Universidade de Aveiro
2020

Departamento de Biologia

Natália Pegoraro Rodrigues

ABORDAGEM MECANISTICA PARA AVALIAR OS EFEITOS DE NANOMATERIAIS NO MEIO AMBIENTE

MECHANISTIC APPROACH TO ASSESS EFFECTS OF NANOMATERIALS IN THE ENVIRONMENT

Universidade de Aveiro
2020

Departamento de Biologia

Natália Pegoraro Rodrigues

ABORDAGEM MECANISTICA PARA AVALIAR OS EFEITOS DE NANOMATERIAIS NO MEIO AMBIENTE

MECHANISTIC APPROACH TO ASSESS EFFECTS OF NANOMATERIALS IN THE ENVIRONMENT

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Janeck Scott-Fordsmand, investigador sénior do Departamento de Biociências da Universidade de Aarhus, Dinamarca, da Doutora Mónica João de Barros Amorim, investigadora principal com agregação da Universidade de Aveiro, Departamento de Biologia e CESAM e do Doutor Tito Trindade, Professor Catedrático do Departamento de Química da Universidade de Aveiro, Portugal.

Apoio financeiro da FCT e do FSE no âmbito do III Quadro Comunitário de Apoio através de uma Bolsa de Doutoramento atribuída a Natália Pegoraro Rodrigues (SFRH/BD/87787/2012)

“Só sabemos com exatidão quando sabemos pouco; à medida que adquirimos conhecimento, instala-se a dúvida”

Goethe

Ao Eduardo e aos frutos do nosso amor:

à Sara,

o Samuel,

à Clara,

à Eva.

o júri

presidente	Doutor Fernando Manuel dos Santos Ramos Professor Catedrático da Universidade de Aveiro
vogais	Doutor Amadeu Mortágua Velho da Maia Soares Professor Catedrático da Universidade de Aveiro
	Doutora Lucia Maria das Candeias Guilhermino Professora Catedratica da Universidade do Porto
	Doutor Augusto Manuel Rodrigues Faustino Professor Associado da Universidade do Porto
	Doutora Mónica João de Barros Amorim (orientadora) Investigadora Principal com agregação da Universidade de Aveiro
	Doutor Tiago Manuel Ferreira Natal da Luz Investigador Doutorado (nível 1) da Faculdade de Ciências da Universidade de Coimbra

agradecimentos

Porque é tamanha bem-aventurança

O dar-vos quanto tenho e quanto posso

Que quanto mais vos pago, mais vos devo

Camões

Muito tenho a agradecer, no entanto, verbalizo aqui, apenas o indispensável.

Aos meus orientadores, pela oportunidade. Em particular, à Dra. Mónica, pela compreensão e paciência; ao Dr. Janeck, pelo acolhimento e atenção em Silkborg; ao Prof. Dr. Tito, pelo acolhimento no Nanolab e colaboração. Aos colegas e amigos, especialmente à Susana, à Ana Cristina e à Jaqueline, pelo carinho e amizade. A FCT, pelo financiamento. À família, em especial, aos meus cunhados, à minha irmã, Maria Luísa e aos meus pais, Sonia e Donizete (*in memoriam*) pela retaguarda, apoio e encorajamento. Um emotivo obrigada ao Eduardo, por tudo; à Sara, por ter tido a maturidade de ajudar a cuidar dos irmãos; aos demais rebentos, naquilo que lhes permitiu a idade, obrigada pelo esforço que fizeram em não solicitarem a mãe, tantas vezes.

A Deus, por guiar a minha vontade no caminho da inteligência, e não o contrário.

palavras-chave Nanomateriais, estágios de desenvolvimento, mecanismos de resposta, sobrevivência, reprodução, crescimento, evitamento, expressão de genes, ciclo de vida, avaliação de risco, invertebrados de solo, prata, grafeno

resumo

Pouco mais de duas décadas se passaram desde o início da manipulação de nanopartículas até a sua produção em massa. No entanto, embora este crescimento tenha ocorrido exponencialmente no que respeita ao desenvolvimento, aplicação e aceitação de mercado, o conhecimento dos potenciais riscos de (eco)toxicidade não acompanharam o mesmo ritmo. Isto se deve, entre outros fatores, à vasta variabilidade de nanomateriais (NMs) e suas possíveis combinações com o meio em que se encontra. Posto isso, e dado como certo a crescente demanda dos NMs, cabe-nos tentar compreender melhor estes novos materiais, bem como o seu potencial impacto para o ambiente e saúda humana.

