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**Toxicity assessment of silver nanoparticles in soils
through standard tests, an integrated battery of
biomarkers at different levels of biological complexity and
in vitro approaches with coelomocytes in *Eisenia fetida***

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I. INTRODUCTION

1. Nanotechnology and engineered nanoparticles

Nanotechnology is one of the most powerful emerging technologies of the end of the twentieth century, which enables the design, characterization, production and application of structures and systems with novel properties and functions due to their size (The Royal Society and The Royal Academy of Engineering, 2004). The size range that holds so much interest is at the nanoscale, where (nano)materials can have different or enhanced properties (e.g catalytic activity, mechanical strength, electrical conductivity, etc.) arising from an increased relative surface area (per unit mass) and, in some cases, quantum effects (Sellers, 2009). Nanomaterials (NMs) can be synthesised with different structures, such as one-dimensional films, thin layers and surface coatings; tubes, wires or rods (in two dimensions); and single particles or crystals, dendrimers, composites and carbon fullerenes at nanoscale in three dimensions (Chen et al., 2011; Royal Commission on Environmental Pollution, 2008; Sellers, 2009). If all external dimensions are in the nanoscale the conditions for a nanoparticle (NP) are given (ISO, 2008). NPs are characterized to have at least 50% of the particles (by number size distribution) with one or more external dimensions in the size range of 1–100 nm (EU, 2011a) and exhibited these new size-dependent properties compared with larger size particles (Fig. 1).

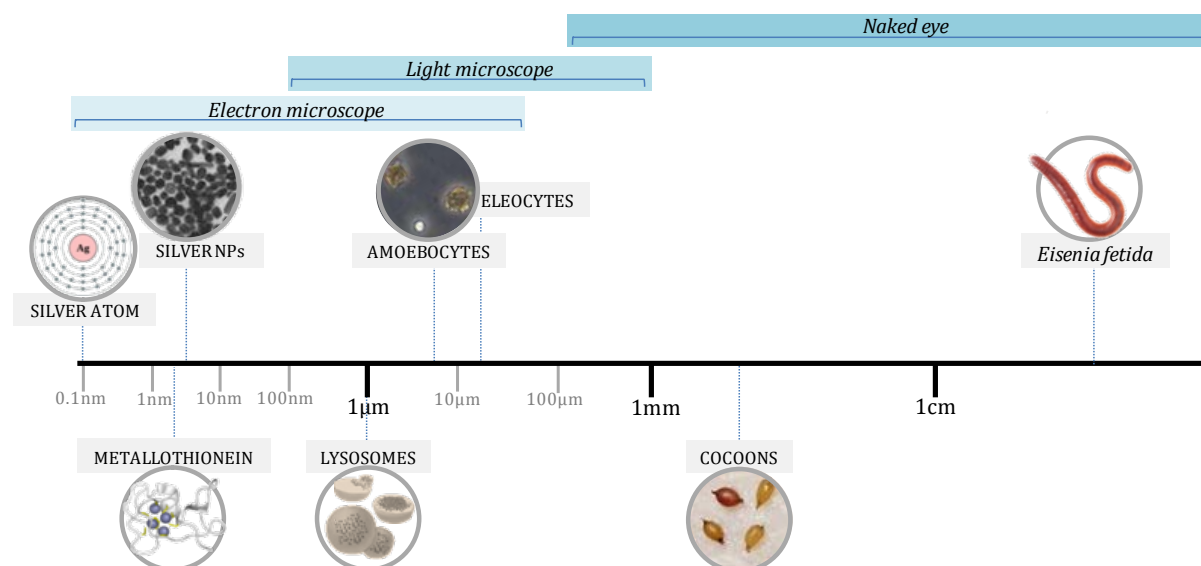


Figure 1. Relative sizes in a logarithmic scale of silver nanoparticles (Silver NPs) and some biological components used in this thesis.

NPs have occurred naturally as products of photochemical and volcanic activities and created by humic materials, algae and bacteria (environmental NPs; Stuart and Compton, 2015). They have also been originated from human activity for thousands of years as products of combustion and industrial processes (incidental NPs; Bleeker et al., 2013). Recently, the intentionally manufactured NPs have attracted the major attention due to the increasing ability of nanoscience and nanotechnology to synthesize and manipulate such materials and to incorporate them in different applications and consumer products. In the latter case, they are often referred to as engineered or manufactured nanoparticles (ENPs or MNPs).

ENPs can be separated based on their chemical composition into carbon-based, inorganic and polymeric, being inorganic NPs further subdivided between oxides (e.g. TiO₂, SiO₂, ZnO, Al₂O₃ and nanomagnetite), elemental metals (e.g. Ag, Fe, Au, nZVI), salts (e.g. metal-phosphates) and the less known aluminosilicates (e.g. zeolites, clays and ceramics) (Nowack and Bucheli, 2007; Sauvé and Desrosiers, 2014). These products of nanotechnology have numerous applications and are being incorporated in many consumer products with medical, domestic, cosmetic, industrial and military uses. In fact, more than 1800 nanotechnology-based products are commercially available nowadays (Vance et al., 2015), around the 15% of all global consumer products (Stuart and Compton, 2015). Further, nanotechnology is considered to have the potential to revolutionise manufacturing industries over the next 10-15 years (OECD, 2015).

1.1. Silver nanoparticles (Ag NPs)

Metal-based NPs (including metal and metal oxides) comprise the majority of MNPs in current use (Tourinho et al., 2012; Vance et al., 2015). Among them, silver nanoparticles (Ag NPs) are of the most frequent. In 2012, Ag NPs were produced at quantities around 55t/year worldwide and at maximum of 10t/year in Europe (Piccinno et al., 2012). Moreover, 438 nanosilver containing products, 24% of the total products containing NMs, were listed in the Nanotechnology Consumer Products Inventory (CPI) in 2015 (Fig. 2). This number has increased gradually in the last years (an increment of 41% from 2011 up to date) and a growing trend is expected for the coming years.

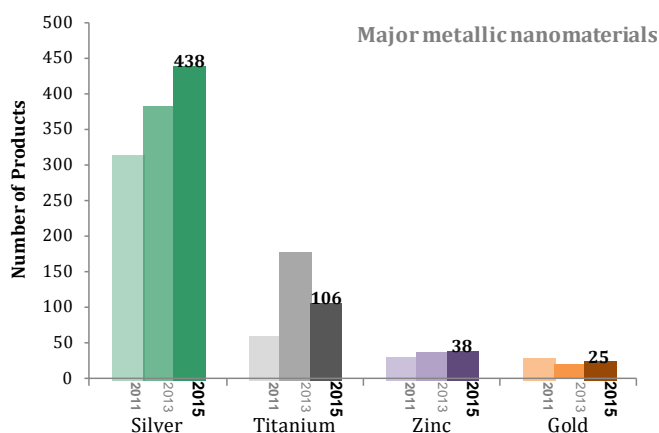


Figure 2. Number of products in the market containing metallic nanomaterials (silver, titanium, zinc and gold) in the years 2011, 2013 and 2015 (Data obtained from *The Nanotechnology Consumer Products Inventory-CPI*, <http://www.nanotechproject.org/cpi/>).

Ag NPs are so popular additives in consumer product due to their unique optical, catalytic and antimicrobial properties (García-Barrasa et al., 2011; Rai et al., 2009). Ag NPs can be found in consumer electronics and for conductivity uses; however, the vast majority of the produced Ag NPs (around 80%) are destined for antimicrobial-coatings (Piccinno et al., 2012). In fact, silver has been for a long time known to have a disinfecting effect and capacity to control bacterial growth, thus, several silver salts and their derivatives have been employed as antimicrobial agents in different biomedical fields, especially for wound healing and burn treatments (Edward-Jones, 2009). Nevertheless, nanosized silver have gained higher commercial and scientific interest in comparison with other silver forms since its large surface area provided better contact with microorganism, increasing biocompatibility. Ag NPs interact with the cell membrane of bacteria, getting attached, interfering with the membrane stability and also penetrating inside, where they can damage sulphur or phosphorus containing compounds like DNA (García-Barrasa et al., 2011; Rai et al., 2009), resulting ultimately in cell death. Hence, Ag NPs can be used as effective growth inhibitors for various microorganisms (i.e. *Escherichia coli*, *Staphylococcus aureus*, *S. epidermidis* and *Listeria monocytogenes*) (García-Barrasa et al., 2011; Kim et al., 2007), making them applicable to diverse medical devices and water treatments (Gong et al., 2007). Their fungicidal activity has been also demonstrated against 18 plant pathogens (e.g. *Fusarium sp.*, *Botrytis cinerea*, etc.) (Kim et al., 2012). Ag NPs can be also found in cleaning agents (e.g. detergents, soaps, wet wipes, etc.), washing machines, textiles (e.g. socks, bedding, sport clothing, etc.), food storage containers, paints and cosmetics (Benn et al., 2008; Piccinno et al., 2012; The Project of emerging Nanotechnologies, 2017-<http://www.nanotechproject.org/cpi/>).

2. Release of Ag NPs into the environment

Since Ag NPs are the most common NP composition for commercialization, they are also the most likely materials to enter into the environment. Consequently, the inputs of Ag NPs into the different environmental compartments are expected to increase, posing concerns regarding risks for ecosystems, living organisms and human health. NMs, including Ag NPs, may end up in aquatic ecosystems and/or soil through many pathways, either from direct release during production or transport, deposition onto landfills as fertilizers (Yu et al., 2013), waste incineration (Jòsko et al., 2013), release from products, or as a material contained within products (Klaine et al., 2012). However, the main sources of Ag NPs in the aquatic and terrestrial ecosystems are the wastewater treatment plants (WWTPs) (Brar et al., 2010; Mueller and Nowack, 2008; Tourinho et al., 2012). Ag NPs used in consumer products and in other industrial applications will be discharged to the sewer system and transported to WWTP. In this context, the amount of released silver strongly depends on the manufacturing process, product use, and disposal (Reidy et al., 2013) and thus, several studies have concerned Ag NPs releases from different materials such as textiles (Benn et al., 2008; Blaser et al., 2008) and paints (Kaegi et al., 2010).

In regard to quantities of Ag NPs reaching the environment, fate models have been carried out in order to calculate predicted environmental concentrations (PECs). These models are based on probabilistic material flow analysis from a life-cycle perspective of ENP containing products (Boxall et al., 2007; Gottschalk et al., 2009; Mueller and Nowack, 2008). Such studies estimated likely concentrations of ENPs in different compartments, including surface waters, WWTP effluents, biosolids, sediments, soils and soils treated with biosolids.

Once released into the environment, Ag NPs may undergo different transformations during their transport in the natural systems (air, water, soil) that will alter their starting characteristics and consequently, change their fate, behavior and ecotoxicology (Tourinho et al., 2012). These transformations will be determined by NPs physical (i.e. size and shape) and chemical (e.g. acid-base character of the surface, solubility) characteristics as well as by their surrounding environment, where Ag NPs could remain as individual particles, aggregate at high ionic strength, oxidize and liberate Ag ions or react with sulfide, chloride or other natural substances (Buzea et al., 2007; Yu et al., 2013). These alterations in the original characteristics of the NPs, apart from on their

intrinsic properties, largely depend on the environmental conditions, involving calcium concentration, ionic strength, cations, pH and organic matter (OM) (Klaine et al., 2008). For instance, soils represent a complex medium for the understanding of the physico-chemical behavior of NPs, taking into consideration the potential interactions of Ag NPs with particulate material and natural colloids in soils solid and aqueous phases.

2.1. Ag NPs deposition onto soils

Ag NPs in particular are predicted to primarily enter terrestrial system (Shoults-Wilson et al., 2011a). In some instances, Ag NPs can directly enter in the terrestrial compartment through accidental spills during synthesis and transformation, soil and water remediation technologies, atmospheric fallouts (i.e. after waste incineration and posterior deposition on land) and agriculture (thought the use of nanopesticides, herbicides and fertilizers) (Blaser et al., 2008; Gottschalk et al., 2009; Shoults-Wilson et al., 2011a). Nevertheless, the major input of Ag NPs to soils is currently through the disposal of WWTP sludges, mainly after their land application or incineration and posterior deposition (Tourinho et al., 2012) (Fig. 3). A large fraction of Ag NPs are predicted to enter WWTP via sewage streams, leached from products and from industrial and household applications, ending up in the sewage sludge as a result of partial elimination during the treatment (Gottschalk et al., 2009; Jósko et al., 2013; Kaegi et al., 2011). The resulting sludge or biosolid is commonly used in many countries as fertilizer or as amendment in agricultural soils since its organic constituents pose beneficial soil conditioning properties (e.g. higher levels of soil OM and microbial activity, improvements in aggregation and structural stability, etc.) (Alloway and Jackson, 1991; Natal-da-Luz et al., 2009). In fact, in Europe the 53% of the produced sewage sludge is applied on agricultural soils as a fertilizer (Kelessidis and Stasinakis, 2012). Further, is estimated that the sewage sludge contribute to inputs of $1 \mu\text{g}/\text{kg}^3$ of Ag to worldwide agricultural land per year (Mueller and Nowack, 2008).

The second most preferable disposal practice in Europe is the incineration of the sludges (21%), so these particles are deposited (dry deposition) or washed out (wet deposition) on soil or water. During the wastewater treatment, Ag NPs may be subjected to various processes leading to their transformation. In this framework, silver sulfide (Ag_2S) NPs are the predominant Ag species found in sewage sludge. Sulfidation of Ag NPs is a common process taking place in WWTPs and more generally in environmental and biological compartments. However, recent studies revealed that Ag NPs spiked to a

WWTP and transformed into Ag₂S NPs during the activated sludge process, will be transformed back into metallic Ag NPs (maintaining the original size) during their incineration (Kaegi et al., 2011). Considering Ag NPs incombustible, Ag NPs will not be eliminated and will reach soils since incineration residues are usually landfilled in Europe. Moreover, the sewage sludge is also directly landfilled (15%), a practice through which Ag NPs could leach into subsoil and groundwater (Blaser et al., 2008).

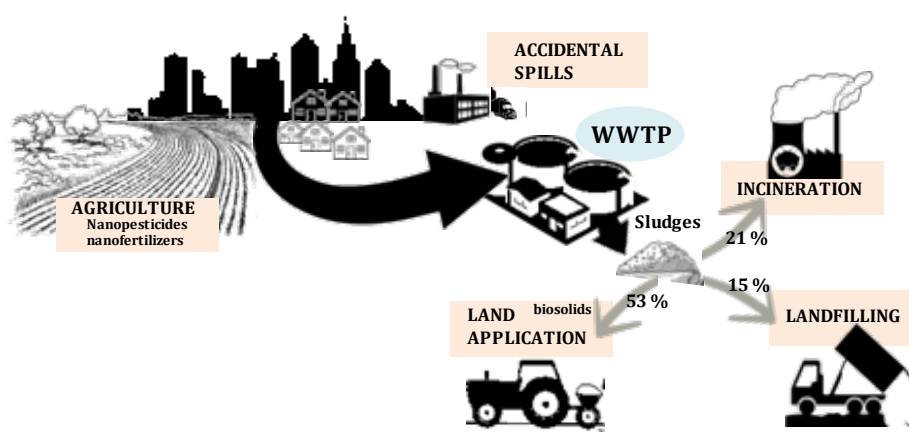


Figure. 3 Possible pathways that could introduce Ag NPs into soils.

At present, there is no available data regarding measurement of Ag NPs in soils due to the absence of analytical methods able to quantify trace concentrations of NPs. Indeed, the quantification of Ag NPs in soil represents a real analytical challenge. Although actual analytical methodology is well equipped to detect environmentally relevant concentrations of traditional pollutants (e.g. trace metals, pesticides, persistent organic pollutants, etc.), NMs detection and characterization in complex matrixes such as soil raised several challenges emerged from the solid-state properties of NMs. These challenges include differentiating NPs from backgrounds natural particles, the use of multiple methods to confirm an accurate detection and achieving sufficiently low detection limits to realistically monitor NPs in environmental compartments (Klaine et al., 2012). Several spectrophotometric analytical methods (e.g. single particle inductively coupled plasma-mass spectrometry-spICP-MS-, UV-vis) have been demonstrated to have potential for environmental detection of NPs (Tuoriniemi et al., 2012). In addition, particle visualization (i.e. scanning electron-SEM-, transmission electron-TEM-, helium ions and atomic force microscopy), size determination (e.g. X-ray, dynamic light scattering-DLS-, fluorescence spectra) and separation techniques (e.g. flow cytometry, ultracentrifugation, field-flow fractionation) might be adaptable to the measurement of NPs in environmental matrixes (Howard et al., 2010). Nevertheless,

through some of these techniques is difficult to distinguish between Ag NPs and silver ions, while methods such as TEM and other particle sizing approaches have difficulty distinguishing and finding low concentrations of particles and could be time consuming and costly (Reidy et al., 2013).

The current lack of information about Ag NPs concentration in soils has resulted in modelling of PECs as a valuable alternative for measurement studies. Models have predicted that Ag NPs in WWTP sludges ranges from 1.31-4.44 mg Ag NPs/kg in Europe (Boxall et al. 2007; Gottschalk et al. 2009), in contrast, the estimations for Ag NP concentration in soils are much lower (Table 1). However, Ag NPs in sludge treated soil reached 0.007 mg/kg in 2012, a value that is expected to be annually enhanced in countries with a high proportion of sludge or sludge incineration residues land-disposal (Gottschalk et al., 2009). Hence, models evidence inputs of Ag NPs in soils, which once reached land would have the potential to contaminate soil (Klaine et al., 2008).

Table 1. Predicted environmental concentrations (PECs) for Ag NPs in sludge, sludge treated soil and soil based on Life Cycle Release Models. PECs are shown as range and mode (the most frequent value).

	Unit	Location	Range	Range details	Mode	Study (Ref)
Sludge	mg/kg	Europe	1.31-4.44	Lower-Upper quantities	1.68	Gottschalk et al., 2009
		UK	0.29-2.90	10%-100% market penetration	-	Boxall et al., 2007
		Switzerland	1.46-6.24	Lower-Upper quantities	1.88	Gottschalk et al., 2009
Soil with sludge	mg/kg	Europe	-	-	0.007	Gottschalk et al., 2009
Soil	µg/kg	Europe	0.02-0.1	Realistic-High emission	-	Mueller & Nowack, 2008
		UK	0.43-4.26	10%-100% market penetration	-	Boxall et al., 2007

2.2. Ag NPs as a contaminant of emerging concern in soils

Soil is one of nature's most complex living systems, described as multicomponent and multifunctional, and with definable operating limits (Kibblewhite et al., 2008). Soil is the basis for food, fuel and fibre production and for many critical ecosystem services including nutrient cycling, carbon sequestration, water purification, climate regulation, biodiversity pool supporter and habitat for organisms (Fig. 4). This ability of soil to deliver ecosystem services is under increasing pressure due to the intensification and competing uses of forestry, cropping, pasture and urbanization, which enhanced rates of soil degradation. In fact, 33% of global soil is moderately to highly degraded in form of erosion, sealing, compaction, salinization, acidification, nutrient depletion and chemical contamination (FAO, 2015).

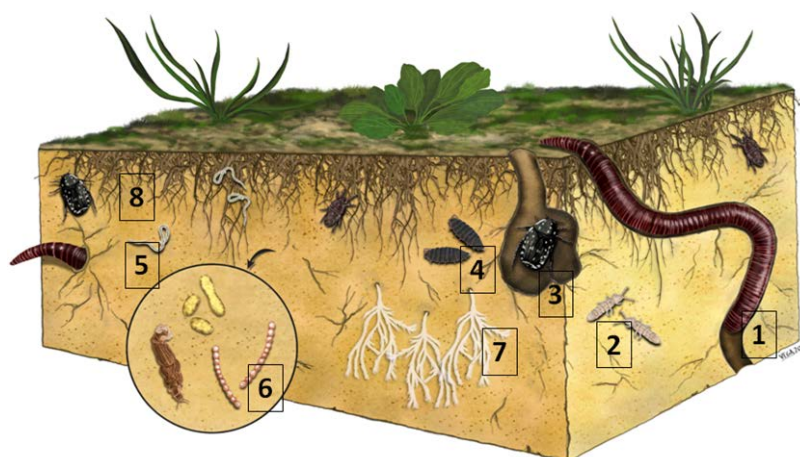


Figure 4. Soils as biodiversity supporters and habitat for numerous organisms: earthworms (1), collembola (2), insects (3), isopods (4), enchytraeus (5), bacteria (6), fungi hyphae (7) and plant roots (8). (Image from Vega Asensio, NorArte illustration studio, www.norarte.es).

The large volume of waste production and disposal (municipal and industrial) together with the handling of chemicals within industrial and commercial activities (e.g. mining, oil extraction and production, power plants etc.) during the past decades have left numerous sites with local soil contamination, which have become a widespread issue globally (Fig. 5A). In Europe there are estimated to be more than 2.5 million potentially contaminated sites, of which 340.000 are expected to be actually contaminated and likely to require risk management measures (i.e. remediation) (Van Liedekerke et al., 2014). In the Basque Autonomous Community (CAPV), as a consequence of a long industrial tradition, 2.7-6.5% of the living surface has been inventoried as contaminated soil in 2012 (*Plan de Suelos Contaminados del País Vasco 2007-2012* of the Basque Government).

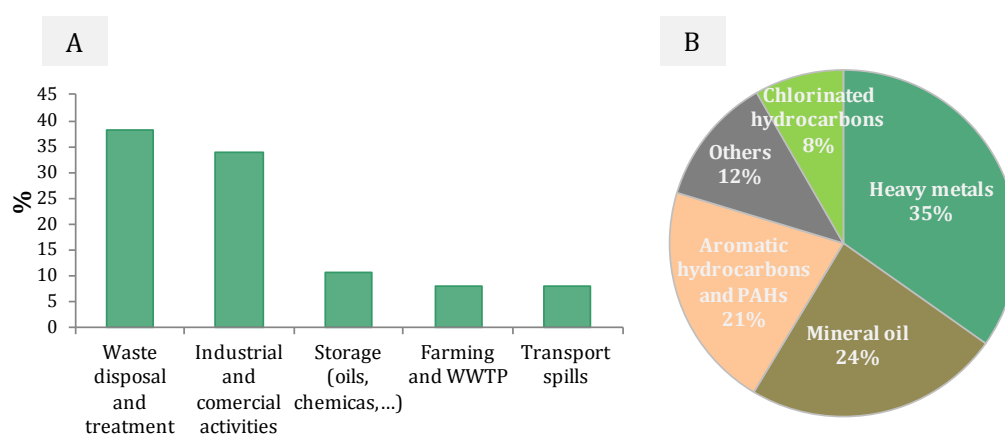


Figure 5. Main contamination sources (A) and kind of contaminants affecting soils (B) in Europe as reported in 2011. (Data obtained from the JRC Report *Progress in the management of contaminated sites in Europe 2014*, Van Liedekerke et al., 2014).

Heavy metals are among the most frequent contaminants found in soils, since, from a historical perspective, gold, silver, copper, tin and lead have been used by humankind for thousands of years (Alloway, 2013). Considerable areas of soil in many parts of the world have had inputs of metals (Fig. 5B), which raised the concern about the uptake via food chain that may result in hazardous effects for humans and ecotoxicological impacts for plants and soil organisms (De Vries et al., 2013). Simultaneously, large numbers of studies on metal contaminated soils in Europe, China and other regions about which relatively little was known until recently have been performed, which provided a wider global perspective on sources, dynamics and effects of metals in soils (Alloway, 2013). In addition, governments have implemented policies to protect human health and environment by measuring metals concentrations in soils and establishing threshold values for the distinction between soils that do not constitute a risk and those that could or currently pose risks for the uses intended (Urzelai et al., 2000).

At the same time that science improved its understanding of current and past contaminants such as metals, nanoscience and nanotechnology experienced huge advances in the synthesis, application and incorporation into products of ENPs. After their increasing use and high levels of commercialization, increasing inputs of NPs into the environment are expected, leading to an emerging concern, since these particles are expected to behave differently from their metallic counterparts. As mentioned before, the current regulation imposes threshold values for metals and organic contaminants but there is no guideline concerning their content in ENPs. The existing scarcity data and the limitations of analytical methods of these materials posed high level of risk uncertainty. Hence, the case of ENPs is quite challenging since they showed potential to pose risks to human health or to the environment while they are not yet subjected to specific regulatory criteria or norms for the protection of human health or the environment (Bleeker et al., 2013). Thus, ENPs, including Ag NPs, could be considered contaminants of emerging concern in soil (Sauvé and Desrosiers, 2014).

As mentioned above, there are no specific regulations for nanotechnologies or NMs in Europe. Instead, the manufacture, use and disposal of NMs are covered by a complex set of consumer and environmental protection regulatory regimes (e.g. REACH-Registration, Evaluation, Authorisation and Restriction of Chemicals-, CLP-Classification and Labelling-, Directive on Industrial emissions-Integrated Pollution Prevention and Control-, etc.). Regulatory instruments like REACH have not been designed for NM products and their applications, so it is a matter of concern that their risks might not be

captured effectively within this framework (Royal commission of Environmental Pollution, 2008). However, The Second Regulatory Review on Nanomaterials (2012) and the REACH Review (2013) concluded that REACH and CLP offer the best possible framework for the risk management of NMs. REACH applies equally to substances for which all or some forms are NMs. The CLP Regulation provides an obligation to notify to ECHA (European Chemical Agency) substances in the forms as placed on the market, including NMs, which meet the criteria for classification as hazardous, independent of their tonnage (Second Regulatory Review on Nanomaterials, 2012). In addition, sector specific regulations (for food, pharmaceuticals, veterinary medicines, pesticides, toys, cosmetics and end-of-life practices) performed risk assessments of NMs through case by case scientific studies. In support to these approaches, harmonization and standardization of measurement and test methods are being promoted through the OECD and by a Commission Mandate to the European Standards Organisations, since accurate information about the toxicity of NMs is important in determining their regulation. In this context, pushed by exposure monitoring and control strategies, nanotoxicology has arise as new discipline to evaluate the health and environmental threats posed by NPs and to enable a safe development of the emerging nanotechnology industry (Bruzea et al., 2007; Donaldson et al., 2004).

3. Nanotoxicology and factors affecting NPs toxicity in soils

From the ecosystems perspective, nanotoxicology addressed the adverse effects of NMs to organisms, populations and communities (Klaine et al., 2012). NPs toxicological effects are complex, since they can be influenced by many factors. Hence, nanotoxicology encompasses the physico-chemical characteristics of NPs, routes and degrees of exposure, biodistribution, cellular and molecular interactions, genotoxicity and regulatory aspects (Arora et al., 2012).

In the terrestrial compartment, together with the NPs characteristics (e.g. size, shape, surface charge, composition, coatings), soil physico-chemical factors (pH, cation exchange capacity-CEC-, clay and OM contents) and the interaction of NPs with the medium (e.g. dissolution, aggregation, sorption to larger particles), will affect NPs behaviour, fate and ecotoxicity (Klaine et al., 2008; Ren et al., 2016; Tourinho et al., 2012).

3.1. Soil physico-chemical factors

Soils are heterogeneous environmental matrixes with varying spatial and temporal gradients of OM, CEC, pH, particle size distribution (soil texture) and water holding capacity (WHC).

OM is formed by chemical and biological decomposition of organic residues, being differentiated into unaltered material and the transformed products (humus). Generally, soil humus is defined as a mixture of colloidal organic compounds with high molecular weights and relatively resistant to decomposition (Nieder et al., 2008). OM plays an important role modulating soil functions, including provision of surface charges (expressed as the cation exchange capacity-**CEC**-), influencing wettability and contributing to nutrient cycling and soil structure maintenance (Kibblewhite et al., 2008). OM has high capacity to chelate positively charged molecules such as metal cations.

Another soils physico-chemical factor affecting metal mobility is the **pH**, since metals become more bioavailable in soils (and soil pore water) when the pH decreases (Sijm et al., 2000). The pH is the factor that greatest affects metal solubility (Giller et al., 1998).

The **particle size distribution** could be divided into three main size grades or separates, sand (2.0-0.05 mm), silt (0.05-0.002 mm) and clay (<0.002 mm) (USDA, 1987). Soil texture will influence, among others, the structure, colour, porosity, CEC and WHC of soils. Soils rich in clay tend to be chemically active (Cornelis et al., 2012), absorbing positively charged molecules (cations) that normally are nutrients for living organisms. The amount of clay present in the soil will also influence its capacity to retain water against the gravity (**WHC**).

These factors will affect NPs behaviour, resulting in NPs dissolution, agglomeration or aggregation, and sorption to surfaces.

3.2. Ag NPs behaviour in soils

Soil represents a complex medium with a solid phase and pore water in which NPs will interact. NPs could travel through soil pores, form aggregates followed by sedimentation or could be sorbed to soil particles and become immobilized (Baalousha 2011). However, Ag NPs dissolution, agglomeration/aggregation and sorption to surfaces (Fig. 6) have been shown to vary with soil composition and characteristics.

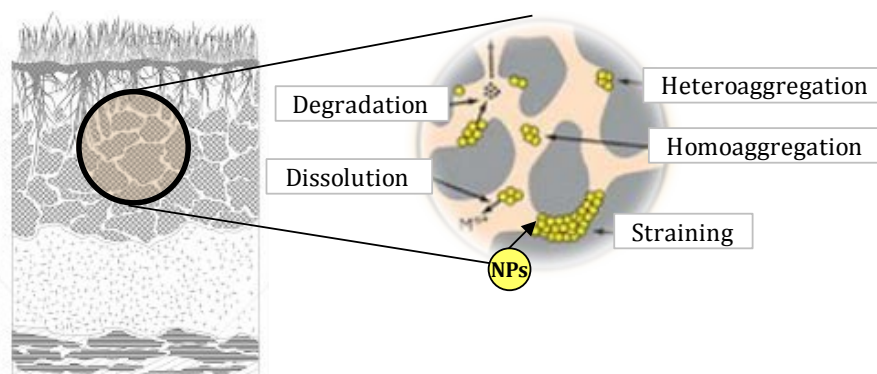


Figure 6. Key processes likely to affect the behaviour, fate and bioavailability of nano-particles in the soil environment (Modified from Batley et al., 2013).

Dissolution occurs when an ion detached from the particle migrates into the solution (Borm et al., 2006). The dissolution of metal based NPs releases ionic species that may be toxic *per se* and thus, both NPs and ionic form need to be considered to better understand the potential effects of NPs (Misra et al., 2012; Tourinho et al., 2012). NPs dissolution and solubility are size dependent, being nanosized materials faster and in a greater extent dissolved in comparison with macroscopic ones. The solubility of Ag and Ag NPs is influenced by soil pH, increasing with the decrease of the pH value in soils (Oromieh, 2011). Thereafter, the dissolved Ag has been stated to tend to bind to clay and organic matter present in soil (Cornelis et al., 2010), since they are abundant in negative charges. The dissolution of NPs can be hindered by the formation of aggregates or agglomerates (Borm et al., 2006).

Aggregation could be defined as the association of primary particles by strong bonding that sediment in the solution while agglomeration means the same but referring to weak bonding associations (Jiang et al., 2009). Physical forces (e.g. Brownian motion, gravity) and NPs size, surface charge, coatings and concentration will affect aggregation/agglomeration rates. In fact, the basic aim of the usage of coating in NPs is to stabilize them against aggregation (Sharma et al., 2014). The aggregation rate of NPs in soil suspensions has been found to be negatively correlated to soil characteristics such as dissolved OM and clay contents (as they cover the surface of NPs and prevents their homo-aggregation with repulsive forces), and positively correlated to the ionic strength, zeta potential and pH (Fang et al., 2009). For instance, greater aggregation has been observed in NPs in high ionic strength medium and at the isoelectric point (zeta potential=0, pH 6) (Jiang et al., 2009). In regard to Ag NPs, the effects of ionic strength were observed for suspensions with pH higher than 7 but for polyvinylpyrrolidone (PVP) coated Ag NPs aggregation was not influenced by increasing ionic strength (El

Badawy et al., 2010). In the soil matrix, aggregation is mainly limited to hetero-aggregation, as NPs-biomolecule conglomerates are mainly produced (Fabrega et al., 2009; Hotze et al., 2010). For instance, PVP coated Ag NPs have been demonstrated to have high affinity for soil solids (VandeVoort and Arai, 2012; Whitley et al., 2013).

The main compartments involved in metal **sorption** are soil OM, clay and oxide surfaces (Fe, Al, Mn...) (Jacobson et al., 2005). Sorption is the attachment or removal of a solute from a solution to a contiguous solid phase (Smith, 1999). Increasing the pH value and clay content of the soils, will increase the sorption of Ag NPs (Cornelis et al., 2010; Van Gestel and Van Dis, 1988), which could be due to the soils higher CEC value or to the finer texture of clay (Hedberg et al., 2015; Oromieh, 2011). This sorption has been demonstrated to be weaker in low ionic strength soils and stronger to soils with high OM concentration (Jacobson et al., 2005).

The behaviour of NPs in soil will control their mobility and their bioavailability to soil organisms (Tourinho et al., 2012), including earthworms.

4. Earthworms for toxicity assessment in soil environments

Among soil organisms, earthworms are abundant, ubiquitous and important for soil processes (Spurgeon et al., 2004). Earthworms, which belong to the order Oligochaeta, comprise roughly 8000 species grouped into 800 genera (Edwards, 2004) and are considered the most abundant animal biomass in the majority of terrestrial ecosystems (Bartlett et al., 2010). They are very versatile and are found in nearly all terrestrial ecosystems (from forest to agricultural ecosystems) (Lavelle et al., 2004), providing many favorable effects on the physical, chemical and biological properties of soil (Bartlett et al. 2010, Edwards 2004). In fact, Darwin (1881) was among the first to include earthworms, in the list of factors responsible for soil formation. Earthworms have been called 'ecosystem engineers' as they change and improve the structure of the soil. They contribute towards mineral weathering, the formation of humus, and burying OM from the surface (Blouin et al., 2013). One of the most important roles of earthworms in soil is their control of humification rates through feeding, burrowing, casting activities and interactions with microorganisms (Bernier, 1998).

Many researchers have studied the role of earthworms in soil ecology by their contribution to soil formation, water regulation, nutrient recycling, plants growth and microorganism population (Blouin et al., 2013; Lavelle et al., 2004). Their potential use for remediation or restoration of soils contaminated with organic and inorganic

contaminants has been also examined (Sinha et al., 2008; Sizmur and Hodson, 2009), together with their use for vermicomposting (Schuldt, 2006). In addition, earthworms have become a model for comparative immunologist with the publication of results that proved the existence of self/nonself recognition by cellular and humoral pathways in earthworms (Bilej et al., 2000, 2010). Due to their susceptibility to chemicals and other unique biological advantages (short life cycle, direct uptake of chemicals by their exterior epidermal surface and exposure via ingestion of soil, among others) much research has been done with earthworms as bioindicators of contamination and toxicity in soil (Nahmani et al., 2007; Paoletti, 1999; Schaefer, 2004; Spurgeon et al., 1994, 2004). Most of the mentioned studies used the widely distributed family of earthworms, *Lumbricidae*, being ecotoxicological research performed mainly in two genera, *Lumbricus* and *Eisenia*. Recently, earthworms have been used for the toxicity assessment of NPs in soil. Such works dealing with Ag NPs toxicity in earthworms used *Lumbricus rubellus*, *L. terrestris*, *Enchytraeus albidus*, *Eisenia andrei* and *E. fetida*, being the latter by far the most commonly employed species.

4.1. *Eisenia fetida* as model organisms

Eisenia fetida (Savigny, 1826) is an epigeic earthworm living in decaying organic matter, in compost and mold (Bilej et al., 2010). It has a short life cycle, hatching from cocoons in 3-4 weeks and reaching maturity in 2 months at 20 °C. It is very prolific (2-5 cocoons/week/worm), is available commercially and can be feed in a wide range of organic waste materials (e.g cow, rabbit and horse manures). It also represents an inexpensive, appropriate and noncontroversial model for experimentation (Bilej et al., 2010). *E. fetida* has been demonstrated to be impacted by soil pollutants and thus, it is broadly used in ecotoxicology and nanotoxicology and it is included as a standard test organism by some international organisations (ISO 1993, 1998; OECD 1984, 2004). Moreover, biomarkers at different levels of biological complexity have been measured in this organism (see 6.1 section). Further, *in vitro* assays with *E. fetida* coelomocytes have been recently optimized in order to assess toxicity of metals (Irizar et al., 2014b, 2015b; see section7).



Taxonomy

Kingdom	Animalia
Phylum	Annelida
Class	Clitellata
Subclass	Oligochaeta
Order	Haplotaxida
Family	Lumbricidae
Genus	Eisenia
Species	<i>E. fetida</i> (Savigny, 1826)

Figure 7. *Eisenia fetida* earthworm and its taxonomic classification ([https://commons.wikimedia.org/wiki/File:Eisenia_foetida_R.H._\(8\).JPG](https://commons.wikimedia.org/wiki/File:Eisenia_foetida_R.H._(8).JPG))

5. Standard toxicity tests with *E. fetida*

A common way to assess the toxicity of pollutants in the environment is by performing laboratory toxicity assays with different organisms, since good correlation has been found between laboratory results and effects in the field (Heimbach, 1992; Maboeta et al., 2004). The first toxicity test in soil was performed at the end of the sixties, using Collembola and earthworms to assess the effects of pesticides in soils (Ghabbour and Imam, 1967). Twenty years later, the first toxicity test with soil invertebrates was internationally standardized by the OECD, using *E. fetida* earthworms and only focusing on short-term (acute) responses like survival (Earthworm Acute Toxicity Test-OECD 207, 1984).

The Acute Toxicity Test-OECD 207 (OECD, 1984) describes two short-term toxicity tests, Paper Contact and Artificial Soil tests, using both methods survival as the unique endpoint. In the Paper Contact test earthworms are exposed to treated filter paper during 2 days, being a useful exposure method for a rapid screening of chemicals and to assess their uptake, biotransformation or other types of mechanistic research (Van Gestel et al., 2012). However, the assessment of the effects produced by the incorporation of toxic substances by ingestion (solid phase) should include toxicity evaluation in a real media. The Artificial Soil test provides a more representative toxicity data of natural exposure of earthworms to chemicals as it is performed in an artificial soil substrate containing 70% sand, 20% clay and 10% sphagnum peat (OECD, 1984). For reasons of standardization and comparison of results, all standardized tests use this OECD artificial soil. However, new standard and natural soils such as LUFAs have started to be used in the last years in order to increase realism (Van Gestel et al., 2012).

The toxicants, once inside the organisms may provoke death or may affect physiological parameters such as growth, assimilation of energy from food, the energetic costs for producing offspring, etc. Thus, complementarily to survival endpoint, toxicity tests using sublethal endpoints like reproduction were standardized for earthworms by OECD (Earthworm Reproduction Test-OECD 222, 2004) and ISO (Determination of effects on reproduction, ISO 11268-2, 1998). The **Earthworm Reproduction Test-OECD 222** has a duration of 28 days in which the earthworms are exposed to the test substance. Afterwards, earthworms are removed from the spiked soil while the cocoons are incubated for another 28 days to enable determining the number of offspring. Despite being focused on the reproductive output, this test also included weight change (growth) of the earthworms.

In addition, standard test guidelines for **avoidance tests** (ISO 17512-1, 2008a) have been recently developed for earthworms. The aim of these tests is to determine the quality of soils and the effects of chemicals on behaviour of *E. fetida* and *E. andrei*.

The acute and sublethal laboratory test guidelines (traditional endpoints) are being applied to new and emerging chemicals as well, especially to determine the toxicity of NPs using earthworms (Heckmann et al., 2011; Lapied et al., 2010; Schlich et al., 2013; Shoults-Wilson et al., 2011b, 2011c).

These above mentioned standardized tests are designed to estimate toxicity values of the chemicals from concentration-response relationships in survival or reproduction of earthworms (lethal concentration-LCx- and effect concentration-ECx-). Therefore, they are considered of great importance for risk assessment and regulation of chemicals (Rodriguez-Ruiz et al., 2014; Spurgeon et al., 1994, 2004). With the same applied purposes, research efforts have been carried out in the framework of modern ecotoxicology to develop and validate biomarkers. In the recent years there has been an increasing interest in the use of biomarkers at different levels of biological complexity in terrestrial invertebrates, including *E. fetida*, for the assessment of potential adverse effects of chemicals in soil ecosystems (Asensio et al., 2013; Irizar et al., 2015a; Kammenga et al., 2000).

6. Target tissues and cells for pollutant handling in earthworms

Earthworms may uptake pollutants from soil and pore water, both through dermal and oral routes. Thus, the first barrier of earthworm against pollutants is their body wall, which consists of an external cuticle and the epidermis (Fig. 8). The earthworm **epidermis** is formed by supporting and basal cells plus mucus-secreting goblet cells, which release mucus over the surface of the earthworm body in order to facilitate the locomotion through soils and prevent desiccation (Lapied et al., 2010). This mucus layer around the **cuticle** is maintained by the secretion of a fluid rich in mucopolysaccharides, proteins and lipid complexes that take part in the humoral immune system (Jamieson, 1981), and is also a way to eliminate metals from their body (Vijver et al., 2003). The integument is highly permeable, serving as respiratory surface, and also as the main uptake route for metals such as cadmium, copper, and lead (Saxe et al., 2001; Vijver et al., 2003).

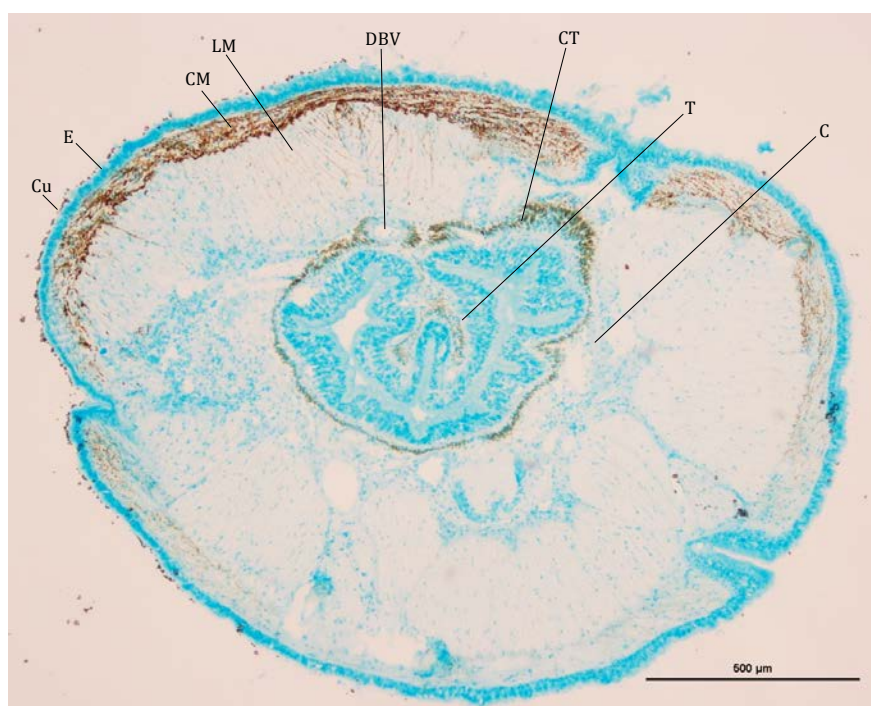


Figure 8. Transverse section of *Eisenia fetida* earthworm stained with Alcian Blue. Cuticle (Cu), Epidermis (E), Circular muscle (CM), Longitudinal muscle (LM), Dorsal blood vessel (DBV), Chloragogenous tissue (CT), Typhlosole (T) and Coelom (C). Scale 500 μm .

Previous studies showed the **chloragogenous tissue**, placed between the digestive epithelium and the coelom (Fig. 8), as the major metal accumulating site (Irizar et al., 2014b; Van Gestel et al., 2012). In addition, the **epithelium of the digestive tract** also exhibits a great ability for metal accumulation (Irizar et al., 2014b; Morgan et al., 2002). In fact, soil ingestion is considered the major entering route of metals in earthworms

(Becquer et al., 2005; Jager et al., 2004) and thus, epithelial cells in the digestive gut are usually targets for biomarker measurements.

Earthworms possess a coelomic cavity that contains coelomic fluid with free floating wandering cells, **coelomocytes**. The earthworm coelomic fluid plays an important role in homeostasis and in immune defenses against external stimuli by means of haemolytic, proteolytic and cytotoxic enzymes (Kurek et al., 2007). This fluid is communicated with the outer environment by a pair of nephridia and dorsal pores per segment (Bilej et al., 2000), and thus, coelomocytes can be in nearly direct contact with pollutants present in soils (Irizar et al., 2015a). Comparable to human leukocytes (Hayashi et al., 2012), coelomocytes are the immune cells of earthworms, and play a pivotal role in recognition and elimination of foreign materials, clotting and wound healing (Cooper, 2002; Kurek et al., 2007).

Among coelomocytes two major subpopulations can be distinguished in earthworms by cytochemical, morphometrical, ultrastructural and functional characteristics (Adamowicz, 2005; Bilej et al., 2000; Engelmann et al., 2004, 2005), amoebocytes and eleocytes (Fig. 9), being the former subgrouped into hyaline and granular amoebocytes.

Hyaline or granular **amoebocytes**, the most numerous coelomocytes (Adamowicz, 2005), represent effector immunocytes with a strong phagocytic and encapsulation activity (e.g. bacteria, fungi, etc.) (Engelmann et al., 2005; Hayashi et al., 2012; Valembois et al., 1985). However, this activity is higher in hyaline amoebocytes, which can adhere and engulf bacteria (Engelmann et al., 2005).

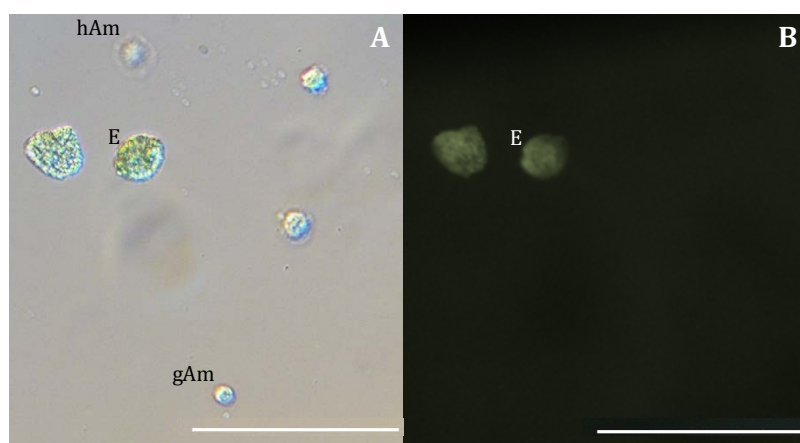


Figure 9. Amoebocytes (hyaline-hAm- and granular-gAm-) and eleocytes (E) of *E. fetida* earthworm (A). Note the autofluorescence of eleocytes (B). Scale 50 μ m.

Eleocytes are detached chloragocytes derived from the chloragogen tissue (Linthicum et al., 1977a) with nutritive functions such as glycogens and lipids production (Bilej et al., 2000, 2010). They do not have phagocytic activity but they participate in the homeostasis and humoral immunity of earthworms (Adamowicz, 2005; Engelmann et al., 2004, 2005) by producing humoral factors such antimicrobial peptides (Bilej et al., 2000, 2010; Cooper et al., 2002). Eleocytes of *E. fetida* exhibited autofluorescence due to the selective accumulation of riboflavin (sourced from their diet, intestinal microflora and other endosymbiotic bacteria), which simplifies their identification (Plytycz et al., 2011; Sulik et al., 2012).

At subcellular level, the **lysosomal system** has been identified as a particular target to assess the toxic effects produced by many contaminants. The lysosomal vacuolar system is involved in the accumulation and degradation of a wide range of substances obsolete in the cell (e.g. biological polymers, proteins, lipids) or taken up by endocytosis (e.g. organic compounds, metals) (Moore, 1985; Viarengo, 1989). Pathological alterations in lysosomes have been especially useful in the identification of adverse environmental impacts.

Since the existence of morphofunctional variability along the earthworms, the selection of the most adequate body region for the measurements of different biomarkers is crucial for accurately assess toxicity in *E. fetida* (Irizar et al., 2014a).

6.1. Biomarkers at different levels of biological complexity in *E. fetida*

Biomarkers can be defined as biological responses to pollutants at molecular, cell and tissue levels that provide early indications of ecosystems health status (McCarthy and Shugart, 1990). Biomarkers forecast effects at higher levels of biological organisation (Spurgeon et al., 2005), and also may provide information on the mode of action of chemicals (Kammenga et al., 2000). These characteristics, together with their simplicity, accuracy and reproducibility, make biomarkers useful tools in nanotoxicology. Current studies with biomarkers in earthworms have been focused on DNA damage (molecular level), cholinesterases, metal binding proteins and enzymatic defense system (biochemical level), lysosomal membrane stability (subcellular level), histopathological alterations (tissue level) and immune responses (Asensio et al., 2007, 2013; Irizar et al., 2014b, 2015a, 2015b; Scott-Fordsmand and Weeks., 2000). Among them, molecular and biochemical level biomarkers have been the most studied after

exposure of *E. fetida* to NPs (Gomes et al., 2015; Hayashi et al., 2013a; Novo et al., 2015; Tsyusko et al., 2012).

Several organic and metallic compounds, including Ag NPs, are known to induce the production of reactive oxygen species (ROS) (McShan et al., 2014). Living organisms, aiming to balance ROS and prevent oxidative stress, have developed antioxidant defence systems with enzymes (e.g. catalase, superoxide dismutase, glutathione peroxidase) and additional protection mechanisms against metal exposure such as metal binding proteins (e.g. metallothioneins).

In regard to antioxidant defense, changes in catalase (CAT) activity after exposure to chemicals are indicators of cellular lesion, and thus are considered as early environmental stress biomarkers (Asensio et al., 2013; Gomes et al., 2015).

