties. Dit resultaat suggereert een onderdrukking van de immuunreactie. Verder werden ook *in vitro* experimenten uitgevoerd met NR8383 rat macrofaag cellen en tri-block copolymeer nanodeeltjes, om de gevoeligheid van verschillende celtypen te vergelijken en om de bruikbaarheid van coelomocyten als testsysteem voor nano-immunotoxiciteit aan te tonen. Uit de *in vitro* experimenten beschreven in dit hoofdstuk werd geconcludeerd dat de afwezigheid van een immuunreactie, zoals geobserveerd in **hoofdstuk 3**, eerder werd veroorzaakt door onderdrukking van het immuunsysteem dan door verhoogde mortaliteit van coelomocyten.

De experimenten die werden uitgevoerd met C_{60}, werden in **hoofdstuk 5** herhaald met zilver nanodeeltjes (AgNP), zowel *in vivo* als *in vitro*. Effecten van *in vivo* blootstelling aan AgNP werden vastgesteld bij nominale concentraties van 0, 1.5 (laag), 15.4 (midden) en 154 (hoog) mg Ag/kg grond, en vergeleken met effecten na blootstelling aan zilvernitraat ($AgNO_3$), bij een nominale concentratie van 15.4 mg Ag/kg grond.
In een vier weken durend reproductie-experiment met volwassen regenwormen produceerden de regenwormen die blootgesteld waren aan de $AgNO_3$ en de hoge AgNP grond significant minder cocons. Daarnaast hadden de regenwormen van de hoge AgNP blootstellingsgroep een lagere gewichtstoename. Bij het monitoren van de nakomelingen van de blootgestelde regenwormen, blootgesteld aan dezelfde concentraties als hun ouders, kwamen bij de hoge AgNP blootstellingsgroep maar twee juvenielen uit hun cocon en die stierven snel. Om deze reden werden alleen de nakomelingen van de andere

blootstellingsgroepen gemonitord op overleving en groei, totdat ze volwassen waren. De individuele parameters van de reproductie- en de nakomelingenexperimenten werden gebruikt om effecten op populatie niveau vast te stellen. Deze analyse liet significant lagere populatie groeisnelheden zien voor de lage en midden AgNP blootstellingsgroepen en de $AgNO_3$ blootstellingsgroep. De hoge AgNP blootstelling zorgde ervoor dat de populatie helemaal niet groeide. De blootgestelde regenwormen, van zowel het reproductie-experiment als van het nakomelingenexperiment, werden ook gebruikt voor een histologische analyse van het weefsel. Uit deze analyse kwam naar voren dat alle AgNP blootstellingen (laag, middel en hoog) weefselschade veroorzaakten, waarbij de schade vooral geobserveerd werd aan de zogeheten 'externe barrières', de cuticula en de darmwand.

Daarnaast werd er ook een *in vitro* experiment uitgevoerd, waarbij een verminderde overleving van de coelomocyten werd vastgesteld bij toenemende AgNP concentraties. Voor AgNP lag de EC_{50} (de concentratie waarbij de overleving van de coelomocyten 50% lager was dan de controle) wel veertien keer hoger dan de EC_{50} voor zilverionen, toegevoegd als $AgNO_3$.

Verder werd in **hoofstuk 5** ook de aanwezigheid en mogelijke *in vivo* blootstelling aan AgNP en $AgNO_3$ geanalyseerd. De resultaten van deze analyse gaven aan dat AgNP zowel afzonderlijk als geaggregeerd in de grond aanwezig waren, en ertoe in staat zijn om voor tenminste elf maanden Ag (ionen en wellicht kleine Ag aggregaten) vrij te laten in het poriewater van de grond. De ionenfractie van Ag in de grond wordt wel gezien als het deel dat (meest) beschikbaar is voor organismen en (grotendeels) verantwoordelijk is voor de geobserveerde toxiciteit van AgNP op bodemorganismen (Coutris et al. 2012; Kool et al. 2011; Shoults-Wilson et al. 2011b). Ter vergelijking werd de aanwezigheid van $AgNO_3$ in de grond ook geanalyseerd en dit metaalzout leek snel op te lossen, zoals wel bekend voor $AgNO_3$ (Ratte 1999). Deze analyse van aanwezigheid en mogelijke blootstelling van AgNP en $AgNO_3$ in de grond was in overeenstemming met de effecten van *in vivo* blootstelling op de regenwormen, waarbij de blootstelling aan AgNP een duidelijker effect van langdurige blootstelling liet zien dan $AgNO_3$. Uit de resultaten van dit hoofdstuk werd geconcludeerd dat blootstelling aan AgNP ernstige gevolgen kan hebben voor populaties van

regenwormen, met de mogelijkheid op immunotoxiciteit, schade aan externe barrières van het lichaam van de regenwormen en een vermindering van groei, reproductie en overleving van juvenielen te veroorzaken.