Neste estudo, o foco de investigação foi avaliar os efeitos de NMs selecionados e entender os mecanismos subjacentes aos efeitos tóxicos em *Enchytraeus crypticus* (Oligoqueta), uma espécie modelo em ecotoxicologia de solo. Sendo o solo uma matriz complexa devido a sua multifacetez, o desafio de compreensão da toxicidade de NMs é proporcional à sua lacuna de conhecimento. Com o objetivo de tentar colmatar algumas destas lacunas, o efeito de dois tipos de materiais foram estudados; prata e grafeno. O primeiro foi testado utilizando três variações de NPs (NM 300K, Ag NM e PVP Ag NM) em comparação com o sal de prata ($AgNO_3$) e o segundo, utilizando duas variações, óxido de grafeno (GO) e óxido de grafeno reduzido (rGO).

Através de uma abordagem multiparamétrica Ag foi o material mais escrutinado, cobrindo todos os estágios de vida de *E. crypticus*, bem como diferentes níveis de organização biológica, desde o nível molecular (expressão de genes), até ao nível do organismo (eclosão, sobrevivência, reprodução e evitamento). Complementarmente, estudamos uma possível alternativa para redução/reversão da toxicidade causada pela prata, através da combinação de Ag NM300K a substâncias contendo grupos thiol em sua cadeia e em comparação com o sua forma não nano. A análise integrada destes resultados permitiu-nos perceber que concentrações sub-letais de Ag, para testes de eclosão em água, pôde prever efeitos de (nano)materiais de prata a longo prazo em solo. Além disso, AgNMs induziram efeitos distintos entre si e em comparação com o $AgNO_3$. A aplicação bem sucedida da conjugação de Ag ao N-acetilcisteína (NAC) e a glutationa reduzida (GSH) para reduzir os efeitos tóxicos da prata é um bom ponto de partida para a sustentabilidade nanotecnológica de Ag. Para o grapheno, foi demonstrado que o teste de ciclo de vida reduzido (rFLC) mostrou ser um bom indicador para prever efeitos a longo prazo através do parametro eclosão associado a medição do tamanho (área) dos juvenis eclodidos. Ademais, distintos efeitos de toxicidade entre GO e rGO foram encontrados: enquanto que GO teve todos os parametros significativamente afetados, a sua forma reduzida (rGO) demonstrou não ter quaisquer efeitos.

Com este trabalho, obtivemos uma melhor compreensão do perfil (eco)toxicólogico dos materiais testados e aprendemos, sobretudo, a importância de se usar uma abordagem multiparamétrica, bem como a atualização/modificação dos protocolos-padrão para avaliação de risco de NMs em solo. Embora não tenha sido o foco desta tese, também descobrimos que *Enchytraeus crypticus* pode ser um organismo adequado para uma primeira triagem da toxicidade de NMs, antes de envolver estudos mais aprofundados usando vertebrados.

keywords Nanomaterials, life stages, mechanisms of response, survival, reproduction, growth, avoidance behaviour, gene expression, full life cycle, risk assessment, soil invertebrates, silver, graphene

abstract Just over two decades have passed from the synthesis of engineered nanoparticles (ENPs) to their mass production. Despite this exponential growth in terms of development, application and market acceptance of ENPs, nano(eco)toxicological knowledge of potential risks have not kept the same pace. This was due, among other factors, to the vast variability of nanomaterials (NMs) and their possible combinations with the surrounding media. Certain that NMs demand will continue to increase, (eco)toxicological research should focus on the understanding of the mechanisms of novel materials and their potential impact on environment and human health.

In this study, the focus of the investigation was to assess the effects of the selected NMs and to understand the mechanisms underlying the toxic responses in *Enchytraeus crypticus* (Oligochaeta), a relevant model species in soil ecotoxicology. Given that soil is a complex matrix due to its multifaceted nature, the challenge of understanding the toxicity of NMs is proportional to its lack of knowledge. In order to try to fill some of these gaps, the effect of two types of materials were studied in soil: silver and graphene. The first being tested on three variations of NPs (NM 300K, Ag NM and PVP - Ag NM) compared to the salt form ($AgNO_3$) and the second, using two variations, graphene oxide (GO) and reduced graphene oxide (rGO).