A number of metals are known to induce metallothioneins and thus, they have been advocated as earthworm biomarkers for a long time. Metallothioneins (MTs) have the capacity to bind a variety of metal atoms (due to their low molecular weight and high cysteine content), participate in homeostasis and detoxification of metals (Brulle et al., 2006) and prevent the organism from oxidative stress (Ribeiro et al., 2015). Hence, oxidative stress could be assessed by measuring the transcription levels (**molecular level**) or the activity (**biochemical level**) of antioxidant enzymes and detoxification mechanisms. In fact, transcription levels of target genes such as those encoding CAT or MTs have been easily measured in earthworm tissues (Asensio et al., 2007; Brulle et al., 2006; Irizar et al., 2014b). Spurgeon et al. (2005) demonstrated that the responses at the molecular level were most sensitive and that genetic tools (genomics, proteomics and transcriptomics etc.) enable a better understanding of molecular mechanisms of action of chemicals. These tools may also help unraveling the mechanisms by which metal-based nanoparticles affect organisms (Van Gestel et al., 2012).

ROS are able to induce genotoxicity (DNA damage), protein carbonylation and membrane oxidation (Piao et al., 2011). The single-cell gel electrophoresis assay or Comet assay is widely used technique to detect DNA damage in individual cells, primarily as strand breakage (Fig. 10). The experimental conditions for this technique were described by Singh et al. (1988) over 20 years ago. For that time onwards this technique has been conducted with coelomocytes of soil invertebrates for the toxicity assessment of organic compounds (Di Marzio et al., 2005; Sforzini et al., 2012) and metals (e.g. cadmium, chromium, nickel) (Di Marzio et al., 2005; Fourie et al., 2007;

Lourenço et al., 2011a; Manerikar et al., 2008; Reinecke and Reinecke, 2004) with satisfactory results. According to Tice et al. (2000), this assay is potentially useful for the screening of DNA-damaging agents.

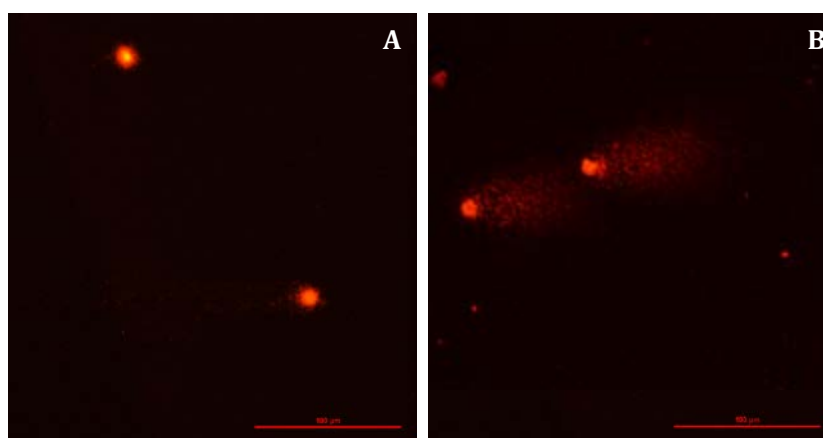


Figure 10. Images of single-cell gel electrophoresis (Comet assay) for untreated coelomocytes (A) and coelomocytes exposed to 50 mM H₂O₂. Note the migration of DNA strands (comet tail) in treated coelomocytes (B). Scale 100 μm.

Cellular level biomarkers in earthworms have been focused on the integrity of the coelomocytes lysosomal membrane. Some authors considered these biomarkers the following more sensitive (after molecular and biochemical biomarkers) to evaluate metal stress in earthworms (Rocco et al., 2011; Spurgeon et al., 2005). The majority of the studies on lysosomal stability used the Neutral Red Retention Time (NRR time) in coelomocytes (Scott-Fordsmand et al., 1998; Weeks and Svendsen, 1996), which has been adapted to spectrophotometric measurement carried out in microplates (Neutral Red Uptake assay-NRU-) (Asensio et al., 2007, 2013; Homa et al., 2003; Irizar et al., 2014b, 2015a, 2015b; Kwak et al., 2014a). Recently, parameters such as the total number and viability of coelomocytes have been also used as biomarkers to assess the impact of metals on earthworms (Homa et al., 2015, Kwak et al., 2014b). In this context, other microplate assays are available to measure cell metabolic activity (MTT, XTT) or viability (Calcein AM) after exposure to chemicals, but they have not been used with earthworm coelomocytes yet. The Calcein AM Viability assay provides a simple, rapid, and accurate method to measure cell viability and cytotoxicity. The calcein acetoxymethyl ester (Calcein AM) permeates live cells and is hydrolyzed by intracellular esterase to calcein, a hydrophilic and strongly fluorescent compound that is well-retained in the cell cytoplasm. Calcein AM has been used to evaluate the esterase activity of several organisms, including microorganisms (Kaneshiro et al., 1993). In the case of

earthworms, Calcein AM has only been used to label earthworm coelomocytes (Kwak et al., 2014b).

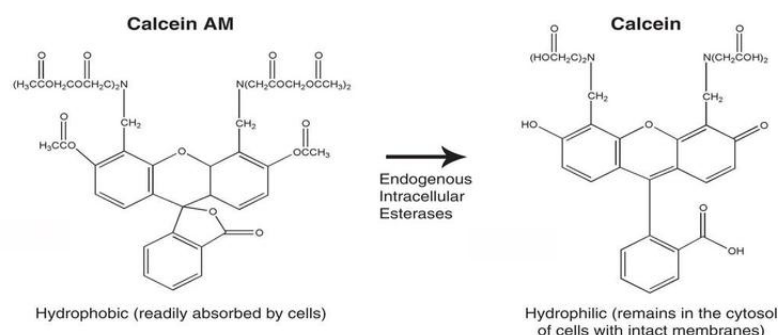


Figure 11. The transformation from Calcein AM (hydrophobic) to Calcein (hydrophilic) by intracellular esterases (From Calcein AM Viability assay-R&D System, Catalog Number 4892-010-k).

At **tissue level**, histological changes such the thickness of the epithelium and the cellular composition (e.g amount of mucous secreting globet cells) have been measured in body wall, gastrointestinal tract and chloragogenous tissue of earthworms exposed to metals, radionuclides and hostile environmental conditions (Amaral et al., 2006; Cunha et al., 2011; Lourenço et al., 2011b). In addition, the intralysosomal metal accumulation in digestive epithelium and chlorogenous tissues have been shown as an effective biomarker of metal exposure through the quantification of black silver deposits (BSD) after the application of autometallography (Amaral et al., 2006). This technique allows the *in situ* localization of metals in tissue sections in a simple and cost-effective way (Soto et al., 1996).

A battery of biomarkers is often used to evaluate the effects of exposure to chemical (Cajaraville et al., 2000). With the aim of summarizing biomarker responses at different level of biological complexity and simplify their interpretation, the **Integrative Biomarker Response Index/n** (IBR/n) has been applied, although mainly in field surveys (Beliaeff and Burgeot, 2002; Marigómez et al., 2013). In fact, IBR index was first applied to study the impacts of organic pollutants in flatfish and mussels from different areas of the Baltic Sea (Beliaeff and Burgeot, 2002). From there on, this approach has been successfully applied in other marine pollution monitoring programs (Broeg and Lehtonen, 2006; Marigómez et al., 2013) to identify temporal and spatial fluctuations in ecosystem health status and their magnitude. In soil studies, the IBR index has been scarcely applied (Asensio et al., 2013). However, other biomarker data integration and interpretation approaches such as the Earthworm Expert System (EES) have been

developed to perform a ranking of the pollutants induced stress syndrome phases in *E. andrei* (Sforzini et al., 2011).

7. Coelomocytes *in vitro*

In vitro approaches are rapid, reliable, cost-effective and reproducible tools for the screening of potentially toxic agents by greatly reducing the number of animals used (Borenfreund and Puerner, 1984). In addition, the exposure of organisms through complex matrixes such as soil could be a challenge when assessing toxicants characterization or their mechanism of toxicity (Hayashi et al., 2012), whereas *in vitro* models, despite limited environmental significance, allow defining and controlling exposure conditions (e.g. dosing, specific targeting).

Commonly, earthworms are exposed *in vivo* to chemicals and subsequently coelomocytes are extruded to assess different endpoints (e.g. NRU, cell viability, oxidative stress etc.) (Asensio et al., 2007, 2013; Homa et al., 2003; Irizar et al., 2014a, 2015a; Scott-Fordsmand et al., 2000). It was 20 years ago that coelomocytes of *L. terrestris* were maintained viable in culture for the first time to study their immune response capacity (Toupin et al., 1977). More recently, primary culture of coelomocytes have been used to evaluate the toxicity of a wide range of metals (Irizar et al., 2014b, 2015b), including Ag NPs (Hayashi et al., 2012, 2013b). These studies seeded coelomocytes in different conventional media (i.e. Leibovitz's L-15 and RPMI-1640 medium), with and without serum, and supplemented with a set of antibiotics (Amphotericin, Penicillin-Streptomycin, Gentamicin).

In order to assess viability, proliferation and cytotoxicity in cell culture, a number of methods have been developed in microplates (Cook and Mitchell, 1989). These methods allow the analysis of many samples rapidly and simultaneously with the aid of plate reader spectrophotometers and fluorescence readers. Cytotoxicity in primary cultures of coelomocytes exposed to metals (Cu, Pb, Ni, Cd) has been measured using the NRU assay (Irizar et al., 2014b, 2015b). For Ag NPs assessment, a counting kit that employs water soluble tetrazolium salts (WST-8) has been recently used (Hayashi et al., 2012).

In addition, flow cytometry has been widely used to analyse the mode of action of pollutants and mixtures in established vertebrate cell lines and primary cultures (Castaño et al., 2000; Gallego et al., 2007). In this technique, particles or cells go through a laser beam (one at a time) and a detector in front of the light beam measures forward scatter (FS, correlated with the size) and several detectors in the side measure side

scatter (SS, correlated with the complexity). Additional detectors measure the fluorescence emitted from positively labelled cells or particles. This technique has been a common approach to study earthworm coelomocytes and to characterize the different subpopulations (Engelmann et al., 2016; Kurek et al., 2007; Plytycz et al., 2007). Using flow cytometry coelomocytes can be separately gated according to their light scatter profiles, forward scatter (cell size) and side scatter (cell granularity/complexity) (Engelmann et al., 2016), and changes in their relative proportion and mortality rates can be recorded (Irizar et al., 2015b). Earthworm coelomocytes can be discriminated between amoebocytes and eleocytes by means of this technique (Fig. 12), being amoebocytes further classified using light or electron microscope. Using this approach, several authors have provided valuable data about the different functional aspects (e.g phagocytosis, proliferation) of earthworm coelomocytes (Cossarizza et al., 1995; Fuller-Espie et al., 2011; Homa et al., 2013). Nevertheless, flow cytometry has been scarcely applied in soil ecotoxicology using coelomocytes retrieved after *in vivo* exposures (Homa et al., 2013; Kwak et al., 2014b; Massicotte et al., 2004; Plytycz et al., 2011) and even less using *in vitro* exposure of coelomocytes (Hayashi et al., 2012; Irizar et al., 2015b; Kwak et al., 2014b; Plytycz et al., 2007). These studies with different earthworm species employed flow cytometric assays with various types of fluorescent probes (e.g. NR, Propidium Iodide-PI-, Calcein AM, DCFDA, acridine orange, etc) to test the cytotoxicity to metals.

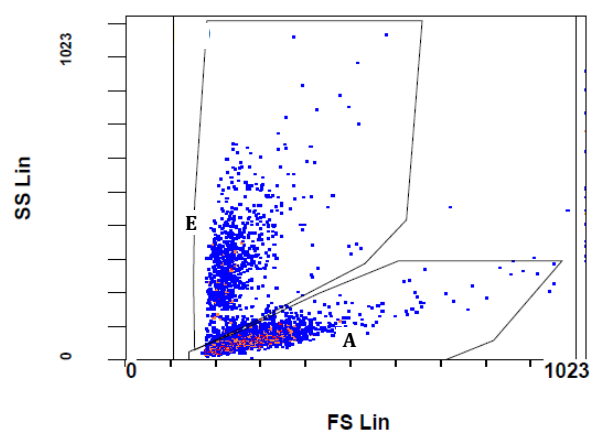


Figure 12. Flow cytometric analysis of *E. fetida* coelomocytes. Distribution by density plot of amoebocytes (A) and eleocytes (E) according to their complexity (SS, Y axis) and size (FS, X axis).

Further, flow cytometry analysis showed a dissimilar sensitivity among coelomocyte subpopulations, being eleocytes more sensitive than amoebocytes when exposed to metals (Homa et al., 2010; Irizar et al., 2015b; Plytycz et al., 2010). Coelomocytes

subpopulation can be physically isolated by means of cell sorting. Engelmann et al. (2016) has just recently done the first example of amoebocyte and eleocyte sorting for post-sort TEM and SEM imaging characterization. In the study subpopulations were sorted with high purity and cell survival after sorting was demonstrated by phagocytosis assay.

8. An overview of the studies regarding Ag NPs toxicity to Oligochaeta

The following table (Table 2) shows an overview of the available studies focused on the toxicity of Ag NPs to Oligochaeta. Literature regarding this topic started to be available in 2010, from there on, the number of publications have slowly increased. The majority of the studies used the species *E. fetida* as model organisms. In regard to the NPs, the reference material NM-300K from the OECD sponsorship program (JRC programme on nanomaterials) were commonly used. In addition, both, uncoated at coated Ag NPs were tested, being PVP the most used coating agent. The vast majority of the studies employed limit test concentrations, being the dose range selected much higher than the concentrations predicted for the environment.

Earthworms were exposed through soils mainly. Among soils, standard (i.e. artificial-OECD-and natural-LUFA and RefeSol-) and field collected soils were used. In order to perform the spiking of soils, wet and dry dosing procedures were followed. In the former, powdered Ag NPs were first suspended in DI water by sonicating (not always) and the resultant suspension was applied to the soils at a range of 40-50% of their WHC. For the latter, Ag NPs were directly mixed (manually) with the dry soil prior to the addition of water. Some studies did the dosing to a carrier soil or to a sub-sample, which afterwards was mixed with the remaining soil. In all cases spiking was carried out individually for each replicate and the stabilization periods ranged from 3 days to 1 week depending on the authors.

Endpoints within the OECD standard tests were the most used ones, based on survival, growth and reproduction. Apart from traditional endpoints, molecular and biochemical biomarkers (changes in gene expression and biochemical measurements) were also studied.

Table 2. An overview of studies on Ag NPs toxicity to Oligochaeta.

Species tested	NPs Size (nm)	NPs Characterization	Concentration ranges	Exposure media	Dosing	Duration	Endpoint	Reference
<i>L. terrestris</i>	8 (a), 20 (b)	(a)-Colloidal -from Purest Colloids (b)-Pristine, powder -from Quantum Sphere	(a) 0-20 mg/l (E1); 0-20 mg/kg (E2); 0-8 mg/kg (E3) (b) 0-100 mg/l (E1); 0-100 mg/kg(E2,E3)	-Water (E1) -Food (horse manure, E2) -Agricultural clay loam soil (E3)	-Suspension in 18 MΩ DI water -Sonication (5 min)	-24h (E1) -2, 4 and 8 w (E2) -4 w (E3)	-Apoptosis in different tissues	Lapied et al., 2010
<i>E. fetida</i>	30-50	-0.2% w/w PVP coated -Zpot:-28.16 mV -from Nanoamor	0-1000 mg /kg	-Sandy loam from Askov experimental station (Denmark)	-Suspension in DI water -Ultra-sonication (30min) -Manually mixed into the soil.	28 d	-Survival -Growth -Reproduction	Heckmann et al., 2011
<i>E. fetida</i>	10, 30-50	-PVP coated -Powder -from NanoAmor	10-1000 mg/kg	-Artificial soil -Yeager sandy loam (YSL) natural soil	-Suspension in 18 MΩ DI water -Sonication (15min, RT) -Applied to soils 50% WHC.	28 d	-Bioaccumulation -Survival -Growth -Reproduction	Shoults-Wilson et al., 2011a
<i>E. fetida</i>	30-50	-PVP and oleic acid coated -Powder -from NanoAmor	10, 100, 1000 mg/kg	-Artificial soil	-Suspension in 18 MΩ DI water -Sonication (15min, RT). -Applied to soils 50% WHC	28 d	-Bioaccumulation -Survival -Growth -Reproduction	Shoults-Wilson et al., 2011b
<i>E. fetida</i>	10, 30-50	-PVP and oleic acid coated -Powder	0-54 mg/kg (environmentally relevant)	-Artificial soil, artificial soil with lower pH -Yeager sandy loam (YSL) natural soil	-Suspension in 18 MΩ DI water -Sonication (15min, RT). -Applied to soils 50% WHC	48 h	-Avoidance	Shoults-Wilson et al., 2011c
<i>E. fetida</i>	20	-Powder -Zpot: -21.1 ± 0.4 mV -from Quantum Sphere	0.77 µg/g	-Food (horse manure) in OECD soil	-Wet spiking	28 d	-Uptake, excretion -Biodistribution	Coutris et al., 2012
<i>E. fetida</i>	80-100	-0.2% w/w PVP coated -from Nanoamor	0-5.91 µg/ml	-Culture medium (RPMI-1640)	-Preparation of a colloidal suspension, then mixed with serum (BSA)	24 h	-Cytotoxicity -ROS -Gene expression -Intracellular accumulation	Hayashi et al., 2012

Table 2. (Continued I)

Species tested	NPs Size (nm)	NPs Characterization	Concentration ranges	Exposure media	Dosing	Duration	Endpoint	Reference
<i>E. fetida</i>	10 (a), 30-50 (b)	-PVP coated -Powder -Zpot -49.5 mV (a) and 35.9 mV (b) -from NanoAmor	100 and 500 mg/kg	-Yeager sandy loam (YSL)	-Suspension in 18 MΩ DI water -Sonication (15min, RT). -Applied to soils 50% WHC	1,3 and 7d	-Gene expression (metal homeostasis and oxidative stress) -Protein oxidative damage -Catalase enzymatic activity	Tsyusko et al., 2012
<i>E. fetida</i>	10	-PEG coated -Colloidal -‘green’ NPs (<i>Thuja</i> leaf extracts)	0-1000 mg/kg	-Natural Soil (Assam, India)	-Wet spiking	30 d	-Survival -Growth -Reproduction	Barua et al., 2013
<i>Enchytraeus albidus</i>	30-50	-0.2% w/w PVP coated -Zpot:-28.6 mV -from Nanoamor	0-1000 mg/kg	-OECD soil	-Dry dosing for each replicate -Manually mixed	2 d, 6 w	-Survival -Reproduction -Gene expression	Gomes et al., 2013
<i>E. fetida.</i>	30-50	-0.2% w/w PVP coated -Powder -Zpot:-28.6 mV -from Nanoamor	500 mg/kg	-Sandy loam from Askov experimental station (batch no. 2031207) (Denmark).	-Dry dosing -Addition of DI water	1, 2, 7, 14d	-Gene expression -Enzymatic activity	Hayashi et al., 2013a
<i>E. andrei</i>	15	-NM-300K (OECD) -Stabilizing agents	-60, 120 and 200 mg/Kg. -15, 30, 60, 120 and 200mg/Kg.	-RefeSol 01A soil	-Application mixing carrier soil (5%) with test soil (95%)	28 d	-Reproduction -Ag content in earthworms and soil (ICP-OES)	Schlich et al., 2013
<i>E. andrei</i>	10	-1% w/w citrate coated -from ABC Nanotech	0-2000 mg/kg	-OECD soil	-Wet spiking for each dose	7 d	-Survival -Cell viability -Cytotoxicity	Kwak et al., 2014a
<i>L. rubellus</i>	15	-NM-300K (OECD)	0-154 mg/kg (E1, E2) 0-1000 µg/ml (E3)	-Sandy loam soil from an experimental organic farm (E1, E2) -Culture medium (E3)	-Wet spiking	4w (E1, E2) 18-20 h (E3)	-Reproduction (E1) -Adult (E1) and juvenile (E2) survival, and growth -Tissue pathology -Cell viability (E3)	Van der Ploeg et al., 2014

Table 2. (Continued II)

Species tested	NPs Size (nm)	NPs Characterization	Concentration ranges	Exposure media	Dosing	Duration	Endpoint	Reference
<i>E. fetida</i>	50	-Uncoated -Powder -from NanoTrade Ltd	0-4395 mg/kg	-LUFA 2.2 soil	-Dry dosing to a sub-sample and then mixed with the remaining soil -1, 9, 30 and 52 w aging	28 d	-Survival -Growth -Reproduction	Diez-Ortiz et al., 2015a
<i>L. rubellus</i>	50	-Uncoated -Powder -from NanoTrade Ltd	100 and 500 mg/kg	-LUFA 2.2 soil	-Dry dosing to a sub-sample and then mixed with the remaining soil -1 w stabilization	Up to 7 d	-Toxicokinetic patters (uptake routes)	Diez-Ortiz et al., 2015b
<i>E. fetida</i>	10	-Uncoated -from Nanoamor	0-1500 mg/kg	-OECD soil	-10 g of sand per replicate were mixed with the Ag NPs and then added to the remainder soil. -Spiking individually per replicate	4 and 28 d	-Biochemical analysis	Gomes et al., 2015
<i>E. fetida</i>	50	-PVP coated -Powder -from Institut Catalá de Nanotecnología (ICN)	0-1758 mg/kg	-LUFA 2.2 soil	-Dry dosing -1 w stabilization	28 d	-Survival -Growth -Reproduction -Transcriptome analysis	Novo et al., 2015
<i>E. crypticus</i>	15	-NM-300K (OECD) -Uncoated	0-225 mg/kg	-LUFA 2.2 soil	-Wet spiking, individually per replicate -3d stabilization	3 and 7 d	-Reproduction -Biochemical analysis	Ribero et al., 2015
<i>E. fetida</i>	15	-NM-300K (OECD) -Uncoated	0-20 µg/ml	-PBS and culture medium (RPMI-1640)	-Suspension in DI water	24 h	-Cytotoxicity -ROS -Gene expression	Hayashi et al., 2016
<i>L. rubellus</i>	20, 35, 50	-bovine serum albumin, chitosan and PVP. coated -from Institut Catalá de Nanotecnología (ICN)	0-250 mg/kg	-Natural soil from a reference experimental organic farm	-Wet spiking -24 h stabilization	28 d	-Bioaccumulation -Survival -Growth -Reproduction	Makama et al. 2016

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II. STATE OF THE ART, HYPOTHESIS AND OBJECTIVES

STATE OF THE ART

In the last years, manufactured nanoparticles (NPs) are being incorporated in many consumer products with several uses. The majority of NPs are metal based and among them silver NPs (Ag NPs) are the most used due to their unique optical, catalytical and antimicrobial properties, which render them high commercial and scientific interest. Concomitantly with the applications of Ag NPs, an increase in the amount of nanosilver entering into the environment is expected. In soils, the major input of Ag NPs is through the disposal of waste water treatment plant (WWTP) sludges, mainly after their land application as fertilizer (biosolids) or incineration and posterior deposition. At present, there is no available data regarding measurements of NPs in the environment due to the limitations of the analytical methods. Nevertheless, estimations based on fate models predicted concentrations that will undoubtedly increase in the forthcoming years due to the high proportion of sludge or sludge incineration residues land-disposal. Therefore, concerns are growing because the novel properties of Ag NPs could pose a risk to human health and to the environment. Toxic effects of Ag NPs have already been demonstrated in the laboratory for many species living in different compartments, but in general terms, very few studies assessing Ag NPs toxicity have involved the terrestrial compartment and soil organisms.

Earthworms play an important role in soil ecosystems and are one of the most studied sentinel taxa in terrestrial ecotoxicology and soil health assessment since their pollutant body burdens reflect environmentally bioavailable pollutant levels and can exert measurable responses and adverse effects. Therefore, the study of effects exerted by Ag NPs on earthworms is crucial to understand the potential impacts of Ag NPs in soils. Among earthworms, *Eisenia fetida* is a model species in toxicology that has been broadly used in standardized OECD toxicity tests (OECD-207-, OECD-222). These conventional tests are aimed to address traditional endpoints such as survival, growth and reproduction after exposure to chemicals, in order to calculate different toxicity indices (LCx and ECx).

Biomarkers at different complexity levels (molecular, biochemical, cellular, tissue) could be also measured in *E. fetida* in order to assess the toxic effects of Ag NPs. These biomarkers can be put together into integrative indexes to summarise and obtain a conclusive outline of the responses of sentinel organisms in order to assess soil health.

The scarce research works dealing with Ag NPs toxicity in earthworms have used Ag NPs in concentration ranges order of magnitude higher than those expected in the environment. In addition, different test soils (artificial and natural) with different physico-chemical characteristics have been used. Such characteristics need to be taken into consideration since would affect the fate and behavior of Ag NPs, and subsequently their accumulation, uptake and toxicity in earthworms.

Recently, the potential of *in vitro* techniques and "omics" technologies is beginning to be harnessed to provide a number of promising applications in nanotoxicology. *In vitro* approaches with primary cultures of earthworm coelomocytes have been successfully developed as cost-effective tools for the screening of potentially toxic agents by greatly reducing the number of animals used and allow defining and controlling exposure conditions (e.g. dosing, specific targeting) opposite to *in vivo* set ups. Among coelomocytes two major subpopulations can be distinguished in *E. fetida* earthworms, amoebocytes and eleocytes that exhibited dissimilar cytochemical, morphometrical, ultrastructural and functional characteristics and also a different behavior against pollutants. These two populations can be distinguished using flow cytometry according to their size, granularity and autofluorescence. Changes in their mortality rate and relative proportion have been scarcely applied in soil ecotoxicology but could help to get deeper knowledge about accumulation, mechanism of action and toxicity of chemical compounds in general and of Ag NPs in particular.

HYPOTHESIS

A reliable screening diagnosis of the toxicity produced by PVP-PEI coated Ag NPs in soils can be achieved after the establishment of the main uptake route (through standard toxicity tests) and through the definition of accurate test soils (artificial vs. natural), the integration of biomarker responses at different levels of biological complexity (molecular, biochemical, cell, tissue, organism), the selection of the most reliable conditions for *in vitro* toxicity testing (conventional and natural culture media, viability assays), and the discrimination of the responses given by two subpopulations of coelomocytes (amoebocytes vs. eleocytes) in *Eisenia fetida* earthworms.

OBJECTIVES

In order to experimentally test this hypothesis, the following objectives were established:

- 1- To determine the **uptake routes** and to assess the **toxicity** of PVP-PEI coated Ag NPs in soil through the implementation of different exposure media based standard toxicity tests (OECD) and cellular biomarkers (coelomocyte number and viability) in *E. fetida* earthworms (**Chapter 1**).
- 2- To perform an **integrative assessment** of the toxic effects exerted by **sublethal concentrations** (close to lethality threshold, and close to environmental predictions based on *in silico* models) of PVP-PEI coated Ag NPs at **different levels of biological complexity** in *E. fetida* earthworms maintained in two widely used standard **soils** (OECD and LUFA 2.3) (**Chapter 2**).
- 3- To compare the toxicity of PVP-PEI coated Ag NPs with the **soluble form** (AgNO₃) at **sublethal concentrations** (close to lethality threshold, and close to environmental predictions based on *in silico* models) in *E. fetida* earthworms, using biochemical endpoints measured in target tissues and cellular and molecular level endpoints (cell number and viability and transcription levels of *cat* and *mt*) **measured in coelomocytes** extruded from exposed earthworms (**Chapter 3**).
- 4- To assess through ***in vitro* approaches** (microplate viability assays, flow cytometry, cell sorting) the toxicity of PVP-PEI coated Ag NPs in primary cultures of *E. fetida* coelomocytes, after a previous selection of an optimal medium for coelomocytes culturing and the most responsive viability assay (**Chapter 4**).

III. RESULTS AND DISCUSSION

CHAPTER 1

**Uptake route and resulting toxicity of silver nanoparticles
in *Eisenia fetida* earthworm exposed through standard
OECD tests**

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Garcia-Velasco, N., Irizar, A., Gandariasbeitia, M., Urionabarrenetxea, E., Soto, M. Toxicity screening of AgNPs and integrative assessment of soil health through biomarker responses in *E. fetida* earthworm at different levels of biological organization. NANOSPAIN 2015. BEC, Bilbao, 10-13 March 2015. Platform and poster presentations (N Garcia-Velasco).

Garcia-Velasco, N., Irizar, A., Marigómez, I., Soto M. Toxicity screening of silver nanoparticles to *Eisenia fetida* earthworm through standardized OECD test and *in vitro* assay with coelomocytes. 24th Annual Meeting of SETAC-Europe, Basel, 11-15 May 2014. Poster presentation.

Abstract

Despite the increasing interest in silver nanoparticles (Ag NPs) toxicity still few works dealt with the hazards of nanosized Ag in soils (either dissolved in pore water or coupled to colloids) although disposal of waste water treatment plant (WWTP) sludges or biosolids in landfills has been reported as the major source of Ag NPs in terrestrial environments. In the present chapter, *Eisenia fetida* was used to assess the toxicity of 5 nm sized PVP-PEI coated Ag NPs in soil through the implementation of different exposure media standard toxicity tests (Paper Contact and Artificial Soil –OECD-207- and Reproduction –OECD-222- tests) together with cellular biomarkers measured in extruded coelomocytes. In order to decipher the mode of action of Ag NPs in soil and the uptake routes in earthworms, special attention was given to the Ag accumulation and distribution in tissues. High Ag accumulation rates, weight loss and mortality due to the disruption of the tegument could be the result of a dermal absorption of Ag ions released from Ag NPs (Paper Contact test). However, autometallography showed metals mainly localized in the digestive tract after Artificial Soil test, suggesting that Ag uptake occurred mostly through soil ingestion. That is, Ag NPs attached to soil particles or colloids seemed to be internalized in earthworms after ingestion of soil and transferred to the digestive gut epithelium where at high doses they have triggered severe effects at different levels of biological complexity.

Key words: Silver Nanoparticle (Ag NP), Soil, *Eisenia fetida*, OECD Standard toxicity test, Uptake route.

Laburpena

Zilar nanopartikulek (Ag NPs) lurzoruetan izan ditzaketen toxikotasunaren ezaguera eskasa da egun. Ag NPak nekazaritzan erabiltzean diren hondakin uren araztegiak (HUA) lokatzen bitartez heldu daitezke lurzorutara eta bertan beren osagaiekin interakzio desberdinak pairatu ditzakete (uretan disolbatu edo koloideekin elkarreraginak jasan ditzakete adibidez). Kapitulu honetan, 5 nm-ko PVP-PEI estalduradun zilar nanopartikulen toxikotasuna aztertu izan da esposizio medio desberdinetan oinarritutako toxikotasun test estandarrek *Eisenia fetida* zizareetan erabiliz (*Paper Contact* eta *Artificial Soil* –OECD-207- eta *Reproduction* –OECD-222- testak). Horietaz gain, biomarkatzaile zelularrak neurtu izan ziren Ag NP pean izandako zizareetatik erauzitako zelomozitoetan. Ag NPak lurzoruetan duten portaera eta zizareetan duten sarrera bide nagusia ezagutu nahian, ehunen zilar metaketa eta metaketa guneei arreta berezia eskaini zitzaion. Zilar metaketa balio altuak, pisu galera eta tegumentuaren haustek eragindako hilkortasuna ikusi ziren dermis bitarteko esposizio pean egondako zizareetan (*Paper Contact test*). Hala ere, lurzoru bitarteko esposizioaren ondoren egindako autometalografiak zilarra liseri-traktuan topatu zuen nagusiki, liseri-traktu bitarteko esposizio bidearen nagusitasuna zehaztuz. Beraz, lurzoru partikula edo koloideetara atxikituriko Ag NPak ingesta bitartez barneratuko dira zizareetan eta behin traktu epitelioan, dosi garaietan, efektuak eragingo dituzte zizareen konplexutasun maila desberdinetan.

Hitz gakoak: Zilar nanopartikula (Ag NPs), Lurzorua, *Eisenia fetida*, OECD toxikotasun test estandarizatuak, Esposizio bidea

Resumen

Es fácilmente constatable que actualmente hay pocos trabajos que se dedican al estudio de la toxicidad de las nanopartículas de plata (Ag NPs) en el ecosistema terrestre, a pesar de que la utilización de lodos de depuradora de aguas residuales (EDAR) en agricultura es su principal vía de entrada a éste compartimento. Hay que tener en cuenta, además, la gran cantidad de interacciones que pueden ocurrir entre estos materiales y los componentes del suelo (ej. disolución en el agua de poro o agregados con coloides). En el presente capítulo se ha evaluado la toxicidad de Ag NPs de 5 nm recubiertas con PVP-PEI en suelo mediante ensayos de toxicidad estandarizados (OECD) en lombrices *Eisenia fetida* utilizando diferentes medios de exposición (*Paper Contact and Artificial Soil test* –OECD-207- y *Reproduction test* –OECD-222-). Además, se han cuantificado biomarcadores celulares en celomocitos extruidos de lombrices expuestas *in vivo* a Ag NPs. Para entender el modo de acción de las Ag NPs en el suelo y su principal ruta de toma por las lombrices se han medido la acumulación y distribución de Ag en tejidos. Se han observado niveles altos de Ag en tejidos tras la toma de Ag (iónica) vía dermis (*Paper Contact test*), y se han determinado una pérdida de peso y mortalidad significativas debidas fundamentalmente a la desestructuración del tegumento. Se ha demostrado la localización mayoritaria de Ag mediante autometalografía en el tracto digestivo de lombrices mantenidas en suelo artificial (*Artificial Soil test*), y se ha demostrado que la ingesta de suelo es la ruta más importante de toma de Ag NPs en condiciones reales. De esta manera, las Ag NPs agregadas a coloides o partículas de suelo serían incorporadas por las lombrices vía ingestión, y ser a continuación transferidas al epitelio del tracto digestivo donde a dosis altas producen efectos severos a diferentes niveles de complejidad biológica.

Palabras clave: Nanopartículas de plata (Ag NPs), Suelo, *Eisenia fetida*, Test estándar de la OECD, Ruta de toma

1. Introduction

In recent years more than 438 consumer products (24% of the total products containing nanomaterials; e.g. detergents, paints, printer inks and textiles) contain silver nanoparticles (Ag NPs) (Vance et al., 2015). These NPs are mainly used in biomedical devices due to their antimicrobial properties (Nowack et al., 2011). Together with the increasing uses and applications of Ag NPs their release into different environmental compartments could occur and thus, the concern about the still scarcely known hazards of nanosized materials is growing considerably. To date, the potential risk of NPs has been studied in aquatic environments mainly, where several reviews have dealt with the environmental fate, exposure routes, and ecotoxicity data addressed through *in vivo* and *in vitro* methodologies (McShan et al., 2014). Such studies included freshwater and marine species such as *Daphnia magna*, *Chlorella kessleri*, *Danio rerio*, *Oncorhynchus mykiss* and *Mytilus galloprovincialis* (Hund-Rinke et al., 2006; Katsumiti et al., 2015; Lacave et al., 2016; Lovern and Klaper, 2006). On the contrary, the effects of NPs on soils have been less investigated despite the great complexity of soil matrix and the potential interactions of soil components with pollutants. These components (organic matter, cations, soil colloids-clay and humic acid-, and water) together with the soil type varying pH and ionic strength factors, may affect the behaviour of NPs, with particular effect on the aggregation (i.e. homoaggregation, heteroaggregation) and subsequent effect on their toxicity to organisms inhabiting soils (Joško and Oleszczuk, 2013). Apart from the soil factors, the basic physicochemical properties of NPs such as chemical composition, shape, size, surface area (coating agent) and charge must be taken into consideration when assessing their toxicity (Tourinho et al., 2012), as these parameters will also influence their fate and behaviour (dissolution, aggregation, agglomeration, etc.) in environmental matrixes. Therefore, determining the toxicity of NPs should take into account not only the character of the NPs as such, but also the soil properties above mentioned.

Among NPs, Ag NPs in particular are predicted to primarily enter terrestrial system (Blaser et al., 2008; Gottschalk et al., 2009; Shoults-Wilson et al., 2011c), mainly through accidental spills during synthesis and transformation, after direct display of NPs in soil and water remediation technologies, atmospheric fallouts (e.g. after waste incineration) and agriculture (through the use of nanopesticides, herbicides and fertilizers). However, the major source of Ag NPs deposition onto soils is currently through the disposal of wastewater treatments plant (WWTP) sewage sludge or biosolids, mainly after their

land application or incineration and posterior deposition (Tourinho et al., 2012). Even more, the predicted concentrations in sewage sludges range from 1.33 to 4.44 mg Ag NPs/kg in Europe based on products life-cycle release models (Gottschalk et al., 2009, 2010).

Ecotoxicological tests to determine the toxicity of NPs to soil organisms can be carried out with earthworms such as *Eisenia fetida* placed in different exposure media (e.g. moisten paper and standard soil). *E. fetida* is a model terrestrial organism, broadly used in standard toxicity tests (OECD, ISO) due to its sensitivity to different toxicants. Although being epigeic and living in decaying organic matter, in compost or mold, the choice of *E. fetida* is pertinent since it represents an inexpensive and non-controversial model for experimentation (Bilej et al., 2010). Additionally, its historical use in standard tests allows the direct comparison of results with reported data. Earthworms are able to uptake chemicals not only by soil ingestion but also from pore water through the outer body wall (Lord et al., 1980). The Paper Contact toxicity test (OECD-207) is an accurate initial screening method to identify toxicity (LC_x and EC_x) and reflects dermal contact exposure, is easy to perform and gives high reproducibility (Zhang et al., 2009; Heckmann et al., 2011). However, the assessment of the effects produced by the incorporation of toxic substances by ingestion (solid phase) via gut requires more detailed testing and should include the assessment of the acute toxicity (Artificial Soil test) and effects on the reproductive output (Reproduction test) in real exposure media (soils).

Apart from assessing effects on survival, body weight and reproduction, and accumulation and tissue distribution of Ag, changes in the immune activity of earthworm coelomocytes can be sensitive indicators too. Content and activity of the coelomic immune cells (coelomocytes) of annelids are known to vary not only between species but also within them depending on the presence of stress factors in soil such as heavy metals (Plytycz and Morgan, 2011). Thus, recently, parameters such as the total number and viability (membrane integrity by Neutral Red Uptake and Retention assays) of coelomocytes have been used as biomarkers to assess the impact of metals on annelids (Asensio et al., 2007, Homa et al., 2015, Irizar et al., 2014b, 2015a). Therefore, the aim of the present chapter is to assess the toxicity of PVP-PEI coated Ag NPs in soil through the implementation of different exposure media based standard toxicity tests (OECD) in *E. fetida* earthworms together with cellular biomarkers measured in coelomocytes. In order to accurately understand the toxicity exerted by Ag NPs and

their mode of action in terrestrial environments a central focus was given to the tissue distribution and uptake routes of Ag in *E. fetida* earthworms.

2. Materials and methods

2.1. Test species

Eisenia fetida earthworms were purchased from a commercial dealer (LOMBRICOR S.C.A., Córdoba, Spain) and set as laboratory culture maintained in containers at 19 ± 2 °C, in darkness and constant humidity. As food source medication-free horse manure was provided when required. The earthworms used for the experiments were all healthy adults, clitellated and of similar size (300-500 mg individual weight).

2.2. Test substances

The tested Ag NPs (NP Ag-2106W purchased from NANOGAP SUB-NM-POWDER, S.A., A Coruña, Spain) were 5.08 ± 2.03 nm sized, polyvinylpyrrolidone-polyethylenimine (PVP-PEI, 3.35:1) coated, with 18.6 ± 7.9 mV Z-potential. Ag NPs were water dispersed, 10 g Ag/L with 104 g PVP-PEI/l. More details on the characterization of Ag NPs are given in Appendix I. The coating agent PVP-PEI was tested separately at the same concentration range and pH as present in the Ag NPs suspension (PVP10-polyvinylpyrrolidone, Mw 10000 and PEI-polyethylenimine, Mw 25000 by LS; both from Sigma-Aldrich).

2.3. Soil preparation and spiking procedure

The OECD artificial soil was prepared following the OECD guideline for testing of chemicals No. 207 (OECD, 1984), consisting the substrate of 70% sand (50% of the particles between 50-200 μm), 20% kaolin clay and 10% sphagnum peat sieved at 2 mm. The pH was then adjusted to 7.0 ± 0.5 by addition of 0.01% calcium carbonate. Dry constituents were mixed, placed in glass containers and moistened to 40% of their water holding capacity (WHC, 21.91%) with suspensions of Ag NPs in distilled water or with distilled water in the case of the control group. After spiking, experimental soils were thoroughly mixed to ensure homogeneous distribution and were left stabilizing during 3 days before the exposure of the earthworms.

2.4. Acute toxicity tests (OECD-207, 1984)

2.4.1. Paper Contact test

Earthworms were kept in moist paper voiding their gut contents (3 h) before being individually placed into glass vials containing filter paper cuts (8 x 5.5 cm) lined in their

sides and previously moistened with 1 ml of PVP-PEI coated Ag NPs in a range of concentrations from 0 to 200 $\mu\text{g}/\text{cm}^2$ (0, 0.02, 0.06, 0.2, 0.6, 2, 6, 8, 16, 20, 66, 200 Ag NPs $\mu\text{g}/\text{cm}^2$). In parallel, another set of earthworms was exposed to PVP-PEI in the same proportion (PVP: PEI, 3.35:1) and pH value (around 7.5) present in the Ag NPs solution. For the control group, vials were treated with 1 ml of distilled water. Ten replicates were done per treatment. Each vial was sealed with a plastic net and maintained in darkness at 19 °C for a period of 48 h. After exposure mortality rate and weight loss were recorded according to earthworm Acute toxicity test (OECD-207). Complementarily, for each treatment the post clitellar region of five depurated worms was dissected out, sectioned transversally in a cryotome (10 μm , Leica CM3000) and stained with hematoxylin/eosin for the examination of histopathological alterations and with Alcian Blue (pH 2.5) to visualize histochemically carboxylated mucopolysaccharids in the tegument. With the remaining earthworms (≤ 5) Ag accumulation and distribution (autometallography) in tissues was determined (see section 2.7).

2.4.2. Artificial Soil test

A total of nine experimental groups were prepared in order to obtain a limit-test concentration range from 0-500 mg Ag NPs/kg soil (0, 0.05, 0.5, 5, 50, 100, 200, 350 and 500 mg/kg). According to the Acute toxicity test (OECD-207) 4 replicates of 750 g (wet weight) were carried out for each treatment. Earthworms previously maintained in non-polluted OECD soil for 24 h were weighed in tens and introduced in the experimental soils during 3 and 14 days, in continuous light and constant humidity. Then, mortality and weight loss were recorded after 3, 7 and 14 days. During the exposure humidity was checked periodically.

2.5. Earthworm Reproduction test (OECD-222, 2004)

Earthworms were exposed to a range of sublethal concentrations of Ag NPs through OECD artificial soil (0, 0.05, 0.5, 5 and 50 mg Ag NPs/kg soil). These concentrations were selected after analysing the results of the Acute toxicity tests and following the same spiking procedure. Adult earthworms were maintained in non-polluted OECD soil for acclimation during 24 h, weighted in tens and placed in containers with 500 g (dry weight) experimental soil. Four replicates were done per treatment. Test was carried out at 19 ± 2 °C under controlled light-dark cycles (8/16 h) and 5 g of medication-free horse manure were provided weekly during the first 4 weeks. After this period, adults were removed from the soils to determine effects on growth and the accumulation of Ag

in tissues (see section 2.7). After the adult removal, their offsprings were kept for another 4 weeks in the same experimental soils and conditions with the exception of the feeding. At day 56, effects on reproduction were assessed by counting cocoons and juveniles using the hand sorting technique twice in all samples (OECD, 2004).

2.6. Silver concentration and pH of experimental soils

At days 3 and 14 of the Artificial Soil test and day 56 of the Earthworm Reproduction test Ag concentration and pH were measured in experimental soils. The real concentration of Ag in soils was quantified following the EPA 3051A method. For that, soil samples (2 g) were acid digested (HNO_3 : HCl, 3:1) in Teflon vessels in a microwave oven, filtered after cooling (0.45 μm , 25 mm, PVDF) and analysed in Inductively Coupled Plasma Mass Spectrometry (ICP-MS, 7700-Agilent Technologies) in the Central Analysis Service of the UPV/EHU (SGIker). For QA/QC, blanks were ensured to be below the limit of quantification ($\text{LOQ} < 0.01 \mu\text{g Ag/l}$) and samples and Certified Reference Materials (CRMs) measurements were replicated with $\text{RSD} < 3\%$, and only measurements within the nominal value of CRMs plus uncertainty were accepted. The CRMs used were sewage sludge 3 (RTC, $100 \pm 9.96 \text{ mg Ag/kg}$) and sewage sludge 4 (Sigma-Aldrich, $64.7 \pm 8.17 \text{ mg Ag/kg}$).

For the measurements of the pH an adaptation of the ISO 10390: 2005 “Soil Quality – Determination of pH in water” was followed. Soil samples from each replicate were mixed with distilled water (1:5), shaken during 1 minute and left to settle for 45 minutes. The process was repeated twice and then the pH of the liquid phase was measured using a calibrated pH-meter (CRISON micro pH 2001).

2.7. Silver accumulation and distribution in earthworms

Ag concentration in tissues was quantified by ICP-MS in the surviving earthworms at the end of each standard toxicity test (Paper Contact test, Artificial Soil test and Earthworm Reproduction test). Depurated earthworms were dried at 120 °C for 48 h (individually after Paper Contact test and in pools of five worms in the case of the Artificial Soil and Reproduction tests), weighted and digested in HNO_3 Tracepur® 69%. Once the concentrated acid was evaporated, pellets were resuspended in 0.01 M HNO_3 Tracepur® and Ag analysed in the Central Analysis Service of the UPV/EHU (SGIker).

Distribution of Ag in earthworm tissues was studied by the implementation of the autometallographical method (Soto et al., 1996). After exposure through Paper Contact

(48 h) and Artificial Soil (days 3 and 14) tests, five earthworms per treatment were placed in Petri dishes with moist filter paper for 24 h to depurate, then were cleaned, dissected out and tissues histologically processed. The dissection was made according to the zonation in the digestive tract described by Irizar et al. (2014a). Following these criteria, a small post clitellar section (around 5 segments) for transversal sectioning and the next section (around 10 segments) for longitudinal sectioning were used for the histological analysis. The portions were immersed in formalin (10% commercial formaldehyde in 0.1 M phosphate buffered saline-PBS- solution with 0.23% NaCl) for 24 h at 4 °C and dehydrated in Leica ASP 300 tissue processor (in 70%, 96%, and 100% ethanol) before being embedded into paraffin blocks. Afterwards, 5 µm sections were obtained in a Leica RM 2125RT microtome. A set of various sections was extended onto slides, dewaxed with xylene and rehydrated through several baths of ethanol (100%, 96% and 70%) and air dried. Autometallography was then carried out with the BBInternational Silver Enhancing Kit for Light and Electron Microscopy (BBI Life Sciences). A mix of initiator and enhancer reagents (1:1) was applied as drops onto each tissue section placed in a moisture chamber to avoid desiccation. After 25 minutes of reaction the slides were washed several times with distilled water, dried, covered with Kaisers' glycerol gelatine and overlaid with a cover slide. Ag tissue distribution was visualized under light microscope as autometallographed Black Silver Deposits (BSDs).

2.8. Coelomocyte number and viability in exposed earthworms

After 3 and 14 days of exposure through artificial soil, from the treatments 0, 0.05, 0.5, 5, 50, 500 mg Ag NPs/kg 5 earthworms were pooled together and their coelomocytes extruded to perform the Calcein AM viability assay. First, earthworms were cleaned with distilled water by softly massaging their body in order to remove any soil particle attached to the tegument or in the posterior part of their digestive tract. Then pools of five individuals were immersed in extrusion solution (0.02% EDTA in PBS with 0.23% NaCl, 1 ml per worm) and were subjected to an electric stimulation with a 9 V battery to allow the release of coelomocytes through dorsal pores (Irizar et al., 2014b). The cell suspensions were transferred to tubes, centrifuged (530 x g, 10 min, 10 °C) and resuspended in 5 ml of PBS for posterior cell counting under light microscope. Neubauer chamber was used to count and adjust the cell density of each pool to 10⁶ cells per ml. Then 2 x 10⁵ coelomocytes per well were seeded in a 96-well microplate (six well per treatment) and were left to stand at 18 °C in darkness for 30 minutes. Afterwards, the microplate was centrifuged (530 x g, 5 min, 10 °C), supernatant removed and cells were

incubated for 40 minutes with 2.5 μM Calcein AM ($n=3$, 100 μl per well). In the remaining wells, instead of Calcein AM, 100 μl PBS were added in order to thereafter subtract the inherent fluorescence of cells. Coelomocytes were washed twice (centrifugation, supernatant removal and addition of 100 μl PBS) and fluorescence was measured at 490 ± 20 nm excitation filter and 520 ± 20 nm emission filter in FLx 800 microplate fluorescence reader.

2.9. Statistical analysis

The statistical analysis of the data was carried out with the aid of the SPSS statistical package (IMB SPSS Statistics 20). Shapiro-Wilk ($n < 30$) and Levene's tests were performed to study normality and equality of variances of the datasets, respectively. One-way ANOVA followed by Tukey's pairwise comparison and Dunnett post hoc test was used as a parametric approach. The non-parametric datasets were analysed with Kruskal-Wallis followed by Dunn's post-hoc test. Statistically significant differences were established at $p < 0.05$ or $p < 0.01$. Pearson's correlation was followed to find the significance of the correlation coefficient (R) and the Probit model to estimate the median lethal concentration (LC_{50}) and the effect concentration for 50% effect (EC_{50}) after OECD standard toxicity tests.

3. Results

3.1. Silver concentration and pH of experimental soils

The real concentrations of Ag in experimental soils did not differ significantly from the nominal concentrations in both Artificial Soil and Reproduction tests (Table 1). Soil pH did not change between treatments and remained stable during time with a neutral value for both tests (Table 1).