Tenslotte werden in **hoofdstuk 6** de bevindingen van het huidige proefschrift bediscussieerd en werden suggesties voor toekomstig onderzoek besproken.

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About the author

Curriculum vitae

Merel van der Ploeg was born September 27 1982 in Bussum. After attending the 'Goois Lyceum' secondary school in Bussum, she started a study Biology at the University of Groningen in 2000. After two years of study, she took a gap year, to backpack through New Zealand and Australia. After this year, she returned to the study Biology, motivated. During her Msc in Marine Biology, she conducted three research projects. The first project was at the Department of Animal Ecology of the University of Groningen, where she studied the trade-off between secondary sexual traits and the immune system of cichlid fish. Subsequently, she did her second research project at the seal rehabilitation centre Lenie 't Hart in Pieterburen, on the transfer of antibodies against morbilli and herpes virus from mother to pup, in common and grey seals. For her third research project she went to the Institute of Ocean Sciences of the Environment Canada in Sydney, Canada, where she studied the effects of natural factors on biomarkers (vitamin A and thyroid hormones) in blood and blubber of seal pups. During her Msc, she also followed courses on 'Science in Business and Policy' and communication skills. After finishing her Msc in 2007, she wanted to gain more knowledge in the field of ecotoxicology, and therefore she started a PhD project on 'Environmental risks of nanoparticles to soil organisms (NanoSoil)' at the department of Toxicology of the Wageningen University in cooperation with Alterra Wageningen UR, in 2008. This project was conducted under the supervision of Prof. dr. ir. Ivonne M.C.M. Rietjens and dr. ir. Nico W. van den Brink and resulted in this thesis. During her PhD she followed postgraduate courses in Toxicology, in order to register as a Toxicologist with the Dutch Society for Toxicology (NVT). She was also involved in several extra-curricular activities at the Wageningen University, including her chairwomanship of the PhD council of the researchschool WIMEK/SENSE (SPC), for three years, and the co-organization of the 2011 PhD study trip of the department of Toxicology, to Switzerland and Italy.

List of publications

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Overview of completed training activities

Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment

C E R T I F I C A T E

The Netherlands Research School for the
Socio-Economic and Natural Sciences of the Environment
(SENSE), declares that

Merel (Janneke Christiane) van der Ploeg

born on 27 September 1982 in Bussum, the Netherlands

has successfully fulfilled all requirements of the
Educational Programme of SENSE.

Wageningen, 30 January 2013

the Chairman of the SENSE board
Prof. dr. Rik Leemans

the SENSE Director of Education
Dr. Ad van Dommelen

The SENSE Research School has been accredited by the Royal Netherlands Academy of Arts and Sciences (KNAW)

KONINKLIJKE NEDERLANDSE
AKADEMIE VAN WETENSCHAPPEN

The SENSE Research School declares that **Ms. Merel van der Ploeg** has successfully fulfilled all requirements of the Educational PhD Programme of SENSE with a work load of 58 ECTS, including the following activities:

SENSE PhD Courses

- Environmental Research in Context
- Research Context Activity: Co-organizing International PhD study trip to Switzerland and Italy for Department of Toxicology, Wageningen University, June 2011
- Risk Assessment

Other PhD Courses

- General Toxicology, Postgraduate Education in Toxicology (PET)
- Epidemiology, PET
- Organtoxicology, PET
- Mutagenisis and Carcinogenesis, PET
- Laboratory Animal Science, PET
- Cell Toxicology, PET
- Molecular Toxicology, PET
- Medical, Forensic and regulatory Toxicology, PET
- Immunotoxicology, PET
- Toxicogenomics, PET
- Project and Time management

Management and Didactic Skills Training

- Member of the SENSE PhD council, 2009-2011
- Supervision of one MSc thesis and one MSc internship
- Supervision of practicals for the MSc courses Food Toxicology and Environmental Toxicology

External training at a foreign research institute

- qPCR with earthworm samples at the National Environmental Research Institute (NERI), Denmark

Oral Presentations

- *Effects of nanoparticles on soil organisms*. SETAC Europe, 31 May - 4 June 2009, Gotenborg
- *Effects of C60 on earthworms Lumbricus rubellus*. 4th International Conference on the Environmental Effects of Nanoparticles and Nanomaterials, 6 - 9 September 2009, Vienna
- *Environmental hazards of nanoparticles to earthworms*. SETAC Europe, 23 - 27 May 2010, Sevilla
- *Effects of nanoparticles on Lumbricus rubellus earthworms*. NanoSENSE Workshop, 27 October 2011, Amsterdam

SENSE Coordinator PhD Education and Research

Mr. Johan Feenstra

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