Through a multiparametric approach Ag was highly screened, covering all the life stages of *E. crypticus*, as well as different levels of biological organization, from the molecular level (gene expression), to the organismal level (hatching, survival, reproduction and avoidance). Complementarily, we study a possible alternative to reduce/reverse the toxicity caused by silver, combining Ag with substances containing thiol groups in its chain and it was compared to non-nano form. The integrated analysis of these results allowed us to realize that sub-lethal Ag concentrations for hatching tests in water, could predict long-term effects of (nano)silver materials in soil. In addition, different AgNMs induced different effects in comparison with $AgNO_3$. Further, the successful application of the conjugation of Ag(NM) to N-acetylcysteine (NAC) and reduced glutathione (GSH) to reduce the toxic effects caused by silver is a good starting point for the nanotechnological sustainability of Ag. For graphene, it was showed that the reduced full life cycle test (rFLC) was a good indicator for predicting long-term effects, through the hatching associated with size (area) measurements of the hatched juveniles. Moreover, a distinct toxicity effect was found between GO and rGO: while GO had all the parameters evaluated significantly affected, its reduced form (rGO) was shown to have no effects.

From this set of experiments, we gained better understanding of the toxicological profile of the materials tested and we learned, above all, the importance of integrative approach as well the updating/modification of standard guidelines for risk assessments of NMs. Although it was not the focus of this thesis, we also found that *Enchytraeus crypticus* showed to be a suitable organism as a starting point for toxicity screening of NMs, before involving in-depth studies using vertebrates.

Contents

List of Figures

Chapter I

Chapter II

Chapter III

Chapter IV

List of Tables

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

Photo by Natália Rodrigues

Chapter I

General Introduction

General Introduction

It is known that the existence of nanomaterials dates back to antiquity (Deguchi et al. 2010; Murphy and Buriak 2015). However, it was from the 1980s that the growth of its conscious use, i.e., aware of its nanometric dimension, became more pronounced (Colvin, 2003; Valiev, 2002), and is currently a part of our daily lives (Kessler, 2011; Parada et al., 2019; Pulit-Prociak and Banach, 2016). Prior to this, in order to modify the properties of non-processed inorganic materials, the most common approach was to control the chemical composition. Nowadays, with the advent of Nanotechnology, new materials have been investigated, whose properties depend intrinsically on size and shape characteristics, that can be fine-tuned at the nanoscale (billionth of the meter: 1x10-9m). These materials present innovative properties that can be explored in several application domains, such as in medicine, green energy, environmental remediation, high performance electronic devices, among many others. In fact, a number of commercial products already incorporate nanomaterials and the tendency is to increase in future technologies that comply with global challenges, justifying the importance to assess technically the potential impact of its exposure for the environmental safety and human health. This will promote the safe use of nanomaterials by providing scientific knowledge that should be the basis of international regulations.

In this context, this thesis focuses on identifying the toxicity and response mechanisms in a soil invertebrate and model species, *Enchytraeus crypticus* (Oligochaeta), exposed to selected nanomaterials (NMs), in order to provide additional information for risk assessment of NMs.

This introductory chapter is divided in six sections and intend to cover the context of the performed studies followed by thesis structure:

1. Nanotechnology and Nanomaterials

2. Nanotoxicology: gaps and challenges

3. Test (nano)materials

4. Test organism

5. Current guidelines – soil ecotoxicology

6. Improving Standard Methods

7. Aim and outline of the thesis

1. Nanotechnology and Nanomaterials

Starting by the definition that has been widely accepted, nanomaterial is "*a material that consists of particles with one or more external dimensions in the size range 1–100 nm for more than 1% of their number"; and/or "has internal or surface structures in one or more dimensions in the size range 1–100 nm"; and/or "has a specific surface area by volume greater than 60 m^2/cm^3, excluding materials consisting of particles with a size lower than 1 nm*" (EUROPEAN COMMISSION, 2011). This definition is still not consensual, since involves some limitations (Maynard, 2011). However, a good complement to the main concept concerning the materials dimensions, is the mention that *nanomaterials have distinct properties as compared to conventional ones due to size and surface effects* (Trindade and Thomas, 2013). Anyway, and apart from that, the set of these materials at a nanoscale represents the backbone unit of Nanotechnology.