3.2. Silver accumulation and distribution in earthworms

The accumulation of Ag in earthworm tissues after Paper Contact toxicity test showed a dose-dependent increase, the maximum values being recorded after exposure to the highest doses of Ag NPs, 6 and 8 $\mu\text{g}/\text{cm}^2$ (140.41 ± 68.27 and 105.91 ± 78.74 $\mu\text{g Ag/g}$, respectively; Table 2).

After 3 and 14 days of exposure in the Artificial Soil test, Ag concentration in earthworm tissues followed an increasing dose-dependent gradient. Exposure to 500 mg Ag NPs/kg for 3 days rendered 29.70 $\mu\text{g Ag/g}$. After 14 days 100% mortality was recorded between 200 and 500 mg Ag NPs/kg (Table 2) and the highest concentrations recorded were

14.10 and 18.20 $\mu\text{g Ag/g}$ after exposure to 50 and 100 mg Ag NPs/kg exposure concentrations, respectively. Earthworms exhibited higher Ag concentrations after 14 days than after 3 days of exposure being the accumulation in earthworms exposed to 50 mg Ag NPs/kg significantly different. Earthworms maintained in Ag NPs polluted soils during the first 28 days of the earthworm Reproduction test exhibited lower Ag accumulation values than the obtained after 14 days of exposure in the Artificial Soil test (Table 2).

After implementation of the autometallography and visualization at the light microscope few BSD were observed in control earthworms when compared with exposed specimens. High amounts of BSDs were observed surrounding the cuticle of earthworms exposed through the Paper Contact test to doses higher than $2 \mu\text{g/cm}^2$, being the highest amount found in $6 \mu\text{g Ag NPs/cm}^2$ dose (Fig. 1b), where Ag accumulation resulted to be the highest too ($140.41 \pm 68,27 \mu\text{g Ag/g}$). After 3 days of exposure in the Artificial Soil test, no differences in the amount of BSDs were observed in the digestive tract epithelium and in the tegument between controls and the lowest Ag NPs exposure concentration ($0.05 \text{ mg Ag NPs/kg}$). Exposure to 50 and 500 mg Ag NPs/kg showed a higher amount of BSDs. Earthworms exposed to 500 mg Ag NPs/kg exhibited BSDs in the apex of the digestive epithelium, in the chloragocytes and attached to the cuticle (Figs. 1C and 1c). After 14 days of exposure, BSDs were observed in the earthworms exposed to 5 and 50 mg Ag NPs/kg being the amount higher at 50 mg Ag NPs/kg in the same cell and tissue compartments mentioned beforehand (Figs. 1D and 1d). Silver deposits were more abundant in the digestive tract epithelium than in the tegument of animals exposed through soil.

3.3. Histopathological alterations and mucopolysaccharid demonstration in the tegument

The observation of histological sections stained with hematoxylin/eosin showed that control earthworms did not exhibit any histopathological alteration (results not shown). Cuticle disruption and epithelial thinning were clearly observed in the tegument of earthworms exposed to concentrations higher than $6 \mu\text{g Ag NPs /cm}^2$ (Paper Contact test, OECD-207), as observed in tissue sections stained with Alcian Blue (Fig. 2). Mucocytes were very conspicuous with an homogenous secretion covering completely the body of control earthworms (Fig. 2A). After exposure to doses higher than $6 \mu\text{g Ag NPs/cm}^2$, a reduced number of mucocytes was visualized (Fig. 2B).

Table 1. Ag concentration (nominal and real values, mg Ag/kg soil) and pH of soils (1) at days 3 and 14 of the Artificial Soil test and (2) at day 56 of the Reproduction test. Values are represented as means \pm standard deviations. (udl: under the detection limit, 0.06 mg/kg).

(1) Artificial Soil test

	Nominal	0	0.05	0.5	5	50	100	200	350	500
Ag (mg / kg soil)	Real 3 d	udl	udl	0.31 \pm 0.03	3.98 \pm 1.23	46.00 \pm 6.96	-	-	-	371.33 \pm 106.82
	Real 14 d	udl	udl	0.36 \pm 0.05	5.67 \pm 2.91	43.93 \pm 2.63	79.13 \pm 3.46	165.25 \pm 8.42	237.5 \pm 45.71	432.00 \pm 43.46
Soil pH	3 d	6.98 \pm 0.1	6.96 \pm 0.03	6.96 \pm 0.04	7.24 \pm 0.06	7.36 \pm 0.03	-	-	-	7.07 \pm 0.14
	14 d	7.07 \pm 0.70	7.05 \pm 0.04	7.01 \pm 0.03	7.09 \pm 0.06	7.03 \pm 0.06	6.75 \pm 0.09	6.79 \pm 0.06	6.91 \pm 0.09	7.18 \pm 0.13

(2) Reproduction test

	Nominal	0	0.05	0.5	5	50
Ag (mg / kg soil)	Real	udl	0.07 \pm 0.03	0.42 \pm 0.09	3.60 \pm 0.76	36.02 \pm 6.63
Soil pH	56d	6.76 \pm 0.08	6.82 \pm 0.02	6.60 \pm 0.23	6.84 \pm 0.04	6.65 \pm 0.10

udl= Under detection limit, 0.06 mg/kg

Table 2. Ag concentration in earthworm tissues ($\mu\text{g Ag/g}$) (1) after 48 h of the Paper Contact test, (2) after 3 and 14 days of the Artificial Soil test, and (3) after 28 days of the Earthworm Reproduction test. Values are represented as means \pm standard deviations. (udl: under the detection limit, $0.03 \mu\text{g/g}$; # 100% mortality). Significant differences respect to control ($p < 0.05$ with Kruskal-Wallis) are represented by asterisks.

(1) Paper Contact test

Ag NPs exposure	(μg/cm ²) (ppm)	0	0.02	0.06	0.2	0.6	2	6	8
		(0)	(0.8)	(2.9)	(8.8)	(29)	(88)	(290)	(362.5)
Ag in tissues (μg / g)	48 h	1.37 \pm 0.26	2.95 \pm 2.16	4.44 \pm 2.30	6.77 \pm 3.29	10.76 \pm 2.53*	14.88 \pm 9.86*	140.41 \pm 68.28*	105.92 \pm 78.74*

(2) Artificial Soil test

Ag NPs in soil (mg/kg)		0	0.05	0.5	5	50	100	200	350	500
Ag in tissues (μg / g)	3 d	udl	udl	0.07 \pm 0.00	1.36 \pm 1.09	0.54 \pm 0.22	-	-	-	29.7 \pm 20.48
	14 d	udl	udl	0.10 \pm 0.05	4.27 \pm 1.23	14.10 \pm 3.92*	18.20 \pm 11.86*	#	#	#

(3) Reproduction test

Ag NPs in soil (mg/kg)		0	0.05	0.5	5	50
Ag in tissues (μg / g)	28 d	udl	0.18 \pm 0.23	udl	0.47 \pm 0.54	3.44 \pm 2.76*

udl= Under detection limit, $0.03 \mu\text{g/g}$

100% mortality

* $p < 0.05$

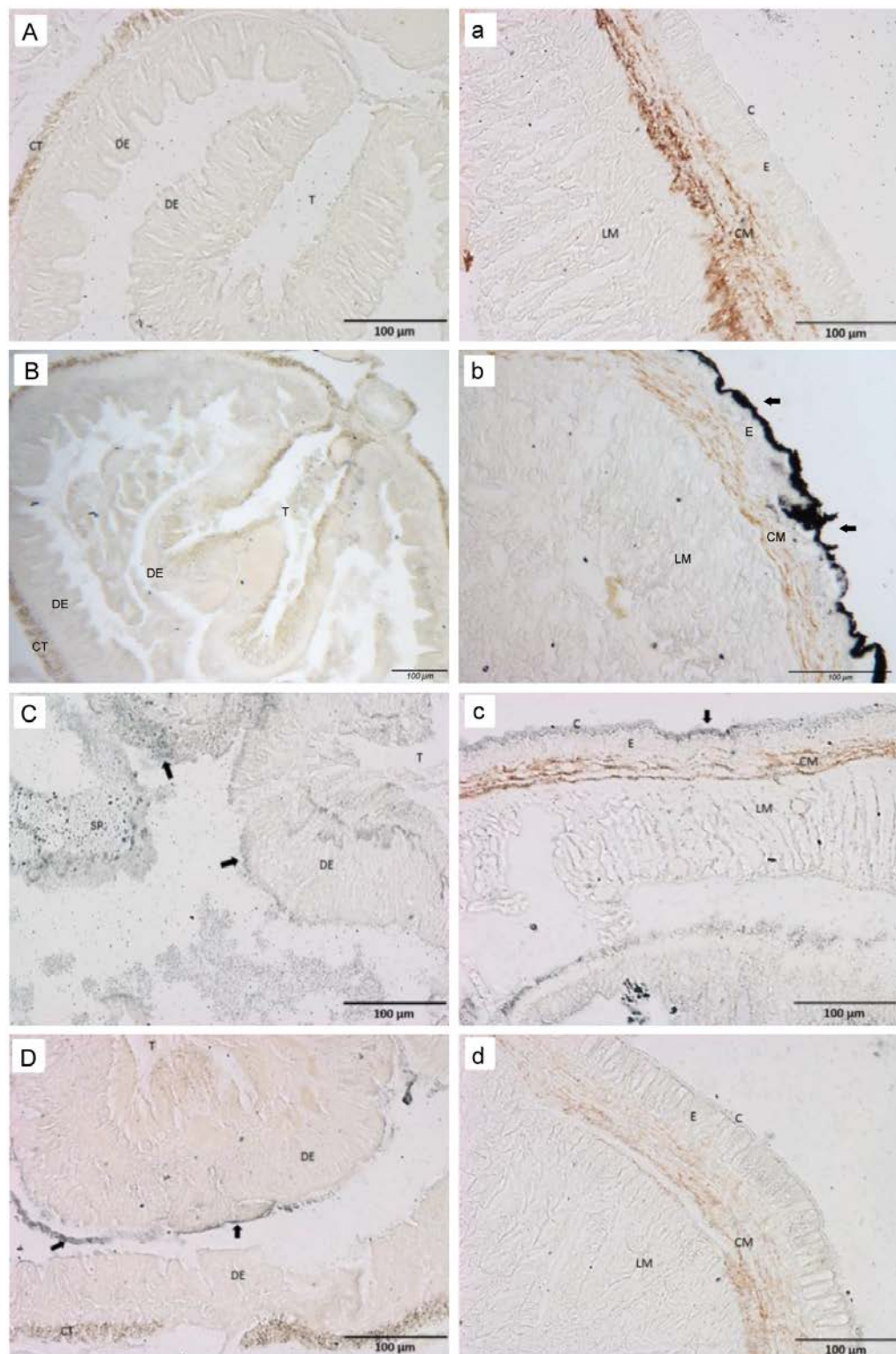


Figure 1. Autometallography in transversal sections of the digestive tract (capital letters) and tegument (small letters) of *E. fetida* earthworms after the Paper Contact and Artificial Soil tests. Control earthworm (A, a); earthworms exposed to 6 μg Ag NPs/cm² through Paper Contact tests (B, b) and exposed to 500 mg Ag NPs/kg during 3 days (C, c) and to 50 mg Ag NPs/kg for 14 days (D, d) through the Artificial Soil Contact test. Black Silver Deposits (BSDs) are labelled with arrows. CT: Chloragogenous tissue; DE: Digestive epithelium; T: Typhlosole; E: Epithelium; C: Cuticle; CM: Circular muscle; LM: Longitudinal muscle; SP: Soil particles in the gut.

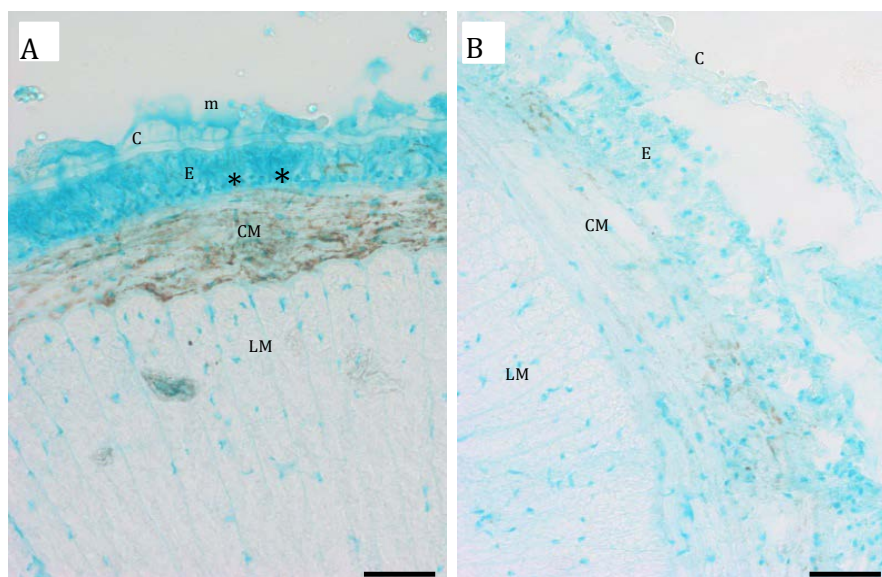


Figure. 2 Demonstration of mucopolysaccharides after Alcian Blue (pH 2.5) staining in the tegument of control earthworms (A) and in earthworms exposed to 6 μg Ag NPs/cm² (B) through the Paper Contact test (OECD-207). Mucocytes are labelled with asterisks and the detached cuticle with an arrow. m: Mucus layer, C: Cuticle, E: Epithelium, CM: Circular muscle, LM: Longitudinal muscle. Scale bar: 50 μm .

3.4. Acute toxicity tests (OECD-207, 1984)

3.4.1. Paper Contact test

Ag NPs caused massive mortality (100%) in earthworms exposed through the Paper Contact toxicity test to concentrations higher than 16 μg Ag NPs/cm². Significant mortalities (50%) were also recorded at lower doses (6 and 8 μg Ag NPs/cm²) where high values of Ag accumulation were observed (140.4 μg Ag/g and 105.9 μg Ag/g, respectively; Table 2). A severe weight loss (>20%) was detected at exposure concentrations higher than 0.06 μg Ag NPs/cm², being significantly different to the control (distilled water moistened paper) from 0.6 μg Ag NPs/cm² onwards (Fig. 3).

PVP-PEI coating agent produced 100% mortality after exposure to 200 μg PVP-PEI/cm² and 10% mortality at 20 μg PVP-PEI/cm². In contrast, the rest of the exposure concentrations did not produce any mortality (Fig. 3). A severe weight loss (47.51%) was recorded after exposure to 20 μg PVP-PEI/cm² while the rest of exposure concentrations produced weight losses ranging 8-12% (Fig. 3).

The LC₅₀ value after Ag NPs exposure was 7.17 $\mu\text{g}/\text{cm}^2$ (equivalent to 315.41 ppm) and the EC₅₀ 0.10 $\mu\text{g}/\text{cm}^2$ (4.49 ppm). The LC₅₀ value for the PVP-PEI coating agent was 15.57 $\mu\text{g}/\text{cm}^2$ (equivalent to 685.08 ppm).

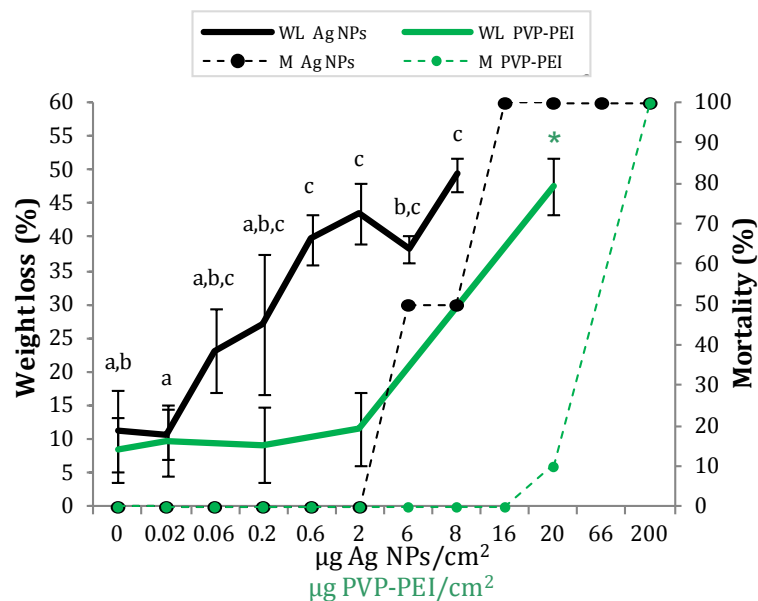


Figure 3. Weight loss (% WL, solid lines) and mortality (%M, dotted lines) of *E. fetida* earthworms after exposure to PVP-PEI coated Ag NPs (black) and PVP-PEI coating agent (green) (0-200 µg/cm²) through the Paper Contact toxicity test. Weight loss values are represented as average ± standard deviations and significant differences (p<0.05 with Kruskal-Wallis) between treatments are represented by letters and asterisk.

3.4.2. Artificial Soil test

After 3 days of exposure to Ag NPs through artificial OECD soil 10% mortality was observed uniquely in the highest concentration (500 mg Ag NPs/kg soil, Fig. 4). Earthworms did not exhibit severe weight loss for the concentrations ranging from 0 - 100 mg Ag-NP/kg soil (<10%). However, after exposure to 500 mg Ag NPs/kg earthworms lost around 25% of their initial weight showing significant differences with the groups exposed to 0.5 and 5 mg Ag NPs/kg (Fig. 4).

After 7 days, 100% mortality was recorded after exposure to the highest concentrations (350 and 500 mg Ag NPs/kg soil), 67.5% after 200 mg Ag NPs/kg and 10% after exposure to 100 mg Ag NPs/kg (Fig. 4). Significant differences in weight loss after 7 days of exposure were only observed at 100 and 200 mg Ag NPs/kg (30-35%) in comparison with 0.5 mg Ag NPs/kg as well as between 200 mg Ag NPs/kg and 5 mg Ag NPs/kg (Fig. 4).

After 14 days, 100% mortality was observed after exposure to 350 mg Ag NPs/kg onwards, 95% after exposure to 200 mg Ag NPs/kg and 10% after 100 mg Ag NPs/kg (Fig. 4). The LC₅₀ value was calculated in 144.20 mg Ag NPs/kg. Significant differences in weight loss could be observed for 50 and 100 mg Ag NPs/kg (20% and 35% respectively) when compared with lower doses (Fig. 4). The calculated EC₅₀ regarding

weight loss at day 14 was 57.62 mg Ag NPs/kg, considering 20% weight loss as a significant effect. No significant differences were observed between exposure days 3, 7 and 14.

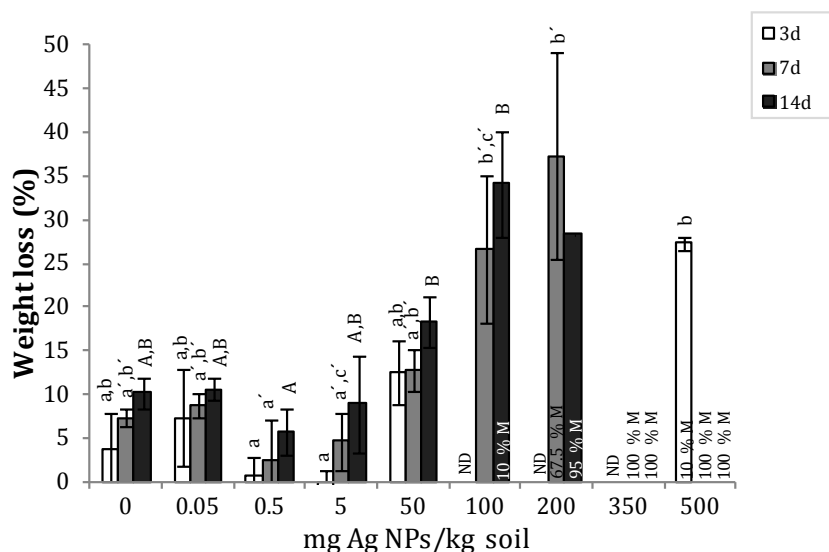


Figure. 4 Weight loss (%) and mortality (%M) of *E. fetida* earthworms exposed to Ag NPs (0-500 mg/kg) through OECD artificial soil during 3 days (white), 7 days (grey) and 14 days (black). Weight loss values are represented as average \pm standard deviations and significant differences ($p < 0.05$ with Kruskal Wallis) between treatments at days 3, 7 and 14 are represented by letters. ND: No Data.

3.5. Earthworm Reproduction test (OECD-222, 2004)

Adult earthworms removed from Ag NPs polluted soils after the first 4 weeks of the Reproduction test gained weight in all treatments, those exposed to the highest dose gaining less weight (50 mg Ag NPs/kg, Fig. 5A). The number of cocoons and juveniles followed a decreasing dose-response trend at increasing exposure doses, being the decrease statistically significant for the number of cocoons and its EC_{50} established at 17.92 mg Ag NPs/kg (Fig. 5B).

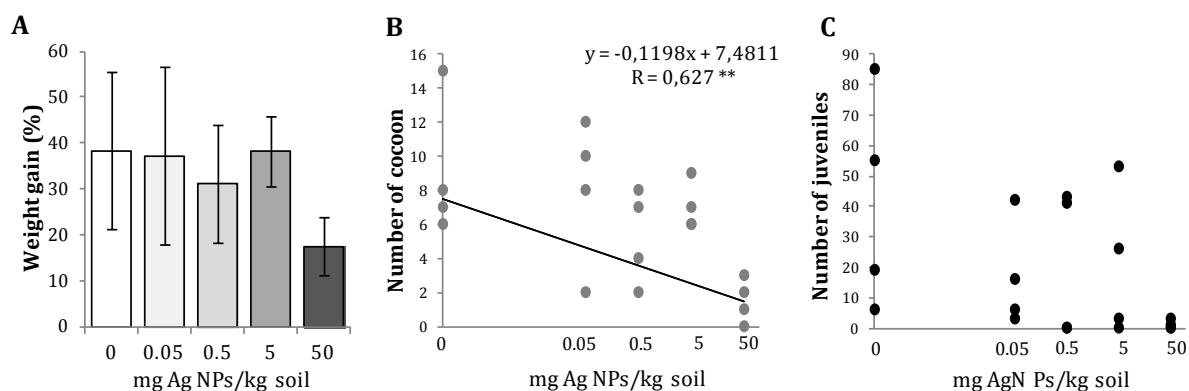


Figure 5. Weight gained (%) by *E. fetida* earthworms after exposure to Ag NPs (0-50 mg/kg) during the first 4 weeks of the Reproduction test (A). Number of cocoons (B) and juveniles (C) counted in the same experimental soils at day 56 of the test. Weigh loss values are represented as average \pm standard deviations. The regression line, its coefficient (R) and the significance of the correlation (**, $p < 0.01$ with Pearson's correlation) are shown for the number of cocoons.

3.6. Coelomocyte number and viability in exposed earthworms

The amount of coelomocytes present in the coelomic fluid extruded from exposed earthworms after the Artificial Soil test showed a decreasing trend at increasing Ag NPs dose (Fig. 6A). Exposures to 5 and 500 mg Ag NPs/kg during 3 days produced a significant reduction of the extruded cell number in comparison with control group (Fig. 6A). After 14 days, the number of coelomocytes presented significant differences between the control and low concentrations of Ag NPs (0.05 and 0.5 mg/kg) with the highest doses (5 and 50 mg Ag NPs/kg) (Fig. 6A).

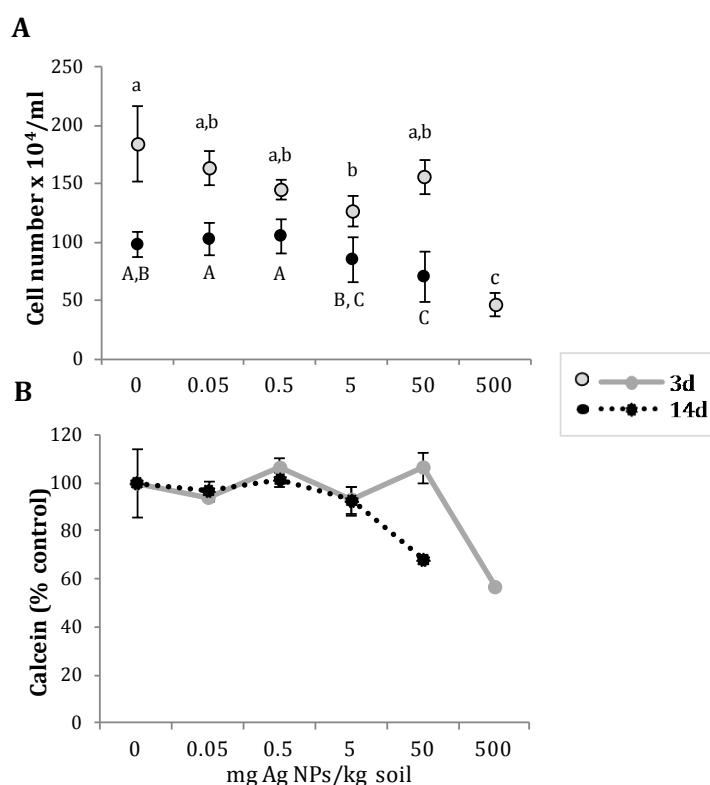


Figure 6. Number of coelomocytes (cell $\times 10^4$ / ml) extruded from pools of 5 earthworms (A) and their viability through Calcein AM Viability assay (Calcein retention in % to the control, B) at days 3 and 14 of the Artificial Soil test. Values are represented as means \pm standard deviations and the significant differences in cell number are represented by letters ($p \leq 0.05$ with Dunnet test).

The Calcein AM viability assay performed in coelomocytes extruded from exposed earthworms showed similar calcein retentions in controls and doses ranging from 0.05

to 5 mg Ag NPs/kg. A decrease occurred after 3 days of exposure to 500 mg Ag NPs/kg and after 14 days exposed to 50 mg Ag NPs/kg (Fig. 6B).

4. Discussion

The uptake of chemicals by earthworms occurs via soil ingestion and from pore water through the outer body wall (Lord et al., 1980). In this work these two main routes were studied when assessing the toxicity of PVP-PEI coated Ag NPs with the aid of standard tests (Paper Contact and Artificial Soil tests-OECD 207- and Reproduction test-OECD 222) performed in different exposure medias (filter paper and soil). During the Paper Contact test, only dermal exposure occurred, while both, dermal (across body wall) and oral (via ingestion and absorption across the digestive gut epithelium) uptakes take place during Artificial Soil and Reproduction tests.

The Paper Contact test evidenced that Ag NPs uptaken by body wall produced a significant weight loss and enhanced mortality in earthworms after exposure to concentration higher than 0.6 μg Ag NPs/cm² for 48 h. Hence, the EC₅₀ was established at 0.10 μg Ag NPs/cm² (equivalent to 4.49 ppm of Ag NPs) and the LC₅₀ at 7.17 μg Ag NPs/cm² (315.41 ppm) (Table 3). The outer part of the earthworm body wall consists of an external cuticle and the epidermis. The cuticle acts as the primary barrier against external mechanical, chemical or biological hazards (Page and Johnstone, 2007). Although being permeable for respiration and perforated by epidermal secretory cell openings, the cuticle offers resistance to abrasion due to the mucus secretion by underlying mucocytes (Lapied et al., 2010). Epithelial mucocytes secrete mucus over the surface of the earthworm body in order to facilitate the locomotion through soils, prevent desiccation and eliminate metals from their body (Lapied et al., 2010; Vijver et al., 2003). It has been previously reported that dermal exposure to environmental stressors can affect the integrity and functionality of the tegument due to enhanced mucous secretion and epithelial disruption (Silva et al., 2016). Likely, the tegument of earthworms exposed to concentrations higher than 6 μg Ag NPs/cm² were severely damaged exhibiting cuticle disruption, epithelial thinning and a reduction in the number of mucocytes in the epithelium. Conversely, the tegument of control earthworms exhibited intact cuticle and functional mucocytes with a homogenous secretion covering completely the surface of the body. It can be concluded that the dermal exposure to Ag NPs produced physical and functional disruption of the tegument as a whole (cuticle and epithelium), resulting in enhanced weight loss in earthworms.

Table 3 Effect concentration for 50% effect (EC₅₀, ppm) and median lethal concentration (LC₅₀, ppm) after Paper Contact and Artificial Soil tests (OECD 207) and Reproduction test (OECD 222).

Substance	Test	Paper Contact	Artificial Soil	Reproduction
	Ag NPs	EC ₅₀ (ppm)	4.49	57.62
LC ₅₀ (ppm)		315.41	144.20	-
PVP-PEI	LC ₅₀ (ppm)	685.08		

In nanotoxicology there is an ongoing challenge regarding whether the toxicity observed is caused by intrinsic properties of the NPs or due to their solubility and ion release, or a combination of both. Hence, dissolution is an important property of NPs which considerably influences their mode of action, and is governed mainly by the chemistry of the coating agents and size. The toxicity resulted after dermal exposure to Ag NPs (Paper Contact test) could be related, at least in part, to the solubility and the uptake of Ag free ions (released from Ag NPs) via the body wall (Vijver et al., 2003; Li et al., 2011). According to manufacturer specifications the 5 nm sized Ag NPs used in the present work were stable in water and were coated by polyvinylpyrrolidone-polyethylenimine (PVP-PEI). PVP has been extensively used as coating agent because modifies the surface of NPs, stabilizing them and preventing ion release and reducing agglomeration (Misra et al., 2012). However, many studies demonstrated that Ag ions can be released from PVP coated Ag NPs (Navarro et al., 2008; Heckmann et al., 2011). Furthermore, particle size has an inverse effect on the dissolution and ion release of NPs (Lapied et al., 2010; Sotiriou and Pratsinis, 2010; Zhang et al., 2011; Ma et al., 2012; Misra et al., 2012), and it is conceivable that the dissolution of 5 nm Ag NPs might occur under present exposure conditions. Thus, it could happen that earthworm were exposed rather to silver ions released to the test media, so Ag NPs would be acting directly as the ions. However, it cannot be discarded whether the effects reported herein are due to Ag NPs, released Ag ions or to both. This controversial point deserves further investigations.

As mentioned before, the role of the coating agent can be a crucial factor since their combination with NPs may influence bioavailability and toxicity. Therefore, in order to discriminate whether the toxicity was exerted by Ag NPs themselves or by the coating agent PVP-PEI, or by the interaction of both, the Paper Contact test was carried out for PVP-PEI agent separately. The results showed that PVP-PEI only caused toxicity at very high doses, being its LC₅₀ of 15.57 µg PVP-PEI/cm² (equivalent to 685.08 ppm), that is, more than two folds higher than the estimated LC₅₀ for PVP-PEI coated Ag NPs (7.17

$\mu\text{g}/\text{cm}^2$, Table 3). Thus, PVP-PEI was less toxic than coated Ag NPs. Likely, Gomes et al. (2013) reported the lack of toxicity due to this compound. However, we would like to suggest that the use of PVP-PEI in the formulation of Ag NPs should be limited since it caused severe weight loss in earthworms exposed to concentrations higher than 290 ppm (Filter Paper test).

The assessment of the effects produced by the incorporation of Ag NPs from the solid (oral uptake) and pore water (dermal uptake) phases requires exposure to soils. In this context, an adequate spiking and homogenising procedure is one of the most crucial issues when characterizing the toxicity of contaminants in soils (Waalewijn-Kool et al., 2012). The chemical analyses carried out in the Ag NPs spiked soils clearly showed that the measured real concentrations did not differ significantly from the nominal concentrations. Similarly, the pH of the soils did not change with time nor between treatments, so was constant and within the optimum range for the experiments, around 7.0. It is well known that metal uptake by earthworms is affected by soil pH, with the highest bioavailability and uptake rates occurring at acid soils (Leveque et al., 2013). However, under the present exposure conditions pH dependent changes in Ag NPs dissolution rates were not expected. Time-dependent Ag accumulation, mortality and weight loss were recorded in earthworms maintained in artificial soil with concentrations higher than 100 mg Ag NPs/kg. Interestingly, the concentrations that produced mortality (LC_{50} 144.20 mg Ag NPs/kg) and severe weight loss (EC_{50} 57.62 mg Ag NP/kg) on the half of the population were much higher than the predicted environmental concentrations for Ag NPs in biosolids of Europe (1.33 - 4.44 mg/kg; Gottschalk et al., 2010). Nevertheless, these lethal and effect concentrations were obtained in laboratory experiments using standard soils, whereas in a real scenario soil components and varying factors may affect the fate and behavior of NPs and subsequently increase their toxicity to organisms inhabiting soils.

Effects exerted by environmental factors (including pollutants) at high levels of biological organisation have the advantage of being ecologically relevant and are widely used tools for the overall assessment of soil health (Lionetto et al., 2012). For instance, effects on reproduction (assessed by standard OECD tests) are one of the most sensitive toxicological parameters due to the fact that even small changes in reproduction can severely affect the survival of the population (Scott-Fordsmand et al., 2008). In the present study, reproduction was also demonstrated to be a more sensitive endpoint compared to mortality and weight loss ($\text{EC}_{50\text{cocoon number}}$ 17.92 mg Ag NPs/kg, Table 3). In

fact, a variety of chemicals (i.e. chlorpyrifos and cadmium) can completely suppress reproduction without producing effects on body size (growth) suggesting that energy is more used for metabolic repair than for the production of offspring (Jager et al., 2006). Accordingly, Ag NPs are able to cause total or partial reproductive failure in Oligochaeta (Gomes et al., 2013; Hekmann et al., 2011; Schlich et al., 2013; Shoults-Wilson et al., 2011a, 2011b; present chapter). However, some of these studies were designed as limit-test toxicity screenings (up to 1000 mg Ag NPs/Kg), whereas presently only sublethal concentrations were employed. Still, alterations in the reproductive output derived from Ag NPs exposure should be closely monitored in soil invertebrates such as earthworms.

E. fetida earthworms exposed to similar concentrations of Ag NPs through soils and through paper contact accumulated nearly the same amounts of Ag, although the time of exposure was far shorter in the latter. These lower accumulation rates recorded in soil could be the result of the lower bioavailability of Ag NPs, probably due to the adsorption and formation of complexes together with clay and organic matter (Coutris et al., 2012; Gomes et al., 2013), which made them less available for absorption. Nevertheless, mortality was much higher in the earthworms that were exposed to Ag NPs in soil. This feature might confirm the existence of two uptake routes for Ag NPs, dermal and soil ingestion. Previous studies have proved that metals such as Cu, Pb, Cd and Zn are absorbed via the dermal route (Vijver et al., 2003), whereas oral exposure is dominant for the assimilation of Ag and Ag NPs in soils (Diez-Ortiz et al., 2015b). Ag NPs contained in soil were incorporated into the digestive epithelium where the low pH of epithelial cell lysosomes could oxidize NPs and induce a series of reactions not occurring outside the organisms (Gomes et al., 2013; Hayashi et al., 2012). These reactions would explain the enhanced toxicity observed under present exposure conditions (digestive impairment and subsequent weight loss and mortality). In fact, the intracellular accumulation of Ag NPs could act as a source of Ag ions mediating oxidative stress and cellular damage *in situ*, a putative mechanism known as the Trojan-horse effect (Limbach et al., 2007).

The combination of standard toxicity tests (Paper Contact, Artificial Soil and Reproduction tests) and the autometallography technique implemented in the present work, was a valuable tool not only to assess the effects exerted by Ag NPs, but also to decipher the uptake routes (dermal or oral) and the target organs where Ag is distributed (digestive epithelium, chloragogenous tissue and cuticle). BSDs (formed around Ag ions released from Ag NPs and/or formed around Ag NPs themselves) were

localized surrounding the cuticle after exposure through Paper Contact test and in the digestive tract (in the apex of the digestive epithelium and in the chloragogenous tissue) and attached to the cuticle in the case of the Artificial soil test. Even though, BSDs were more abundant in the digestive epithelium, which proves that Ag uptake occurred mostly through soil ingestion rather than via dermal exposure, being the latter more related with the absorption of free ions or chemicals present in the pore water of the soils (Spurgeon et al., 2006). Evidencing this, Leveque et al. (2013) reported that the absolute uptake of metals was higher via the digestive tract than via the dermal route when metal concentration was high in the solid phase and low in the aqueous phase. Diez-Ortiz et al. (2015a) measured Ag concentrations in the pore water of soils spiked with Ag NPs, distinguishing the percentage of particulate (intact Ag NPs and silver associated to colloids) and dissolved Ag following ultrafiltration. After one week of aging, 0.15% Ag (respect to the Ag measured in soil) was recorded in the pore water phase and the dissolved ions fraction scarcely reached 0.05% (Diez-Ortiz et al., 2015a). Hence, Ag NPs would form complexes with the solid phase of the soils and they would be internalized by earthworms through ingestion to then interfere with the correct functioning of the gut epithelium, thus, affecting on the earthworm survival.

Autometallography showed that metals were located in the digestive epithelium primarily, however, the cells comprising the chloragogenous tissue (chloragogen cells) could have accumulated Ag NPs too. Chloragocytes derived from the chloragogenous tissue are part of the immune system of earthworms (coelomocytes) and thus, they exhibit a well-developed lysosomal system (Peeters-Joris, 2000), known to accumulate metals. It has been reported that the number of coelomocytes varies after exposure to metals (Plytycz and Morgan, 2011; Podolak et al., 2011), in most of the cases being reduced as a result of changes in the permeability of the cell membrane that leads to diminished cell viability. Likely Ag NPs largely reduced the number and viability of coelomocytes extruded from exposed earthworms at the highest exposure concentrations. Coelomocytes are involved in eliminating foreign material by phagocytosis and encapsulation (amoebocytes) and they also synthesize and secrete cytotoxic components into the coelomic fluid (chloragocytes or eleocytes), causing haemagglutination, opsonisation as well as lysis of non-self material (Bilej et al., 2010). Recent *in vitro* test with coelomocytes demonstrated the selective intracellular accumulation of Ag NPs in the amoebocyte subpopulation and their role as scavengers of Ag NPs, effecting cytokine release and even death of the cell (Hayashi et al., 2012).

Thus, a phagocytic uptake of Ag NPs may have occurred, which explains the observed toxicity (reduced number and viability of extruded coelomocytes) and the consequent dysfunction of the immune system at cellular level (Plytycz and Morgan, 2011).

One of the most important aims of applying biomarkers in ecotoxicology, like the viability of coelomocytes in earthworms, is to detect changes at low concentrations and short exposure periods, which can be linked with effects occurring at longer exposure times and have an effect on high levels of biological complexity (organism and population level). In this work, the toxicity assessment of coelomocytes at 50 mg Ag NPs/kg after 14 days of exposure showed a negative effect on the cells without noticeable effects on mortality at organism level. However, longer exposure periods to that Ag NP concentration (56 days) caused a strong reduction in reproduction, evidencing the valuable usefulness of the assessment of the immune system in earthworm-based ecotoxicity tests as early-response biomarker.

5. Conclusions

The standard toxicity tests (OECD) in *E. fetida* earthworms have provided relevant toxicity data for soil ecotoxicology and in this case, they also allowed the understanding of the behaviour of 5 nm sized PVP-PEI coated Ag NPs in two different exposure conditions. This way, the effects recorded after exposing earthworms to high doses of Ag NPs through the Paper Contact test (high Ag accumulation rates and weight loss and mortality due to the disruption of the tegument), could be the results of a dermal absorption of Ag ions released from Ag NPs. So this preliminary test could be representative of the uptake and resulting toxicity in earthworms of chemicals dissolved in the pore water of real soils. However, the implementation of autometallography indicated that in real situations where earthworms are exposed to the Ag NPs in soil media, Ag NPs are internalized mainly via ingestion of soil, and are then uptaken by the digestive gut epithelium causing severe effects on survival, growth and reproduction. Complementarily, the cell biomarkers measured in the coelomocytes of exposed earthworms offered rapid and accurate information since they were able to predict impairments caused by Ag NPs in higher complexity and exposure levels, demonstrating the suitability and usefulness of cell level biomarkers in nanotoxicology. In addition, it must be pointed out that the toxicity showed in this study could be exerted by the Ag NPs or released Ag ions, but not from the PVP-PEI coating agent.

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CHAPTER 2

Integrative assessment of the effects produced by silver nanoparticles at different levels of biological complexity in *Eisenia fetida* maintained in two standard soils (OECD and LUFA 2.3)

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Abstract

There is a potential risk to increase the release of silver nanoparticles (Ag NPs) into the environment. For instance, in soils receiving sludge models estimate 0.007 mg Ag NPs/kg that will annually increase due to sludge or sludge incineration residues land-disposal. Thus, the concern about the hazards of nanosilver to soils and soil invertebrates is growing. Studies performed up to now have been focused in traditional endpoints, used limit range concentrations and employed different soil types that differ in physico-chemical characteristics. In the present chapter, effects of PVP-PEI coated Ag NPs have been measured at different levels of biological complexity in *Eisenia fetida* earthworm, exposed for 3 and 14 days to high but sublethal (50 mg Ag NPs/kg) and close to modelled environmental concentrations (0.05 mg Ag NPs/kg). Since characteristics of the exposure matrix may limit the response of the organisms to these concentrations, experiments were carried out in OECD and LUFA soils, the most used standard soils. High but sublethal concentrations of Ag NPs increased catalase activity and DNA damage in earthworms maintained in OECD soils for 14 days while in LUFA 2.3 soils produced earlier effects (weight loss, decrease in cell viability and increase in catalase activity at day 3). At day 14, LUFA 2.3 soil (containing low clay and organic matter-OM-) could have provoked starvation of earthworms, masking Ag NPs toxicity. The concentration close to modelled environmental concentrations produced effects uniquely in LUFA 2.3 soil. Accurate physico-chemical characteristics of the standard soils are crucial to assess the toxicity exerted by Ag NPs in *E. fetida* since low clay and OM contents can be considered toxicity enhancers.

Keywords: Silver nanoparticle (Ag NP), biomarker, biological complexity level, standard soil, Integrated Biomarker Response/n index.

Laburpena

Zilar nanopartikulen (Ag NPs) sarrera ekosistemetan gertu dago, araztegi-tako lokatzak jasotzen dituzten lurretan 0.007 mg Ag NPs/kg aurreikusten direlarik. Kontzentrazio hau urtero emendatzeko aurreikusia dago eta ondorioz, Ag NPek eragin dezaketen kalteen gaineko ardurak piztu dira. Ag NPen toxikotasuna lurzorutan ezagutzeko diharduten lanek helburu tradizionalak, kontzentrazio altuak eta ezaugarri desberdinak dituzten lurzoruak erabili dituzte orain arte. Kapitulu honetan, PVP-PEI estalduradun Ag NPen efektuak *Eisenia fetida* zizarearen konplexutasun maila biologiko desberdinetan neurtu dira. Horretarako zizareak 3 eta 14 egunez mantendu ziren kontzentrazio altu baina azpiletalean (50 mg Ag NPs/kg) eta ingurumenean aurreikusten diren eragin gertu dauden kontzentrazioetan (0.05 mg Ag NPs/kg). Era berean, Ag NPen toxikotasunean esposizio medioak eragina izan dezakeenez, zizareen esposizioa bi lurzoru estandarretan egin zen, OECD eta LUFA. Ag NP kontzentrazio altuek katalasa aktibitatearen emendatzea eta DNAn kalteak eragin zituzten OECD lurretan 14 egunez izandako zizareetan. LUFA 2.3 lurzoru-ean efektu goiztiarragoak ikusi ziren (pisu galera, zelula bideragarritasunaren beherakada eta katalasa aktibitatearen emendioa 3 egunetan). 14 egunean, LUFA 2.3 lurzoru-ean (buztin eta materia organiko gutxiago) behatutako efektuak, Ag NPek eragindakoak baino, zizareek jateari utzi ziotelako izan ziren. Ingurumenean aurreikusten diren eragin gertu dauden kontzentrazioen (0.05 mg Ag NPs/kg) pean izandako zizareak LUFA 2.3 lurzoru-ean bakarrik erakutsi zituzten kalteak. Ag NPen toxikotasuna lurzoru-ean aztertzerako orduan, lurzoru estandarren ezaugarri fisiko-kimikoen ezaguera garrantzi handikoa da, jakinda buztin eta materia organiko gutxiren presentzia toxikotasunaren indartzaile direla.

Hitz gakoak: Zilar nanopartikula (Ag NP), biomarkatzailea, konplexutasun maila biologikoa, lurzoru estandarra, IBR/n

Resumen

Existe un riesgo potencial de aumentar la entrada de nanopartículas de plata (Ag NPs) en los ecosistemas. Así se ha estimado un incremento anual de 0.007 mg Ag NPs/kg en suelos que reciben descargas de lodos de depuradora o residuos de su incineración. De forma simultánea crece la preocupación respecto a posibles daños que estas Ag NPs puedan provocar en el ecosistema terrestre y en los invertebrados que lo habitan. Los estudios realizados hasta el momento para evaluar los efectos de las Ag NPs en suelo se han centrado fundamentalmente en objetivos tradicionales utilizando concentraciones elevadas y empleado suelos con características físico-químicas muy diferentes. En el presente capítulo, se han estudiado los efectos de las Ag NPs (recubiertas de PVP-PEI) a diferentes niveles de complejidad biológica en la lombriz *Eisenia fetida*. Para ello se expusieron durante 3 y 14 días a una concentración alta pero subletal (50 mg Ag/kg) y a otra concentración próxima a la estimada para el medio ambiente (0.05 mg Ag/kg). Dado que las características del medio de exposición pueden limitar las respuestas de los organismos ante las Ag NPs, las exposiciones se realizaron en los dos suelos estándares más comunes, OECD y LUFA. La exposición a altas concentraciones de Ag NPs provocó un incremento en la actividad de catalasa y daño en el ADN en lombrices mantenidas en suelo OECD durante 14 días. En suelo LUFA 2.3 se observaron efectos más tempranos (pérdida de peso, bajada de viabilidad celular e incremento en la actividad de catalasa en el día 3). Los efectos observados en las lombrices tras 14 días de exposición en el suelo LUFA 2.3 (menor contenido en arcillas y materia orgánica) podrían estar enmascarados por la falta de ingesta de suelo. La concentración de Ag NPs próxima a la estimada para el medio ambiente (0.05 mg Ag NPs/kg) produjo efectos únicamente en el suelo LUFA 2.3. Se ha concluido que las características físico-químicas de los suelos estándares son cruciales para evaluar correctamente la toxicidad de las Ag NPs en *E. fetida*, dado que contenidos bajos en arcilla y materia orgánica pueden potenciar la toxicidad de las Ag NPs en el suelo.

Palabras clave: Nanopartícula de plata (Ag NP), biomarcador, nivel de complejidad biológica, suelo estándar, IBR/n.

1. Introduction

Nanotoxicity studies focused on the effects of Ag NPs on soils are becoming more frequent, dealing most of them with survival, growth and reproduction measurements in soil invertebrates (Diez-Ortiz et al., 2015a; Garcia-Velasco et al., 2016; Gomes et al., 2013; Heckmann et al., 2011; Lapied et al., 2010; Schlich et al., 2013; Shoults-Wilson et al., 2011a, 2011b, 2011c; Van der Ploeg et al., 2014; Chapter 1). This way, standard toxicity tests with earthworms (promoted by the Organisation for Economic Co-operation and Development, OECD) have provided relevant toxicity data for soils spiked with Ag NPs. In fact, in the previous chapter (Chapter 1), Ag NPs were demonstrated to be uptaken mainly by soil ingestion and once absorbed by the digestive gut epithelium caused severe effects at organism level in the earthworm *Eisenia fetida*.

In order to obtain toxicity data, sandy loam is used in the tests as soil matrix, but depending on the authors, commercial (6.9% of the studies focused on NPs toxicity with terrestrial invertebrates used soils from this source), field collected (from reference sites, 31%) or standard (62.1%) soils are selected, which differ in pedological and physico-chemical characteristics, including pH, cation exchange capacity (CEC), water holding capacity (WHC), clay and organic matter (OM) contents. These soil type varying physico-chemical properties may affect the behaviour of Ag NPs as well, with particular consequence on their aggregation/agglomeration and dissolution and subsequent effect on their bioavailability and toxicity to soil organisms (Joško and Oleszczuk, 2013). Among standard soils OECD and LUFA substrates are the most commonly used ones, being both in accordance with the OECD principles of GLP (Good Laboratory Practice) and recommended and included in other related guidelines such as the German JKI (Julis-Kühn-Institut). In both OECD and LUFA soils a wide range of experiments have been conducted with *E. fetida* earthworm, an organism broadly used in standard toxicity tests (OECD, ISO). Further, due to its sensitivity to different toxicants, different biomarkers are measured at all levels of biological complexity.

Apart from assessing traditional endpoints (survival, growth and reproduction), some authors have measured biochemical and molecular parameters in *E. fetida* earthworm exposed to Ag NPs (Gomes et al., 2015; Hayashi et al., 2013a; Novo et al., 2015; Tsyusko et al., 2012). For instance, Ag NPs are known to induce the production of reactive oxygen species (ROS) (McShan et al., 2014) and living organisms, aiming to balance ROS and prevent oxidative stress, have developed antioxidant defence systems with enzymes

(e.g. catalase -CAT-) and additional protection mechanisms against metal exposure such as metal binding proteins (e.g. metallothioneins -MT-). Nanosilver has been demonstrated to induce oxidative stress in *E. fetida* as well (Gomes et al., 2015; Hayashi et al., 2013a; Tsyusko et al., 2012) and changes in the transcription levels of *cat* or *mt* have been easily measured after the exposure to other metals (Brulle et al., 2006). ROS are able to induce genotoxicity (DNA damage), protein carbonylation and membrane oxidation (Piao et al., 2011). Nevertheless, the information available regarding oxidative stress and DNA damage (though the Comet assay) in soil invertebrates exposed to Ag NPs is still scarce.