Richard Feynman, Nobel Laureate in Physics, was a pioneer in introducing the concept of Nanotechnology, in a lecture, in 1959 (Feynman., 1960) though never mentioning the term "nano". Later, the invention of the Scanning Tunneling Microscope (STM), patented in 1980 by Gerd Binnig e Heinrich Rohrer, Physics Nobel in 1986 (Martins and Trindade, 2012) and Atomic Force Microscope (AFM), invented in 1985 by Gerd Binnig, Calvin Quate and Christoph Gerber (Binnig, 1988), allowed us to know and see the nano world (size, thickness of particles, etc.). The invention of these two techniques were the starting point for a new dimension to chemistry, physics and biology, as, for instance, the discovery of fullerenes molecule (C60), in 1985 by Harold Kroto, Robert Curl and Richard Smalley also Nobel Laureates (Smalley, 1997) which opened the doors to the nano world.

Motivated by the potential benefits that Nanotechnology can offer, the scientific community, governments and companies around the world, have converged to investigate this domain, leading to heavy financial investments, in the order of 10 billion dollars/year globally (Bhushan, 2017). From then on, the United States became the first nation to establish a formal initiative, the National Nanotechnology Initiative (NNI) in 2001, to advance on nanoscale science. Further, more than 60 nations established similar programs to NNI (Sargent, 2016), causing a strong competition for global leadership with varying degrees of investment and different established priorities by nation. Fortunately, Nanotechnology has the advantage of being able to respond to important current challenges, complementary to a wide number of conventional technologies, given its unique multidisciplinary capacity (Krug and Wick, 2011). Additionally, nanotechnologies have served as a catalyst to move to a common language and knowledge, helping us to constantly teach ourselves to be much broader.

More than half a century later, given a huge dimension reached, what seemed to be a visionary technology from Feynman (1960), today it is part of our daily lives. Despite of nanoparticles (NPs) occur naturally in several products, their artificial synthesis, named engineered nanoparticles (ENPs) currently, cover all areas, from electronics to health, and most of them, involves improvements to existing products, such as 1) Electronics applications: transistors, have gotten smaller and smaller through Nanotechnology, flash memory chips for smart phones and thumb drives, ultra-responsive hearing aids, antimicrobial/antibacterial coatings on keyboards and cell phone casings; 2) Energy Applications: solar panels have been converted sunlight to electricity more efficiently, energy efficiency as more efficient lighting systems, lighter and stronger vehicle chassis materials for the transportation sector, lower energy consumption in advanced electronics; 3) Environmental Remediation: NPs are involved to clean industrial water pollutants in ground water through chemical reactions, allowing less costs than with traditional methods, engineers have developed a thin film membrane with nanopores for energy-efficient desalination, which allows water filtration five times greater than current conventional filters; 4) Medical and Healthcare Applications: better imaging and diagnostic tools, earlier diagnosis, more individualized treatment options, better therapeutic success rates, NPs can help to deliver medication directly to cancer cells and minimize the risk of damage to healthy tissue; 5) many other products of everyday materials life (NNI website).

However, after more than 20 years of nanoscience research and development, applications of Nanotechnology are delivering both in expected and unexpected ways, on the promise to benefit society. Feynman said: "*let's build from the atom*". Being nature built from a wise grouping of atoms and molecules, when studying the nano-metric scale, pertinent not only for inventions, but also allowing us to get closer to understanding the complex system of life. In this context, it is desired that humanity evolve at the same pace as science, making good use of the fantastic features that Nanotechnology allows us to glimpse.

2. Nanotoxicology: gaps and challenges

Driven by the huge benefits that nanotechnology has brought us, an accelerated production, development and use was generated worldwide. However, Nanotechnology is not just beneficial, as it also presents risks depending on the type of use. Therefore, it should be assessed in the specific context of application and its adverse effects should be investigated. There are already several studies reporting this fact (Edward Davidson, 2012; Kittler et al., 2010; Reidy et al., 2013; van der Ploeg et al., 2014). Because of its specific properties, for example, the enormous specific surface area when compared to the bulk form, or because its nanometric dimension, potentially more capable of entering and interacting with media than other compounds would not be able to do, their potential impact arouses many questions.

Although we do not know exactly the impact caused by its use, it is guaranteed that this increase in consumption, causes release and interaction with the environment. This fact is reason enough to investigate the potential implications for the society and everything related to it (Rasmussen et al., 2016a; Reidy et al., 2013). In this context, the scientific community, regulators and governs increasingly turn their attention to attempt to decipher the potential impact of this high demand, with heavy research investment and hundreds of thousands of publications across the world (Amorim et al., 2016; Bradford et al., 2009; Lopes et al., 2014; Rahman et al., 2009; Schlich et al., 2013).