Recently, parameters such as the total number and viability (membrane integrity by Neutral Red Uptake and Retention assays) of coelomocytes have been used as biomarkers to assess the impact of metals on annelids (Asensio et al., 2007; Garcia-Velasco et al., 2016; Homa et al., 2015; Irizar et al., 2014b, 2015a; Kwak et al., 2014a). In Chapter 1, cell biomarkers measured in the coelomocytes of exposed earthworms offered rapid and accurate information and were able to predict impairments caused by Ag NPs at higher complexity levels. Thus, the measurement of changes in the immune activity of earthworm coelomocytes can be a sensitive indicator of Ag NPs toxicity.

In most of the studies dealing with the above mentioned endpoints, earthworms were exposed to concentrations orders of magnitude higher than those expected in the environment. The major source of Ag NPs deposition onto soils is through the disposal of waste water treatment plant (WWTP) sludges, mainly after their land application as fertilizers or incineration and posterior deposition (Tourinho et al., 2012). During the wastewater treatment, sulfidation of Ag NPs is a common process taking place being silver sulfide (Ag_2S) NPs the predominant silver species found in sewage sludge and thereafter in soils. Nevertheless, Kaegi et al. (2011) demonstrated that Ag NPs spiked to a WWTP and transformed into Ag_2S NPs during the activated sludge process, were transformed back into Ag NPs (maintaining the original size) during their incineration. Thus, Ag NPs could reach soils in their original state since incineration residues are usually landfilled in Europe (Kelessidis and Stasinakis, 2012). Predicted environmental concentrations (PECs for Europe, based on a probabilistic material flow analysis from a life-cycle perspective of nanomaterials containing products-*in silico* models-) in WWTP sludges ranges from 1.31 to 4.44 mg Ag NPs/kg (Boxall et al., 2007; Gottschalk et al., 2009), in contrast, the estimations for Ag NP concentration in soils are much lower (0.0001-0.00426 mg/kg; Boxall et al., 2007; Mueller and Nowack 2008). However, Ag

NPs in sludge treated soil reached 0.007 mg/kg in 2012, a value that is expected to be annually enhanced in countries with a high proportion of sludge and sludge incineration residues land-disposal (Gottschalk et al., 2009).

Hence, the present work aims to (a) assess the toxicity of PVP-PEI coated Ag NPs at sublethal concentrations (including close to environmental predictions) in *E. fetida* earthworms at different levels of biological complexity and (b) to compare the toxicity exerted by Ag NPs in two widely used standard soils (OECD and LUFA 2.3). For those purposes earthworms were maintained during 3 and 14 days in OECD and LUFA 2.3 soils previously spiked with 0.05 and 50 mg Ag NPs/kg. These concentrations were selected from predictive modelling values in soils amended with WWTP sludges or sludge incineration residues (0.05 mg Ag NPs/kg) and from the survival and reproductive output data scored in the previous screening (50 mg Ag NPs/kg; Garcia-Velasco et al., 2016; Chapter 1), respectively. At each exposure time, Ag concentration was measured in test soils and earthworm tissues, survival and weight loss were checked and coelomocyte number and viability were recorded. DNA damage was also assessed in coelomocytes extruded from exposed earthworms with the aid of the Comet assay. In addition, catalase and metallothionein protein and transcription levels were measured. A parallel study using the same concentration used herein and aiming to compare silver forms (Ag NPs vs. AgNO₃) is presented in Chapter 3. Thus, in the present work, attention was given to the exposure matrix dependant toxicity rather than to the silver form. All the mentioned endpoints were included in a Principal Component Analysis (PCA) and in the Integrated Biomarker Response/n index (IBR/n) in order to give an integrative and conclusive idea of the effects posed by Ag NPs at different complexity levels of *E. fetida*.

2. Materials and methods

2.1. Test species

Eisenia fetida earthworms were purchased from a commercial dealer (LOMBRICOR S.C.A., Córdoba, Spain) and set as laboratory culture maintained in containers under controlled conditions of temperature (19 ± 2 °C) and humidity. As food source medication-free horse manure was provided when required. The earthworms used for the experiment were all adults (clitellated) of similar size (300-500 mg weight).

2.2. Test nanoparticles

Polyvinylpyrrolidone-polyethylenimine (PVP-PEI, 3.35:1) coated Ag NPs (NP Ag-2106W) were purchased from NANOGAP (SUB-NM-POWDER S.A., A Coruña, Spain). Ag NPs were water dispersed (10 g Ag/L with 104 g PVP-PEI/L), 5.08 ± 2.03 nm average size and with a Z-potential of 18.6 ± 7.9 mV. Particle size distribution and zeta potential determinations (in distilled water) through Dynamic Light Scattering were provided by NANOGAP in their certificate of analysis (Appendix I).

2.3. Test soils and spiking procedure

Two different standard soils were used, the artificial OECD soil and the natural soil LUFA 2.3. The OECD soil was prepared following the guideline for testing of chemicals No. 207 (OECD 1984), the substrate consisting of 70% sand (50% of the particles between 50-200 μm), 20% kaolin clay and 10% sphagnum peat sieved at 2 mm. The pH was then adjusted to 7.0 ± 0.5 with addition of powdered calcium carbonate (0.01%). LUFA 2.3 soil was purchased from the LUFA Speyer Institute (Germany). Soils were spiked with 0.05 mg Ag NPs/kg soil (close to predicted environmental concentration) and with 50 mg Ag NPs/kg soil (high but sublethal dose according to previous experiments in Garcia-Velasco et al., 2016; Chapter 1). Soils were placed in glass containers and moistened with Ag NPs dispersions or distilled water (in control groups) to 40% of their water holding capacity (WHC, Table 1) to obtain the final wet weight of 750 g. After spiking, test soils were thoroughly mixed to ensure a homogeneous distribution of the Ag NPs and were left stabilizing during 3 days before the exposure of the earthworms.

Table 1. Main characteristics of OECD and LUFA 2.3 soils: Type, pH value (in dH₂O and 0.01 M CaCl₂), CEC (Cation exchange capacity, meq/100g), WHC (Water holding capacity, %) and clay, sand and OM (Organic matter, %) contents.

	OECD	LUFA 2.3
Soil type	Artificial	Natural sandy loam
pH (dH₂O)	7.00	7.50
pH (0.01 M CaCl₂)	5.88	6.80
CEC (meq/100g)	10.80	10.9
WHC (%)	21.9	37.3
Clay (%)	20	9 [#]
Sand (%)	70	65 [#]
OM (%)	10	1.88 [*]

[#] Particle size distribution according to United States Department of Agriculture (USDA)

^{*}Organic matter calculated as 2 x percent organic carbon

2.4. Experimental set up

Earthworms previously maintained in non-spiked OECD and LUF 2.3 soils for 24 h were weighed in tens and introduced in test soils (10 earthworms per container, 4 containers per treatment) during 3 and 14 days in continuous light and 19 °C. During the exposure no food was supplied and humidity was checked periodically. After exposure, for each endpoint earthworms were dissected out according to the zonation described by Irizar et al. (2014a).

2.5. Test soil characterization

At days 3 and 14 Ag concentration and pH were measured in test soils. The measured concentration of Ag in soils was quantified following the EPA 3051A method. Briefly, soil samples (2 g) were acid digested (HNO_3 : HCl, 3:1) in Teflon vessels in a microwave oven (MW CEM-Mars 5), filtered after cooling (0.45 μm , 25 mm, PVDF) and analysed in Inductively Coupled Plasma Mass Spectrometry (ICP-MS, 7700-Agilent Technologies) in the Central Analysis Service of the UPV/EHU (SGIker). For QA/QC, the same set of processes and Certified Reference Materials (CRM) as in Chapter 1 were used.

The protocol for pH measurement was adapted from the ISO 10390: 2005 “Soil Quality – Determination of pH in water”. Soil samples from each replicate were mixed with distilled water (1:5), shaken during 1 minute and left to settle for 45 minutes. The process was repeated twice and then the pH of the liquid phase was measured using a calibrated pH-meter (CRISON micro pH 2001).

2.6. Organism level endpoints: mortality, weight loss and Ag accumulation in tissues

After exposure mortality rate and weight loss were recorded in earthworms according to the earthworm Acute toxicity test (OECD-207). Complementarily, Ag concentration in earthworm tissues was quantified by ICP-MS (7700 Agilent) at days 3 and 14. Depurated (left on wet filter paper for 24 h to void gut content) and cleaned earthworms (n=5) were dried in pools at 120 °C for 48 h, weighted and digested in HNO_3 Tracepur® 69%. Once the concentrated acid was evaporated, pellets were resuspended in 0.01 M HNO_3 Tracepur® and Ag quantified in the Central Analysis Service of the UPV/EHU (SGIker). The Detection limit (DL) was 0.03 $\mu\text{g/g}$.

2.7. Cellular endpoints: coelomocytes number, viability and genotoxicity

Earthworms were cleaned with distilled water by softly massaging their body in order to remove any soil particle attached to the tegument or in the posterior part of their digestive tract. Then pools of seven individuals were immersed in extrusion solution (0.02% EDTA in PBS with 0.23% NaCl, 1 ml per worm) and were subjected to an electric stimulation with a 9 V battery to allow the release of coelomocytes through dorsal pores (Irizar et al., 2014b). Cell suspensions were transferred to tubes, centrifuged (530 x g, 10 min, 10 °C in Allegra X-30R Beckman Coulter) and resuspended in PBS for posterior cell counting under light microscope. Neubauer chamber was used to count and adjust the cell density of each pool to 10^6 cells per ml. Then 2×10^5 coelomocytes were seeded per well in a 96-well microplate (7 well per treatment) and were left to stand at 18 °C in darkness for 30 minutes. Afterwards, the microplate was centrifuged (530 x g, 5 min, 10 °C), supernatant removed and cells were incubated for 40 minutes with 2.5 μ M Calcein AM (Molecular Probes® ThermoFisher Scientific, 100 μ l per well, 4 wells per treatment). In the remaining wells, instead of Calcein AM, 100 μ l PBS were added in order to thereafter subtract the inherent fluorescence of cells. Coelomocytes were washed twice (centrifugation, supernatant removal and addition of 100 μ l PBS) and fluorescence was measured at 490 ± 20 nm excitation filter and 520 ± 20 nm emission filter in a FLx 800 microplate fluorescence reader.

Comet assay was performed as described by Singh et al. (1988) with slight modifications. Earthworms (n=5) were left in moist filter paper during 24 h to dehydrate and coelomocytes were extruded from each individual using the method described above. Cell suspension (5×10^5 cells/ml) were diluted (1:2) in 0.5% low melting point agarose (LMPA) and 80 μ l transferred to slides previously coated with 1% normal melting point agarose (NMPA). Then slides were kept on ice for 10 minutes in order to allow cell layer to solidify. Afterwards, slides were immersed in lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% DMSO, pH 10.0) for 1 h at 4 °C in darkness, washed in dH₂O and incubated in alkaline solution (1 mM EDTA, 300 mM NaOH, pH>13.0; 20 min) for posterior electrophoresis (300 mA, 19 V; 20 min). Slides were then washed in neutralization buffer (0.4 M Tris-HCl, pH 7.5; 10 min) and cells fixed in methanol. For the analysis, slides were stained with 10 μ l of ethidium bromide (20 μ g/ml) and DNA migration scored (in 100 coelomocytes per sample) in Olympus BX50 fluorescence microscope with the aid of the Komet 5.5 image analysis system (Kinetic Imaging). Results were represented by % of tail DNA.

2.8. Biochemical endpoints: MT concentration and CAT activity

Metallothionein (MT) concentration in earthworms was quantified spectrophotometrically by determining the sulphhydryl (SH) residue content after Ellman's reaction (DTNB; 5,5-dithiobis-2-nitrobenzoic acid) (Viarengo et al., 1997). Briefly, the post-clitellar portion of earthworms (n=5) were weighed and homogenized in three volumes of homogenization buffer (pH 8.6) containing 0.006 mM leupeptine, 0.5 mM PMSF (phenylmethylsulphonyl fluoride) and 0.01% β -mercaptoethanol as a reducing agent. Homogenates were ultracentrifuged (30.000 x g, 20 min, 4 °C in Optima™ L-90K Beckman Coulter) and precipitated with ethanol/chloroform. Then the MT enriched fraction was resuspended in 0.25 M NaCl and 1 N HCl containing 4 mM EDTA, followed by the addition of a known amount of DTNB reagent in a high ionic strength medium to completely denature MTs. Samples (300 μ l) were placed in 96-well microplates (4 replicates) and absorbance was read at 412 nm in Multiskan Thermo Scientific Spectrophotometer using reduced glutathione (GSH) as standard. Data (μ g MTs/g earthworm ww) were expressed in % relative to control.

Catalase (CAT) activity was determined measuring decrease in absorbance at 240 nm due to hydrogen peroxide (H_2O_2) consumption (Claiborne, 1985). The pre-clitellar portion of earthworms (n=5) was weighed and homogenized in five volumes of homogenization buffer (TVBE, 1 mM sodium bicarbonate, 1 mM EDTA, 0.1% ethanol and 0.01% Triton X-100, pH 7.4). Absorbance was measured in 96-well UV Flat Bottom microplates using Multiskan Thermo Scientific Spectrophotometer. 4 replicates were added per sample (5 μ l of sample and 295 μ L of freshly prepared KH_2PO_4 and H_2O_2 solution) and a standard curve prepared with different volumes of TVBE and KH_2PO_4 and H_2O_2 solution was included as well. CAT activity (μ mol H_2O_2 / min.mg protein) was expressed in % relative to control.

Total protein content was estimated according to Lowry et al. (1951) with the aid of the DC™ Protein Assay (Bio-Rad), using bovine γ -globulin as standard.

2.9. Molecular endpoints: *mt* and *cat* transcription levels

Quantifications of metallothionein (*mt*) and catalase (*cat*) transcription levels were done in the post-clitellar body (~100 mg) of earthworms (n=6) that were dissected out and homogenized with silica beads in a Precellys™ 24 homogenizer (Bertin Technologies) at 6 m/s for 20 s twice. Total RNA was extracted using the TRIzol® (Invitrogen, ThermoFisher Scientific) method and purified using the RNase-Free DNase

Set (QIAGEN®). After measuring final concentration and purity spectrophotometrically (Epoch BioTek), 1 µg of total RNA was retro-transcribed into cDNA using the AffinityScript Multiple Temperature kit (Agilent Technologies). cDNA was then amplified in reactions (final volume of 20 µL) containing 2 µL of sample, 10 µL of SYBR Green (Roche), primer pairs at set concentrations (Table 2) and RNase free water. *mt* and *cat* transcription levels were quantified in a ViiA 7 by Life Technologies (AB Applied Biosystems) in 384 well plates. qPCRs were run as follows: 2 min at 50 °C, 10 min at 95 °C and 40 cycles of 15 s at 95 °C followed by 1 min at melting temperature (Table 2). Efficiency was determined running a standard curve and specificity of each reaction was certified by verifying the presence of a single peak in the melting curve plot. Three replicates were run per sample as well as RT minus controls and non-template controls. The amount of input cDNA per sample was used to normalize genes transcription data. For that a Δ CT formula adapted from the $\Delta\Delta$ CT normalization method was used (Rojo-Bartolomé et al., 2016a). cDNA concentration of each sample was measured with the Quant iT OliGreen ssDNA assay Kit (Life Technologies). Briefly, diluted samples (50 µL, 1/50) were placed in 96-well clear bottom microplates, the reagent was added (50 µL) and fluorescence was measured at 485 ± 20 nm excitation and 528 ± 20 emission in a FLx 800 (BioTek) microplate fluorescence reader.

Relative Quantification (RQ) of the transcription levels was calculated using a plate calibrator to obtain the Δ CT, the efficiency (E) of the PCR and the amount of cDNA (in ng) used in each reaction:

$$RQ = (1+E)^{-\Delta CT} / \text{ng cDNA}$$

The average of control earthworms in each exposure time (3 and 14 days) was used as calibrator.

Table 2. Primer sequences (Fw: Forward, Rv: Reverse), melting temperatures (°C), amplicon length (bp) and primer concentrations used for the specific amplification of metallothionein (*mt*) and catalase (*cat*) genes by qPCR in earthworms.

Gene (Acc. number)		Primer sequence (5'-3')	Melting T. (°C)	Amplicon length (bp)	Primer conc. (pmol)
<i>mt</i> (AJ236886)	Fw	AAATGCTCGGCTGGTTCGT	55.5	103	6.0
	Rv	TGATGACAGAGTTCCGTATTTC			
<i>cat</i> (DQ286713)	Fw	GCCGACGGAGAAGCTGTGTA	59.0	125	12.5
	Rv	TAAAGGTCACGGGTCCGCATAG			

2.10. Integrated Biomarker Response/n (IBR/n)

Integrative Biological Response (IBR) index was calculated with the aim of integrating alterations at different level of biological complexity, following the procedure described by Beliaeff and Burgeot (2002). The 5 most representative biomarkers were used for this purpose ranging from molecular level to organism level changes: *cat* transcription levels (*cat* expression), DNA damage, CAT activity, viability of extruded coelomocytes and weight loss in earthworms. Such selection was performed according to Garmendia et al. (2011) and Asensio et al. (2013), selecting biomarkers at different levels of biological complexity and ordering them accordingly, to provide an integrative view of the effects posed by Ag NPs on earthworms. Five biomarkers would be representative of all the studied biological complexity levels (molecular, biochemical, cellular, organism). Finally, as the IBR value depends on the number of applied biomarkers, the IBR/n was obtained dividing IBR by the number of biomarkers applied (n=5) (Marigómez et al., 2013).

2.11. Statistical analysis

The statistical analysis of the data was carried out with the aid of the SPSS statistical package (IMB SPSS Statistics 23). Shapiro-Wilk and Levene's tests were performed to study normality and equality of variances of the datasets, respectively. In order to compare treatments in each soil, one-way ANOVA followed by Tukey's pairwise comparison and Dunnett's test was used as a parametric approach and the non-parametric datasets were analysed with Kruskal-Wallis followed by Dunn's post-hoc test. Differences between exposure times were explored with Student's t (parametric) and Mann-Whitney U (non-parametric) tests. In all cases significant differences were established at $p < 0.05$.

The average values of each endpoint were plotted together in a Principal Component Analysis (PCA) in order to observe the distribution of the different Ag NP concentrations and exposure times in each soil. The two dimensional PCA was based on Euclidean distances and was performed in previously standardized and normalized variables.

3. Results

3.1. Test soil characterization

The real concentration of silver in OECD soils did not significantly differ (either between treatments and exposure times) from the nominal concentration (Table 3). In LUFA 2.3 soil, Ag traces were detected in non-spiked soils in both exposure times, being the nominal concentration values at the same range as those measured in 0.05 mg Ag NPs/kg spiked soils (Table 3). In soils dosed with 50 mg Ag NP/kg real concentrations were very close to the nominal concentration in both test soils and exposure times. Similarly, the pH did not change between treatments and only showed a weak decrease in OECD soil after 14 days, still with a neutral value (6.5-6.9).

3.2. Organism level endpoints: mortality, weight loss and Ag accumulation in tissues

Mortality was not detected in earthworms maintained in OECD soil. In LUFA 2.3 soil mortality was observed in the highest Ag NP concentration at both exposure times (10% after 3 days and 7.5% after 14). The weight loss of earthworms maintained in OECD soil did not differ between treatments (Fig. 1A). In LUFA 2.3 soils a significant weight loss was observed after exposure to the highest dose (50 mg Ag NPs/kg) for 3 days. At day 14 higher weight losses were observed in comparison to day 3, mainly in control and 0.05 mg Ag NPs/kg spiked soils. At this exposure time severe weight losses (>20%) occurred in all treatments (Fig 1A).

Earthworms maintained in Ag NP spiked soils exhibited Ag accumulation, with the highest Ag concentration in earthworms exposed to 50 mg Ag NPs/Kg (Table 4). At this dose earthworms accumulate more Ag in OECD soil than in LUFA 2.3 soil, especially after 14 days of exposure (13.88 vs. 6.61 $\mu\text{g Ag/g}$ earthworm, respectively). In LUFA 2.3 soil higher Ag concentrations were detected in earthworms maintained in non-spiked and 0.05 mg Ag NPs/kg dose during 14 days in comparison with OECD soil (0.07 and 0.18 $\mu\text{g Ag/g}$, respectively for each treatment).

Table 3. Ag concentration (nominal and real values, mg/kg soil) and pH (in dH_2O) of OECD and LUFA 2.3 test soils at days 3 and 14. Values are represented as means \pm standard deviations of four soil samples. (udl: under the detection limit, 0.03 mg/kg).

Nominal (mg Ag NPs/kg soil)	Time	OECD soil			LUFA 2.3 soil		
		0	0.05	50	0	0.05	50
Real (mg Ag/kg soil)	3 d	udl	0.11 \pm 0.02	46.32 \pm 1.36	0.17 \pm 0.04	0.12 \pm 0.01	43.58 \pm 2.55
	14 d	udl	0.12 \pm 0.01	41.13 \pm 1.68	0.13 \pm 0.01	0.14 \pm 0.04	34.44 \pm 17.87
pH (in dH_2O)	3 d	7.70 \pm 0.03	7.32 \pm 0.01	7.10 \pm 0.15	7.55 \pm 0.08	7.58 \pm 0.03	7.74 \pm 0.09
	14 d	6.42 \pm 0.04	6.75 \pm 0.00	6.89 \pm 0.01	7.36 \pm 0.19	7.60 \pm 0.06	7.83 \pm 0.13

Table 4. Ag concentration in earthworm tissues (μg Ag/g) after 3 and 14 days of exposure in OECD and LUFA 2.3 test soils. Values represented concentrations measured in pools (n=5). (udl: under the detection limit, 0.03 $\mu\text{g/g}$).

Exposure (mg Ag NPs/kg soil)	Time	OECD soil			LUFA 2.3 soil		
		0	0.05	50	0	0.05	50
Ag in tissues (μg Ag/g earthworm)	3d	udl	0.06	2.01	udl	udl	1.32
	14d	udl	0.04	13.88	0.07	0.18	6.61

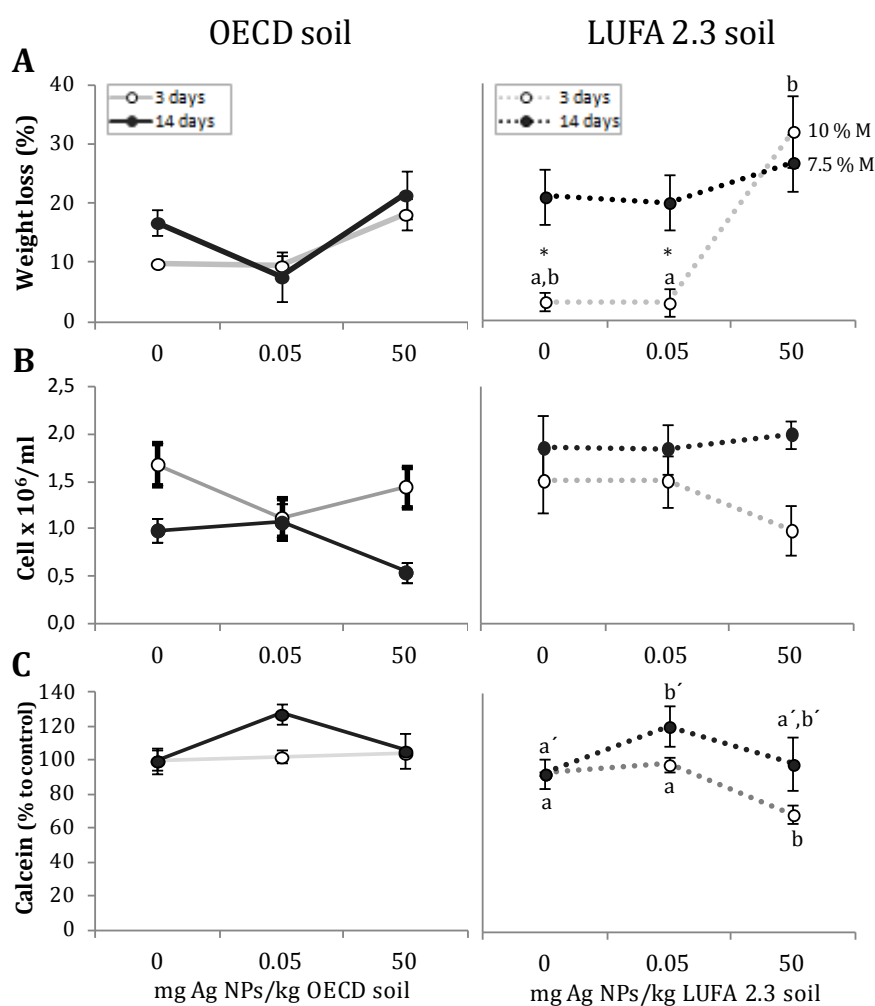


Figure 1. Weight loss (%), A), number of extruded coelomocytes (cell x 10⁶/ ml, B) and cell viability through Calcein AM Viability assay (Calcein retention in % relative to the control, C) in earthworms maintained in OECD and LUFA 2.3 soils spiked with Ag NPs (0, 0.05 and 50 mg Ag NPs/kg) during 3 and 14 days. Values are represented as means ± standard deviations and the significant differences are represented by asterisk for exposure times ($p \leq 0.05$ with Mann-Whitney U) and by letters for exposure doses ($p \leq 0.05$ with Kruskal-Wallis).

3.3. Cellular endpoints: coelomocytes number, viability and genotoxicity

The exposure of earthworms to 50 mg Ag NPs/kg in OECD soil during 14 days produced a trend of reduction in the number of extruded coelomocytes. In earthworms maintained in LUFA 2.3 soil the non significant cell number decrease was noticeable after 3 days of exposure (Fig. 1B). After the Calcein AM viability assay the number of viable cells was stable after exposure to Ag NPs in OECD soils (Fig. 1C). A significant decrease in the number of viable cells could be only appreciated after 3 days of exposure to the highest Ag NPs dose in LUFA 2.3 soil (Fig. 1C).

DNA damage in coelomocytes of earthworms exposed during 14 days in OECD soil followed a dose dependent response when increasing Ag NPs concentration (Fig. 2). In LUFA 2.3 soil, no significant differences were found between treatments (Fig. 2).

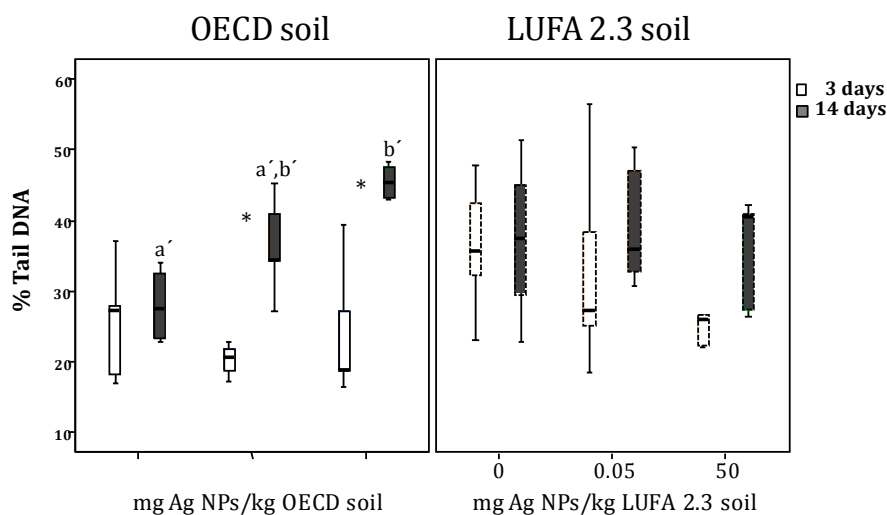


Figure 2. DNA damage (% Tail DNA) after Comet assay in earthworms maintained in OECD and LUFA 2.3 soils spiked with Ag NPs (0, 0.05 and 50 mg Ag NPs/kg) during 3 and 14 days. Values are represented as means \pm standard deviations and the significant differences are represented by asterisk for exposure times ($p \leq 0.05$ with Student's t) and by letters for exposure doses ($p \leq 0.05$ with Tukey's Test).

3.4. Biochemical endpoints: MT concentration and CAT activity

MT concentration in earthworms maintained in both test soils during 3 days did not show differences between treatments. After 14 days MT protein levels tend to decrease in earthworms maintained in test soils spiked with the highest Ag NPs dose and in LUFA 2.3 soil also after exposure to 0.05 mg Ag NPs/kg (Fig. 3A).

An increase of CAT activity was recorded in earthworms exposed to the highest dose, in OECD soil after 14 days and in LUFA 2.3 soil after 3 days (Fig. 3B).

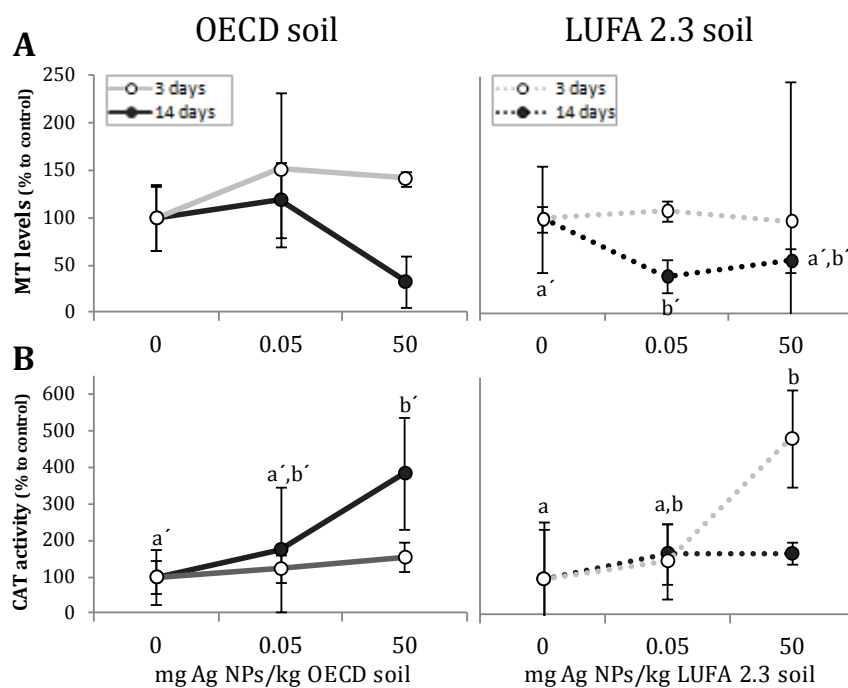


Figure 3. Metallothionein (MT) protein concentration (in % relative to the control, A) and catalase (CAT) activity (in % relative to the control, B) in earthworms maintained in OECD and LUFA 2.3 soils spiked with Ag NPs (0, 0.05 and 50 mg Ag NPs/kg) during 3 and 14 days. Values are represented as means \pm standard deviations and the significant differences are represented by letters ($p \leq 0.05$ with Kruskal-Wallis).

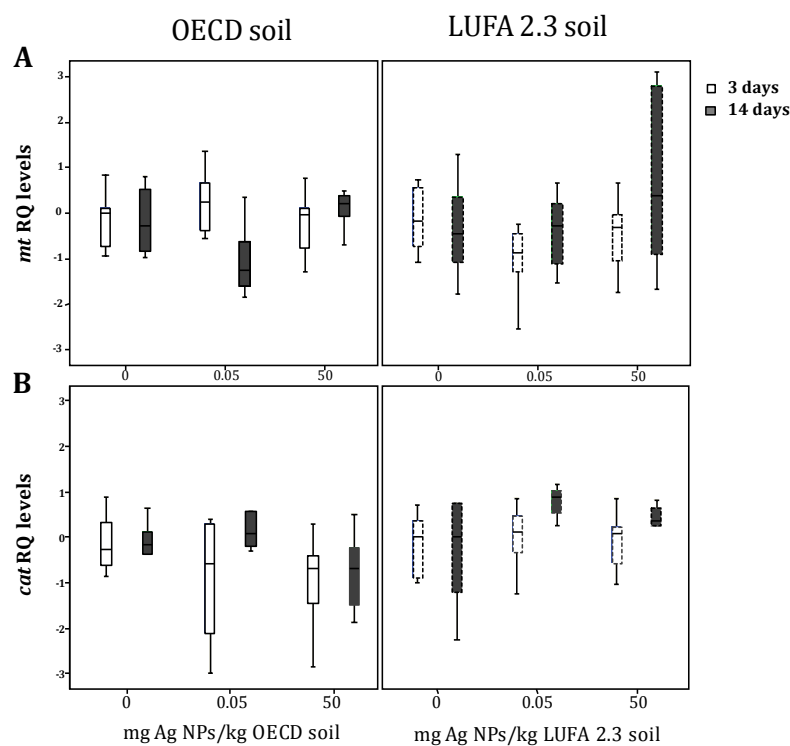


Figure 4. Relative Transcription (RQ) levels of metallothionein (*mt*) (A) and catalase (*cat*) (B) in earthworms maintained in OECD and LUFA 2.3 soils spiked with Ag NPs (0, 0.05 and 50 mg Ag NPs/kg) during 3 and 14 days. Values are represented as means \pm standard deviations.

3.5. Molecular endpoints: *mt* and *cat* transcription levels

mt and *cat* transcription levels were not altered in earthworms maintained in both test soils (Fig. 4A, 4B).

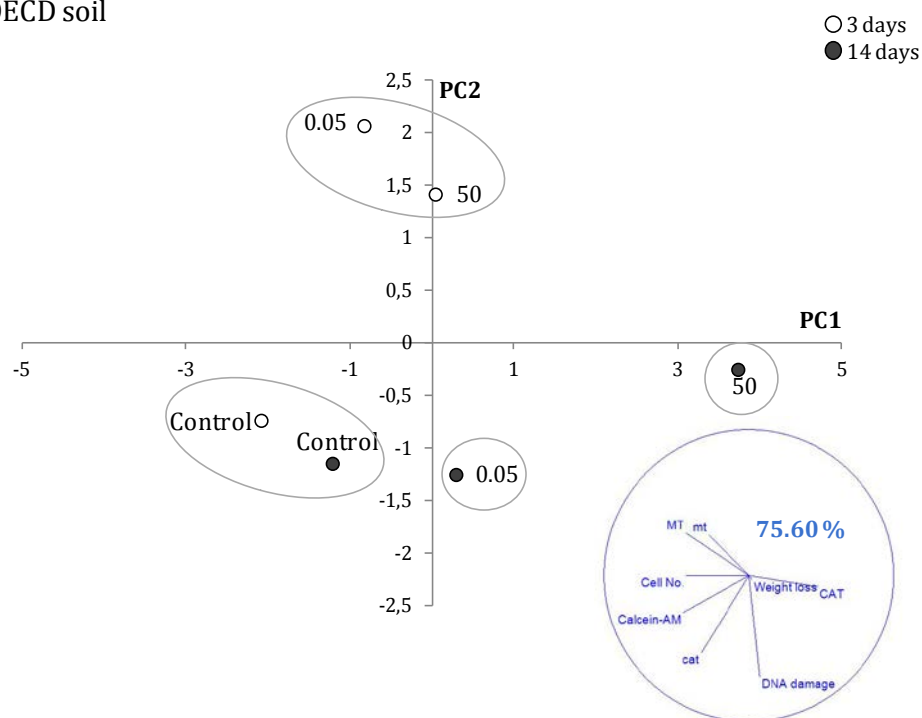
3.6. Principal Component Analysis (PCA) in test soils

In the PCA scatter plots performed for each test soil, the variation explained by the two main components reached 75.60% in OECD soil and 85.00% in LUFA 2.3 soil. In OECD soil, CAT activity and coelomocyte number and viability were the endpoints that presented higher correlation (0.479 and -0.432, -0.456; positively and negatively correlated, respectively) with the first component (PC1), while the second component (PC2) was correlated with changes in metallothionein protein (0.289) and transcription levels (0.278) and DNA damage (-0.697) (Fig. 5A).

Based on these variables, control earthworms of both exposure times were characterized by low weight loss and low CAT activity, and high cell number and viability. The group conformed by the controls was distinguished from earthworms exposed to 0.05 and 50 mg Ag NPs/kg during 3 days, which presented higher values in metallothionein protein and transcription levels (Fig. 5A). Remaining treatments (exposure to 0.05 and 50 mg Ag NPs/kg during 14 days) were individually discriminated as they were characterized by high CAT activity and a reduced coelomocyte number and viability (Fig. 5A). These effects were higher in 50 mg Ag NPs/kg soil after 14 days exposure (Fig. 5A).

In LUFA 2.3 soil, CAT activity and DNA damage were the endpoints that presented higher correlation (0.421 and -0.457, respectively) with PC1, while PC2 was correlated with changes in weight loss (0.563) and MT protein concentration (-0.602) (Fig. 5B). The display of the different treatments and exposure times showed that the control groups of both exposure times together with the close to predicted environmental concentration (0.05 mg Ag NPs/kg) after 3 days of exposure were grouped together. This cluster was distinguishable from the earthworms exposed to the highest concentration (50 mg Ag NPs/kg) during 3 days, explained by the higher CAT activity of the latter, and also from the close to predicted environmental concentration and high dose at longer exposure times (14 days) as they were characterized by higher losses in weight and depletion in MT protein levels (Fig. 5B).

A OECD soil



B LUFA 2.3 soil

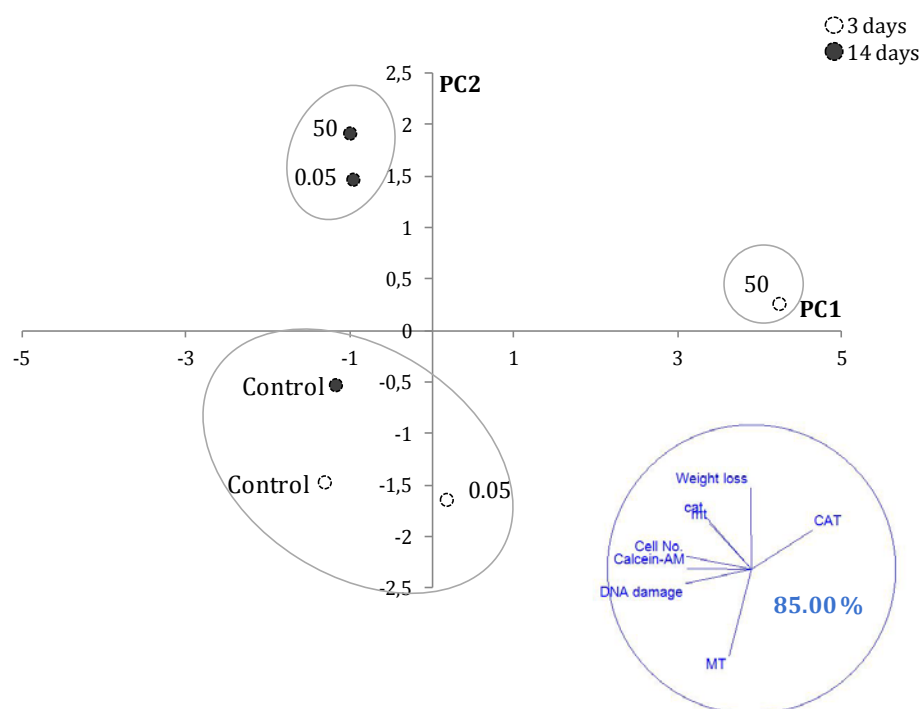


Figure 5. Two-dimensional Principal Component Analysis (PCA) scatter plot including all biomarkers (Weight loss, Coelomocyte No., Cell Viability, MT concentration, CAT activity, DNA damage and *mt* and *cat* transcription levels) measured in earthworms maintained in OECD (A) and LUFA 2.3 soils (B) spiked with Ag NPs (0, 0.05 and 50 mg Ag NPs/kg) during 3 and 14 days. The variation explained by the two main components reached 72.60% in OECD soil analysis and 85.00% in LUFA 2.3 soil. Different experimental clusters ($p < 0.05$) are marked. The circle located in the bottom and left part explains the distribution of the used variables (biomarkers).

3.7. Integrated Biomarker Response/n (IBR/n)

In OECD soil, the IBR index did not reflect effects after exposure to Ag NPs during short exposure times (3 days) (Fig. 6A). At longer times (14 days) earthworms exposed to the highest dose (50 mg Ag NPs/kg) presented the highest IBR index, indicating highly affected earthworms, explained by increase in CAT activity and DNA damage (Fig. 6A). In LUFA 2.3 soil, earthworms were affected by Ag NPs after 3 days of exposure to the highest dose and after 14 days of exposure to close to predicted environmental concentrations and high doses, demonstrated by the reduction of the coelomocyte viability and enhancement in weight loss and CAT activity (Fig. 6B).

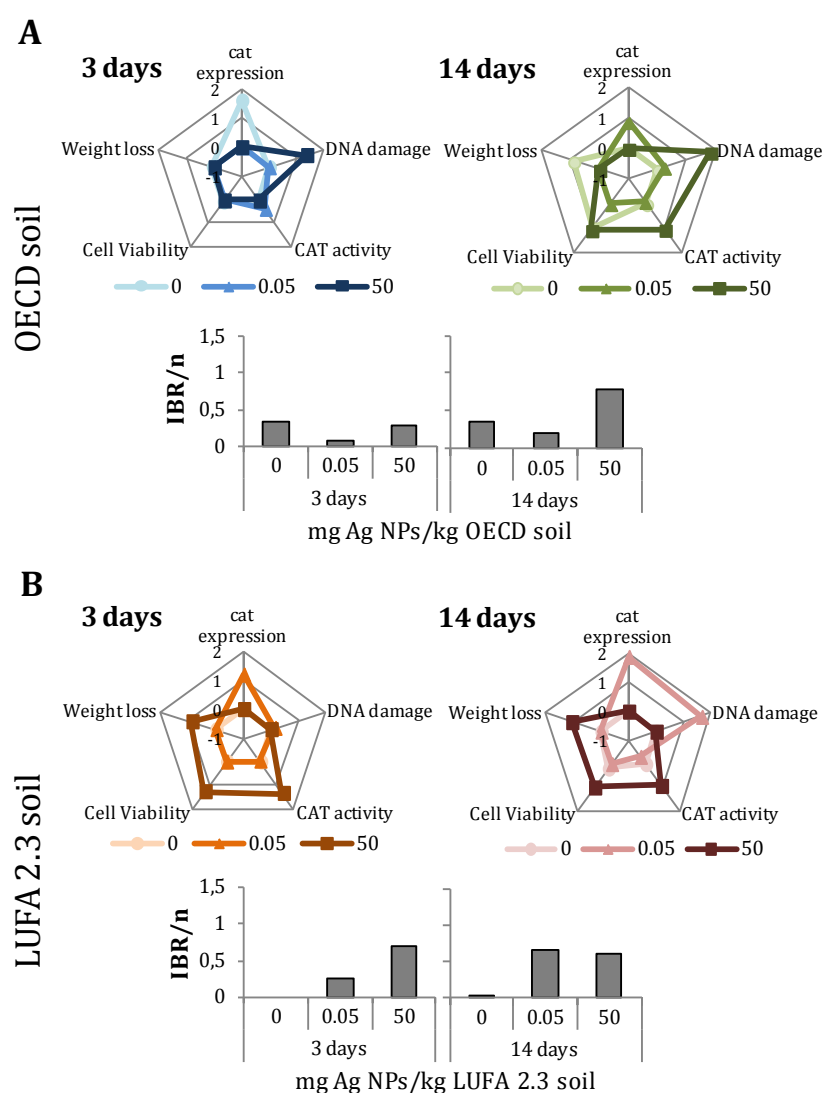


Figure 6. Integrative Biomarker Response (IBR/n) in earthworms maintained in different test soils (OECD, A and LUFA 2.3 soils, B) dosed with Ag NPs (0, 0.05 and 50 mg Ag NPs/kg soil) during 3 and 14 days. For the analysis the most responsive biomarkers (5) were employed measured at different levels of biological complexity: *cat* transcription levels (*cat* expression), DNA damage, CAT activity, viability of extruded coelomocytes and weight loss in earthworms.

4. Discussion

The study of the toxicity exerted by Ag NPs in soil has been focused on traditional endpoints and has used limit test range concentrations that did not reflect real conditions. In the last years, the effects of Ag NPs at biochemical and molecular levels of *E. fetida* earthworms have also been assessed (Tsyusko et al., 2012; Hayashi et al., 2013a; Gomes et al., 2015; Novo et al., 2015), but still high exposure concentrations were used in those studies. The present chapter has been mainly focused on the exposure matrix dependant toxicity rather than in the comparison between silver forms (see Chapter 3). In fact, different soil matrices with different physico-chemical characteristics have been employed for assessment purposes difficulting the direct comparison among studies. Presently, the toxicity posed by Ag NPs in soil was measured at different levels of biological organisation using a battery of biomarkers in *E. fetida*. Such responses were measured in earthworms exposed to sublethal concentration of Ag NPs (including close to environmental predictions) in two commonly used standard soils.

Soil physico-chemical characteristics are known to directly affect the fate and behaviour of pollutants, and subsequently their accumulation, uptake and toxicity in earthworms (Irizar et al., 2015a; Lukkari et al., 2004; Nahmani et al., 2007; Shoultz-Wilson et al., 2011a, 2011b, 2011c). The soils used in the current study differed in clay and OM contents mainly, being the percentages lower in LUFA 2.3 soil compared to OECD substrate. In control and 0.05 mg Ag NPs/kg exposure treatments Ag accumulation was higher in earthworms maintained in LUFA 2.3 soil than in OECD soil due to the Ag traces quantified in this commercial soil that have been previously detected by Waalewijn-Kool et al. (2014). Irrespective of this, the measured Ag concentrations did not differ from the nominal concentration evidencing and accurate spiking and homogenization procedure. Ag NPs could be expected to be more available and highly accumulated by earthworms in LUFA 2.3 soil due to its lower clay and OM content in comparison with OECD soil, however, after exposure to the highest dose, Ag concentration was higher in earthworms maintained in the latter. This fact and weight losses below 20% recorded in earthworms maintained in OECD soil could reveal a major feeding activity in this soil, through which earthworms would have uptaken soil-Ag NPs complexes (Lukkari et al., 2004). In contrast, earthworms maintained in LUFA 2.3 soil would have presented lower soil ingestion, which also explains the higher weight loss (at the high dose after 3 days and enhanced in all the treatments after 14 days and mortality (at high dose)

observed. In fact, diminished feeding activities followed by lower metal accumulation in earthworms have been previously reported in soils with low OM content (Irizar et al., 2015a). The behaviour of metal NPs in soils (i.e. aggregation/agglomeration, sorption to surfaces or dissolution to the ionic metal) is influenced by soil pH, isoelectric point and the presence of granulometric clay and OM content (Cornelis et al., 2012). The different clay and OM contents of the soils used herein would have influenced Ag NPs behaviour as well, and consequently, their bioavailability and uptake for earthworms would be different. As mentioned above, in OECD soils Ag NPs could be bounded to soil particles and coated by OM which would suppress dissolution (Cornelis et al., 2012; Klitzke et al., 2015), being the primary uptake form soil ingestion. In LUFA 2.3 soils, Ag could be dissolved in soil pore water due to the absence of OM and incorporated by dermal uptake. Assuming that NPs suspended in pore waters are more widely dispersed and that they could be subjected to earlier oxidation processes, being more bioavailable to organisms (Di Toro et al., 1991; Klaine et al., 2008), it makes sense that effects (at organism level) of Ag NPs on LUFA 2.3 were greater than in OECD soils after 3 days of exposure to the highest dose. Nevertheless, after 14 days of exposure in this soil with less clay and poor in OM, Ag NPs toxicity could be masked by the starvation of earthworms under those conditions. So far, it is unclear to which extent Ag NPs were coupled to soils or dissolved in pore water, this point will deserve further investigations.

Accordingly, effects at cellular level (i.e reduction in the viability of extruded coelomocytes at day 3) occurred earlier in earthworms in LUFA 2.3 soils. As stated before, it could happen that the higher fraction of dissolved Ag in the pore water of this soil have entered through dorsal pores (Irizar et al., 2015a) and impact on the permeability of the cell membrane, leading to a significantly diminished coelomocyte viability. Several studies reported that the number of coelomocytes decreases after exposure of earthworms to metals (Homa et al., 2010; Plytycz and Morgan, 2011; Podolak et al., 2011) and this effect has also been described after exposure to Ag NPs but at concentrations higher than the ones used presently (Garcia-Velasco et al., 2016; Chapter 1). Coelomocytes are involved in eliminating foreign material by phagocytosis and encapsulation (amoebocytes) and they synthesize and secrete cytotoxic components into the coelomic fluid (leucocytes), causing opsonisation and lysis of non-self material (Bilej et al., 2000, 2010). Therefore, the reduction in the viability of coelomocytes exerted by Ag NPs in LUFA 2.3 soil could be posing a dysfunction of the immune system at cellular level (Plytycz and Morgan, 2011) that could lead to physiological alterations

together with effects at higher levels of biological organisation (weight loss and mortality). The fact that effects at cellular level were only significant in earthworms maintained in LUFA 2.3 soils and exposed to the highest dose during 3 days, and not at longer exposure periods, could be due to changes in the subpopulation ratios of coelomocytes. In fact, coelomocytes are a heterogeneous group of cells (Adamowicz, 2005) where amoebocytes appeared to be more sensitive against Ag NPs (Hayashi et al., 2012). Thus, the dissimilar responses between amoebocytes and eleocytes after Ag NPs exposure could be interfering with the cell number and viability responses of the whole coelomocyte population.

Apart from earthworm coelomocytes, MTs are also involved in metal homeostasis and detoxification (Homa et al., 2010). MTs involvement in earthworm protection against metal pollution has been described in many studies (Asensio et al., 2007; Brulle et al., 2006; Demuynck et al., 2007; Homa et al., 2010; Irizar et al., 2014a, 2015a). Accordingly, MTs have been studied (at protein and transcription levels) in earthworms subjected to metallic NPs, especially after exposure to Ag NPs (Gomes et al., 2015; Hayashi et al., 2013a; Tsyusko et al., 2012). Presently, it seems that MT levels were not affected at short exposure times but tended to reduce in OECD and LUFA 2.3 soils after 14 days of exposure to the highest dose of Ag NPs. Interestingly, significant inhibition effects were only recorded on LUFA 2.3 soils spiked with low Ag NP concentration (0.05 mg Ag NPs/kg). The lack of MT induction and the increase of CAT activity may resemble the onset of oxidative stress mechanisms posed by Ag NPs, which seems to occur earlier in LUFA 2.3. In fact, cells have a complex defence system, including non-enzymatic scavengers (MTs) or enzymatic ones such as CAT. On the other hand, MT concentrations can be influenced by other factors than metal exposure, such as the animal condition and growth, developmental stages, and environmental stimuli, like starvation (Dallinger, 1996). Thus, the depletion of MTs recorded after 14 days of exposure to the concentration close to environmental predictions in LUFA 2.3 soil and to the high dose in OECD soil could be related to weight losses above 20%, which limited their capability of response against metals or due to an onset of oxidative stress. CAT activity in *E. fetida* has been previously reported to be activated in short exposure periods (4 days) and inhibited in long periods (28 days) of exposure to Ag (Gomes et al., 2015). Presently, CAT activity increased after exposure to the highest concentration, at day 14 in OECD soil and at day 3 in LUFA 2.3 soil. Thus, in OECD soil earthworms CAT responses would need longer exposure periods, probably due to a less availability of Ag in this media

richer in clay and OM. In LUFA 2.3 soil CAT activity was not enhanced at longer exposure times, even if higher Ag body burdens were measured in earthworms at day 14, which could be associated, once again, to the starvation of earthworms. Overall, these results highlight the idea of oxidative stress as a mechanism of toxicity of Ag NPs in *E. fetida*, which by means of ROS can damage cell components including DNA, proteins and membranes (Piao et al., 2011). Moreover, nanosilver is known to interact with DNA, alter its conformation and subsequently, induce genotoxicity (McShan et al., 2014). Presently, DNA damage was only recorded in coelomocytes of earthworms maintained in OECD soil during 14 days, following a dose dependant response, which is in accordance with the depletion trend observed in coelomocyte number and with the increase in CAT activity recorded. In contrast, earthworms maintained in LUFA 2.3 soils did not show significant differences in DNA damage between treatments as they were characterized by high variability. Nevertheless, a trend of higher genotoxicity was observed in comparison to OECD soils, maybe due to the dissimilar Ag availability of the test soils.

The lack of alterations in the transcription levels of *mt* and *cat* could be due to the selection of the post-clitellar body of earthworms to perform this endpoint. The consideration of the zonation when applying biomarkers for toxicity assessment has been reported to reduce the intrinsic variability that results from the morphofunctional heterogeneity that exists along the body axis of annelids. However, for *cat* expression levels, high variability was recorded along the post-clitellar body of earthworms (Irizar et al., 2014a). Other studies dealing with molecular endpoints in nanosilver exposed *E. fetida* used the whole earthworm for the RNA extraction with successful results (Hayashi et al., 2013a; Tsyusko et al., 2012). Nevertheless, much higher concentrations of Ag NPs were employed in those studies. The identification of target tissues to assess the Ag NPs posed oxidative stress at molecular level and the clarification of its mechanisms of action will deserve thorough investigations in the future.

Both the PCA and the IBR/n index revealed earlier toxic effects in soil with lower clay and OM content (LUFA 2.3). In fact, in OECD soil Ag NPs exerted oxidative stress and DNA damage in earthworms uniquely after 14 days of exposure in the high dose while the same parameters were already affected at shorter exposure times (3 days) and even at lower exposure doses (0.05 mg Ag NPs /kg) after 14 days in LUFA 2.3 soil. These approaches were able to give an integrative and conclusive idea of the effects posed by

Ag NPs at different complexity levels of *E. fetida*, in differing doses, exposure times, and standard soils.

5. Conclusions

PVP-PEI coated Ag NPs exerted toxicity at different levels of biological complexity in *E. fetida* earthworms, being weight loss, reduction in the viability of coelomocytes, increase in CAT activity and DNA damage the major effects recorded, which reinforce oxidative stress as a mechanism of toxicity of Ag NPs. High but sublethal concentrations of Ag NPs (50 mg Ag NPs/kg) caused increase in CAT activity and DNA damage in OECD soils after 14 days while in LUFA 2.3 soils produced earlier effects (weight loss, decrease in cell viability and increase in CAT activity at day 3). At day 14, the characteristics of this natural soil (lower clay and OM contents) could have provoked starvation of earthworms, masking Ag NPs toxicity. The concentration of Ag NPs close to environmental predictions (0.05 mg Ag NPs/kg) did not produce significant effects in OECD soils, while exposure in LUFA 2.3 soil posed weight losses above 20% and depletion of MTs at day 14. Therefore, the lower clay and organic matter contents of LUFA 2.3 soils in comparison to OECD soil can be considered as a toxicity enhancer to Ag NPs toxicity.

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CHAPTER 3

Responses to silver nanoparticles and silver nitrate in a battery of biomarkers measured in target tissues and in coelomocytes of *Eisenia fetida* earthworms

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Abstract

The current use and development of applications with silver nanoparticles (Ag NPs) could lead to potential inputs of these NPs to soils. Consequently, it is crucial to understand the ecotoxicological risks posed by Ag NPs in the terrestrial compartment. In the present chapter, the effects produced by PVP-PEI coated Ag NPs were assessed in *Eisenia fetida* earthworms in comparison with the soluble form (AgNO₃). Earthworms were exposed for 1, 3 and 14 days to high but sublethal (50 mg Ag/kg) and close to modelled environmental concentrations (0.05 mg Ag/kg) and at each exposure time, apart from mortality and weight loss of individuals, metallothionein (MT) protein concentration and catalase (CAT) activity were quantified in earthworm tissues. In addition, cellular and molecular level endpoints (cell viability, subpopulations relative number and transcription levels of metallothionein-*mt*- and catalase-*cat*-) were measured in coelomocytes extruded from exposed earthworms. Despite the lack of effects in traditional endpoints (mortality and weight loss), Ag NPs and AgNO₃ posed changes at lower levels of biological complexity (biochemical, cellular and molecular levels). Both Ag forms induced similar changes in the metal detoxification mechanism (MT, *mt*) and in the antioxidant response system (CAT, *cat*) of *E. fetida*. In contrast, Ag form dependant cytotoxicity and alterations in the subpopulations (eleocytes and amoebocytes) relative number were recorded in extruded coelomocytes. Complementarily, the use of coelomocytes to assess molecular level endpoints represented a relevant alternative for development of non-invasive biomarkers.

Keywords: Silver nanoparticle (Ag NP), Silver nitrate (AgNO₃), *Eisenia fetida*, metal detoxification, antioxidant response, coelomocyte cytotoxicity.

Laburpena

Zilar nanopartikulen (Ag NPs) egungo erabilerek eta aplikazio desberdinen garapenek material hauen sarrera eragin dezakete lurzoruan. Beraz, Ag NPek lurzoruko ekosistemetan sortu ditzaketen arrisku toxikologikoen ikertzeak berebiziko garrantzia du egun. Kapitulu honetan, PVP-PEI estalduradun Ag NPen efektuak zilar nitratoarekin (AgNO_3) alderatu ziren *Eisenia fetida* zizarean. Horretarako, zizareak 1, 3 eta 14 egunez mantendu ziren kontzentrazio altu baina azpiletalean (50 mg Ag /kg) eta ingurumenean aurreikusten diren eragin gertu dauden kontzentrazioetan (0.05 mg Ag/kg). Esposizio denbora desberdinetan zizareen hilkortasuna eta pisu galera neurtzeaz aparte, metalotioneina (MT) kontzentrazio eta katalasa (CAT) aktibitatea kuantifikatu ziren zizareen ehunetan. Horretaz gain, biomarkatzaile zelular eta molekularrak (zelulen bideragarritasuna, azpipopulazioen proportzio erlatiboa, eta metalotioneina-*mt*- eta katalasa-*cat*- transkripzio mailak) neurtu ziren esposizio pean izandako zizareetatik erauzitako zelomozitoetan. Ag NP and AgNO_3 zizareetan hilkortasuna eta pisu galera sortu ez bazuten ere, zizareen konplexutasun maila baxuagoetan (maila biokimiko, zelular eta molekularra) efektuak eragin zituzten. Bi zilar formek antzeko aldaketak eragin zituzten *E. fetidaren* metal detoxifikazio (MT, *mt*) eta erantzun antioxidatzailearen (CAT, *cat*) sistemetan. Bestalde, Ag formaren menpeko zitotoxizitatea eta azpipopulazioen (amebozito eta eleozito) proportzio erlatiboan aldaketak behatu ziren erauzitako zelomozitotan. Era berean, maila molekularreko biomarkatzaileen neurketarako zelomozitoen erabilera aukera aproposa dela ikusi izan da.

Hitz gakoak: Zilar nanopartikula (Ag NP), Zilar nitratoa (AgNO_3), *Eisenia fetida*, metal detoxifikazioa, erantzun antioxidantea, zelomozitoen zitotoxizitatea.

Resumen

El actual uso y desarrollo de aplicaciones con nanopartículas de plata (Ag NPs) pueden producir una entrada de éstas en el suelo. Por esta razón, es crucial conocer los riesgos toxicológicos de las Ag NPs en el compartimento terrestre. En el presente capítulo, se han comparado los efectos causados por las Ag NPs recubiertas de PVP-PEI y los producidos por la forma soluble de plata (AgNO₃) en *Eisenia fetida*. Para ello se expusieron las lombrices durante 1, 3 y 14 días a una concentraciones alta pero subletal (50 mg Ag/kg) y a otra concentración próxima a la estimada para el medio ambiente (0.05 mg Ag/kg). En cada tiempo de exposición, aparte de cuantificar la mortalidad y pérdida de peso en los individuos, se determinaron la concentración de metalotioneinas y la actividad catalasa en tejidos. Además, se midieron biomarcadores a nivel celular y molecular (viabilidad celular, número relativo de celomocitos, y niveles de transcripción de metalotioneínas -*mt*- y catalasa-*cat*-) en celomocitos extruidos de lombrices expuestas *in vivo*. A pesar de no observarse mortalidad o pérdida de peso significativas, las Ag NPs y AgNO₃ causaron cambios a niveles más bajos de complejidad biológica (niveles bioquímico, celular y molecular). Los cambios en los sistemas de detoxificación de metales (MT, *mt*) y respuesta antioxidante (CAT, *cat*) fueron similares tras la exposición de las lombrices a Ag NPs y AgNO₃. Por el contrario, se observaron diferencias dependientes de la forma de Ag en la citotoxicidad y en el número relativo de celomocitos (amebocitos y eleocitos). De forma complementaria, se concluye que el uso se celomocitos puede ser una buena alternativa para desarrollar biomarcadores no invasivos.

Palabras clave: Nanopartícula de plata (Ag NP), Nitrato de plata (AgNO₃), *Eisenia fetida*, detoxificación de metales, respuesta antioxidante, citotoxicidad de celomocitos.

1. Introduction

The wide range of current and potential future applications exhibited by silver nanoparticles (Ag NPs) has made them one of the most commonly used nanomaterials (Dubey et al., 2015; Vance et al., 2015). Due to these applications and to the massive disposal of sewage sludge released from waste water treatment plants (WWTP, one of the major sources of Ag NPs in soils), Ag NPs might have the potential to severely affect soil health (Shoults-Wilson et al., 2011c; Tourinho et al., 2012). However, the potential risk of Ag NPs in soils has been poorly investigated in comparison with aquatic environments. Even if fewer studies have involved the effects of Ag NPs on terrestrial organisms, the number of studies carried out with earthworms has increased during the last five years (Diez-Ortiz et al., 2015a, 2015b; Gomes et al., 2013, 2015; Hayashi et al., 2012; Heckmann et al., 2011; Kwak and An, 2015; Novo et al., 2015; Schlich et al., 2013; Shoults-Wilson et al., 2011a, 2011b, 2011c; Tsyusko et al., 2012).

Earthworms play an important role in terrestrial ecosystems (e.g. decomposition and nutrient recycling) and therefore, the study of effects exerted by Ag NPs on them is crucial to understand the potential impacts of NPs in soils. In this context, standard toxicity tests with *Eisenia fetida* earthworm (OECD 1984, 2004) are aimed to address traditional endpoints such as survival or weight loss in order to calculate different toxicity indices (LC_x and EC_x). Furthermore, tissue, cellular or molecular level biomarkers could be also quantified in target tissues of *E. fetida* in order to assess the exposure degree or the toxic effects of pollutants. For instance, metallothioneins (MTs), low molecular weight proteins, with high cysteine content (up to 30%) that enables to bind a variety of metal atoms (Asensio et al., 2007; Brulle et al., 2006), participate in homeostasis of essential metals and in the detoxification of toxic trace metals (Brulle et al., 2006) and may prevent oxidative stress (Ribeiro et al., 2015). Ag NPs are known to cause oxidative stress in terrestrial invertebrates by the production of highly reactive oxygen species (ROS) that can damage cell components including DNA, proteins and membranes (Yang et al., 2011). Cells, in order to protect themselves from ROS, have developed complex defence systems including non-enzymatic scavengers and antioxidant enzymes such as catalase (CAT). A change in CAT activity is an indicator of a cellular lesion after exposure to chemicals, and thus it is considered as an early environmental stress biomarker (Asensio et al., 2013; Gomes et al., 2015).

Biomarkers can be measured in earthworm tissues or even in target cells as coelomocytes. Coelomocytes are the immune cells of earthworms and play a pivotal role in recognition and elimination of foreign materials and are involved in clotting and wound healing (Cooper, 2002; Kurek et al., 2007). Coelomocytes compose a heterogeneous cellular group that circulates in fluid-suspension in the coelomic cavity. Based on cytomorphometric, ultrastructural and cytochemical properties two major cell subpopulations are distinguished, amoebocytes and eleocytes, being the former subgrouped into hyaline and granular amoebocytes (for detailed descriptions see Adamowicz, 2005). Changes in coelomocytes viability and subpopulation ratios in earthworms exposed to xenobiotics or subjected to different types of stress reflect alterations in the earthworms immune response and in the general health status (Adamowicz and Wojtaszek, 2001; Di Marzio et al., 2005; Homa et al., 2003; Irizar et al., 2015b). Hence, these cellular parameters have been proposed as biomarkers of general stress in soil toxicity assessment (Homa et al., 2003; Irizar et al., 2015b; Olchawa et al., 2006). Regarding lower levels of biological organization, Ag NPs are known to alter the transcription of genes involved in the abovementioned pathways in *E. fetida*: oxidative stress, detoxification and immune signalling (Hayashi et al., 2013a; Tsyusko et al., 2012). Transcription levels of target genes such those encoding CAT or MT have been easily measured in earthworm tissues (Asensio et al., 2007; Brulle et al., 2006; Irizar et al., 2014a; Chapter 1). However, the utilization of immune cells (coelomocytes) to assess molecular level endpoints would represent a relevant alternative for the development of non-invasive biomarkers in more controllable and reproducible test systems than whole animals.

The aim of the present chapter was to assess the toxicity of PVP-PEI coated Ag NPs in earthworms, *E. fetida*, in comparison with the soluble form of the metal (AgNO₃). For this purpose, earthworms were exposed for 1, 3 and 14 days to high but sublethal (50 mg Ag /kg) and close to modelled environmental concentrations (0.05 mg Ag /kg) in the form of Ag NPs and AgNO₃. At each exposure time, apart from mortality and weight loss of individuals, MT protein concentration and CAT activity were quantified in earthworm tissues. In addition, cellular and molecular level endpoints (cell viability, subpopulations relative number and transcription levels of *cat* and *mt* genes) were measured in coelomocytes extruded from exposed earthworms.

2. Materials and Methods

2.1. Test species

Eisenia fetida earthworms (350–500 mg fresh weight) used for the experiments were healthy adults, clitellated and obtained from the stock population provided by a commercial dealer (LOMBRICOR S.C.A., Córdoba, Spain). Earthworms were maintained in the laboratory under controlled conditions of temperature (19 ± 2 °C), darkness and constant humidity. As food source medication-free horse manure was provided when required.

2.2. Test substances

Polyvinylpyrrolidone-polyethylenimine (PVP-PEI, 3.35:1) coated silver nanoparticles (NP Ag-2106W) were purchased from NANOGAP (SUB-NM-POWDER, S.A., A Coruña, Spain). Ag NPs were water dispersed (10 g Ag/L with 104 g PVP-PEI/L), 5.08 ± 2.03 nm average size and with a Z-potential of 18.6 ± 7.9 mV. Particle size distribution and zeta potential determinations through Dynamic Light Scattering were provided by NANOGAP CoA (Appendix I). High grade (>99% purity) AgNO₃ was purchased from Sigma-Aldrich.

2.3. Artificial soil preparation, contamination and characterization

The OECD artificial soil was prepared following the OECD guideline 207 (OECD, 1984). The artificial soil contained 70% sand (50% of particles were between 50-200 µm), 20% kaolin clay and 10% sphagnum peat sieved at 2 mm. pH was adjusted to 6.0 ± 0.5 by addition of 0.01% calcium carbonate. Dry constituents were mixed, placed in glass containers and moistened to 40% of their water holding capacity (WHC, 21.91%) with suspensions of Ag NPs and solutions of AgNO₃ in distilled water or with distilled water in the case of the control group. The same concentration used in Chapter 2 were chosen, sublethal (50 mg Ag /kg, according to previous experiments in Garcia-Velasco et al., 2016; Chapter 1) and close to modelled environmental concentrations (0.05 mg Ag /kg). After spiking with the corresponding silver form, experimental soils were thoroughly mixed to ensure a homogeneous distribution of the metal. Then soils were stabilized during 3 days before adding earthworms previously acclimated (24 h) to OECD soil. Earthworms (n=20) were exposed to unpolluted soil (control) and to soils spiked with Ag NPs or AgNO₃ during 1, 3 and 14 days. At the end of each Ag exposure, weight loss was assessed in earthworms and Ag quantification and pH measurements were carried out in experimental soils at day 14. The real concentration of Ag in soils was quantified

following the EPA 3051A method and analysed in Inductively Coupled Plasma Mass Spectrometry (ICP-MS, 7700-Agilent Technologies) in the Central Analysis Service of the UPV/EHU (SGIker). Detection limit (DL) was 0.03 mg/kg. For the measurements of the pH an adaptation of the ISO 10390: 2005 “Soil Quality – Determination of pH in water” was followed.

2.4. Concentration of metallothioneins (MTs)

Metallothionein (MTs) concentration was determined in earthworms by the spectrophotometric method described by Viarengo et al. (1997). In order to perform pools, the post-clitellar portion of 3 earthworms were weighed and homogenized in three volumes of 0.5 M sucrose and 20 mM Tris-HCl buffer (pH 8.6) containing 0.006 mM leupeptine and 0.5 mM phenylmethylsulfonylfluoride, as an antiproteolytic agents, and 0.01% β -mercaptoethanol, as a reducing agent. Homogenates were ultracentrifuged (30.000 x g, 20 min, 4 °C) and precipitated with ethanol/chloroform. Three pools were done per treatment and exposure time. MTs concentration was quantified by spectrophotometric titration of the sulfhydryl residues using the Ellman’s reagent (5,50-dithiobis-2-nitrobenzoic acid) with reduced glutathione (GSH) as standard. Samples were centrifuged for 5 minutes (530 x g, 4 °C) and the supernatant (300 μ l) was added in 96-well microplate wells. Each sample was replicated four times. Finally, absorbance was measured at 412 nm in a microplate reader Multiskan Thermo Scientific Spectrophotometer. Data (μ g MTs/g earthworm ww) were expressed in % relative to control.

2.5. Catalase (CAT) activity

Catalase (CAT) activity was determined measuring decrease of absorbance at 240 nm due to hydrogen peroxide consumption (Claiborne, 1985). The pre-clitellar portion of 5 earthworms were weighed and homogenized in five volumes of homogenization buffer (TVBE, 1 mM sodium bicarbonate, 1 mM EDTA, 0.1% ethanol and 0.01% Triton X-100, pH 7.4) in order to obtain pools. Two pools per treatment and exposure time were used. Absorbance was measured in 96-well UV Flat Bottom microplates and using a microplate reader Multiskan Thermo Scientific Spectrophotometer. Four replicates were added per sample and a standard curve was also included in the plates. Total protein content was estimated according to Lowry et al. (1951) with the aid of the DC™ Protein Assay (Bio-Rad), using bovine γ -globulin as standard. CAT activity (mM of H₂O₂/mg of protein/min) was expressed in % relative to control.

2.6. Coelomocyte extrusion, viability and subpopulations relative number

Before the extrusion earthworms were left in moist filter paper during 24 h to void gut contents. Coelomocytes were collected using a non-invasive extrusion method (Di Marzio et al., 2005). Briefly, four pools (of 5 organisms) per treatment and exposure time were placed into tubes containing 2 ml of 5% ethanol phosphate buffered saline solution (0.1 M, PBS) per individual and incubated for 1 min. Extruded coelomocyte suspensions were washed in PBS twice (530 x g, 10 min, 4 °C). Final pellets were resuspended in 2 ml of PBS and cells were counted using a haemocytometer (Neubauer chamber). Three replicate slides were analyzed per pool for viability and subpopulations relative number measurements. Cytotoxicity was expressed as the percentage of non-viable cells measured with 0.4% Trypan Blue. Additionally, the population of coelomocytes was characterized as eleocytes or amoebocytes according to their morphology (Adamowicz, 2005).

2.7. *cat* and *mt* transcription levels in extruded coelomocytes

The same coelomocyte pools used to record viability and subpopulations relative number (4 pools- from 5 individuals- per treatment and exposure time) were employed to quantify *cat* and *mt* transcription levels. Coelomocytes (2×10^6 cell pellets) were homogenized in TRIzol® (Invitrogen, ThermoFisher Scientific USA) using silica beds in a HYBAID RiboLyser (FP120-HY-230) for 45 s at maximum speed. Total RNA was extracted following the manufacturer's protocol (TRIzol® method). RNA purity and integrity were spectrophotometrically checked. RNA was purified using the RNase-free DNase Set (Qiagen®) following manufacturer's indications and 1 µg of total RNA was retro-transcribed into cDNA using the AffinityScript Multiple Temperature kit (Agilent Technologies). *Cat* and *mt* transcription levels were quantified in a 7300 Real Time PCR System (Applied Biosystems, ThermoFisher Scientific) using FastStar Universal SYBR Green Master mix (Roche). Each reaction (final volume 20 µL) contained 2 µL of sample (previously diluted at 1/100), 0.25 µL of 25 pmol primer pair (Table 1), 7.75 µL of RNase free water, and 10 µL of SYBR Green (Roche). qPCRs were run as follows: 2 min at 50 °C, 10 min at 95 °C; and 40 cycles at 95 °C (15 s) followed by each melting temperature (Table 1) (45 s). Efficiency was determined running a standard curve and specificity of each reaction was determined by the melting curve where a single peak was identified in all dissociation curves, confirming the production of a single amplicon per primer set. In all cases, a control without template was run for quality assessment. The specific

amplification of each amplicon was also checked by sequencing both PCR products. Relative Quantification (RQ) of the transcription levels was calculated using a plate calibrator to obtain the Δ CT, the efficiency (E) of the PCR and the amount of cDNA (in ng) used in each reaction:

$$RQ = (1+E)^{-\Delta CT} / \text{ng cDNA}$$

Amount of cDNA was determinate by using QuantiT OliGreen ssDNA assay Kit following manufacturer's procedure. RQ values were represented relative to the average of control earthworms in each exposure time (1, 3 and 14 days).

Table 1. Primer sequences (Fw: Forward, Rv: Reverse), melting temperatures ($^{\circ}$ C) and the expected amplicon length (bp) for the specific amplification of metallothionein (*mt*) and catalase (*cat*) by qPCR in earthworms.

Gene (Acc. number)		Primer sequence (5'-3')	Melting T. ($^{\circ}$ C)	Amplicon length (bp)
<i>mt</i> (AJ236886)	Fw	AAATGCTCGGCTGGTTCGT	55.5	103
	Rv	TGATGACAGAGTTCCGTATTTC		
<i>cat</i> (DQ286713)	Fw	GCCGACGGAGAAGCTGTGTA	59.0	125
	Rv	TAAAGGTCACGGGTTCGCATAG		

2.8. Statistical analysis

Normal distribution of data was assessed using the Shapiro-Wilk's test and homogeneity of variance was tested using the Bartlett's test. Significant differences ($p < 0.05$) with respect to the control were based on the non-parametric Kruskal-Wallis test followed by the Dunn's post hoc test. Differences between Ag forms were explored with Student's t (parametric) and Mann-Whitney U (non-parametric) tests. All statistical analysis was performed using Statistica v. 8 (StatSoft).

3. Results

3.1. Ag concentration and pH of soils

Real concentrations of Ag in experimental soils were similar to nominal concentrations with the exception of the 0.05 mg AgNO₃/kg experimental group that showed 0.53 mg Ag/kg as real concentration (Table 2). Soil pH remained around 6 during the experiment for all the exposure groups.

Table 2. Nominal and real Ag exposure concentrations (mg /kg soil) and pH of experimental soils. Detection limit (DL) was 0.03 mg/kg.

Nominal concentration (mg/kg)		Real concentration (mg Ag/kg)	pH (in aH ₂ O)
Control	0	0.05	5.93
Ag NPs	0.05	0.09	5.99
	50	48.40	5.89
AgNO ₃	0.05	0.53	6.00
	50	50.30	6.11

3.2. Weight loss

Control and exposed earthworms lost similar weight (15-17%) during the experiment, regardless of the Ag form (Ag NPs and AgNO₃) and time (1, 3 and 14 days).

3.3. Concentration of metallothioneins (MTs)

MTs concentration did not change during the experiment time in control earthworms. MT levels significantly increased with respect to controls after exposure to both concentrations (0.05 and 50 mg/kg) of Ag NPs and AgNO₃ (Fig. 1). The major increases were recorded at day 3. No differences in MT concentration were found between Ag forms at day 1 and 3. After 14 days of exposure, MT concentrations appeared to be significantly higher after exposure to Ag NPs at the highest dose (50 mg/kg).

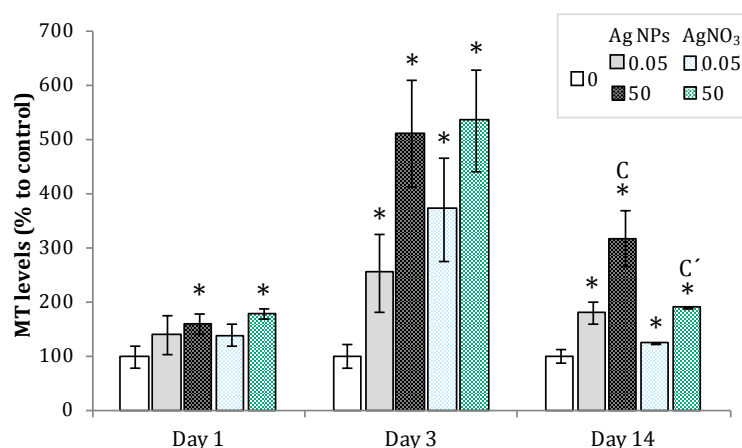


Figure 1. Metallothioneins (MTs) concentration (% relative to the control) in organisms exposed to Ag NPs and AgNO₃ spiked soils (0.05 and 50 mg/kg) and unpolluted soil (0) for 1, 3 and 14 days. Mean values and standard deviations are shown. The statistically significant differences with respect to the control group ($p < 0.05$ with Kruskal-Wallis) are represented by asterisk. Letter and letter plus apostrophe pairs (C, C') indicate significant differences ($p < 0.05$) between both Ag forms.

3.4. Catalase (CAT) activity

CAT activity did not show alterations during the experiment in control organisms (Fig. 2). In earthworms exposed to both concentrations (0.05 and 50 mg/kg) of Ag NPs and AgNO₃ CAT activity was significantly enhanced in comparison to controls up to day 3 (Fig. 2). After 14 days of exposure, activity was reduced, especially after exposure to the highest dose of both Ag forms (Fig. 2). CAT activity was significantly higher in earthworms exposed to AgNO₃ than to Ag NPs at day 14, only at 0.05 mg/kg concentration.

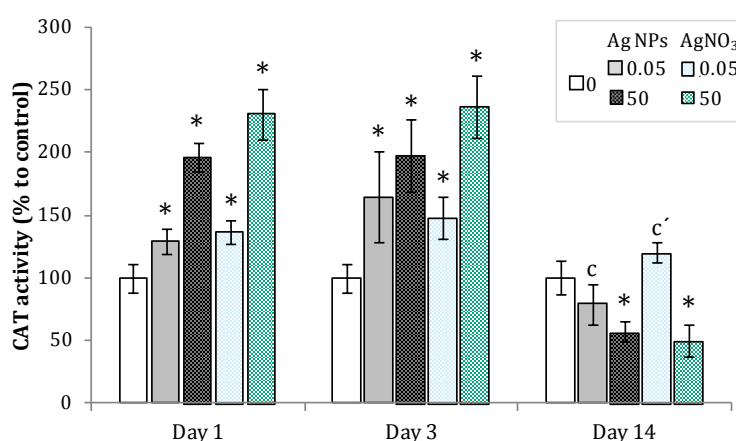


Figure 2. Catalase activity (CAT, in % relative to the control) in organisms exposed to Ag NPs and AgNO₃ spiked soils (0.05 and 50 mg/kg) and unpolluted soil (0) for 1, 3 and 14 days. Mean values and standard deviations are shown. The statistically significant differences with respect to the control group ($p < 0.05$ with Kruskal-Wallis) are represented by asterisk. Letter and letter plus apostrophe pairs (C, C') indicate significant differences ($p < 0.05$) between both Ag forms.

3.5. Coelomocytes viability and subpopulations relative number

Percentage of non-viable coelomocytes in control earthworms remained in the same value along the experimental period. Percentage of non-viable coelomocytes increased significantly in earthworms exposed to both forms of Ag (NPs or salts) (Fig. 3A). Exposure to Ag NPs produced significantly higher cell mortality than AgNO₃ for the same exposure concentration and time.

The number of eleocytes (Fig. 3B) in control earthworms and in earthworms exposed to the predicted environmental concentration (0.05 mg/Kg) of Ag NPs was similar all along the experiment (Fig. 3B). However, after 3 and 14 days of exposure to the highest concentration of Ag NPs (50 mg/kg) the total number of eleocytes increased in comparison to controls. Eleocyte number tended to decrease after exposure to 0.05 mg AgNO₃/kg for 3 and 14 days. Exposure to the highest dose (50 mg/kg) of AgNO₃ significantly decreased the number of eleocytes in comparison to control groups.

Significant differences were found in eleocyte number when comparing Ag forms (Fig. 3B).

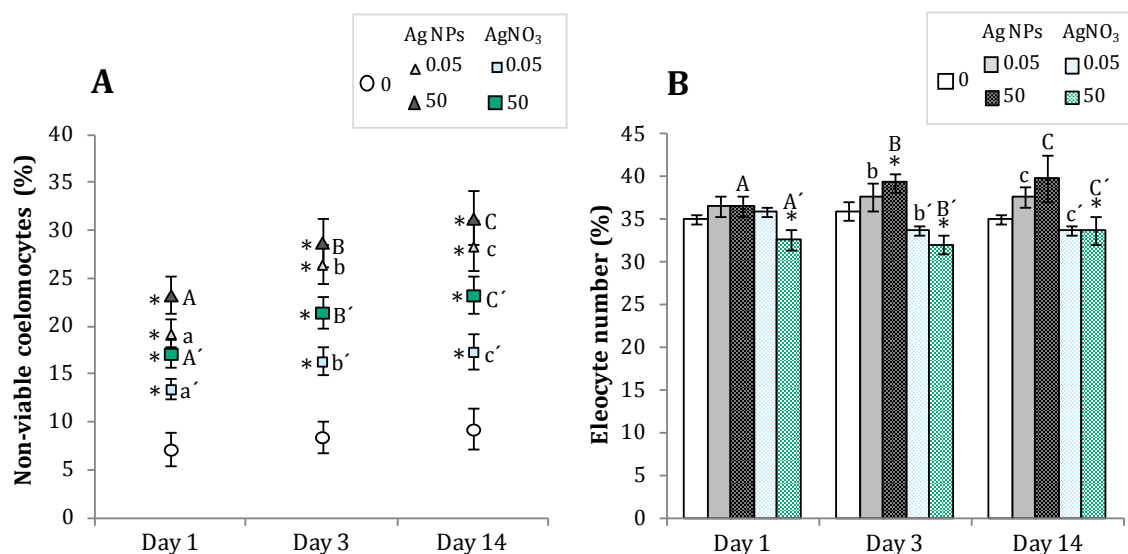


Figure 3. Non-viable coelomocytes (Trypan Blue 0.4%, A) and number of eleocytes (%), B) after 1, 3 and 14 days of exposure to Ag NPs and AgNO₃ (0.05 and 50 mg/Kg) spiked soils and unpolluted soil (0). Values are represented as mean values and standard deviation. Statistically significant differences with respect to the control group ($p < 0.05$ with Kruskal-Wallis) are represented by asterisk. Letter and letter plus apostrophe pairs indicate significant differences ($p < 0.05$) between both Ag forms for day 1 (a, A), day 3 (b, B) and day 14 (c, C).

3.6. *cat* and *mt* transcription levels in extruded coelomocytes

Overall, *cat* and *mt* transcription levels were higher in coelomocytes extruded from earthworms exposed to both forms of Ag (Ag NPs and AgNO₃) during 1 and 3 days in comparison to control coelomocytes (Fig. 4). These differences were enhanced after the exposure to the highest concentration of Ag (50 mg/kg). After 14 days, Ag NPs caused increase of *cat* transcription levels while no significant differences were found in *mt* at this time. After exposure to AgNO₃, both genes were significantly up-regulated at low doses (0.05 mg/Kg) while at the highest exposure concentration a significant inhibition was observed for *mt* (Fig. 4A). In the case of *cat*, transcription levels decreased up to the control values after 14 days of exposure to the highest dose of AgNO₃ (Fig. 4B). Alterations in *mt* and *cat* transcription levels were significantly higher after AgNO₃ exposure than after Ag NPs exposure at days 1 and 14 after exposure to the low dose.

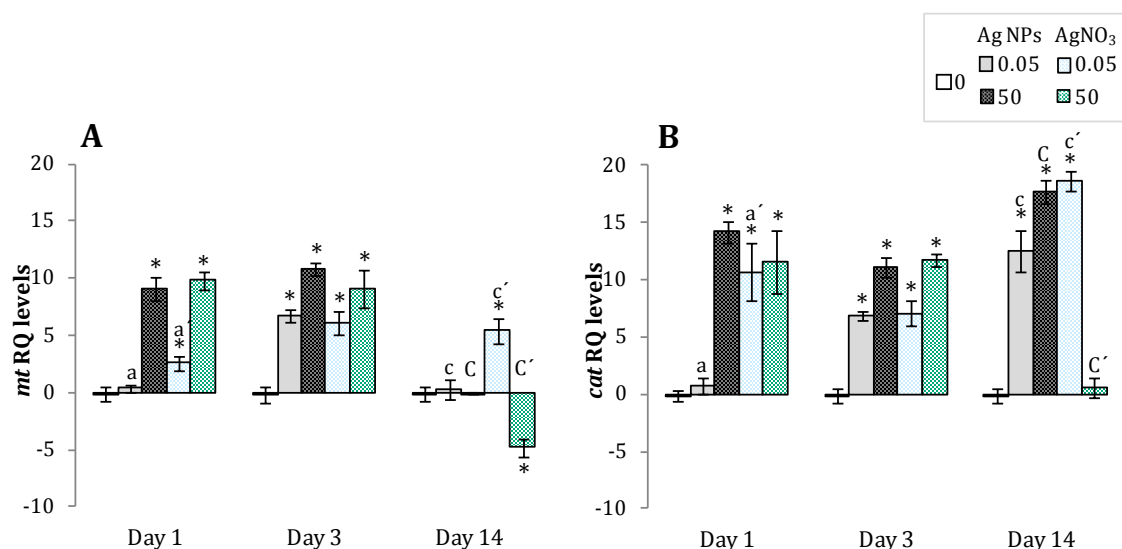


Figure 4. Relative transcription levels of *mt* (A) and *cat* (B) in coelomocytes extruded from control earthworms and from earthworms exposed to Ag NPs and AgNO₃ (0.05 and 50 mg/kg) spiked soils for 1, 3 and 14 days. Relative Quantification (RQ) is represented by means and standard deviations. Statistically significant differences with respect to the control group are indicated by asterisk ($p < 0.001$). Letter and letter plus apostrophe pairs indicate significant differences ($p < 0.05$) between both Ag forms for day 1 (a, A), day 3 (b, B) and day 14 (c, C).

4. Discussion

An adequate spiking and homogenizing procedure is one of the most crucial issues when characterizing the toxicity of pollutants in soils (Waalewijn-Kool et al., 2012). Presently, Ag concentrations measured in both Ag NPs and AgNO₃ spiked soils did not differ from the nominal concentrations, with the exception in 0.05 mg AgNO₃/kg treatment (0.53 mg Ag/kg as real concentration, probably due to inhomogeneity/contamination of the soil sample collected), indicating overall validity of the spiking protocol used herein. In this context, it is noteworthy that chemical analyses carried out on experimental soils ensured the exposure of earthworms to different known concentrations of Ag (NPs or salt) for up to 14 days. This exposure may exert physiological responses at different levels of biological complexity, possibly altering earthworm fitness, and ultimately changing their populations or community densities. Aiming to measure these effects, standard toxicity tests (OECD, ISO) with *E. fetida* earthworms are based on short and long-term experiments and traditional endpoints (Moser and Römbke, 2009). However, presently earthworms appeared to be unaffected at high levels of biological organization (organism level) since severe weight losses (>20%) were not recorded after Ag NPs and AgNO₃ exposures. It seemed that exposure concentrations and duration (or both) were not high enough to produce significant somatic effects at the organism level. Accordingly, in Chapter 1 was proved that the EC₅₀

for weight loss after 14 days of exposure (EC_{50} = 57.62 mg Ag NPs/kg) was higher than the highest dose used in the present chapter.

Complementary to the classical toxicity endpoints, changes in health status can be detected at lower levels of biological complexity, which can forecast effects in more ecologically relevant parameters. In fact, even if the weight loss of earthworms remained unaltered after exposure to both silver forms, MT concentrations significantly increased in comparison with controls after exposure to low and high doses of Ag. This might suggest a possible activation of the metal detoxification mechanism that involves these metal quenching proteins, as Ribeiro et al. (2015) found for *Enchytraeus crypticus* after exposure to both silver forms. Hence, Ag would be selectively bound to MTs and the resulting Ag-MT complexes would be sequestered into lysosomes (Garcia-Velasco et al., 2016; Marigómez et al., 2002; Chapter 1). Thus, MT could participate in Ag removal, helping to prevent oxidative stress, mainly at short exposure times (day 3). In fact, biochemical responses are known to be time dependent, and therefore, 3 days could be enough to scavenge silver. A similar pattern was reported for *E. fetida* after exposure to carbon nanotubes, where MT concentration increased significantly at the third day of exposure followed by a decrease at longer exposure times (Calisi et al., 2016). In contrast, Gomes et al. (2015) observed that MTs in *E. fetida* were not affected by Ag NPs or AgNO₃ even at higher exposure concentrations than the ones used in the present work. These controversial results can be the effect of different concentrations and exposure times. In any case, it can be concluded that there is no full discrimination between the two Ag forms regarding MT levels.

Accordingly with Gomes et al. (2015) CAT activity in *E. fetida* was activated in short exposure periods (1 and 3 days) and inhibited after long periods of exposure to both Ag forms. This inhibition has been previously demonstrated in earthworms after exposure to metals by producing relevant quantities of superoxide anions (Irizar et al., 2014a). Recent studies evidence oxidative stress (with temporal changes) as a mechanism of toxicity after exposure to both silver forms in *E. fetida* earthworms (Hayashi et al., 2012, 2013; Tsyusko et al., 2012) and present results reinforce this idea. However, such studies showed that antioxidant responses to AgNO₃ started earlier than to Ag NPs, which could be related to oxidation time (quicker ion release) or a slower uptake of Ag NPs or due to a complexation with the soil matrix of the nanoform. Presently, CAT activity did not show a clear dissimilar patten between the two Ag forms.

The interaction of NPs or released ions with thiol groups of vital enzymes and proteins affects cellular processes and ultimately can lead to cell death (Hayashi et al., 2012; Levard et al., 2012). Cytotoxicity and subpopulations relative number were determined in coelomocytes extruded from organisms exposed to both Ag forms to assess coelomocytes viability. Coelomocytes are involved in eliminating foreign material by phagocytosis and encapsulation (amoebocytes) and they also synthesize and secrete cytotoxic components into the coelomic fluid (chloragocytes or eleocytes), causing lysis of non-self material (Bilej et al., 2000, 2010). Several studies have reported that the number of coelomocytes varies after exposure to metals as a result of changes in the permeability of the cell membrane that leads to diminished cell viability (Irizar et al., 2015b; Podolak et al., 2011). Similarly, Irizar et al. (2014b) found that *in vivo* and *in vitro* exposure to sublethal concentrations of metals (Pb, Ni, Cd, Cu) provoked a dose-dependent decrease in Neutral Red Uptake capacity due to damage in the coelomocytes membrane. Likewise, after *in vivo* exposure to Ag NPs and AgNO₃, non-viable coelomocytes increased following a dose and time trend. Both silver forms could have released ions to soil pore water that would have entered through the dorsal pores of the earthworms tegument (Garcia-Velasco et al., 2016; Irizar et al., 2015a; Chapter 1) and impact in the permeability of the cellular membrane (McShan et al., 2014) of coelomocytes, causing the observed cytotoxicity. The degree of Ag NPs solubilisation seems to be crucial to exert biological effects. Moreover, the toxicity of Ag NPs has been principally attributed to bioavailable Ag⁺ ions (Van Aerle et al., 2013). However, it cannot be discarded in which form remained Ag NPs and AgNO₃ under present exposure conditions, in pore water as particulate form, soluble salts or insoluble Ag (nano)clusters or bound to soil particles conforming heteroaggregates.

The response of the different subpopulations (amoebocytes and eleocytes) was dependant of the Ag form. In fact, according to coelomocytes viability and the subpopulations relative number, the exposure to Ag NPs (or/and to Ag ions released from them) provoked a more marked toxicity than exposure to AgNO₃. This could be related with the target cell for each Ag form, hence Ag NPs enhanced the mortality of amoebocytes (increased the relative number of eleocytes), while AgNO₃ posed a decrease in eleocytes. Likewise, recent *in vitro* tests with coelomocytes demonstrated the selective intracellular accumulation of Ag NPs in the amoebocyte subpopulation and their role as scavengers of Ag NPs, effecting cytokine release and even death of the cell (Hayashi et al., 2012). Thus, a phagocytic uptake of Ag NPs may have occurred in

amoebocytes, followed by intracellular particle oxidation which can produce cellular damage (Hayashi et al., 2012; Limbach et al., 2007). The intracellular accumulation of Ag NPs could act as Ag⁺ source that is known as Trojan horse effect (Limbach et al., 2007). In contrast, studies with metal salts (e.g. CdCl₂) demonstrated eleocytes to be more sensitive than amoebocytes (Irizar et al., 2015b) and the same could happen with Ag salts (AgNO₃). Hence, both Ag forms caused cytotoxicity in coelomocytes but dissimilar sensitivities were recorded among subpopulations depending on the Ag form.

Apart from assessing metal detoxification (MT) and antioxidant response (CAT) in earthworm tissues, advances in molecular biology propelled the use of a new family of biomarkers based on the analysis of transcription levels of stress-related genes. In this framework, changes in the transcription levels of target genes such those encoding CAT or MT have been easily measured in earthworms subjected to Ag NPs (Chapter 2) and to NP and salt Ag forms (Hayashi et al., 2013a; Tsyusko et al., 2012). In all these studies gene expression was measured in tissues whereas presently transcription levels were, for the first time, recorded in isolated coelomocytes extruded from exposed earthworms. According to the results obtained at biochemical level, *mt* transcription levels increased at days 1 and 3 followed by an inhibitory response after 14 days of exposure to the highest concentration. Equally, Tsyusko et al. (2012) pointed out that the highest number of significant changes in the levels of expression of *mt* in *E. fetida* exposed to both Ag NPs and AgNO₃ occurred at short exposure periods (up to 3 days) as can be expected for a early warning biomarker of metal exposure. It can be concluded that the transcription of *mt* is involved in short term homeostasis mechanisms for Ag exposure. Previous works dealing with *cat* expression in *E. fetida* showed different regulation patterns and temporal variation maybe due to a different bioavailability of the Ag in the media or related to the Ag NPs concentration and characterization (i.e. coating agent and size) (Hayashi et al., 2013a; Tsyusko et al., 2012). Nevertheless, the dissimilar changes in eleocyte and amoebocyte number after Ag NPs and AgNO₃ exposure found in the present study should be taken into consideration when analyzing transcription level profiles of the whole coelomocyte population. In fact, the basal transcription level of each gene in each subpopulation might be different. Thus, subpopulation specific gene transcription profiles are found relevant for further studies including cell sorting techniques. However, the results obtained at transcription level in coelomocytes of earthworms exposed to Ag NPs and AgNO₃ were able to reflect responses at higher levels of biological complexity and thus, the utilization of these

immune cells to assess molecular level endpoints represents a relevant alternative for development of non-invasive biomarkers.

5. Conclusions

Despite the lack of effects in traditional endpoints (mortality and weight loss), Ag NPs and AgNO₃ posed changes at lower levels of biological complexity. Both Ag forms induced similar responses in most of the endpoints (significant changes in the metal detoxification mechanism and in the antioxidant response system). In contrast, at cellular level cytotoxicity was higher after exposure to Ag NPs but, dissimilar sensitivities were recorded among coelomocytes subpopulations depending on the Ag form, suggesting a different mode of action of nanoparticulate/salt/ionic Ag depending on the target cell.

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CHAPTER 4

Selection of an optimal culture medium and the most responsive viability assay to assess Ag NPs toxicity with primary cultures of *Eisenia fetida* coelomocytes

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Garcia-Velasco, N., Irizar, A., Goñi de Cerio, F., Etxebarria, J., Marigómez, I., Soto M. Determination of toxicity thresholds exerted by AgNPs on earthworm coelomocytes (eleocytes, amoebocytes) through viability and cytotoxicity bioassays combined with flow cytometric analysis. 25th Annual Meeting of the SETAC-Europe, Barcelona, 3-7 May 2015. Poster presentation.

Abstract

Earthworm coelomocytes have become a target system in ecotoxicology due to their sensitivity against a wide range of pollutants, including silver nanoparticles (Ag NPs). Presently, several *in vitro* approaches with primary cultures of coelomocytes have been used to test the toxicity and the dissimilar response of amoebocyte and eleocyte subpopulations after PVP-PEI coated Ag NPs and AgNO₃ exposures. In this framework, the maintenance of coelomocytes in an optimal culture medium and the selection of the most responsive assay are of utmost importance to obtain reliable data and to accurately assess Ag NPs toxicity. Thus, primary cultures of coelomocytes were maintained in widely used conventional media (Leibovitz's L-15, Basal Medium Eagle, RPMI-1640) and in a natural medium based on freshly extruded coelomic fluid. Afterwards coelomocytes were exposed to PVP-PEI coated Ag NPs, the coating agent (PVP-PEI) and AgNO₃ (0-100 mg/l) and after 24 h flow cytometric analyses were used to assess mortality of coelomocytes and changes in the relative proportion of subpopulations, amoebocytes and eleocytes. In addition, viability was assessed in microplates by Neutral Red Uptake (NRU), Cell Proliferation WST-1 and Calcein AM Viability assays. Finally, in order to better understand the behaviour of both cell-types after Ag NP exposure, amoebocytes and eleocytes were sorted and Ag concentration measured in both isolated subpopulations. Our results showed that the coelomic fluid occurred to be the optimal medium for coelomocytes maintenance and Ag NPs toxicity assessment due to the lower mortality quantified by flow cytometry, but its methodological limitations made RPMI-1640 medium the best option among conventional media for coelomocytes culturing and for the development of microplate assays. NRU and WST-1 assays exhibited large unavoidable interferences with the absorbance wavelengths of the exposure media, while Calcein AM viability assay was the most accurate and responsive to assess the effects produced by Ag NPs (and AgNO₃) exposure. According to this assay, Ag NPs posed a gradual decrease in coelomocytes viability starting at 10 mg/l. The LC₅₀ for Ag NPs was established at 30.48 mg/l in RPMI-1640 medium. Ag NPs appeared to be more toxic than AgNO₃ (LC₅₀ 43.38 mg AgNO₃/l) for coelomocytes, which could be mediated by a dissimilar uptake of the different Ag forms. Nevertheless, the observed cytotoxicity cannot be attributable to its coating agent PVP-PEI. Exposure to Ag NPs caused selective cytotoxicity in amoebocytes, which correlated with the Ag concentrations measured in sorted amoebocytes and reinforced the idea of dissimilar sensitivities among amoebocytes and eleocytes.

Key words: Silver nanoparticle (Ag NP), *in vitro*, coelomocytes primary culture, culture medium, microplate assay, flow cytometry, cell sorting.

Laburpena

Zizareen zelomozitoak itu zelulak bilakatu dira ekotoxikologian kutsatzaile ugariaren aurrean (zilar nanopartikulak-Ag NPs- barne) azaldu duten sentikortasunagatik. Kapitulu honetan, zilar nanopartikulek eragindako toxikotasuna eta zelomozito azpipopulazioen (amebozito eta eleozito) erantzun desberdinak aztertu dira zelomozito hazkuntza primarioetan *in vitro* proba desberdinak erabiliz. Horretarako, aurretiaz zelomozito kultibo primarioen mantenurako hazkuntza medio optimoaren eta erantzun esangarriak eskaintzen dituen entsegu sentikorrenaren aukeratzeak garrantzi handikoak dira. Hortaz, zelomozitoen kultibo primarioak hazkuntza medio konbentzionaletan (Leibovitz's L-15, Basal Medium Eagle, RPMI-1640) eta zeloma barrunbetik erauzitako fluido naturalean prestatu ziren. Ondoren, zelomozitoak PVP-PEI estalduradun Ag NP, estaldura konposatu (PVP-PEI) eta AgNO_3 pean izan ziren (0-100 mg/l) 24 orduz. Denbora horren ostean, zelulen hilkortasuna eta azpipopulazioen (amebozitoak eta eleozitoak) proportzio erlatiboan aldaketak kuantifikatu ziren fluxu zitometro analisi bitartez. Horretaz gain, Gorri Neutroaren metaketa (NRU), zelulen proliferazio WST-1 eta Kaltzeina AM bideragarritasun testak burutu ziren mikroplaketan. Azkenik, zelomozito azpipopulazioek Ag NP esposiziopean duten portaera ulertzeko asmoz, amebozito eta eleozitoak banatu egin ziren eta isolatutako azpipopulazioetan Ag metaketa neurtu zen. Fluxu zitometroan neurtutako zelomozitoen hilkortasunari begiratuta fluido zelomikoa medio optimotzat hartu bazitekeen ere, medio honek muga metodologikoak aurkeztu zituen. Beraz, horren ordeztu RPMI-1640 medio konbentzionala aukeratu zen, zelomozitoen hazkuntza eta mikroplaka entseguak egiteko mediorik aproposena izanik. NRU eta WST-1 entseguen eta esposizio medioen absorbantzien artean interferentziak aurkitu ziren. Kaltzeina AM bideragarritasun testak, aldiz, erantzunik sentikorrenak eskaini zituen. Azken honen emaitzei erreparatuta, Ag NPek zelomozitoen bideragarritasunean 10 mg/l kontzentrazioan hasitako jeitsiera graduala eragin zuten. RPMI-1640 medioan eta Ag NPs esposiziopean mantendutako zelomozitoen LC_{50} balorea 30.48 mg/l-koa izan zen. Ag NP pean izandako zelomozitoen toxikotasuna AgNO_3 pean izandakoena (LC_{50} 43.38 mg AgNO_3 /l) baino handiagoa izan zen, toxikotasun maila zilar forma desberdinen metaketa bidearen menpekoa izan zitekelarik. Hala ere, behatutako toxikotasuna ezin zaio PVP-PEI estaldurari esleitu. Ag NPen esposizioak zitotoxizitate selektiboa eragin zuen amebozitoetan, isolatutako amebozitoen kuantifikatutako zilarren edukiarekin bat etorritik, eta amebozito eta eleozitoen Ag-arekiko sentikortasun ezberdina dutela frogatzen du.

Hitz gakoak: Zilar nanopartikula (Ag NP), *in vitro*, zelomozito hazkuntza primarioa, hazkuntza medioa, mikroplaka entseguak, fluxu zitometroa, zelulen banaketa.

Resumen

Los celomocitos de lombriz son considerados como células diana en ecotoxicología debido a su sensibilidad frente a diversos contaminantes, incluidas las nanopartículas de plata (Ag NPs). En el presente capítulo se han empleado diferentes ensayos *in vitro* con cultivos primarios de celomocitos para evaluar la toxicidad y determinar las respuestas de los amebocitos y eleocitos frente a la exposición a Ag NPs (recubiertas de PVP-PEI) y AgNO_3 . Para evaluar adecuadamente la toxicidad de estos compuestos, se considera que tanto el mantenimiento de los celomocitos en un medio de cultivo óptimo como la validación del ensayo más sensible son factores de gran importancia. Con ese objetivo, se prepararon cultivos primarios de celomocitos en medios de cultivo convencionales (Leibovitz's L-15, Basal Medium Eagle, RPMI-1640) y en un medio natural, el líquido de la cavidad celómica de las lombrices. Posteriormente, se expusieron los celomocitos mantenidos en los medios anteriormente mencionados a diferentes concentraciones de Ag NPs recubiertas (PVP-PEI) y a AgNO_3 (0-100 mg/l). Pasadas 24 horas, se midieron mortalidad y los cambios en la proporción relativa de amebocitos y eleocitos mediante citometría de flujo. Además se realizaron ensayos de viabilidad celular en microplaca: Retención de Rojo Neutro (NRU), Proliferación celular WST-1 y Calceina AM. Con el objetivo de entender el comportamiento de los diferentes tipos celulares expuestos a Ag NPs, los amebocitos y eleocitos fueron aislados mediante técnicas de separación celular y se cuantificó su contenido en Ag. A pesar de que el medio natural (fluido celómico) resultó ser el óptimo para el mantenimiento de los celomocitos en cultivo ya que produjo la menor mortalidad en los análisis de citometría de flujo, éste presentó limitaciones metodológicas importantes. Por ello se seleccionó el medio RPMI-1640 como la mejor opción entre los medios de cultivo convencionales para el mantenimiento de celomocitos y para aplicar ensayos en microplaca. Los ensayos NRU y WST-1 mostraron interferencias con la absorbancia de los medios de exposición, mientras que el ensayo con Calceina AM resultó ser el más sensible para evaluar los efectos de las exposiciones a Ag NPs (y AgNO_3). Mediante el ensayo de viabilidad Calceina AM se observó un descenso gradual en la viabilidad de los celomocitos a partir de 10 mg/l. El valor LC_{50} para Ag NPs fue establecido en 30.48 mg/l para células mantenidas en el medio RPMI-1640. Se observó una mayor toxicidad de las Ag NPs en comparación con AgNO_3 (LC_{50} 43.38 mg AgNO_3 /l) en los celomocitos, pudiendo estar la toxicidad mediada por una diferente vía de acumulación dependiendo de la forma de Ag. Se descartó que la toxicidad observada fuese debida al recubrimiento PVP-PEI. La exposición de celomocitos a Ag NPs causó citotoxicidad selectiva en los amebocitos, coincidiendo con una mayor acumulación de Ag en este tipo celular y reforzando así la idea de diferente sensibilidad entre amebocitos y eleocitos.

Palabras clave: Nanopartícula de plata (Ag NP), *in vitro*, cultivo primario de celomocitos, medio de cultivo, ensayo en microplaca, citometría de flujo, separación celular.

1. Introduction

A number of *in vivo* assays with earthworms have been carried out to assess the effects produced by silver nanoparticles (Ag NPs) in soils, dealing most of them with traditional endpoints (avoidance, survival, growth and reproduction) (Garcia-Velasco et al., 2016; Heckmann et al., 2011; Lapied et al., 2010; Schlich et al., 2013; Shoults-Wilson et al., 2011b, 2011c; Chapter 1). Even if scarcely, biomarkers measured at different levels of biological complexity (biochemical and molecular levels mostly) have also been used (Gomes et al., 2013, 2015; Hayashi and Engelmann 2013; Novo et al., 2015; Tsyusko et al., 2012). In regard to cellular level, earthworm immune cells (coelomocytes) have become an increasingly studied target system in ecotoxicology. In fact, several studies concerned the effects of polluted soils by measuring coelomocytes number, viability and activity in earthworms exposed *in vivo* (Hayashi et al., 2012; Homa et al., 2003; Irizar et al., 2015a; Kwak et al., 2014b; Scott-Fordsmand and Weeks, 2000). Such measurements have been recently included in *in vivo* studies dealing with Ag NPs toxicity assessment in soils (Curieses et al., 2017; Garcia-Velasco et al., 2016; Kwak et al., 2014a; Chapters 1, 2 and 3). Even more, molecular endpoints measured in coelomocytes of earthworms (i.e. *Eisenia fetida*) exposed to Ag NPs *in vivo* are less invasive biomarkers that can represent an alternative to those measured at higher levels of biological complexity (Curieses et al., 2017; Chapter 3). Nevertheless, coelomocytes are not an homogeneous group of cells in the coelomic cavity of *E. fetida* earthworms, two main subpopulations, eleocytes and hyaline or granular amoebocytes being distinguished by cytochemical, morphometrical, ultrastructural and functional characteristics (Adamowicz, 2005; Bilej et al., 2000, 2010; Engelmann et al., 2004, 2005). Regarding their function, amoebocytes participate in phagocytosis and encapsulation of foreign particles (e.g. bacteria, fungi, etc.) (Engelmann et al., 2005; Hayashi et al., 2012; Valembois et al., 1985) and eleocytes are detached chloragocytes derived from the chloragogen tissue (Linthicum et al., 1977a) that contribute to homeostasis and humoral immunity in earthworms (Adamowicz, 2005; Engelmann et al., 2004, 2005). Eleocytes of *E. fetida* possess autofluorescence due to the selective accumulation of riboflavin, which simplifies their identification (Plytycz et al., 2011).

Both subpopulations behave differently after exposure to Ag NPs (Curieses et al., 2017; Chapter 3) and the accumulation, together with the intracellular mechanism of action of NPs in each cell type is still unclear. *In vitro* models could help to get deeper knowledge about these issues.

Recently, *in vitro* approaches with primary cultures of coelomocytes have been developed as cost-effective tools with promising applications in toxicity assessment of chemicals (Hayashi et al., 2012, 2013b; Irizar et al., 2014b, 2015b, Madhusudhan et al., 2009). However, these methods require an accurate composition of the culture medium to achieve good experimental reproducibility and to ensure that the responsiveness of cells against toxicants is not compromised by culture conditions (Brunner et al., 2010). Different commercial culture media (e.g. Leibovitz's L-15, Basal Medium Eagle-BME-, RPMI-1640 medium, etc.) have been used to evaluate the toxicity of chemical compounds in cellular models, which could lead to different biological toxicity values that difficult comparisons. As an alternative to the use of conventional culture media, Hayashi et al. (2013, 2013b) suggested the maintenance of *E. fetida* coelomocytes in a medium supplemented with cell-free coelomic fluid in order to increase their viability. The use of coelomic fluid would allow obtaining more realistic responses from coelomocytes exposed to Ag NPs since these cells reside on it, being this cell-fluid combination essential for homeostasis and immune defence functions (Kurek et al., 2007).

Together with the culture media composition the selection of a responsive assay to assess cytotoxicity is of great importance. Existing studies with earthworm coelomocytes have investigated the effects of toxicants on the lysosomal stability using Neutral Red Retention assay (NRRT, Scott-Fordsmand and Weeks, 2000; Scott-Fordsmand et al., 1998; Weeks and Svendsen, 1996; Yuk et al., 2012), which has been adapted to spectrophotometric measurement carried out in microplates (Asensio et al., 2007, 2013; Homa et al., 2003; Irizar et al., 2014b, 2015a, 2015b; Kwak et al., 2014a). However, the relevance of other microplate viability assays, based on tetrazolium salts (Cell Proliferation WST-1, MTT) and fluorescence (Calcein AM), is still low in primary cultures of coelomocytes and even lower in toxicity testing of Ag NPs.

Another approach to test the cytotoxicity in coelomocytes is flow cytometric analysis (Brousseau et al., 1997; Engelmann et al., 2016; Homa et al., 2013), which have been applied in coelomocytes extruded after *in vivo* exposures (Bilej et al., 1990; Brousseau et al., 1997; Homa et al., 2013, Vernile et al., 2007) and slightly in primary cultures of coelomocytes (Fugère et al., 1996; Hayashi et al., 2012, 2013b; Irizar et al., 2015b). Flow cytometric assays use various fluorescent dyes to detect dead cells and apoptosis (i.e propidium iodide and 7-aminoactinomycin D), mitochondrial membrane potential and mass (i.e., rhodamine 123, acridine orange) and reactive oxygen species (i.e

Dichlorofluorescein) (Kwak et al., 2014b). In addition, with the aid of flow cytometry, amoebocytes and eleocytes can be easily distinguished according to their forward and side scatter profiles (size and granularity) and to the green fluorescence, and thereafter, their dissimilar sensitivities against compounds (including Ag NPs) can be analyzed. Moreover, these subpopulations can be physically isolated by means of cell sorting, an issue that has been newly performed by Engelmann et al. (2016) with cell characterization purposes. The sorting of coelomocytes exposed to Ag NPs (followed by chemical analysis) would allow to better understand accumulation pattern and subsequent toxicity in each subpopulation.

Hence, the aim of the present work is to accurately assess (through *in vitro* approaches) Ag NPs toxicity in primary cultures of *E. fetida* coelomocytes, (a) by selecting an optimal medium for coelomocytes culturing and, (b) by determining the most responsive viability assay. For that, primary cultures of coelomocytes were maintained in widely used conventional media (Leibovitz's L-15, BME, RPMI-1640) and in a natural medium based on freshly extruded coelomic fluid. Afterwards coelomocytes were exposed to PVP-PEI coated Ag NPs, the coating agent (PVP-PEI) and AgNO₃ (0-100 mg/l) and after 24 h flow cytometric analyses were used to assess mortality of coelomocytes (with propidium iodide, 5 µg PI/ ml) and changes in the relative proportion of amoebocytes and eleocytes (recorded by their forward and side scatter profiles and green fluorescence). In addition, viability was assessed in microplates by Neutral Red Uptake (NRU), Cell Proliferation WST-1 and Calcein AM Viability assays. In order to better understand the behaviour of both cell-types after Ag NP exposure, amoebocytes and eleocytes were sorted and Ag concentration measured in both isolated subpopulations.

2. Materials and methods

2.1. Test species

Eisenia fetida earthworms were purchased from a commercial dealer (LOMBRICOR S.C.A., Córdoba, Spain) and set as laboratory culture maintained in containers under controlled conditions of temperature (19 ± 2 °C) and humidity. As food source medication-free horse manure was provided when required. The earthworms used for the harvesting of coelomocytes were all adults (clitellated) of similar size (300-500 mg individual weight).

2.2. Test substances

Polyvinylpyrrolidone-polyethylenimine (PVP-PEI, 3.35:1) coated Ag NPs (NP Ag-2106W) were purchased from NANOGAP (SUB-NM-POWDER S.A., A Coruña, Spain). Ag NPs were water dispersed (10 g Ag/l with 104 g PVP-PEI/l), 5.08 ± 2.03 nm average size and with a Z-potential of 18.6 ± 7.9 mV. More details on the characterization of Ag NPs are given in Appendix I. The coating agent PVP-PEI was tested separately at the same concentration range and pH as present in the Ag NPs suspension (PVP10-polyvinylpyrrolidone, Mw 10000 and PEI-polyethylenimine, Mw 25000 by LS; both from Sigma-Aldrich). High grade (>99% purity) AgNO₃ was purchased from Sigma-Aldrich.

2.3. Culture media preparation

2.3.1. Conventional culture media

Leibovitz's L-15 (L4386 Sigma-Aldrich, powder, 13.8 g/l) and Basal Medium Eagle (BME, B9638 Sigma-Aldrich, powder, 9.2 g/l) were prepared in MQ water with 0.35% HEPES (A14777 Alfa Aesar). RPMI-1640 medium (R7388 Sigma Aldrich) was purchased liquid with 20 mM HEPES. After having adjusted the pH of the different media to 7.4, these were filtered through 0.22 µm filter and a set of antibiotics was supplemented (1% Amphotericin B-250 µg/ml-, 1% Penicillin/Streptomycin-10.000 units penicillin/10 mg streptomycin/ml- and 0.5% Gentamicin sulphate-10 mg/ml-). All the processes were performed in a flow chamber (Cultair BC100, Cultek) and sterile material was used.

The composition (inorganic salts, aminoacids, vitamins and others) of the above mentioned conventional media (Leibovitz's L-15, BME and RPMI-1640) are illustrated in Table 1.

2.3.2. Coelomic fluid based medium

Earthworms were first left for depuration in moistened filter paper (24 h) and cleaned with distilled water. Coelomic fluid was then extruded by suctioning in the coelomic cavity with the aid of a syringe and needle (0.4 × 20 mm, Henke Sass Wolf GmbH). PBS was used during the process to lubricate the needle. Once extruded, the aspirated coelomic fluid was filtered through 0.45 µm filter to discard any cell in suspension and antibiotics were added (1% Amphotericin B-250 µg/ml-, 1% Penicillin/Streptomycin-

10.000 units penicillin/10 mg streptomycin/ml- and 0.5% Gentamicin sulphate-10 mg/ml-). About 100 μ l of coelomic fluid were extruded from each earthworm.

Table 1. Composition (inorganic salts, aminoacids, vitamins and others) and some specifications of the different conventional media (Leibovitz's L-15, BME and RPMI-1640) used for coelomocyte culturing (from Sigma-Aldrich Product Information sheets).

		Leibovitz's L-15	BME	RPMI-1640
		L4386 (g/L)	B9638 (g/L)	R7388 (g/L)
PRODUCT SPECIFICATION	Osmolality (mOs/kg)	305 - 338	229 - 253	252 - 278
	with 2.2 g/L NaHCO ₃	-	273 - 301	-
	pH	7.3-7.9	5.6 - 6.2	7.3-7.7
	with 2.2 g/L NaHCO ₃	-	7.4-8.0	-
	Appearance (Turbidity)	Clear	Clear	Clear
	Appearance (Form)	Powder	Powder	Solution
	Sterility by USP guidelines	Pass	Pass	Pass
	Endotoxin Level	<_ 1.0 EU/ml	<_ 1.0 EU/ml	<_ 1.0 EU/ml
	Galactose Concentration	0.81 - 0.99 g/l	-	-
	Total Glucose	-	1.0 g/l	2.0 g/l
Cell Culture Test	Pass	Pass	Pass	
INORGANIC SALTS	CaCl ₂ •2H ₂ O	0.140	0.265	-
	Ca(NO ₃) ₂ • 4H ₂ O	-	-	0.1
	MgCl•6H ₂ O	0.093	-	-
	MgSO ₄ (anhyd)	0.09767	0.09767	0.04884
	KCl	0.4	0.4	0.5
	KH ₂ PO ₄ (anhyd)	0.06	-	-
	NaCl	8.0	6.8	6
	Na ₂ HPO ₄ (anhyd)	0.19	-	0.8
	NaH ₂ PO ₄ (anhyd)	-	0.122	-
	AMINO ACIDS	L-Alanine	0.225	-
L-Arginine (free base)		0.5	-	-
L-Arginine•HCl		-	0.021	0.2
L-Asparagine (anhyd)		0.25	-	0.05
L-Aspartic Acid		-	-	0.02
L-Cysteine (free base)		0.12	-	-
L-Cystine•2HCl		-	0.01565	0.0652
L-Glutamine		0.3	0.292	0.3
L-Glutamic Acid		-	-	0.02
Glycine		0.2	-	0.01
L-Histidine		0.25	0.008	0.015
L-Isoleucine		0.125	0.026	0.05
L-Leucine		0.125	0.026	0.05
L-Lysine		-	-	-
Monohydrochloride		0.0937	0.03647	0.04
L-Methionine		0.075	0.0075	0.015
L-Phenylalanine		0.125	0.0165	0.015
L-Proline		-	-	0.02
Hydroxy-L-Proline		-	-	0.02
L-Serine		0.2	-	0.03
L-Threonine		0.3	0.024	0.02
L-Tryptophan		0.02	0.004	0.005
L-Tyrosine (free base)	0.3	-	-	
L-Tyrosine•2Na•2H ₂ O	0.02595	-	0.02883	
L-Valine	0.1	0.0235	0.02	

Table 1. (Continued)

	Leibovitz's L-15 L4386 (g/L)	BME B9638 (g/L)	RPMI-1640 R7388 (g/L)
VITAMINS	p-Aminobenzoic Acid		0.001
	Choline Chloride	0.001	0.001
	D-Biotin	-	0.001
	Flavin		
	Mononucleotide•Na	0.0001	-
	Folic Acid	0.001	0.001
	myo-Inositol	0.002	0.002
	Niacinamide	0.001	0.001
	DL-Pantothenic Acid•½Ca	0.001	0.001
	Pyridoxine•HCl	0.001	0.001
	Thiamine Monophosphate•HCl	0.001	-
	Thiamine•HCl	-	0.001
	Riboflavin	-	0.0001
	Vitamin B12		-
	OTHERS	D-Galactose	0.9
D-Glucose			1.0
Phenol Red•Na		0.011	0.011
Pyruvic Acid•Na		0.55	-
Glutathione (reduced)		-	-
HEPES		-	-
^{ADD} Sodium Bicarbonate	-	2.2	2

2.3.3. Exposure media

Exposure media were prepared from the previous culture media by adding Ag NPs, the coating agent (PVP-PEI) and AgNO₃, and performing serial dilutions. Triton X-100 (0.2%) containing media were also prepared to be used as positive control. All the steps were performed in a flow chamber (Cultair BC100, Cultek) and sterile material was used during the whole process.

2.4. Characterization of the exposure media

The size of Ag NPs in the different exposure media (conventional- Leibovitz's L-15, BME and RPMI-1640-and natural-coelomic fluid- with 10 mg Ag NPs/l) was determined by Dynamic Light Scattering (DLS, ZetaSizer Nano Series ZS, Malvern, Worcestershire). DLS measurements were carried out after 2 and 24 h of the exposure media preparation at 19 °C. Size distribution was determined by number, selecting the size ranges that included >99% of the measured particles.

Absorbance spectra of conventional and natural media with 0, 1, 10 and 100 mg/l PVP-PEI coated Ag NPs and AgNO₃ (only in coelomic fluid and Leibovitz's L-15) were

measured with a Multiskan™ GO Microplate spectrophotometer (Thermo Fisher Scientific) at 200-1000 nm wavelength range.

2.5. Primary culture of coelomocytes

Previous to the harvesting of coelomocytes *E. fetida* earthworms were left for depuration on wet filter paper (24 h) and were cleaned with distilled water by softly massaging their body in order to remove any soil particle attached to the tegument or in the posterior part of their digestive tract. Clean earthworms were immersed in extrusion solution (0.02% EDTA in PBS-0.1 M Na-Phosphate Buffered Saline- with 0.23% NaCl, 1 ml per worm) and were subjected to an electric stimulation (9 V) to allow the release of coelomocytes through dorsal pores (Irizar et al., 2014b). The cell suspension was centrifuged (530 x g, 10 min, 10 °C), washed in PBS with antibiotics (1% Amphotericin B-250 µg/ml-, 1% Penicillin/Streptomycin-10.000 units penicillin/10 mg streptomycin/ml- and 0.5% Gentamicin sulphate-10 mg/ml-) and counted in a haemocytometer (Neubauer chamber) under light microscope to adjust the cell density to 10⁶ cells per ml. Cells were then resuspended in conventional (Leibovitz's L-15, BME and RPMI-1640 medium) and natural (coelomic fluid) media supplemented with antibiotics. Afterwards, coelomocytes suspended in the different culture media were seeded in 24-well plates (5 x 10⁵ cells/well) for flow cytometry analysis and in 96-well microplates (2 x 10⁵ cells/well) to perform viability assays. Primary cultures of coelomocytes were left to stand in a cell incubator (CO₂ free) at 18 °C for 24 h.

2.6. Coelomocyte exposure to Ag NPs, PVP-PEI and AgNO₃

After the stabilization, coelomocytes seeded in 24-well plates were exposed to 0, 1, 10, 100 mg/l PVP-PEI coated Ag NPs and AgNO₃ for flow cytometric analysis. The latter exposure (AgNO₃) was performed only in coelomocytes maintained in coelomic fluid.

Coelomocytes seeded in 96-well microplates were exposed to PVP-PEI coated Ag NPs, the coating agent PVP-PEI and AgNO₃ (0, 0.01, 1, 10 and 100 mg /l medium) during 24 h for posterior viability assessment. Triton X-100 (0.2%) treated cells were used as positive control in the microplate assays since they were nonviable coelomocytes showing no mitochondrial activity.

Coelomocytes cultured in the optimal medium (defined after 2.7 and 2.8 sections) for a posterior sorting and chemical analysis were exposed to PVP-PEI coated Ag NPs (0, 0.0001, 1, 10, 100 mg/l medium).

All exposures were carried out by replacing the medium in the wells with exposure medium, doing 4 replicates per dose for flow cytometry and sorting techniques and 7 for viability assays.

2.7. Coelomocytes mortality and changes in the relative proportion of subpopulations by flow cytometric analysis

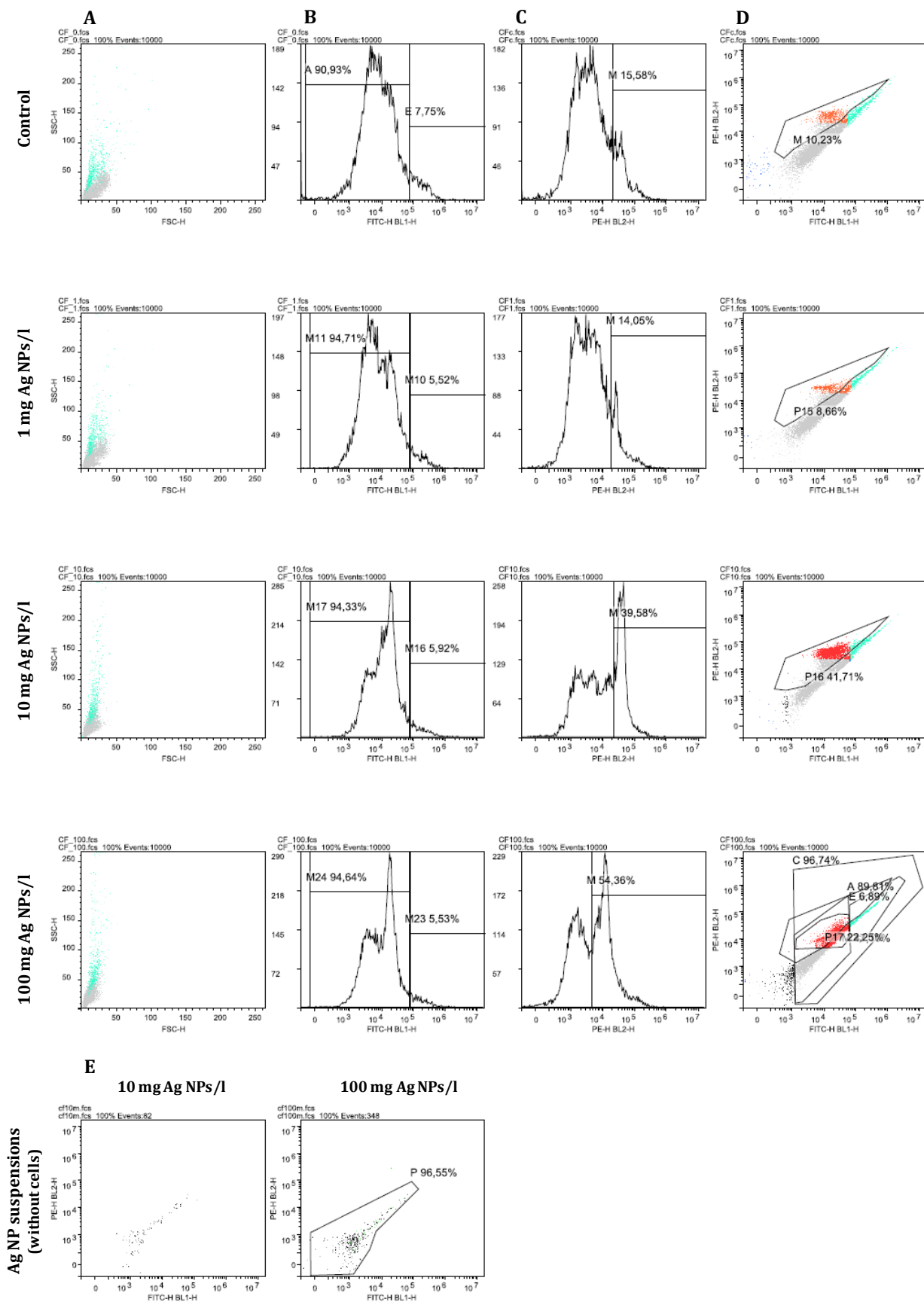
Wells containing exposed coelomocytes were grouped in 2 pools per treatment (0, 1, 10, 100 mg/l Ag NPs and AgNO₃) and were introduced in a NovoCyte® Flow Cytometer (ACEA Biosciences, Inc.). In one of the pools propidium iodide (PI, 5 µg/ml, Molecular Probes) was added in order to detect death coelomocytes by red fluorescence at 513/617 nm (PE BL2). PI only penetrates the cells with damaged membranes, and binds to nucleic acids of the double strand in a stoichiometric manner. Mortality was detected through PI fluorescence and determined by histogram (C column in Fig. 1a, 1b; SM-Fig. 1c, 1d, 1e).

Coelomocytes in the second pool were used to determine the relative proportion of the different subpopulations (amoebocytes and eleocytes) using their forward (FSC, cell size) and side scatter (SSC, cell granularity/complexity) characteristics. Green fluorescence (488 nm excitation/525 nm emission, FITC BL1) detection was also used to record eleocytes due to their autofluorescence (B column in Fig. 1a, 1b; SM-Fig. 1c, 1d, 1e).

In order to avoid the occurrence of false counting of particles due to the aggregation/agglomeration of Ag NPs or precipitation of Ag, after exposure to 10 and 100 mg Ag/l mortality and relative proportion of amoebocytes and eleocytes were assessed by density plots (D column in Fig. 1a, 1b; SM-Fig. 1c, 1d, 1e) after subtraction of particles detected in the exposure media without cells (E line in Fig. 1a, 1b; SM-Fig. 1c, 1d, 1e).

During the probes, at least 10.000 events per single determination were collected and analyzed. The collected data were analyzed in Flowlogic™ Flow cytometry analysis software (Inivai Technologies) (Fig. 1, SM-Fig. 1). By means of flow cytometry, the optimal culture medium was established by comparing the mortality of control coelomocytes in the different media.

(a) Coelomic fluid and Ag NPs exposure



(b) Coelomic fluid and AgNO₃ exposure

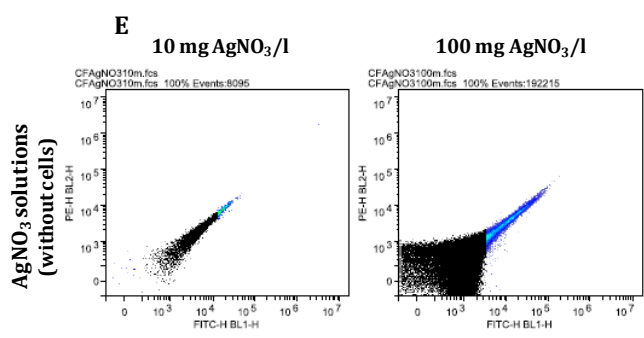
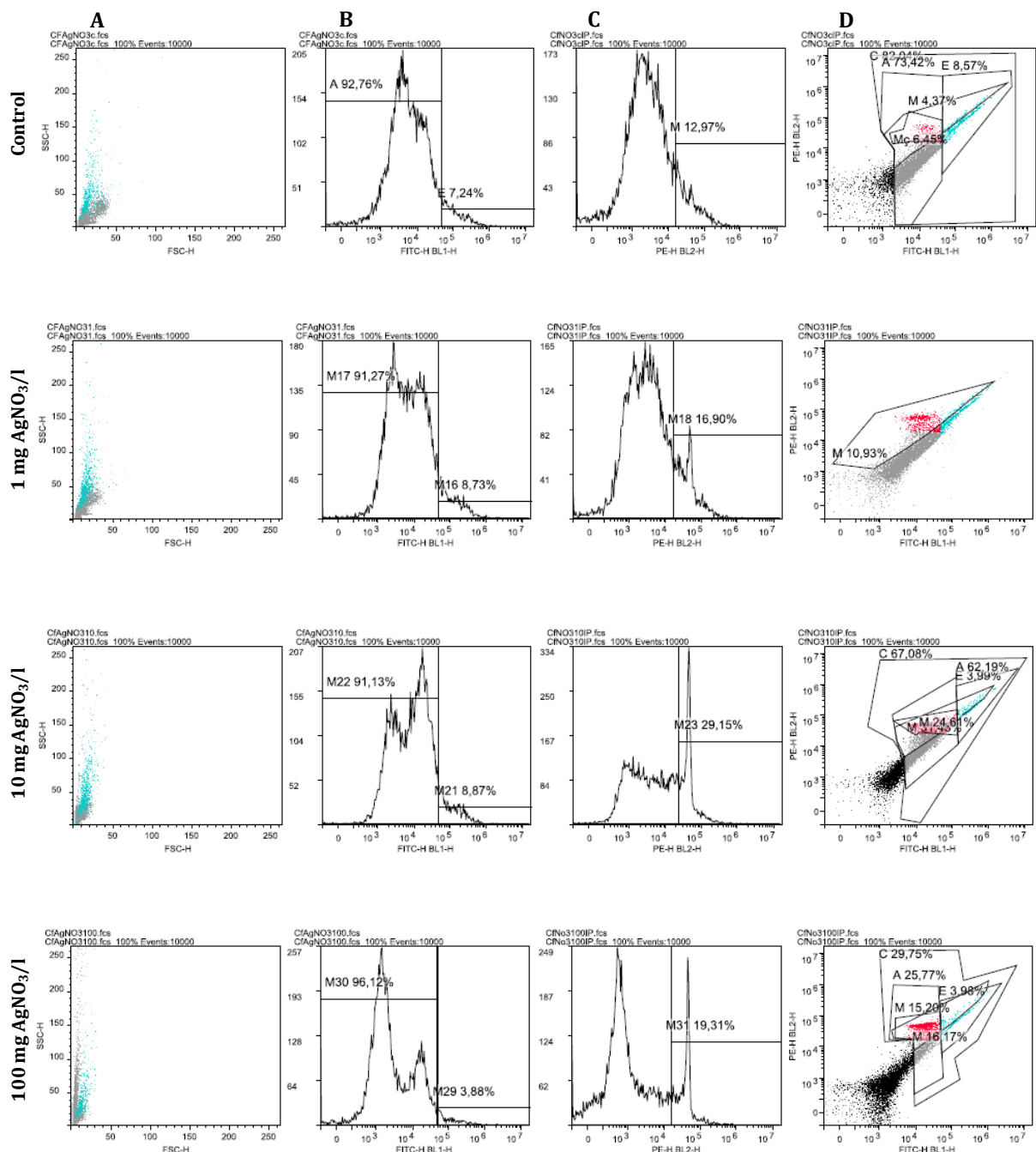


Figure 1. Analysis of the data with Flowlogic™ to establish subpopulation distribution and mortality in coelomocytes maintained in coelomic fluid and exposed to PVP-PEI coated Ag NPs (a) and AgNO₃ (b). Amoebocytes (grey dots) and eleocytes (blue dots) distribution was established according to their complexity (SSC, Y axis) and size (FSC, X axis) features (represented by density plot, A column) and according to their autofluorescence in green (FITC histogram, B column). Mortality of the whole coelomocyte population (%) was detected through PI fluorescence (PE histogram, C column). In the case of 10 and 100 mg/l exposure concentrations, amoebocyte and eleocyte relative number and mortality of coelomocytes were assessed by density plot (amoebocytes in grey, eleocytes in blue and dead coelomocytes in red, D column) after having subtracted the particles detected by density plot in the exposure media without cells (E line).

2.8. Coelomocytes viability through microplate assays

After exposure, coelomocytes viability was assessed in microplates through Neutral Red Uptake (NRU), Cell Proliferation WST-1 (Water soluble tetrazolium salts, WST-1) and Calcein AM viability assays. Each assay was replicated three times. Thereafter the optimal culture medium and the most accurate viability assay were selected according to the sensitivity and the responsiveness of coelomocytes against Ag NPs. For that, the median lethal concentration values (LC₅₀) calculated from the most responsive assay were used.

For NRU assay, exposure media were removed from wells and coelomocytes were incubated with NR solution (0.05% in PBS, 200 µl per well, 7 replicates) for 30 minutes in darkness. Then, several washes were done (centrifugation at 530 x g, 5 min, 10 °C; supernatant removal and addition of 200 µl PBS) to completely remove the dye. After washing, 100 µl of NR extraction solution (50% acetic acid, 1% ethanol, 49% dH₂O) were added to the wells in order to withdraw the dye retained within lysosomes. Absorbance was measured at 540 nm in a microplate reader spectrophotometer (Multiskan Spektrum, Thermo Scientific).

WST-1 assay was performed by adding 20 µl of the reagent (Roche Diagnostics GmbH, Mannheim; 7 replicates) into the wells containing 200 µl exposure medium so the reagent was 10 times diluted. Absorbance was measured ($Abs_{450nm} - Abs_{690nm}$, Multiskan Spektrum, Thermo Scientific) after 3 h and data was presented as relative to the control (in %).

In Calcein AM viability assay, microplate was centrifuged (530 x g, 5 min, 10 °C), exposure media were removed and cells were incubated for 40 minutes with 2.5 µM Calcein AM (Molecular Probes® ThermoFisher Scientific, 100 µl per well, 4 replicates) in darkness. In the remaining wells, instead of Calcein AM, 100 µl PBS were added to thereafter subtract the basal fluorescence measured in the wells, derived from

riboflavin containing eleocytes. Coelomocytes were washed twice (centrifugation, supernatant removal and addition of 100 μ l PBS) and fluorescence was measured at 490 ± 20 nm excitation filter and 520 ± 20 nm emission filter in FLx 800 microplate fluorescence reader. Fluorescence micrographs were taken from washed microplate wells in which Calcein AM was added.

2.9. Cell sorting and Ag quantification in isolated amoebocytes and eleocytes

Coelomocytes cultured in the optimal culture medium (defined after 2.7 and 2.8 sections; 24-well plates, 5×10^5 cells/well) and exposed to Ag NPs (0, 0.0001, 1, 10, 100 mg/l medium) were pooled per treatment, analyzed in flow cytometry and sorted (BD FACSAria™ III) according to their forward, side scatter and fluorescence characteristics. Data acquisition and analysis were performed in FACSDiva software (version 6.1.3). Collected amoebocytes and eleocytes were maintained in PBS and centrifuged ($530 \times g$, 5 min). Cell pellets were then dried in the heating block (80 - 130 °C) and acid digested (HNO_3) for posterior silver quantification (Flame-AAS, Perkin Elmer Analyst 4100).

2.10. Statistical analysis

The statistical analysis of the data was carried out with the aid of the SPSS statistical package (IMB SPSS Statistics 23). Datasets were analysed with Kruskal-Wallis followed by Dunn's post-hoc test and significant differences were established at $p < 0.05$. In order to estimate the median lethal concentration (LC_{50}) the Probit model was used.

3. Results

3.1. Characterization of the exposure media

The size (nm) of PVP-PEI coated Ag NPs in the different exposure media (10 mg/l) appeared to be higher than 5.08 ± 2.03 nm (the average size of the particles provided by NANOGAP). Ag NPs size after 2 h in the different conventional media (Leibovitz's L-15, BME, RPMI-1640) was similar, ranging from 7.9-22.7 nm (Table 2). Ag NPs size after 2 h in coelomic fluid ranges from 12.7-30.5 nm (Table 2). After 24 h of the exposure media preparation, the size of Ag NPs remained in the same range with exceptions in BME in which Ag NPs size increased and coelomic fluid where decreased (Table 2).

Table 2. Size ranges (nm) of PVP-PEI coated Ag NPs (10 mg Ag NPs/l) after 2 and 24 h in conventional (Leibovitz's L-15, BME and RPMI-1640) and natural (coelomic fluid) media measured by Dinamic Light Scattering (DLS).

DLS-Size (nm)	2 h	24 h
Leibovitz's L-15	8.7-18.0	7.9-17.3
BME	7.9-17.4	12.0-26.8
RPMI-1640	10.3-22.7	8.4-18.3
Coelomic Fluid	12.7-30.5	11.3-24.0

Absorbance spectra of the different exposure media containing 100 mg/l of Ag NPs showed a peak at 405-420 nm wavelengths (Fig. 2). This peak was not visible in culture media without Ag or in AgNO₃ containing coelomic fluid and Leibovitz's L-15 medium (Fig. 2).

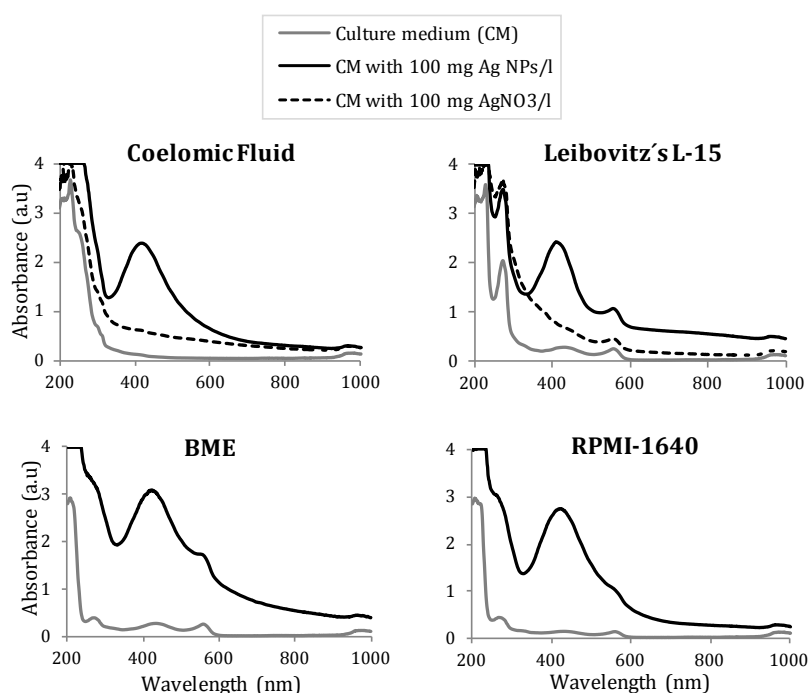


Figure 2. Absorbance spectra of the different culture media (conventional-Leibovitz's L-15, BME and RPMI-1640-and natural-coelomic fluid-, grey line) with PVP-PEI coated Ag NPs (black line) and with AgNO₃ (dotted-line) at 100 mg/l concentration.

3.2. Coelomocytes mortality and changes in the relative proportion of subpopulations by flow cytometric analysis

Coelomocytes mortality in controls and after exposure to 1 mg/l was lower in coelomic fluid in comparison with the conventional media (Fig. 3A). Coelomocytes seeded in coelomic fluid showed a mortality of 16% in controls while in conventional media mortality reached 33-39%. Cells maintained in coelomic fluid showed a gradual increase in mortality starting at 10 mg Ag NPs/l exposure (Fig. 3A). A decrease in eleocytes

relative number was detected after exposure to concentrations higher than 1 mg Ag NPs/l (Fig. 3B).

Coelomocytes maintained in Leibovitz's L-15 and BME and exposed to Ag NPs showed an increase in mortality after exposure to 10 mg/l (55.6% and 54.6%, respectively) (Fig. 3A). The relative number of eleocytes was enhanced in these media after exposure to 10 mg/l (Fig. 3B).

In RPMI-1640 medium, a gradual increase was observed in both, coelomocytes mortality and eleocytes relative number at increasing exposure concentration (Fig. 3A, 3B).

Mortality in coelomocytes maintained in coelomic fluid and exposed to AgNO_3 followed a dose response increase (Fig. 3C). The relative number of eleocytes decreased to 5.94% after exposure to 10 mg AgNO_3 /l and at the highest dose increased up to 13.37% (Fig. 3D).

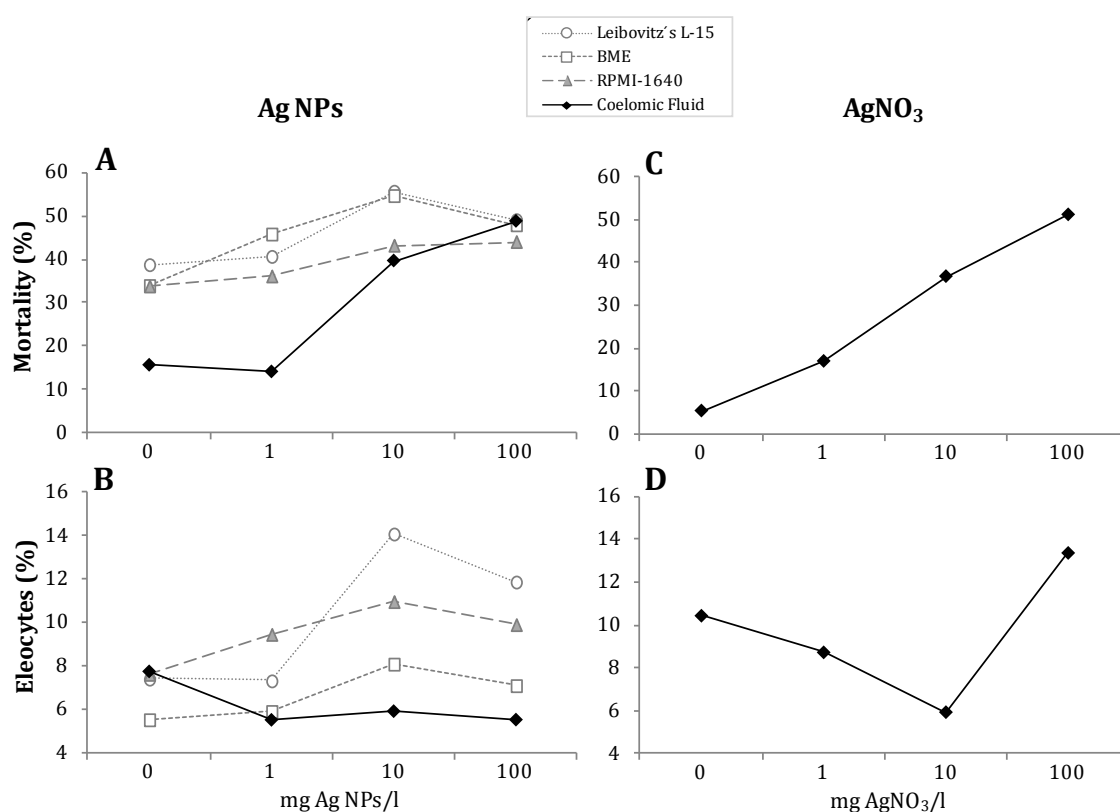


Figure 3. Mortality of total coelomocytes (%; A,C) and the relative number of eleocytes (%; B,D) measured by flow cytometry in primary cultures of coelomocytes maintained in conventional (Leibovitz's L-15, BME and RPMI-1640) and natural (coelomic fluid) media and exposed to PVP-PEI coated Ag NPs (A,B) and AgNO_3 (data for coelomic fluid only; C,D).

3.3. Coelomocytes viability through microplate assays

Absorbance values measured in NRU and WST-1 assays exhibited unavoidable interferences with the absorbance wavelengths of the different exposure media (Fig. 4, Fig. 5). Both assays showed an increase after exposure to the highest dose (100 mg/l). This increase was due to the appearance of exposure medium and dye deposits (reddish) strongly attached to the bottom of the wells (even after thorough washing) after performing NRU assay (Fig. 4C) and due to the higher colour intensity of the exposure media at 100 mg Ag NPs/l in WST-1 (Fig. 6).

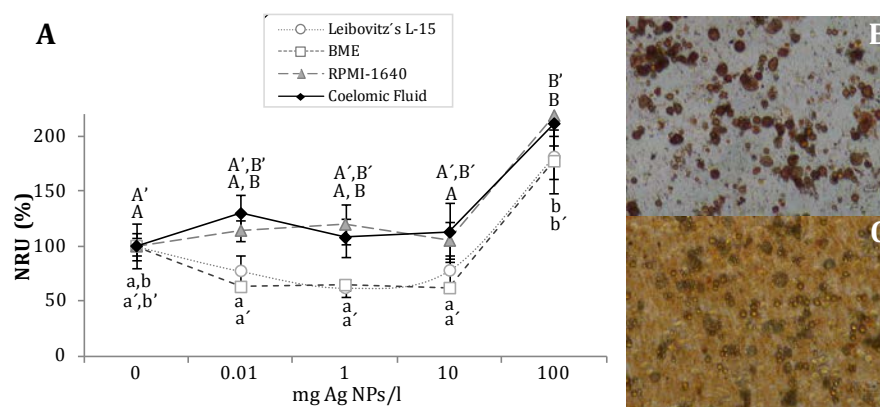


Figure 4. NRU (in % relative to the control, A) in primary cultures of coelomocytes maintained in conventional (Leibovitz's L-15, BME and RPMI-1640) and natural (coelomic fluid) media and exposed to PVP-PEI coated Ag NPs. Values are represented as means \pm standard deviations and the significant differences ($p \leq 0.05$ with Kruskal-Wallis) are represented by letters (a',b' for Leibovitz's L-15; a,b for BME; A,B for RPMI-1640 and A',B' for coelomic fluid). Micrographs showed untreated (B) and 100 mg Ag NPs/l exposed (C) coelomocytes in Leibovitz's L-15 medium after the washing steps of the NRU assay. Note the NR retention in control coelomocytes (B), the absence of dye within Ag NPs exposed cells and the reddish deposits in the bottom of the well (C).

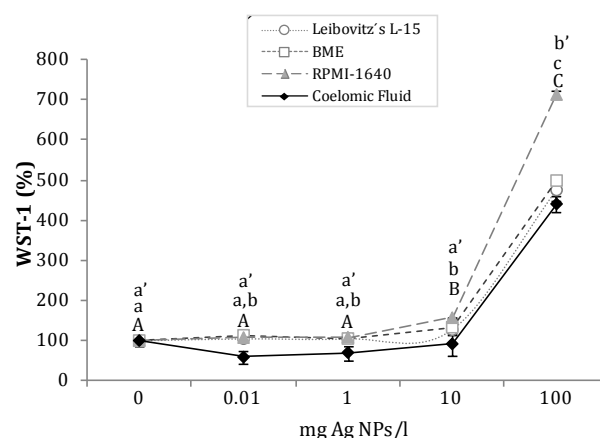


Figure 5. Cell Proliferation Reagent WST-1 (in % relative to the control) in primary cultures of coelomocytes maintained in conventional (Leibovitz's L-15, BME and RPMI-1640) and natural (coelomic fluid) media and exposed to PVP-PEI coated Ag NPs. Values are represented as means \pm standard deviations and the significant differences ($p \leq 0.05$ with Tukey's test) are represented by letters (a',b',c' for Leibovitz's L-15; a,b,c for BME; A,B,C for RPMI-1640).

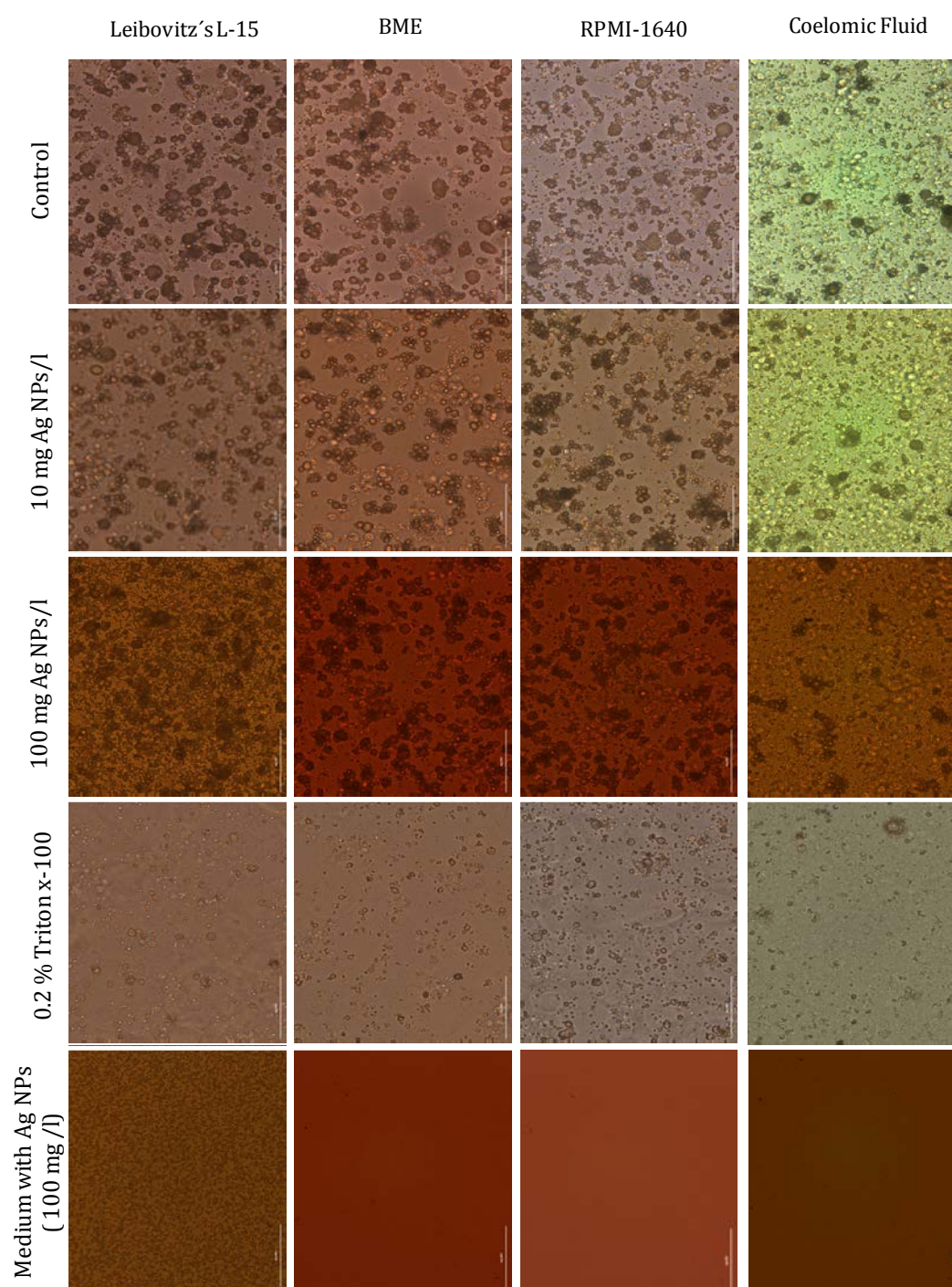


Figure. 6. Micrographs showing primary cultures of coelomocytes maintained in conventional (Leibovitz's L-15, BME and RPMI-1640) and natural (coelomic fluid) media and exposed to PVP-PEI coated Ag NPs (10 and 100 mg/l) and Triton X-100 (0.2%). In the last line 100 mg Ag NPs/l exposure media without coelomocytes. Scale bar 100 μ m. Note the different colour intensity of the exposure media when increasing Ag NPs concentration and the formation of aggregates in Leibovitz's L-15 with Ag NPs (100 mg/l), not noticeable in the rest of the media.

The calcein retention was not measurable in coelomic fluid maintained coelomocytes due to the fluorescence feature of this (natural) medium (Fig. 7). The Calcein AM viability assays performed in coelomocytes maintained in Leibovitz's L-15 and exposed to Ag NPs and AgNO₃ did not show differences among doses (Fig. 8A, 8C). Coelomocytes seeded in this medium showed the lowest calcein retention capacity after PVP-PEI exposure followed by Ag NPs and AgNO₃ exposures (Fig. 8B, Fig. 9A). The median lethal concentration (LC₅₀) in Leibovitz's L-15 maintained coelomocytes was 78.42 mg/l after PVP-PEI exposure. For both Ag forms, the LC₅₀ values were higher than the exposure doses used (>100 mg/l, Table 3).

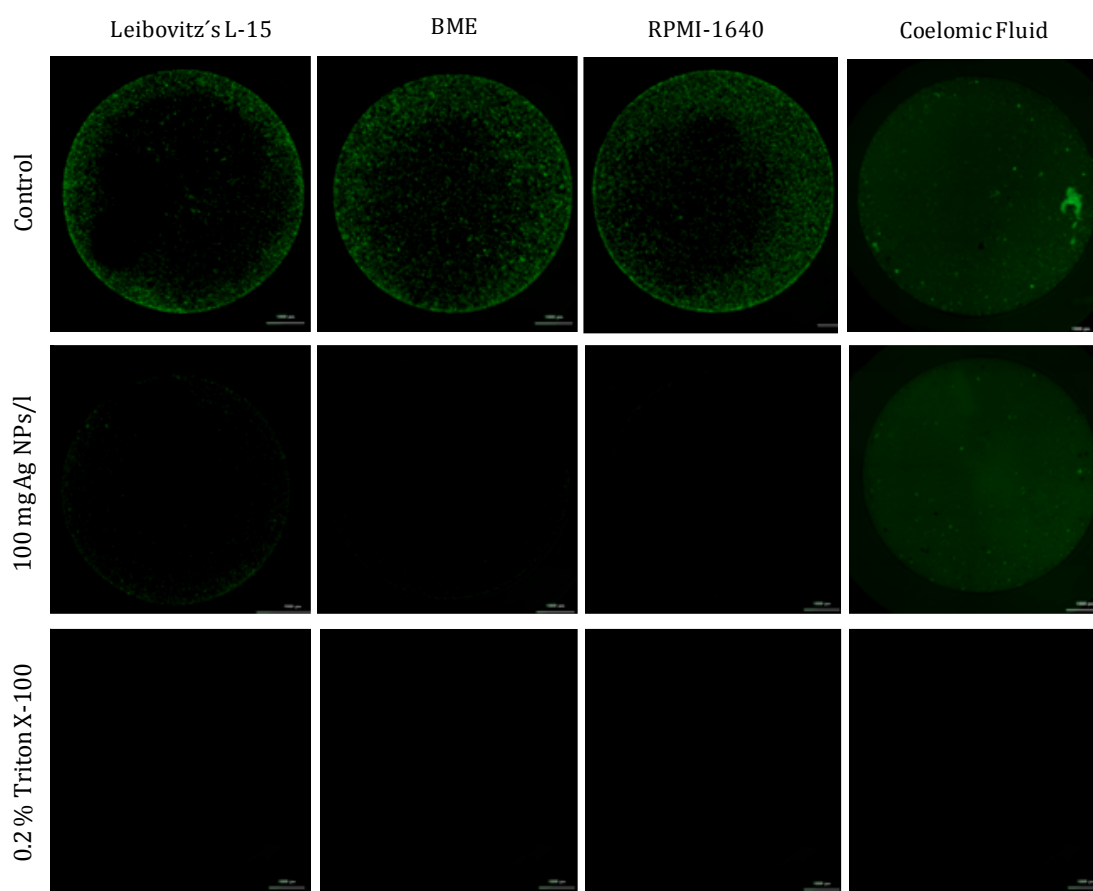


Figure 7. Fluorescence micrographs of microplate wells after Calcein AM viability assay in untreated (control), Ag NPs (100 mg/l) and Triton X-100 (0.2%) exposed coelomocytes in conventional (Leibovitz's L-15, BME and RPMI-1640) and natural (coelomic fluid) media. Scale bar 1000 μ m. Note the high autofluorescence of the coelomic fluid based medium.

Coelomocytes cultured in BME and RPMI-1640 media and exposed to both Ag forms revealed a significant decrease in calcein retention starting after exposure to 10 mg/l (Fig. 8A, 8C). The lowest calcein retention was found after Ag NPs exposure (Fig. 9B, 9C), estimating the LC₅₀ value at 36.68 in BME and at 30.48 mg/l in RPMI-1640 (Table 3).

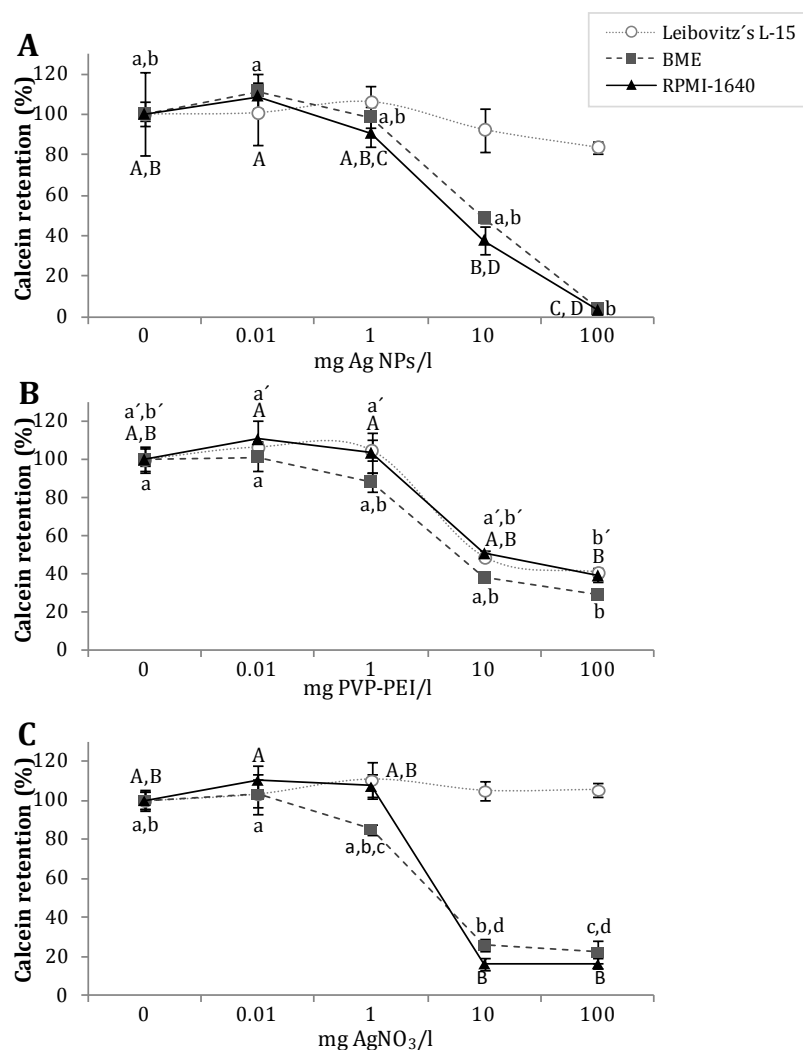


Figure 8. Cell viability (Calcein AM viability assay, represented by calcein retention in % relative to the control) in primary cultures of coelomocytes maintained in conventional media (Leibovitz's L-15, BME and RPMI-1640) and exposed to PVP-PEI coated Ag NPs (A), the coating agent (PVP-PEI, B) and AgNO₃ (C). Values are represented as means \pm standard deviations and the significant differences ($p \leq 0.05$ with Kruskal-Wallis) are represented by letters (a',b' for Leibovitz's L-15; a,b,c for BME and A,B for RPMI-1640).

After exposure to AgNO₃, coelomocytes retained more calcein than after exposure to Ag NPs at the same concentration (Fig. 9B, 9C) and the LC₅₀ values were calculated around 45 mg/l in both media (48.08 mg/l in BME and 43.38 mg/l in RPMI-1640, Table 3). After exposure to the coating agent PVP-PEI, significant decreases in calcein retention only occurred in the highest concentration (100 mg/l, Fig. 8B). The LC₅₀ values after PVP-PEI exposure were 58.72 mg/l in BME and 76.25 mg/l in RPMI-1640 medium (Table 3).

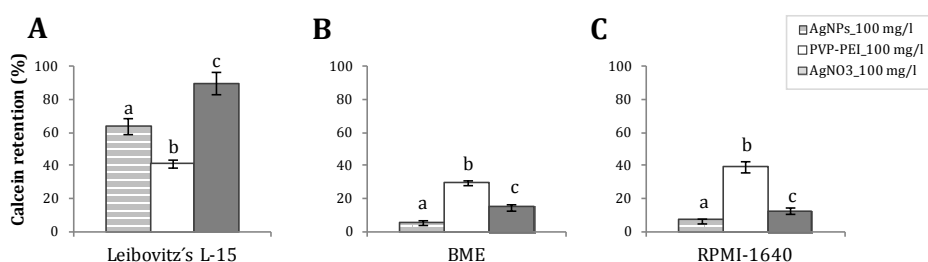


Figure 9. Calcein retention capacity (% respect to the control) of coelomocytes maintained in Leibovitz's L-15 (A), BME (B) and RPMI-1640 (C) and exposed to 100 mg/l of PVP-PEI coated Ag NPs, coating agent (PVP-PEI) and AgNO₃. Values are represented as means \pm standard deviations and the significant differences ($p \leq 0.05$ with Kruskal-Wallis) are represented by letters.

Table 3. Median lethal concentration (LC₅₀, mg/l) calculated after Calcein AM Viability assay in coelomocytes cultured in different conventional media (Leibovitz's L-15, BME and RPMI-1640) and exposed to PVP-PEI coated Ag NPs, the coating agent (PVP-PEI) and AgNO₃.

LC ₅₀ (mg/l)	Ag NPs	PVP-PEI	AgNO ₃
Leibovitz's L-15	>100.00	78.42	>100.00
BME	36.68	58.72	48.08
RPMI-1640	30.48	76.25	43.38

3.4. Cell sorting and Ag quantification in isolated amoebocytes and eleocytes

Sorting and posterior Ag quantification were performed in coelomocytes maintained in RPMI-1640 medium, since this medium was selected as the optimal (more sensitive LC₅₀ values were obtained with RPMI-1640 in comparison with the rest of the tested conventional media). Ag concentration in sorted amoebocytes showed a gradual increase, significantly different from 10 mg Ag NPs/l exposure on. At the highest exposure concentrations (10 and 100 mg/l) amoebocytes accumulate 0.52 ± 0.00 and 1.00 ± 0.01 $\mu\text{g Ag}/10^6$ cells, respectively (Fig. 10). In sorted eleocytes Ag concentration appeared to be higher (Fig. 10).

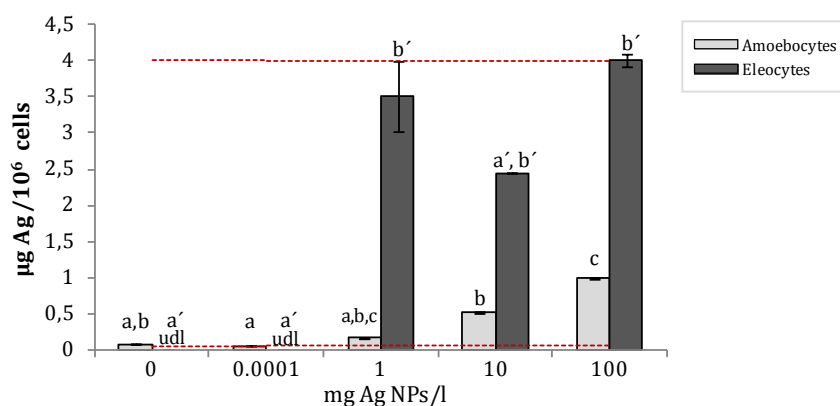


Figure 10. Silver concentration ($\mu\text{g Ag}/10^6$ cells, AAS) in sorted amoebocytes (light grey) and eleocytes (dark grey) previously maintained in RPMI-1640 medium and exposed to PVP-PEI coated Ag NPs. Values are represented as means \pm standard deviations and the significant differences ($p \leq 0.05$ with Kruskal-Wallis) are represented by letters. Detection limit range: 0.05-4 $\mu\text{g Ag}/10^6$ cells (dotted lines), udl: under detection limit.

4. Discussion

Earthworm coelomocytes have become a target system in immune response studies (Toupin et al., 1977) and in ecotoxicology (Hayashi et al., 2012; Homa et al., 2003; Irizar et al., 2014b; Scott-Fordsmand and Weeks, 2000) due to their sensitivity against a wide range of pollutants, including Ag NPs (Curieses et al., 2017; Garcia-Velasco et al., 2016; Kwak et al., 2014a; Chapters 1, 2 and 3). Presently, *in vitro* approaches (viability assays in microplate, flow cytometry, cell sorting) with primary cultures of coelomocytes have been used as rapid, cost-effective and reproducible tools to test the toxicity and the dissimilar response of amoebocyte and eleocyte subpopulations after exposure to PVP-PEI coated Ag NPs and AgNO_3 . In this framework, the maintenance of coelomocytes in an optimal culture medium and the selection of the most responsive assay to accurately assess Ag NPs toxicity are of utmost importance.

The use of coelomocyte cultures for toxicity screenings of compounds likely to end up in soils is convenient since these cells could give an idea of the effects occurring at higher levels of biological complexity (i.e. organism and population levels) in earthworms subjected to polluted soils. Moreover, coelomocytes are easily retrieved from the coelomic cavity via the dorsal pores (Stankiewicz and Plytycz, 1998), reducing the number of animals in experimentation and avoiding their killing. Despite these high potentials of *in vitro* techniques with coelomocytes, culturing conditions for this cell model have been poorly investigated. In the present work, the coelomic fluid appeared to be the best medium for the maintenance of coelomocytes, indicated by the lower mortality of control coelomocytes in comparison with the conventional media. This fact

could be due to the presence of proteins and enzymes in the coelomic fluid, which could render a better or quicker adaptation to external stimuli (i.e. trauma provoked by the extrusion from earthworms and seeding in plates) due to their role in homeostasis and immune defense of earthworms (Kurek et al., 2007). Among the haemolytic proteins, the so-called *EFAF* (*Eisenia fetida andrei* factors) are characterized by two glycoproteins secreted by eleocytes that participate in the cytotoxic activity of the coelomic fluid (Bilej et al., 2010). Thus, the use of coelomic fluid as culture medium would imply the presence of haemolytic proteolytic and cytotoxic enzymes that are active against foreign materials (Bundy et al., 2001), allowing to obtain more realistic responses from coelomocytes exposed to Ag NPs. However, the extrusion of coelomic fluid by suction is tremendously time consuming and the number of individuals needed to obtain the minimum amount of coelomic fluid is very high (> 20 earthworms per plate). In conclusion, despite its accurate properties for cell maintenance the use of coelomic fluid is not suitable to perform *in vitro* testing with coelomocytes and the use of a conventional medium is recommended.

Toupin et al. (1977) stated the use Leibovitz's L-15 medium supplemented with fetal bovine serum and antibiotics and similarly, Irizar et al. (2014b) employed the same medium but without serum. In the latter study, primary cultures with coelomocytes were optimized with this medium for their application in soil toxicity testing. In the present work, coelomocytes maintained in Leibovitz's L-15 (serum free) showed higher mortality (by flow cytometry) and lower responsiveness against both Ag NPs and AgNO₃ using the Calcein AM viability assay in comparison with BME and RPMI-1640 media. In fact the LC₅₀ values obtained for Ag NPs and AgNO₃ were higher than the exposure concentrations used in the present work. In contrast, coelomocytes seeded in the other conventional culture media (BME and RPMI-1640 medium) and exposed to both Ag forms revealed a clear decrease in calcein retention, showing a major sensitivity of the cells responding to Ag in these media. Although the responses of the exposed coelomocytes were similar in BME and RPMI-1640 media, cells in the latter showed even more sensitive responses against both Ag forms, indicated by the lower LC₅₀ values obtained in the Calcein AM viability assay. BME was formulated for nutritional requirements of mouse L fibroblasts and HeLa cells (Eagle, 1955) whereas currently is employed in cell cultures of marine invertebrates such as molluscs hemocytes (Gómez-Mendikute et al., 2003; Katsumiti et al., 2015) but has not been used with earthworm coelomocytes beforehand. The lower cytotoxicity showed by exposed coelomocytes

could be also due to the higher size of Ag NPs (up to 26.8 nm after 24 h) in this medium that could have minimized solubility. In fact, higher size Ag NPs has been demonstrated to have lower ion release rates than smaller ones, considering ions responsible or enhancers of toxicity (Sotiriou and Pratsinis, 2010).

Conversely, RPMI-1640 medium has been widely used to maintain earthworm coelomocytes in culture (Engelmann et al., 2016; Hayashi et al., 2012, 2013b) and furthermore, studies using this medium dealt with similar endpoints to those carried out herein (e.g. Ag NPs toxicity assessment, flow cytometric probes, coelomocyte sorting etc.), allowing comparisons. Unlike Leibovitz's L-15 and BME, RPMI-1640 presented more amino acids (i.e. L Arginine, L-Aspartic acid, L-Cystine, L-proline, etc.) and vitamins (i.e. myo-Inositol, B12 etc.) together with the highest quantity of glucose (2 g/l) in its composition (Table 1). In one hand, these amendments in medium could be beneficial for the accurate maintenance of cells in primary cultures. On the other hand, the different components of the medium could have changed the physicochemical characteristics (i.e. size) of the Ag NPs and subsequently, their toxic behaviour in each medium could be altered. The size of Ag NPs in the different exposure media was higher than the average size of the particles in distilled water provided by NANOGAP, which makes sense considering the NP-protein interactions under *in vitro* test conditions (Hayashi et al., 2012). However, the size of Ag NPs did not differ between the different exposure media (excepting BME after 24 h) and thus, the highest sensitivity obtained from exposed coelomocytes in RPMI-1640 medium was not mediated by the characteristics of Ag NPs in that exposure medium. Therefore, the components present in RPMI-1640 medium appeared to be more suitable for coelomocytes in culture and thus, allowed to perform an accurate assessment of Ag NPs toxicity. Further, the information regarding the optimal composition of the medium (Table 1) could result useful to better understand the nutrient requirements of these cells with high *in vitro* potentials. Animal serum of different origins is frequently added to chemically defined basal media for cell growth and to stimulate metabolism and proliferation (Brunner et al., 2010). In this case, the supplementation with serum was not considered since viability was high in serum-free media during 3 days without replenishment (Irizar et al., 2014b) and being demonstrated that Ag NPs dispersion is stable in media with and without serum (Hayashi et al., 2012).

Apart from using an optimal culture medium for coelomocytes, the selection of the most convenient and responsive assay is necessary to obtain reliable data and to accurately

assess Ag NPs toxicity. Cytotoxicity assays in microplate allow many samples to be analyzed rapidly and simultaneously (Weyermann et al., 2005) and thus, in the present chapter, NRU, WST-1 and Calcein AM viability assays were compared for Ag NPs toxicity assessment in coelomocytes. Metals and other xenobiotics have been demonstrated to act upon coelomocyte membranes, where they cause structural and physiological changes as lysosomal fragility and release of acid hydrolases (Engelmann et al., 2004) and subsequently, reduction in the capacity to uptake supravital dyes (neutral red; Irizar et al., 2014b, 2015b) or to transform products such as formazan salts (WST-1, WST-8; Hayashi et al., 2012) or calcein by intracellular enzymes. The Calcein AM is a fluorescence intensity based assay while NRU and WST-1 are colorimetric assays in which absorbance in specific wavelengths was measured. Interferences were not noticed between the absorption wavelengths of the colorimetric assays used in this study (540 nm and 450 nm, respectively) and Ag NPs (405-420 nm in exposure media containing 100 mg/l of Ag NPs). However, the absorbance values obtained after NRU and WST-1 assays did not entirely represent the dye retained within lysosomes or the transformed product. In spite of the washing steps, exposure medium and dye deposits in the bottom of the wells appeared to interfere with the absorbance measurements in NRU assay at the highest concentrations (10, 100 mg Ag NPs/l). Thus, this assay showed high absorbance values despite the absence of dye within exposed coelomocytes. Similarly, in WST-1 the highest absorbance values were measured at 100 mg Ag NPs/l exposure wells where the exposure medium reached the highest colour intensity. The occurrence of these interferences at the highest exposure concentrations (10 and 100 mg Ag NPs/l) could be related to the presence of Ag NPs aggregates/agglomerates or Ag precipitates in the exposure media (and consequently, in the bottom of the wells). In fact, it has been previously reported that Ag NPs aggregate in media with a high electrolyte content, so in culture media Ag has rich opportunities to form AgCl complexes (Kittler et al., 2010; Zhang et al., 2013). Moreover Kittler et al. (2010) found that PVP stabilized Ag NPs rapidly agglomerate and precipitate in serum-free cell culture medium. This fact has been confirmed herein by flow cytometric probes, which demonstrate the presence of Ag NPs aggregates or Ag precipitates in all the exposure media (without cells) with 10 and 100 mg/l of Ag NPs and AgNO₃. Moreover, microscopic observations allow distinguishing micrometric range precipitates in Leibovitz's L-15 culture medium with 100 mg Ag NPs/l.

Therefore, both NRU and WST-1 provided no reliable results about Ag NPs toxicity, at least at the highest exposure concentrations. Conversely, the NR based assays (i.e. NRU and NRRT) in earthworm coelomocytes have been found to be reliable, dose-related, and practical in the assessment of the adverse effects of metal contamination at the subcellular level of different earthworm species from different habitats (Asensio et al., 2007, 2013; Irizar et al., 2014b, 2015a; Scott-Fordsmand et al., 1988; Weeks and Svendsen, 1996). Nevertheless, Diogène et al. (1997) suggested that for *E. fetida* species coelomocytes in particular, the NRU may not be a suitable parameter to evaluate, since slow uptake and high standard deviation were observed as also recorded in Kwak et al. (2014a) for *E. andrei*. WST-1 assay is a stable and rapid method, newly validated for mouse primary lung fibroblast (Vietti et al., 2013). Thus, the possibility that the procedure followed herein for *E. fetida* coelomocytes and Ag NPs exposure need to be optimized is feasible.

Hence, among the tested assays, the Calcein AM viability assay appeared to be the most responsive, showing more clear effects produced by Ag NPs (and AgNO₃) exposures and providing simple, rapid, and accurate cell responses.

According to Calcein AM Viability assay, Ag NPs posed a gradual decrease in coelomocytes viability starting at 10 mg/l concentration. The LC₅₀ value for coelomocytes maintained in RPMI-1640 medium and exposed to Ag NPs was established at 30.48 mg/l, being the toxicity higher after exposure to Ag NPs than after AgNO₃ exposure (LC₅₀ 43.38 mg/l). Most of the *in vivo* studies (Gomes et al., 2013, 2015; Shoults-Wilson et al., 2011c), and part of *in vitro* ones (Hayashi et al., 2012), comparing the toxicity of both Ag forms stated AgNO₃ to be more toxic than Ag NPs to *E. fetida* earthworms/coelomocytes. In contrast, significantly higher cytotoxicity of Ag NPs has been reported in coelomocytes extruded from exposed earthworms (Curieses et al., 2017; Chapter 3). A similar response was observed in A549 cells exposed to Ag NPs with silver ions fractions below 2.6% (Beer et al., 2012). Furthermore, Studer et al. (2010) showed that CuO NPs had a higher toxicity than dissolved copper ions, suggesting that the plasma membrane functioned as a natural barrier for Cu but not for Cu nanoform, which once taken up by the cell was dissolved within lysosomes through the mechanism described as Trojan horse (Limbach et al., 2007). The same mechanism cannot be discarded for Ag NPs. Hence, the toxicity of the Ag NPs could be mainly caused by the nanoform *per se* (Fabrega et al., 2009a; Navarro et al., 2008) and partly by released Ag ions (Beer et al., 2012; Gomes et al., 2015), or by both forms acting together considering

the Trojan Horse effect (Hayashi et al., 2012). In any case, the toxic effects could not be attributable to its coating agent PVP-PEI since has been reported not to be toxic (Beer et al., 2012; Garcia-Velasco et al., 2016; Gomes et al., 2013; Chapter 1) and, moreover, being its LC₅₀ in coelomocytes more than two folds the value calculated for Ag NPs.

Exposure to Ag NPs caused changes in the subpopulation ratios of coelomocytes, reinforcing the idea of dissimilar sensitivities among amoebocytes and eleocytes (Curieses et al., 2017; Hayashi et al., 2012; Irizar et al., 2015b; Chapter 3). In RPMI-1640 medium, even low concentrations of Ag NPs (1 mg/l) provoked an increase in the relative number of eleocytes, which is related to a major mortality of amoebocytes. This fact is in agreement with Hayashi et al. (2012) who reported a selective cytotoxicity and preferential phagocytic uptake of Ag NPs in amoebocytes. Such cytotoxicity in amoebocytes correlated with the Ag concentrations measured in sorted amoebocytes, which followed a dose response fashion, 0.16, 0.52 and 1.00 µg Ag/10⁶ cells after exposure to 1, 10, 100 mg Ag NPs/kg, respectively. In contrast, Ag accumulation in eleocytes was higher (>2.45 µg Ag/10⁶ cells) but did not follow any gradual trend. The Ag concentration values showed in a previous study in coelomocytes exposed to Ag NPs were closer to those measured in amoebocytes (0.5 and 0.25 µg Ag/10⁶ cells after exposure to 2 and 4 mg Ag NPs/l, respectively) (Hayashi et al., 2012). Therefore, a selective intracellular accumulation of Ag NPs could happen in amoebocyte subpopulation, followed by intracellular release of Ag ions that mediated cellular damage; whereas in eleocytes, Ag NPs could be adhered to the cellular membrane, no entering the cell. This could explain the absence of toxicity in eleocyte subpopulation despite the presence of Ag after Flame-AAS analysis. Nevertheless, through the flow cytometric and sorting techniques used herein we were not able to distinguish between internalized Ag NPs or nanosilver adhered to the cellular membrane, but these approaches provided valuable information about the dissimilar response of amoebocyte and eleocyte against Ag NPs. Moreover, this work is one of few examples performing coelomocytes sorting successfully for further analysis (Flame-AAS).

5. Conclusions

In the present chapter several *in vitro* approaches (viability assays in microplate, flow cytometry, cell sorting) with primary cultures of coelomocytes have been successfully used to test the toxicity and the dissimilar response of amoebocyte and eleocyte subpopulations after PVP-PEI coated Ag NPs and AgNO₃ exposures. The coelomic fluid

occurred to be the optimal medium for coelomocytes maintenance and Ag NPs toxicity assessment though flow cytometry, but its methodological limitations made RPMI-1640 medium the best option among conventional media for coelomocytes culturing and for the development of microplate assays. NRU and WST-1 assays exhibited no reliable results due to the large unavoidable interferences with the absorbance wavelengths of the exposure media, while Calcein AM viability assay was the most accurate and responsive to assess the effects produced by Ag NPs (and AgNO₃) exposure. According to this assay, Ag NPs posed a gradual decrease in coelomocytes viability starting at 10 mg/l concentration, establishing the LC₅₀ value in RPMI-1640 medium at 30.48 mg/l. Ag NPs appeared to be more toxic than AgNO₃ (LC₅₀ 43.38 mg AgNO₃/l) for coelomocytes which could be mediated by a dissimilar uptake of the different Ag forms. Nevertheless, the observed cytotoxicity cannot be attributable to its coating agent PVP-PEI. Exposure to Ag NPs caused selective cytotoxicity in amoebocytes, which correlated with the Ag concentrations measured in sorted amoebocytes and reinforced the idea of dissimilar sensitivities among amoebocytes and eleocytes.

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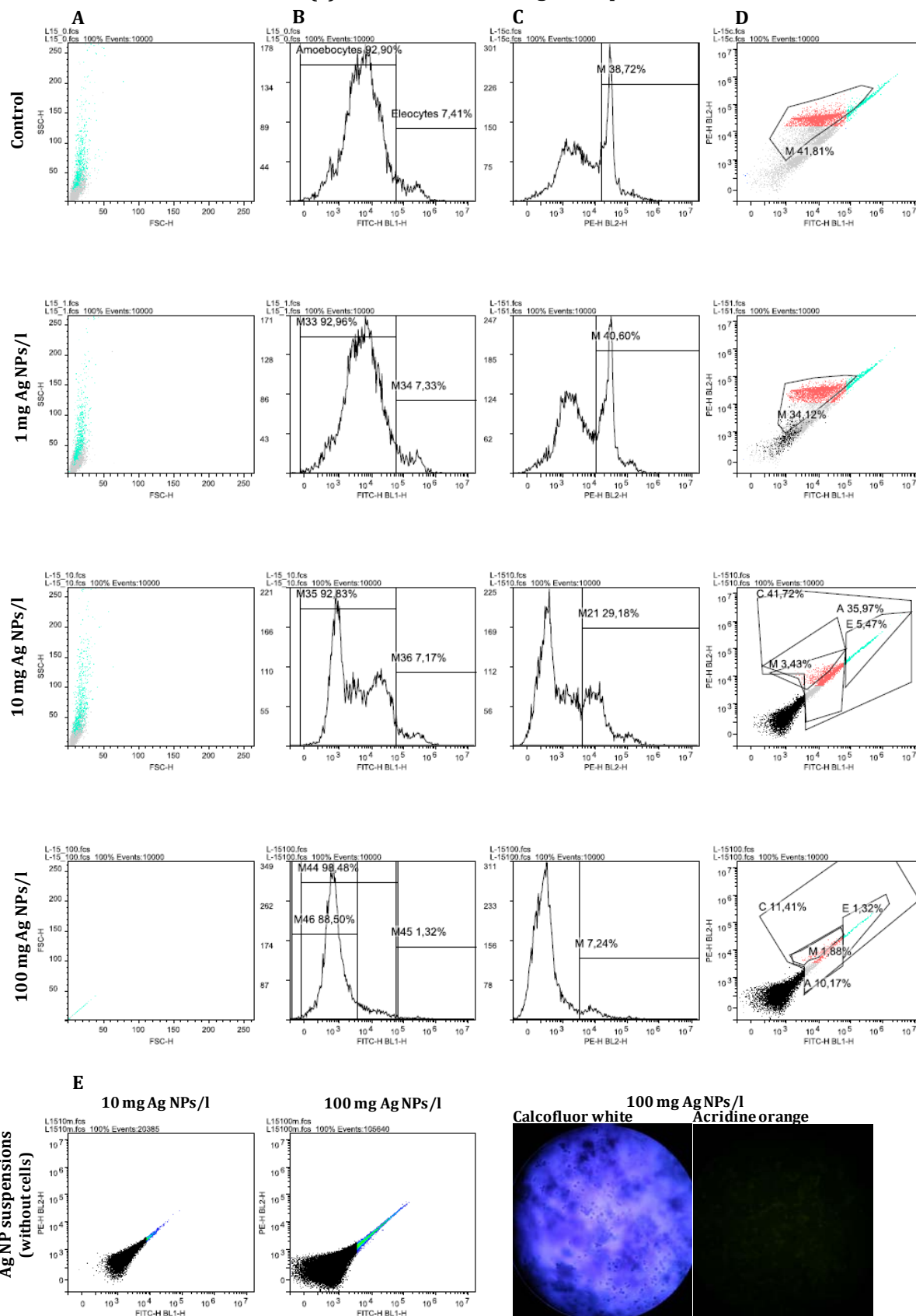
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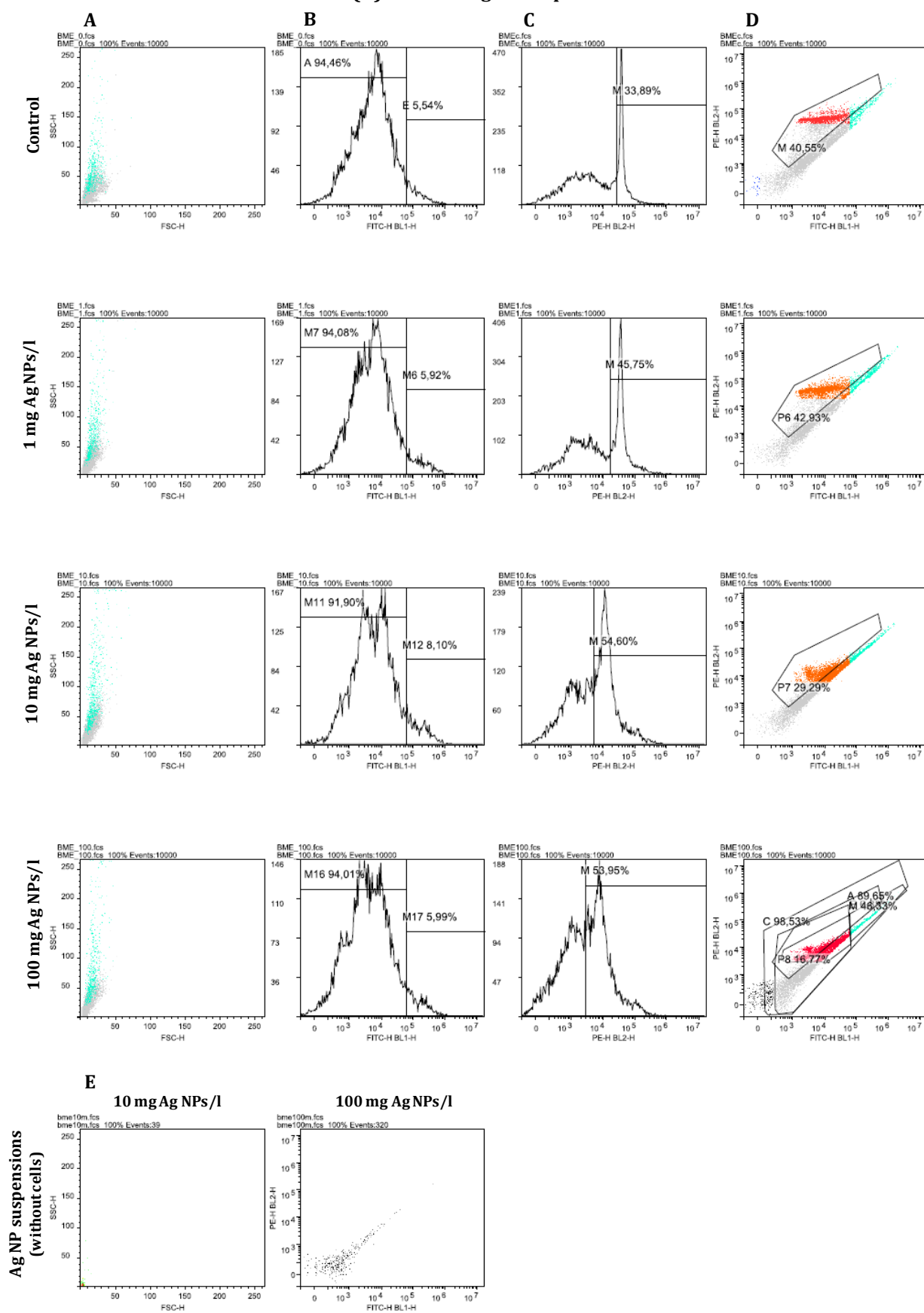
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Supplementary material

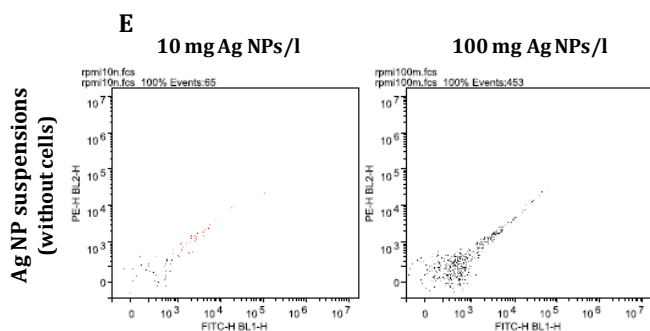
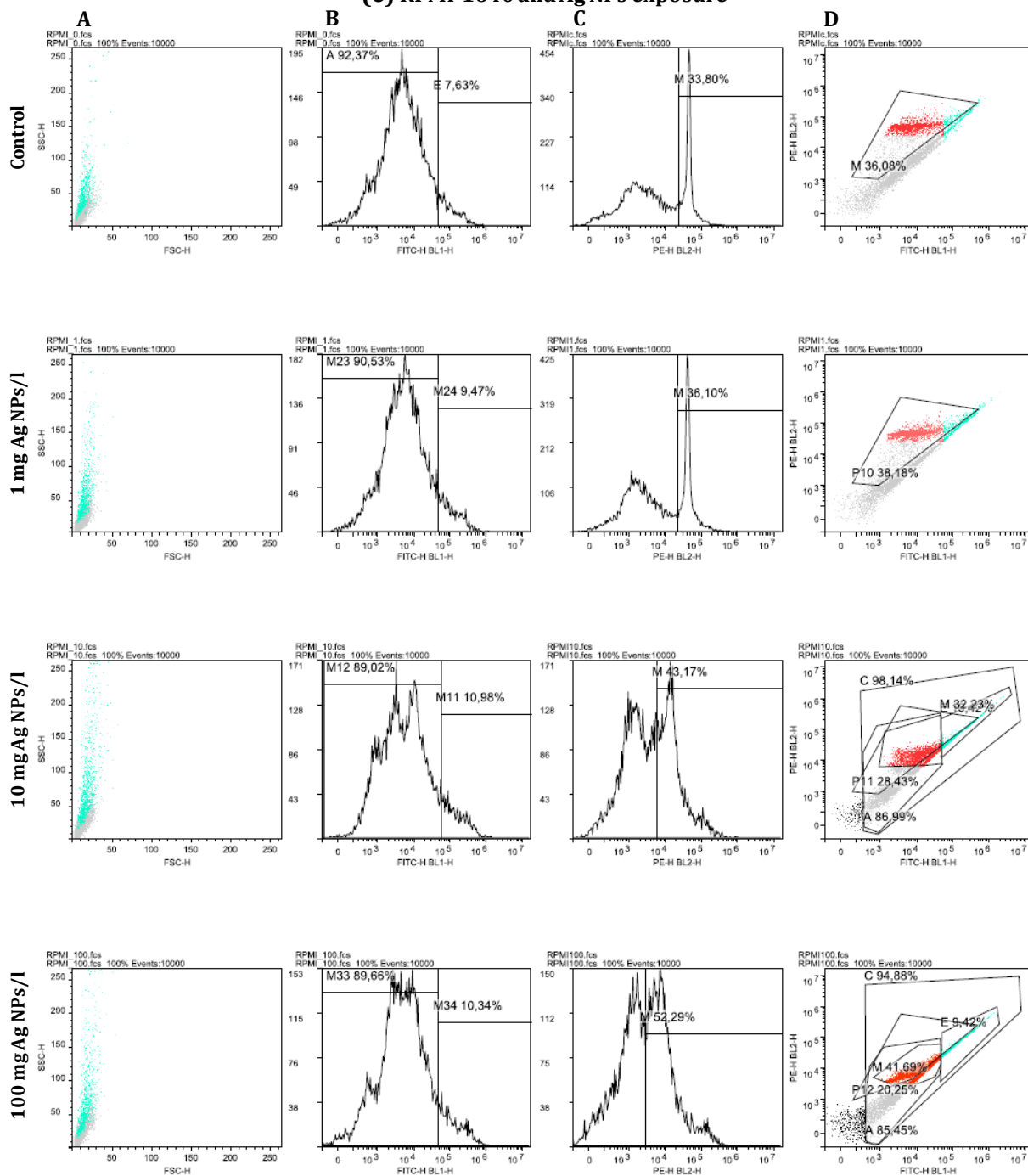
(c) Leibovitz's L-15 and Ag NPs exposure



(d) BME and Ag NPs exposure



(e) RPMI-1640 and Ag NPs exposure



SM-Figure 1. Analysis of the data with Flowlogic™ to establish subpopulation distribution and mortality in coelomocytes maintained in conventional media (Leibovitz's L-15-c-, BME-d- and RPMI-1640 medium-e-) and exposed to PVP-PEI coated Ag NPs. Amoebocytes (grey dots) and eleocytes (blue dots) distribution was established according to their complexity (SSC, Y axis) and size (FSC, X axis) features (represented by density plot, A column) and according to their autofluorescence in green (FITC histogram, B column). Mortality of the whole coelomocyte population (%) was detected through PI fluorescence (PE histogram, C column). In the case of 10 and 100 mg/l exposure concentrations, amoebocyte and eleocyte relative number and mortality of coelomocytes were assessed by density plot (amoebocytes in grey, eleocytes in blue and dead coelomocytes in red, D column) after having subtracted the particles detected by density plot in the exposure media without cells (E line).

IV. GENERAL DISCUSSION

Silver nanoparticles (**Ag NPs**) are the most frequently used nanomaterials (NMs), with 438 nanosilver containing products (24% of the total products containing NMs; e.g. textiles, detergents and cosmetics) (Vance et al., 2015) and numerous applications in electronics and biomedicine due to their well-documented antimicrobial properties (Nowack et al., 2011). These properties that make Ag NPs so attractive to commercialization have also lead to a great concern due to the expected increasing inputs of Ag NPs into the environment (Yu et al., 2013) when there is still a gap of knowledge regarding their release, fate, behavior and toxicity on the biota. In soils, the major sources of Ag NPs are the disposal of waste water treatment plant sludges or their incineration and posterior deposition of residues (Tourinho et al., 2012). The impacts exerted by Ag NPs are being extensively studied in the different environmental compartments, including **soils** although in a less extent, where different test organism, soil types, exposure concentrations and endpoints have been employed for assessment purposes in the frame of **nanotoxicology**.

In this thesis work the toxicity assessment of PVP-PEI coated Ag NPs in soil was carried out using *Eisenia fetida* earthworms. The accuracy of this earthworm species for soil health assessment could be controversial since it is epigeic, living in decaying organic matter, in compost or mold. Nevertheless, it represents a commercially available, inexpensive and easily cultured model for experimentation (Bilej et al., 2010). These aspects together with their biological advantages (e.g. short life cycle, prolific, direct uptake of chemicals by dermis, oral uptake) and susceptibility to pollutants have made *E. fetida* broadly used in standard toxicity tests (OECD, ISO). Previous works assessing Ag NPs toxicity in earthworms used *E. fetida*, *E. andrei*, *Enchytraeus albidus*, *Lumbricus rubellus* or *L. terrestris*, being *E. fetida* by far the most commonly used species. Among the total of studies published in the last 5 years dealing with Ag NPs toxicity in earthworms only few chose an endogeic earthworm (*L. rubellus*, Diez-Ortiz et al., 2015b; Makama et al., 2016; Van der Ploeg et al., 2014) limiting the comparison with present results.

Presently, standard toxicity test (OECD), the integration of a battery of biomarkers at different levels of biological organization and *in vitro* approaches with coelomocytes have been carried out with *E. fetida* in order to obtain relevant toxicity data regarding PVP-PEI coated Ag NPs. Beforehand, the main uptake route, an accurate test soil for its maintenance and the optimization of the conditions for *in vitro* approaches with

coelomocytes were defined to ensure a reliable screening diagnosis of these Ag NPs in *E. fetida*.

OECD tests with *E. fetida* are focused on acute (Acute toxicity test-207, OECD 1984) and chronic (Reproduction test-222, OECD, 2004) bioassays, using lethal and sublethal endpoints, respectively. Acute toxicity tests are useful for screening of chemicals and can be carried out placing earthworms in different exposure media (Paper Contact test and Artificial Soil test). Since earthworms are able to uptake chemicals by soil ingestion (oral uptake) and from pore water through the outer body wall (dermal uptake) (Lord et al., 1980), the Paper Contact test reflects a dermal uptake while both, dermal and oral uptakes can be observed during Artificial Soil and Reproduction tests. Hence, apart from providing toxicity data, these tests allowed understanding the behaviour of Ag NPs in two different exposure conditions (aqueous and soil matrices), the main **uptake route** in earthworms and the resulting toxicity (Chapter 1). The Paper Contact test suggested the dermal absorption of Ag ions released from Ag NPs through pore water of soils, which produced the disruption of the tegument and enhanced weight loss and mortality. The exposure in artificial soil revealed the ingestion as the main uptake route of Ag NPs in real conditions. Therefore, in a real scenario in which Ag NPs would enter soils by the disposal of sewage sludges, earthworms would be good candidates to ingest this material since many species feed on organic debris on the surface (Coutris et al., 2012; Kiser et al., 2009). Once ingested, a fraction of the Ag NPs could be processed in the earthworm gastro-intestinal tract to again be dispersed throughout the entire soil profile through faeces. The remaining Ag NPs could be internalized by the digestive gut epithelium, producing severe effects at organism level. For instance, in this work affection to survival, growth and reproduction have been proven with different toxicity values: LC₅₀ 144.20 ppm, EC₅₀ 57.62 ppm, EC₅₀ 17.92 ppm; respectively

For an accurate assessment of NPs toxicity the intrinsic properties such as chemical composition, shape, size and surface area (coating agent) must be taken into consideration (Tourinho et al., 2012). Among them, the formulation of the **coating agent** can be a crucial factor that influence on toxicity. According to manufacturer specifications the Ag NPs used herein were stable in water as they were coated by polyvinylpyrrolidone-polyethylenimine (PVP-PEI) (Appendix I). PVP has been extensively used as coating agent for Ag NPs (Hayashi et al., 2012; Heckmann et al., 2011; Shoults-Wilson et al., 2011a; Tsyusko et al., 2012), stabilizing them and

preventing ion release and reducing agglomeration (Misra et al., 2012). The lack of toxicity due to PVP has been previously reported (Beer et al., 2012; Gomes et al., 2013) and presently it has been confirmed that the observed toxicity was not attributable to PVP-PEI. In fact, both the Paper Contact test (Chapter 1) and *in vitro* assays in microplate (Chapter 3) showed that PVP-PEI toxicity was two folds lower than the toxicity exerted by PVP-PEI coated Ag NPs. Thus, the effects observed all over this thesis work would be due to the Ag NPs itself (or to the released ions) but not to the coating agent PVP-PEI.

Together with NPs characteristics, soil physico-chemical factors (pH, CEC, WHC, clay and OM contents) and the interaction of NPs with the medium (e.g. dissolution, aggregation or agglomeration, sorption to larger particles), will affect NPs behaviour, fate and toxicity to earthworms (Joško and Oleszczuk, 2013, Klaine et al., 2008; Ren et al., 2016; Tourinho et al., 2012). Therefore, in Chapter 2 experiments were carried out in two widely used standard **test soils**, OECD and LUFA 2.3 soils, which mainly differ in OM and clay contents. The same exposure concentration of Ag NPs produced earlier and stronger effects in soils with low clay and organic matter contents (LUFA 2.3), probably due to a dissimilar speciation and bioavailability of Ag NPs and also to a different behavior of the earthworms depending on the soil substrate. In OECD soils Ag NPs could be coupled to the solid phase of the soil and coated by OM, which would suppress dissolution (Cornelis et al., 2012; Klitzke et al., 2015); whereas in LUFA 2.3 soils, Ag could be dissolved in soil pore water and subjected to earlier oxidation processes (Di Toro et al., 1991; Klaine et al., 2008). In addition, even if *E. fetida* is considered tolerant to many soil types, allowing the testing in different soils (Lanno et al., 2004), earthworms maintained in LUFA 2.3 soil for 14 days showed severe weight losses probably due to the starvation of earthworms, which masked the real toxicity of Ag NPs. For further studies the implementation of an Avoidance test (ISO 17512-1, 2008a) could help determining the accuracy of soils regarding behaviour of *E. fetida*. Irrespective of this, the integration of the responses at different levels of biological complexity in *E. fetida* allowed discriminating the toxic effects exerted by PVP-PEI coated Ag NPs in standard soils with different physico-chemical characteristics (Chapter 2). In conclusion, OECD standard soil occurred to be more appropriate test soil to assess Ag NPs toxicity using *E. fetida* due to the accuracy of its physico-chemical characteristics and thus, this soil was used in the following experiment aiming to compare the toxicity effects produced by Ag NPs and the soluble form (AgNO₃) (Chapter 3).

The toxicity values obtained by means of standard toxicity tests in OECD soils reinforce reproduction output as the most sensitive and ecologically relevant endpoint to assess Ag NPs toxicity in Oligochaeta (Gomes et al., 2013; Hekmann et al., 2011; Schlich et al., 2013; Shoults-Wilson et al., 2011a, 2011b, Chapter 1). Nevertheless, a standard toxicity test such as earthworm Reproduction test-222 (OECD, 2004) has limitations when the amount of soils to be tested is high and when a rapid assessment is required due to its duration (8 weeks). Moreover, toxic effects not detectable through traditional endpoints (mortality and weight loss) could happen at lower biological complexity levels, as it occurred after exposing earthworms to Ag NPs and AgNO₃ (Chapter 3). In addition, the concentrations used in standard toxicity tests are normally orders of magnitude higher than those modelled for the environment. Thus, in Chapter 2 and 3 the toxicity of PVP-PEI coated Ag NPs was assessed applying a battery of **biomarkers at different levels of biological complexity** at sublethal concentrations, including those close to environmental predictions. All concentrations were selected from the survival and reproductive output data scored from standard tests in Chapter 1 (50 mg Ag NPs/kg) and from *in silico* model predictions (Gottschalk et al., 2009) in soils amended with WWTP sludges or sludge incineration residues (0.05 mg Ag NPs/kg). The use of biomarkers in ecotoxicology allows detecting changes at low concentrations and short exposure periods. In OECD soil **high but sublethal concentrations** of Ag NPs (50 mg Ag NPs/kg) caused significant increases in catalase activity and DNA damage in OECD soils after 14 days, reinforcing the idea of oxidative stress and genotoxicity as relevant mechanisms of toxicity produced by Ag NPs in earthworms (McShan et al., 2014). The selected biomarkers can be put together in integrative indexes to have a conclusive idea about the toxicity exerted by chemical compounds or test media. Thus, the IBR index, particularly used in marine monitoring programmes, allowed the discrimination of the responses as function of time (3 vs. 14 days) and to distinguish the overall toxicity of different exposure concentrations. Therefore, the integration of the responses at different levels of biological complexity of *E. fetida* provide a reliable and conclusive outline of the toxicity exerted by PVP-PEI coated Ag NPs at different exposure conditions.

After exposure of earthworms to **close to predicted environmental concentrations** of Ag NPs (0.05 mg Ag NPs/kg) significant effects were not observed in *E. fetida* maintained in OECD soil (Chapter 2). However, as stated in Chapter 3 certain alterations regarding metal detoxification mechanisms (MT, *mt*) and in antioxidant responses (CAT,

cat) were observed at the same concentration (0.05 mg AgNPs/kg), which could be due to a dissimilar pH of the OECD soils (6.42-6.89 vs. 5.89-5.99 in Chapter 2 and Chapter 3, respectively). Accordingly, it has been proven by many authors that metal solubility and speciation are highly pH dependent (Leveque et al. 2013; Spurgeon et al., 2006) and thus, in a moderately acidic soil (OECD in Chapter 3) the bioavailability of Ag NPs could lead to an increase in metallothionein levels and catalase activity. This fact proves, once again, that soil characteristics influence more in the toxicity than the own NP features (Shoults-Wilson et al., 2011a).

In nanotoxicology there is an ongoing challenge regarding whether toxicity is caused by the nanoform or due to NPs solubility and ion release, or a combination of both. Previous reports indicate that toxicity of Ag NPs may be caused by released Ag ions (Hayashi et al., 2013a; Gomes et al., 2015), however Ag NPs specific effects have also been demonstrated (Fabrega et al., 2009a). Furthermore, the release of ions from Ag NPs has been proved not to be the responsible of the avoidance effect in *E. fetida* (Tourinho et al., 2012). In order to better understand the potential effects of Ag NPs, both NPs and ionic form need to be considered (Misra et al., 2012; Tourinho et al., 2012) and thus, the design of experiments comparing the effects of Ag NPs and the **soluble form** (AgNO₃) are strongly recommended. The majority of these works showed earlier and more toxic effects after silver salts exposure, which could be related to oxidation time (quicker ion release) or a slower uptake of Ag NPs or due to a less mobility of the nanoform (Coutris et al., 2012; Hayashi et al., 2013a; Shoults-Wilson et al., 2011b; Gomes et al., 2015). However, as presently observed PVP-PEI coated Ag NPs and AgNO₃ induced similar changes in metal detoxification mechanisms and in antioxidant responses of *E. fetida* after exposure to high but sublethal (50 mg/kg) and to close to environmental (0.05 mg/kg) concentrations. In order to decipher to which extent dissolved ions are the responsible of the observed toxicity ionic Ag can be measured in exposure media (soils -solid and pore water phases- and culture media) through ultracentrifugation and ultrafiltration techniques (Diez-Ortiz et al., 2015a) or ion selective electrode (ISE) (Gomes et al., 2013). Diez-Ortiz et al. (2015b) revealed ten times higher Ag concentrations in pore water samples in soil spiked with ionic Ag than in soil spiked with Ag NPs at the same total nominal Ag concentrations (100 mg/kg). However, at nominal concentrations below 50 mg Ag/kg (concentrations used herein) it has not been possible to detect ionic Ag in soil pore water of spiked soils.

In contrast with the responses observed at biochemical level, cytotoxicity was higher after exposure to PVP-PEI coated Ag NPs and, dissimilar sensitivities were recorded among coelomocytes subpopulations depending on the Ag form. Recent *in vitro* tests with coelomocytes exposed to metal salts (e.g. CdCl₂, CuCl₂, PbCl₂) demonstrated eleocytes to be more sensitive than amoebocytes (Irizar et al., 2015b), and the same was observed herein after exposure to Ag salts (AgNO₃) (Chapter 3 and Chapter 4). In contrast, a major sensitivity of amoebocytes was shown after exposure to Ag NPs, which is supported by their selective intracellular accumulation of Ag NPs and their role as scavengers of Ag NPs found by Hayashi et al. (2012). Thus, a phagocytic uptake of Ag NPs may have occurred in amoebocytes, followed by intracellular particle oxidation which can produce cellular damage (Trojan horse effect; Hayashi et al., 2012; Limbach et al., 2007).

In order to get deeper knowledge about these issues *in vitro* approaches were performed with primary cultures of coelomocytes. However, first of all, optimal culture conditions and *in vitro* tests were selected in order to ensure an accurate response of cells against Ag NPs. RPMI-1640 medium was the best option among conventional media for coelomocytes culturing and for the development of microplate assays. Calcein AM viability assay was the most accurate and responsive test to assess the effects produced by Ag NPs (and AgNO₃) exposure. According to this assay, Ag NPs caused selective cytotoxicity in amoebocytes, which correlated with the Ag concentrations measured in sorted amoebocytes and reinforced the idea of dissimilar sensitivities against particulate or dissolved metals among amoebocytes and eleocytes. Further studies based on Transmission Electron Microscopy (TEM) of isolated subpopulations would help ensuring this aspect. Nevertheless, *in vitro* approaches (viability assays in microplate, flow cytometry, cell sorting) with primary cultures of coelomocytes provide valuable information about PVP-PEI coated Ag NPs toxicity and about the dissimilar response of amoebocyte and eleocyte against Ag NPs. Moreover, as far as we know, this work is one of few examples performing coelomocytes sorting successfully for further analysis (Flame-AAS).

Even if the ecological relevance of biomarkers measured at low levels of biological complexity has been questioned, presently endpoints measured in coelomocytes retrieved from exposed *E. fetida* (cell viability and transcription levels-*mt* and *cat*-quantification) offered rapid and accurate information to predict impairments caused at longer exposure times and higher complexity levels (Chapter 1 and Chapter 3). In fact,

the concentration causing depletion on coelomocytes viability correlated with the concentration in which reproductive failure occurred. Even more, molecular endpoints measured in coelomocytes of earthworms exposed to Ag NPs *in vivo* were less invasive and represent an alternative to those measured at higher levels of biological complexity. In addition, even if it is a challenge to link *in vitro* approaches with more ecologically relevant endpoints (such reproduction and growth), the toxicity indices (LC₅₀, EC₅₀) obtained in this thesis at different levels (Chapter 1, Chapter 4) followed the same pattern, indicating the high potential of *in vitro* assays with coelomocytes for accurate diagnosis, screening and toxicity testing of emerging chemicals such as Ag NPs.

This thesis work represents a scientific based goal approach for the diagnosis of toxic effects exerted by Ag NPs soil using *E. fetida*. The toxicity data obtained from this thesis work would be helpful for the development of risk assessment strategies and establishment of regulatory criteria for the protection of the environment by environmental protection regulatory regimes (EPA, CLP, ECHA, REACH). Nevertheless, these lethal and effect concentrations were obtained in laboratory experiments using standard soils, whereas in a real scenario soil components and varying factors may affect the fate and behaviour of NPs and subsequently increase their toxicity to organisms inhabiting soils. Hence, in order to increase realism, future experiments should be designed with real soils in which WWTP sludges have been applied with amendment purposes. The same endpoints applied in this work could be used in those experiments. A challenging future of possibilities is open.

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V. CONCLUSIONS AND THESIS

CONCLUSIONS

- 1- The combination of standard toxicity tests (OECD-207 and OECD-222) with cellular biomarkers provided relevant toxicity data and allow to understand the behavior and to decipher the uptake routes of PVP-PEI coated Ag NPs in *Eisenia fetida* earthworms under two different exposure conditions, aqueous and soil matrices.
 - a. The Paper Contact test suggested the dermal absorption of Ag ions released from Ag NPs through pore water of soils that produced the disruption of the tegument and enhanced weight loss and mortality.
 - b. The exposure in artificial soil revealed the ingestion as the main uptake route of Ag NPs that were internalized by the digestive gut epithelium producing severe effects on survival (LC₅₀ 144.20 ppm), growth (EC₅₀ 57.62 ppm) and reproduction (EC₅₀ 17.92 ppm).
 - c. The toxicity of the coating agent PVP-PEI seemed to be meaningless.
- 2- The integration of the responses at different levels of biological complexity of *E. fetida* provides a reliable and conclusive outline of the toxicity exerted by PVP-PEI coated Ag NPs at different exposure times, at environmentally relevant concentrations in standard soils with different physico-chemical characteristics. The same exposure concentration of Ag NPs produced earlier and stronger effects (weight loss, reduction in the viability of coelomocytes, increase in catalase activity and DNA damage) in soils with low clay and organic matter contents (LUF 2.3).
- 3- PVP-PEI coated Ag NPs and AgNO₃ induced similar changes in metal detoxification mechanisms and in antioxidant responses of *E. fetida* after exposure to high but sublethal (50 mg/kg) and to close to environmental (0.05 mg/kg) concentrations. In contrast, cytotoxicity was higher after exposure to PVP-PEI coated Ag NPs but, dissimilar sensitivities were recorded among coelomocytes subpopulations depending on the Ag form, suggesting a different mode of action of nanoparticulate/salt/ ionic Ag depending on the target cell.
- 4- Cell viability and transcription levels (*mt* and *cat*) quantified in coelomocytes retrieved from *E. fetida* earthworms exposed *in vivo* to PVP-PEI coated Ag NPs offered rapid and accurate information to predict impairments caused at higher

complexity levels. Thus, the utilization of coelomocytes to assess cellular and molecular level endpoints represents a relevant alternative for development of non-invasive biomarkers.

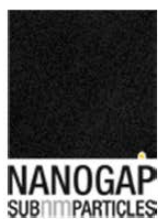
5- *In vitro* approaches (viability assays in microplate, flow cytometry, cell sorting) with primary cultures of coelomocytes have been successfully used to test the toxicity and the dissimilar responses of amoebocyte and eleocyte subpopulations after exposure to PVP-PEI coated Ag NPs. RPMI-1640 medium was the best option among conventional media for coelomocytes culturing and for the development of microplate assays. Calcein AM viability assay was the most accurate and responsive test to assess the effects produced by Ag NPs (and AgNO₃) exposure. According to this assay, Ag NPs were more toxic than AgNO₃ for coelomocytes (LC₅₀ 30.48 mg Ag NPs/l vs. 43.38 mg AgNO₃/l). Exposure to Ag NPs caused selective cytotoxicity in amoebocytes, which correlated with the Ag concentrations measured in sorted amoebocytes and reinforced the idea of dissimilar sensitivities against nanoparticulate or dissolved metals among amoebocytes and eleocytes.

THESIS

An accurate diagnosis of the toxicity exerted by PVP-PEI coated Ag NPs in soils has been achieved in *Eisenia fetida* earthworms, after establishing the main uptake route and the definition of accurate test soil, using standard tests together with the integration of biomarker responses at different levels of biological complexity and the implementation of *in vitro* approaches with coelomocytes.

VI. APPENDIX I

CHARACTERIZATION OF Ag NPs



CERTIFICATE OF ANALYSIS

NGAP NP Ag-2106-W Silver nanoparticles

Chemist: Noelia Durán

Product: Aqueous dispersion of silver nanoparticles stabilized with PEI and PVP.

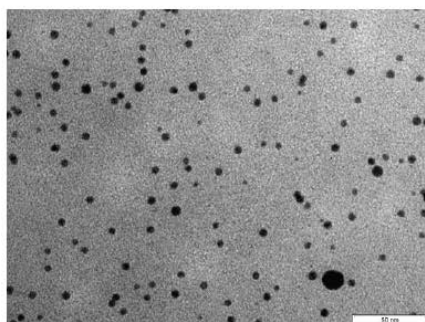
Batch: 08.02.11

Date: 19.04.2013

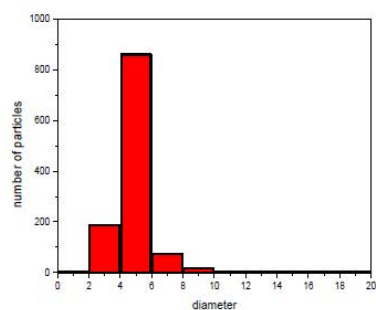
2106-W	Size distribution	Product Form	Concentration	Colour
	4,9 ± 1,4 nm	Aqueous solution	10 g/L Ag	Black, Yellow (when diluted)
	Volume	Density	Density of particles	Storage
	1L	1,031 g/ml	≈ 8,7·10 ¹⁸ part/l	At room temperature

Characterization:

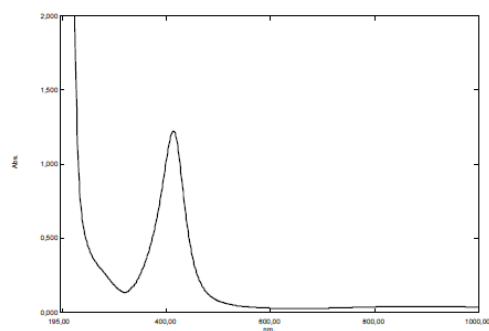
TEM



Size distribution



UV-Visible



Noelia Durán

VII. APPENDIX II

PROTOCOLS

1. Histology

1.1. Non-fixed samples

1.1.1. Hematoxylin-eosin (H/E) staining

1.1.2. Alcian Blue pH 2.5 staining

1.2. Fixed samples

1.2.1. Autometallography

2. Coelomocyte extrusion

2.1. Cell counting and viability (Trypan Blue)

3. Cell viability assays in microplate

3.1. Neutral Red Uptake (NRU)

3.2. Cell proliferation kit WST-1

3.3. Calcein AM viability assay

4. Metallothioneins (MTs) measurement by spectrophotometry

5. CAT activity measured by H₂O₂ consumption

5.1. Total protein quantification in microplate

6. Comet assay with earthworm coelomocytes

7. Gene transcription levels by Real-Time qPCR

7.1. Tri-reagent protocol (total RNA extraction)

7.2. RNA Purification through RNase-Free DNase Set

7.3. First-Strand cDNA Synthesis protocol (Retro Transcription-RT-)

7.4. Establishment of primer conditions for PCR

7.5. cDNA concentration through Quant iT OliGreen ssDNA assay Kit

7.6. Relative Quantification (RQ) level calculation

1. Histology

1.1. Non-fixed samples

- a. Prior to the dissection, left earthworms voiding their gut contents during 24 h (deuration) and clean them thoroughly with dH_2O to avoid soil particles
- b. Dissect out the post-clitellar sections of earthworms, freeze them in liquid nitrogen and store (-80 °C) samples
- c. Cut transversal sections of the frozen earthworms in a cryotome (10 μ m, -24 °C Leica CM3000)
- d. Maintain tissue sections at -40 °C (no longer than 72 h) to perform the stainings

1.1.1. Hematoxylin-eosin (H/E) staining

- Move slides containing unfixed 10 μ m cryostat sections from -40 °C to 4 °C for 10 min and then keep them at room temperature for other 10 min
- Put them in distilled H_2O for 5 min
- Stain sections in Harris hematoxylin (HHS128 Sigma-Aldrich) for 4min
- Wash them in dH_2O for 4 min
- Wash them in dH_2O (10 sec x 3)
- Put them in acid alcohol for 10 s
- Wash them in dH_2O for 5 min
- Put them in acid lithium carbonate for 10 s
- Wash them in dH_2O for 1 min
- Stain sections in eosin yellowish alcoholic solution 1% (256879 Panreac) for 1 min
- Mount slides in glycerine (Kaisers' glycerol gelatin)

1.1.2. Alcian Blue pH 2.5 staining

Alcian Blue (A3157 Sigma-Aldrich)	1 g
dH_2O	97 ml
Acetic acid glacial (Scharlau AC0343)	3 ml

- Move slides containing unfixed 10 μ m cryostat sections from -40 °C to 4 °C for 10 min and then keep them at room temperature for other 10 min
- Put them in distilled H_2O
- Stain sections in Alcian Blue (pH 2.5) for 30 min
- Wash them in dH_2O (10 sec x 3)
- Mount slides in glycerin (Kaisers' glycerol gelatin)

1.2. Fixed samples

- a. Prior to the dissection, left earthworms voiding their gut contents during 24 h (depuration) and clean them thoroughly with dH_2O to avoid soil particles
- b. Dissect out the post-clitellar sections (~5 segments), immerse them in formalin (10% commercial formaldehyde in 0.1 M phosphate buffered saline solution-PBS- with 0.23% NaCl, 1 l per 20 samples) and keep at 4 °C for 24 h
- c. After 24 h remove formalin and store histological samples in 70% alcohol at 4 °C
- d. Fixation and dehydration in Tissue processor (Leica ASP 300):

70% ethanol	1 h
96% ethanol	1 h
96% ethanol	1 h
100% ethanol	1 h
100% ethanol	1 h
100% ethanol/xylene	1 h
xylene	1 h
xylene	1 h
paraffin	2 h
paraffin	2 h

- e. Embed samples in paraffin to obtain paraffin blocks
- f. Cut tissue sections (5 μ m) with the aid of a rotary microtome (Leica RM 2125RT)
- g. Dewax with xylene, rehydrate through several baths of ethanol (d step in reverse) and air dry slides

1.2.1. Autometallography

BBinternational Enhancing Kit (BBI Life Sciences)
 Mix Initiator and Enhancer reagents (1:1)

- Applied as drops into each tissue section and place in a moisture chamber to avoid desiccation
- Check the reaction in control and treated samples and stop when it is visible (20-25 min).
- Wash them in dH_2O (10 sec x 3)
- Mount slides in glycerin (Kaisers' glycerol gelatin)

2. Coelomocyte extrusion

(Irizar *et al.*, 2014b)

A. Equipment and Reagents

Equipment:

- Centrifuge
- Light microscope
- Laboratory balance
- Glass Petri dishes
- 9V batteries
- Pasteur
- Tweezers
- Falcon tubes of 15 and 50 ml
- Pipettes (and multichannel pipette)
- Cell counter or hemocytometer (neubauer chamber)



Reagents:

- Distilled water (dH_2O)
- $Na_2HPO_4 \cdot 12H_2O$ (71649 Sigma or similar)
- $NaH_2PO_4 \cdot H_2O$ (Panreac 131965 or similar)
- NaCl (Sigma S-9888 or similar)
- EDTA (Sigma E-6758 or similar)
- Trypan blue solution 0.4% (Sigma T8154 or similar)

B. Preparation of solutions

- *Phosphate buffer saline -PBS-, pH 7.4 (for 1 l)*

$Na_2HPO_4 \cdot 12 H_2O$	28.98 g in 808 ml dH_2O
$NaH_2PO_4 \cdot H_2O$	2.65 g in 192 ml dH_2O
NaCl	2.32 g

Dissolve the sodium phosphates separately, mix them and then add sodium chloride. Check pH to be about 7.4 and store solution in the fridge, but use it at room temperature

- *Extrusion Fluid, pH 7.3 (100 ml)*

Dissolve 0.02 % EDTA in PBS and adjust to pH 7.3.

Store solution in the fridge, but use it at room temperature

C. Procedure

- a. Clean the worms with dH₂O to remove any particle of soil
- b. Gently massage the earthworms to remove intestine contents
- c. Place a pool of 5 worms per group in a glass Petri dish with 5 ml extrusion fluid (1ml/worm) and retrieve the cells by an electric shock with a 9 V battery. Coelomocytes will be extruded through dorsal pores

2.1. Cell counting and viability (Trypan Blue)

- d. Transfer the cell suspension to a 15 ml Falcon tube
- e. Centrifuge at 530 xg for 10 min at 4 °C
- f. Resuspend the pellet in 5 ml of PBS (1 ml/earthworm)
- g. Mix trypan blue solution (0.4%) with coelomocytes and count death/alive cells in a haemocytometer under an inverted microscope. Use a dilution factor of 2:

10 µl of cell solution + 10 µl trypan blue solution (0.04%)

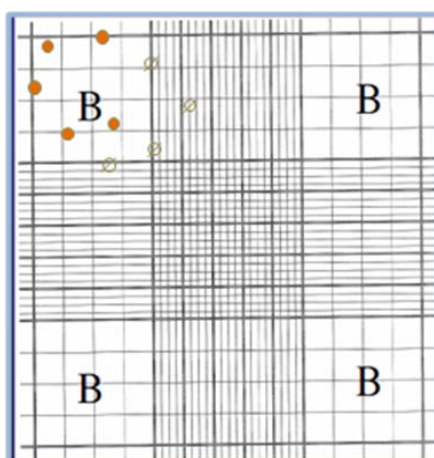
Total number of cells per ml:

Total n° of **alive** cells/ml = $(B1+B2+B3+B4)/4 \times 10^4 \times \text{dilution factor}$

+

Total n° of **dead** cells/ml = $(B1+B2+B3+B4)/4 \times 10^4 \times \text{dilution factor}$

Viability % = Total n° of alive cells per ml / Total number of cells per ml



3. Cell viability assays in microplate

- a. Adjust cell solution to 10^6 cells per ml
- b. Place 2×10^5 alive cells on each well of a 96-well microplate. 8 replicates per group
- c. Leave coelomocytes to stabilizing and attaching to the plate for 30 min at RT
- d. Centrifuge at $530 \times g$ for 5 min at 4°C .

3.1. Neutral Red Uptake (NRU) *(Irizar et al., 2014b)*

A. Equipment and Reagents

Equipment

- Centrifuge, equipped with rotors for microplates
- Pipettes (and multichannel pipette)
- 96-well microplates
- Inverted microscope
- 96-well microplate spectrophotometer

Reagents

- Distilled water
- Neutral Red dye (Sigma N-7005 or similar)
- Ethanol (Panreac 131086 or similar)
- Acetic acid glacial (Scharlau AC0343 or similar)

B. Preparation of solutions

- Neutral Red Stock Solution (NRS) (10 ml)

Dissolve 5 mg Neutral Red dye in 10 ml distilled water (Neutral Red 0.5 %)

Centrifuge NRS ($530 \times g$, 5 min) and use the supernatant

Keep in darkness and use it freshly made

- Neutral Red Working Solution (NRW) (20 ml)

Dissolve 2 ml NRS in 18 ml PBS (Neutral Red 0.05%)

Keep in darkness and use it freshly made

- Extraction Solution (for 100 ml)

Acetic acid glacial	1 ml
Ethanol	50 ml
dH ₂ O	49 ml

Store solution in the fridge, but use it at room temperature

C. Procedure

- a. Remove the supernatant by suction and replace with 200 μl of NRW (0.05%).
Make the negative control by adding 200 μl of NRW to empty wells
- b. Incubate the plate at room temperature for 30 min in darkness

- c. Centrifuge at 350 x g for 5 min at 4 °C
- d. Wash the plate with PSB until no colour is visible in the negative control, removing the supernatant and replacing it with 100 µl PBS
- e. Solubilise the dye uptaken by coelomocytes by adding 100 µl of extraction fluid. Incubate at room temperature for 5 min
- f. Shake the plate and read the absorbance at 540 nm in a microplate reader spectrophotometer

Calculations

Substrate the mean of the negative control to all group means. Supposing that the control group has the maximum retention capacity (100%), the relative values are compared using proper statistical analysis

3.2. Cell proliferation kit WST-1

A. Equipment and Reagents

Equipment

- Centrifuge, equipped with rotors for microplates
- Pipettes (and multichannel pipette)
- 96-well microplates
- Inverted microscope
- 96-well microplate spectrophotometer

Reagents

- Cell proliferation reagent (Roche Diagnostics GmbH, Mannheim)

B. Procedure

- a. Add the reagent 10 times diluted to the wells (20 µl of the reagent into the wells containing 200 µl volume). Do the same in well containing medium without cells
- b. Incubate the plate at room temperature for 3 h in darkness
- c. Read the absorbance at $Abs_{450nm} - Abs_{690nm}$ in a microplate reader spectrophotometer

Calculations

Substrate the mean of the wells containing exposure media without cells to the cell containing wells. Supposing that the control group has the maximum transformation of formazan (more metabolically active cells) (100%), the relative values are compared using proper statistical analysis

3.3. Calcein AM viability assay

A. Equipment and Reagents

Equipment

- Centrifuge, equipped with rotors for microplates
- Pipettes (and multichannel pipette)
- 96-well microplates
- Inverted microscope
- 96-well microplate fluorescence reader

Reagents

- Calcein-AM (Molecular Probes® ThermoFisher Scientific)

B. Preparation of solutions

-Calcein-AM Working Solution (2 ml)

5 μ l Calcein-AM in 1,995 ml PBS (2.5 μ M Calcein-AM)

Keep in darkness and use it freshly made

C. Procedure

- Remove the supernatant by suction and replace with 100 μ l of Calcein-AM working solution in 4 wells. In the remaining wells, instead of Calcein-AM, 100 μ l PBS were added to ensure that the loss of cells during wash steps was alike in all treatments
- Incubate the plate at room temperature for 40 min in darkness.
- Centrifuge at 350 x g for 5 min at 4 °C.
- Wash the plate twice with PBS, removing the supernatant and replacing it with 100 μ l PBS.
- Read the fluorescence at 490 \pm 20 nm excitation filter and 520 \pm 20 emission filter.

Calculations

Substrate the mean of the wells incubated with PBS to all group means. Supposing that the control group has the maximum calcein retention capacity (100%), the relative values are compared using proper statistical analysis.

4. Metallothioneins (MTs) measurement by spectrophotometry

(Viarengo et al., 1997)

A. Preparation of solutions

-Homogenization buffer (pH 8.6, 50 ml)

8.557 g sucrose (0.5M)
0.157 g Tris-HCl (0.02M)
0.006 mM Leuptyne (150 µl)
0.5 mM PMSF (Phenylmethylsulphonyl fluoride, 0.004 g PMSF in 75 µl d H₂O)
0.01% β-mercaptoethanol (5µl)

-DTNB (5,5-dithiobis-2-nitrobenzoic acid, pH 8, 20 ml)

0.2 M Na- phosphate (1.43g)
2 M NaCl (1.16 g)
43 mM DTNB *Freshly made

B. Procedure

a. Homogenization

- Weight a pool of ~ 0.5 g
- Homogenize in 3 volumes of homogenization buffer

b. Centrifugation

- Centrifuge at 18000 rpm (30000 xg) for 20 min at 4 °C
- Transfer the supernatant to a centrifuge tube
- Add 1.05 ml of cold (-20°C) absolute ethanol and 80 µl of chloroform and vortex
- Centrifuge at 1500 rpm (530 x g) for 10 min at 4°C
- Collect the supernatant (1.8 ml minimum) and transfer to a new centrifuge tube
- Add 40 µl of 37% HCl, 10µl of RNA and 3 volumes of cold ethanol (5.55 ml)
- Store the tubes sealed with paraffin at -20 °C for 1h
- Recentrifuge at 1500 rpm (530 x g) for 10 min and discard the supernatant
- Wash the pellet with 2 ml ethanol/chloroform/homogenizing buffer (-20°C) (87:1:12)
- Centrifuge at 1500 rpm (530 x g) for 10 min and discard the supernatant
- Dry pellets at RT

c. Resuspension of the MT enriched fraction (for 0.5 g sample)

- Add to the pellet 150 µl of 0.25 M NaCl and 150µl 1N HCl/4mM EDTA
- Vortex and stir to resuspend the pellet
- Add 4.2 ml DTNB solution and centrifuge for 5 min at RT at 1500 rpm (530 xg)

d. DTNB assay: Standard curve

	GSH stock solution	0.25 M NaCl	1N HCl	DTNB	Total vol.
Blank	-	150 µl	150 µl	4.2 ml	4.5 ml
10	10 µl	140 µl	150 µl	4.2 ml	4.5 ml
20	20 µl	130 µl	150 µl	4.2 ml	4.5 ml
40	40 µl	110 µl	150 µl	4.2 ml	4.5 ml
80	80 µl	70 µl	150 µl	4.2 ml	4.5 ml

GSH: Reduced glutathione

- Centrifuge samples at 1500 rpm for 5 min at RT

- e. -Add 300 μ l of the serial dilutions (standard curve) and of sample (4 replicates in both)
- f. Measure absorbance at 412 nm in a microplate reader spectrophotometer

5. CAT activity measured by H₂O₂ consumption

(Claiborne, 1985)

A. Preparation of solutions

-TVBE buffer (pH 7.4, 1l)

1 nM NaHCO₃ (84 mg)
 1mM EDTA (10 ml 0.1 M EDTA-3.802 g in 100 ml dH₂O-)
 0.1% Abs Ethanol (1 ml)
 0.01% Triton X-100 (100 μ l)

-Potassium dihydrogen phosphate (KH₂PO₄, 50 mM)

KH₂PO₄ 3.402 g
 d H₂O 500 ml

- 20.2 mM H₂O₂ in KH₂PO₄*Freshly prepared

H₂O₂ 46 μ l
 KH₂PO₄ 20 ml

B. Procedure

a. Homogenization

- Weight the samples
- Homogenize in 5 volumes of TVBE homogenization buffer

b. Prepare serial dilutions for the standard curve

	TVBE (μ l)	H ₂ O ₂ in KH ₂ PO ₄ (μ l)	mM H ₂ O ₂	Volume well (μ l)	μ mol/well
1	0	1000	20.28	300	5.9826
2	400	600	12.168	300	3.58956
3	600	400	8.112	300	2.39304
4	800	200	4.056	300	1.19652
5	900	100	2.028	300	0.59826
6	950	50	1.014	300	0.29913
7	975	25	0.507	300	0.149565
8	1000	0	0	300	0

- c. Add 5 μ l of the homogenized sample (4 replicates) in a 96 wells UV microplate

- d. Add 300 μl of the calibration solutions to perform the standard curve (4 replicates)
- e. Add 295 μl H_2O_2 in KH_2PO_4 to the wells containing 5 μl samples
- f. Measure absorbance at 240 nm in a microplate reader spectrophotometer 2-10 min after the addition of H_2O_2 in KH_2PO_4 to the samples. Read absorbance every 22 s during 4 min

Calculations

Select the kinetic section with the higher slope from the standard curve and use it for all the samples and replicates. Activity is represented as $\mu\text{mol H}_2\text{O}_2/\text{mg protein}/\text{min}$. Total protein content was estimated according to Lowry et al. (1951)

5.1. Total protein quantification in microplate

(Lowry et al., 1951)

(Bio-Rad DC Protein Assay)

- a. Preparation of serial dilutions for the Standard curve (For a bovine γ -globuline-Bio-Rad 500-0005- concentration of 1.5 mg/ml)
0.00 mg/ml (Homogenation buffer)
0.15 mg/ml (5 μl of standard + 45 μl of homogenation buffer)
0.30 mg/ml (10 μl of standard + 40 μl of homogenation buffer)
0.60 mg/ml (20 μl of standard + 30 μl of homogenation buffer)
1.00 mg/ml (36 μl of standard + 18 μl of homogenation buffer)
1.50 mg/ml standard
- b. Add 5 μl per well of the serial dilutions or sample (4 replicates) in 96-well microplates
- c. Add 25 μl **Reagent A** (Bio-Rad DC) to each well with the aid of a repetitive pipette. If the homogenization buffer contains detergent, 20 μl of **Reagent S** (Bio-Rad DC) per ml of **Reagent A** (Bio-Rad DC) need to be added.
- d. Add 200 μl of **Reagent B** (Bio-Rad DC) to each well with the aid of a repetitive pipette
- g. Wait for 15 min and measure absorbance at 750 nm in a microplate reader spectrophotometer

6. Comet assay with earthworm coelomocytes

(Singh et al., 1988 with modifications)

A. Reagents

- Ethanol
- Guayacol Gliceril eter (G5627 Aldrich)
- H₂O₂(H1009 Sigma)
- Normal melting point agarose (NMPA, A4679)
- Low melting point agarose (LMPA, A9414)
- Tris (Trizma base T4661)
- Triton X-100 (X100 Sigma-Aldrich)
- Dimethyl sulfoxide DMSO (276855 Sigma-Aldrich)
- NaOH (Riedal-de Hasen 30620)
- Ethidium bromide (E1510 Sigma)
- NaCl (Sigma S-9888 or similar)
- EDTA (Sigma E-6758 or similar)
- Phosphate buffer saline (PBS, see Appendix II-3)

B. Solutions

1% NMPA (1g agarose in 100ml PBS)

0,5% LMPA (100mg LMPA in 20ml PBS) at 37°C

-Lysis solution pH10

2,5 M NaCl
100 mM EDTA
10 mM Tris (pH 10)
1% Triton X-100 (added fresh*)
10% DMSO (added fresh*)

-Electrophoretic buffer (pH> 13, 3l)

1 mM EDTA
300 mM NaOH

-Neutralisation buffer

0.4 M Tris-HCl, pH 7.5

C. Procedure

- a. Coat the slides adding a layer of 1% NMPA one day before. Let it solidify
- b. Coelomocytes extrusion:

-Place the earthworms in a glass Petri dish with moistured filter paper to deparate (void gut contents) for 24 h

- Place each earthworm in a Petri dish with 1 ml extrusion solution and retrieve the cells by an electric shock with a 9 V battery. Coelomocytes will be extruded through dorsal pores
 - Transfer the cell suspension to a 15 ml Falcon tube
 - Centrifuge at 350 x g for 10 min at 4°C
 - Resuspend the pellet in 2 ml of cold PBS
 - For a positive control resuspend the cell suspension in 50 mM H₂O₂ (in PBS) for 30min. Centrifuge at 200 g, 5 min and resuspend in PBS.
 - *Keep the cell suspensions on ice.
- c. Dilute (1:2) the cell suspension in 0.5% LMPA at 25-37 °C and transfer 80 µl of the cell suspension to a slide having a thin layer of solidified 1% agarose. Cover with a coverslip of 18 x 18 mm and left on ice for 10 min to allow the 2nd layer to solidify.
 - d. Remove the coverslip
 - e. Cell lysis
 - Add 1ml Triton X-100 and 10 ml DMSO to 100ml of lysis solution (4°C) (or equivalent volumes). Mix thoroughly
 - Immerse the slides in Lysis solution in a staining jar for at least 1h at 4°C in darkness.
 - Wash the slides in dH₂O
 - f. Alkaline incubation and electrophoresis
 - Place the slides in the electrophoresis tank immersed in alkaline electrophoresis solution forming complete rows (gaps filled with blank slides) and wait for 20-25 min in darkness. The tank should be leveled and gels just covered
 - Connect the electrophoresis across the platform in the same buffer at 300mA (19V) during 20 min.
 - g. Neutralisation
 - Wash the slides with the neutralization buffer for 10 min
 - h. Fix the cells in methanol (-20 °C) 3 min
 - i. Stain the slides with 10 µl of 20 µg/ml ethidium bromide

D. Analysis

Analyse the DNA migration in a fluorescence microscope.
Analyse 100 cells for sample (image analysis program)
% Tail DNA (100-head % DNA)

7. Gene transcription levels by Real-Time qPCR

7.1. Tri-reagent protocol (Total RNA extraction)

- 1-Prepare extraction tubes containing silica beds (up to 0.25 ml line), 1 ml TRIzol® (Invitrogen, ThermoFisher Scientific) and ~100 mg sample.
- 2-Homogenize at 6 m/s-for 20 s twice (or more)
- 3-Transfer the homogenate to 1.5 ml eppendorf using filter tips
- 4-Allow to stand 5 min at RT
- 5-Add 200 µl chloroform per ml Trizol and shake manually (15 s)
- 6-Allow to stand at RT for 2 min
- 7-Centrifuge at 12000 x g (12 rcf) for 15 min at 4 °C
- 8-Transfer the upper aqueous phase to a new eppendorf carefully not to include the white precipitate
- 9-Add 500 µl isopropanol (cold) per 1ml Trizol
- 10-Allow to stand at RT for 10 min
- 11-Centrifuge at max speed for 10 min at 4 °C
- 12-Remove supernatant. Pellet should be visible
- 13-Wash pellet with 70% cold ethanol (from the freezer) 1ml
- 14-Centrifuge at max speed (7.4 rcf) for 5 min at 4 °C. Ensure that all the ethanol is removed. Pulse down several times if required
- 15-Air dry pellet for 10 min in ice (leave lids open)
- 16-Add 10-100 µl RNase DNase free water, leave tubes on ice and resuspend
- 17-Put tubes on 57.7 °C heat block for 2 min
- 18-Measure RNA concentration and purity (keep samples in ice):

Blank: 60 µl RNase DNase free H₂O

Sample: 1 µl sample + 60 µl RNase DNase free H₂O
- 18-Store at -80 °C

7.2. RNA Purification through RNase-Free DNase Set

- 1-Select a volume with a maximum of 100 µg RNA and adjust the sample to a volume of 100 µl with RNase-free water
- 2-Add 350 µl Buffer RLT and mix well by pipetting
- 3-Add 250 µl ethanol (96-100%) and mix well by pipetting
- 4-Transfer the sample (700 µl) to an RNeasy Mini spin column placed in a 2 ml collection tube. Close the lid and centrifuge for 15 s at >8000 g (>10000 rpm). Discard the flow-through
- 5- Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid and centrifuge for 15 s at >8000 g (>10000 rpm). Discard the flow-through

6-Prepare DNase I incubation mix by adding 10 µl DNase I stock solution to 70 µl Buffer RDD. Mix by gently inverting the tube

7-Add DNase I incubation mix solution (80 µl) to the RNeasy spin column and keep at 20-30 °C 15 min

8-Add 350 µl Buffer RW1 to the RNeasy spin column. Close the lid and centrifuge for 15 s at >8000 g (>10000 rpm). Discard the flow-through

9-Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid and centrifuge for 15 s at >8000 g (>10000 rpm). Ensure that ethanol is added to Buffer RPE before use. Discard the flow-through

10-Add 500 µl Buffer RPE to the RNeasy spin column. Close the lid and centrifuge for 2 min at >8000 g (>10000 rpm). After centrifugation carefully remove the RNeasy spin column from the collection tube so that the column does not contact the flow-through

11-Optional: Place the RNeasy spin column in a new 2 ml collection tube. Close the lid and centrifuge for 1 min at full speed

12-Place the RNeasy spin column in a new 1.5 ml collection tube. Add 30-50 µl RNase-free water. Close the lid and centrifuge for 1 min at >8000 g (>10000 rpm) to elute the RNA.

7.3. First-Strand cDNA Synthesis protocol (Retro Transcription-RT-)

1- Measure RNA concentration

2-Prepare the following mix (RNA/primer) in sterile microcentrifuge tubes:

3 µl Random Primers (0.1 µg/µl)
1 ng-5 µg total RNA
Up to 15.7 µl RNase-free H₂O

Every component must be vortex before use

3-Incubate tubes in thermocycler (V=15.7 µl)

5 min → 65 °C
10 min → RT

4-Prepare the following mixture (calculations for `samples+1`) and add 4.3 µl per sample

2 µl 10xAffinityScript RT **Buffer**
0.8 µl **dNTP** mix
0.5 µl **RNase Block** Ribonuclease Inhibitor
1 µl AffinityScript Multiple Temperature **RT**

5-Incubate tubes in thermocycler (V=20 µl)

10 min → 25 °C
60 min → 49 °C
15 min → 70 °C
∞ → 4 °C (until PCR amplification)

7.4. Establishment of primer condition for qPCR

A. Confirmation of primer specificity (Agarose gel electrophoresis)

1- Prepare the following mixture (calculations for `4 samples+1`):

H ₂ O RNase/DNase free	208 µl
X10 PCR Buffer	25 µl
MgCl ₂ 50nm	7.5 µl
dNTP	5 µl
Primer Fw	1 µl
Primer Rv	1 µl
Taq	2.5 µl

2- Mix 48 µl of the mixture with 2 µl cDNA

3- Incubate tubes in termocycler (V=50 µl)

2 min → 94 °C	} 30-35 cycles
30 s → 94 °C	
30 s → *Primer °C	
30 s → 72 °C	
8 min → 72 °C	
∞ → 4°C	

4- Prepare the gel:

1.5 g agarose
100ml TAE buffer

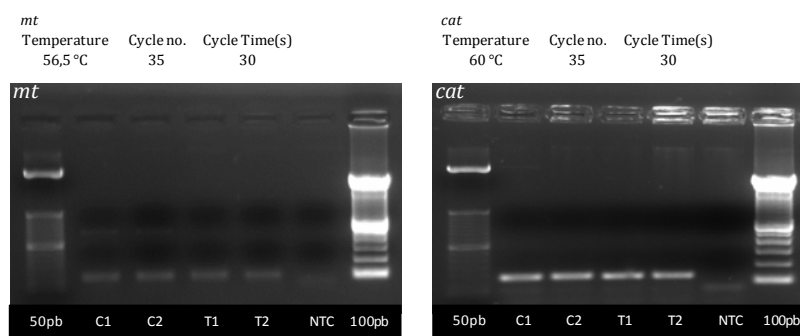
- Warm it up in the microwave and let it temper before adding 10 µl Ethidium Bromide
- Assemble the gel plate, add the solution (avoiding bubbles) and create wells on the plate
- Wait for solution to solidify (15-30 min)
- Remove the gel carefully and place it in a gel box (wells in the negative electrode-black)
- Slowly pour 1x TAE buffer adding enough solution to cover the wells

5- Load 4 µl Ladder (50pb), 1 µl Loading buffer + 7 µl sample, and 1 µl Loading buffer + 4 µl Ladder (100pb)

6- Do the migration at 100 V and 400 mA for 30 min

7- Specificity verification through sequencing of a single amplicon (SGIKer Genomic service)

Agarose gel electrophoresis



C1 and C2: Coelomocytes (pool of 5 earthworm) samples
T1 and T2: Earthworm tissue samples
NTC: Non template controls

B. Concentration of the primer and dilution of the samples for qPCR***mt***

-Pure sample

-6 pmol sample

1- Prepare the MASTER MIX (calculations for the number of wells):

10 μ l SYBR Green

0.03 μ l Fw Primer

0.03 μ l Rv Primer

7.94 μ l H₂O

2- Load in the plate 2 μ l sample and add 18 μ l MIX:

Standard curve: Pure sample, 1/10, 1/100, 1/1000, 1/10000 (3 replicates each)

Samples (Pure) (3 replicates each)

NTC (6 wells)

3-Place a film on, shake the plate and centrifuge at 1000 rpm for 1 min

3-Run the qPCR as follows

2 min \rightarrow 50 °C

10 min \rightarrow 95 °C

15 s \rightarrow 95 °C

1 min \rightarrow 55.5 °C

40 cycles

cat

-Pure sample

-12.5 pmol sample

1- Prepare the MASTER MIX (calculations for the number of wells):

10 μ l SYBR Green

0.06 μ l Fw Primer

0.06 μ l Rv Primer

7.88 μ l H₂O

2- Load in the plate 2 μ l sample and add 18 μ l MIX:

Standard curve: Pure sample, 1/10, 1/100, 1/1000, 1/10000 (3 replicates each)

Samples (Pure) (3 replicates each)

NTC (6 wells)

3-Place a film on, shake the plate and centrifuge at 1000 rpm for 1 min

4-Run the qPCR as follows

2 min \rightarrow 50 °C

10 min \rightarrow 95 °C

15 s \rightarrow 95 °C

1 min \rightarrow 59 °C

40 cycles

7.5. cDNA concentration through Quant iT OliGreen ssDNA assay Kit

1-Defrost the reagents of the kit at RT in darkness

2-Prepare M13 Oligonucleotide working solution:

(x50 → x1) (1 µl M13 in 49 µl H₂O)

3-Prepare the standard curve using M13 working solution

	H ₂ O (µl)	M13 (µl)	ng/ µl
1	50	0	0
2	49.5	0.5	0.02
3	45	5	0.2
4	25	25	1
5	0	50	2

3-Prepare the standard curve using M13 working solution

4-Prepare Quant-IT™ OilGreen® working solution reagent (calculations per well):

0.25 µl R in 49.75 µl H₂O

5-Dilute the samples to be around 0.02-0.2 ng cDNA per well (1/50)

6-Add in each well of a 96-well Clear Bottom microplates 50 µl sample and 50 µl Quant-IT™ OilGreen® working solution reagent (3 replicates)

7-Mix and wait 2-5 min at RT protected from light

8-Measure fluorescence at 485 ± 20 nm and 528 ± 20 emission in a microplate fluorescence reader

Calculations

The standard deviation among replicates should not surpass %5. Subtract the fluorescence value of the blank to samples and use the corrected data to generate a standard curve (samples fluorescence-y axis- vs. M13 Oligonucleotide concentration-x axis-). Obtain cDNA concentration from the equation of the linear regression (R>0.9). Take into consideration the dilution factor used for cDNA quantification and calculate the amount of cDNA placed in each well of the qPCR plate.

7.6. Relative Quantification (RQ) levels calculation

a. Relative Quantification (RQ) of the transcription levels was calculated with the following formula:

$$RQ = (1+E)^{-\Delta CT} / \text{ng cDNA}$$

-where Efficiency (E) was obtained using the slope of the linear regression of the standard curve in the PCR

$$E = [10^{(-1/\text{slope})}] - 1$$

- $\Delta CT = CT(\text{sample}) - CT(\text{plate calibrator})$ *in 386 wells plate calibrator=0

-Divided by the amount of cDNA (in ng) used in each reaction

b. The average of the controls RQ values is used as calibrator

c. Use Log₂ for the graphical representation of the RQ values

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Eskerrik asko