Despite this, until this moment, risk assessment (RA) procedures for nanomaterials are still far from (consensual) regulation (ISO - AWI TS 21633; ISO - TS 22082, under development). The wide variety of synthesis methods, the colossal possibilities of combining and interacting NMs with different biotic and abiotic factors that surrounding them, became a challenge (eco)toxicological evaluation of NMs and further comparison between studies. These facts contribute to the lack of adequate protocols and regulations that establish methodologies for nanotoxicological assessments.

When dealing with soil, the challenge is even greater due to the complexity of its multifaceted matrix (Amorim et al., 2016; Cornelis et al., 2014; Shoults-Wilson and Reinsch, 2011). In this case, the scientific community face several fundamental practical challenges. Basic obstacles as standard spiking procedures, soil balance, final water content (establish a common value between 40-60%) and characterisation *in situ*, need to be overcome before deciding on the recommendations.

The last point deserves a special attention, since the characterisation of NMs is an essential aspect for any study regarding environmental or health impact in order to establish a causal link between exposure and effect. Many publications have referred the current difficulty/impossibility of characterisation in soil matrix (Amorim et al., 2016; Scott-Fordsmand et al., 2016). Some of the challenges are not only the huge variability of NMs and their possible combinations with the medium, as mentioned above, but especially the difficulty of distinguishing the anthropogenic materials (low concentration) when compared to the high concentrations of natural constituents, also with nanometric size (Navratilova et al., 2015; Wagner et al., 2014)). Hence, NMs characterisation in soil is hindered by the current lack of proper techniques and/or equipment difficult such task (Amorim et al., 2016).

3. Test (nano)materials

Basically, throughout the experiments we screened the toxicity of two types of nanomaterials: 1) metal-based NPs (Ag) and a metal salt, $AgNO_3$ and 2) carbon-based nanomaterials (rGO and GO). These materials are briefly explained bellow.

Silver

For more than six millennia, silver has been used as an antimicrobial agent and it was very important for these purposes until the introduction of chemically prepared antibiotics (Hill and Pillsbury, 1940; Alexander, 2009). However, due to the increase of bacterial resistance to such conventional antibiotics and the possibility of using less Ag, through replacement with silver nanoparticles having the same antibacterial effect (Chernousova and Epple, 2013), a renew interest for silver as antimicrobial agent has emerged. As such, more than half a century later, we are able to explore properties and a colossal range of new applications for Ag nanomaterials.

In fact, due to specific properties, such as 1) small particle size versus enormous specific surface area; 2) high capacity to adsorb biomolecules and interact with biological systems; 3) capacity to generate reactive oxygen species (Reidy et al., 2013), silver is one of the most used NMs worldwide (Piccinno et al., 2012), as well as one of the most studied NPs ever (Giese et al., 2018), belonging to one of the eleven NMs list of the Working Party on Manufactured Nanomaterials by OECD Testing Programme (OECD, 2016a). Their applications ranges vary from disinfecting agents to medical devices, water treatment, conductivity, imaging, among many others.

Given this wide range of silver NM applications, a naturally concern are emerging regarding their overuse, especially knowing the silver's antimicrobial activity. Having said that, questions are raised about the environmental impact and conclusions go through the need for assessing the risk—benefit of such applications.

Assuming the indisputable importance of studying silver, the tested materials included three different types of AgNPs: polyvinylpyrrolidone (PVP)-coated AgNPs, referred here as PVP - AgNM; non-coated AgNPs, referred here as NC - AgNM and Ag NM300K (JRC Repository) and their salt form, $AgNO_3$. Ag NM300K deserves special attention due to be a reference material, being present in a variety of studies and projects including the OECD WPMN Sponsorship Programme.

Graphene

Graphite is one of the oldest and most used natural materials in the world. Now it has been widely used as a starting material for designing other types of carbon-based nanomaterials (CBM), including the nanotubes of single or multiple wall, fullerenes, nanodiamonds and graphene and its derivatives, such as graphene oxide (GO and reduced graphene oxide (rGO) (Cha et al., 2013).

Graphene is the name given to a two-dimensional (2D) structure, consisting of an atom thick monolayer of covalent bound carbon atoms, arranged in a structure similar to a beehive (Geim and Novoselov, 2009). Due to its physical and chemical properties, it is the thinnest and strongest material ever seen in the universe. There are several derivatives of graphene and considering the number of layers and the chemical modification performed, the graphene family include few or multiple layered graphene oxide (GO) and reduced graphene oxide (rGO), all having different properties from each other (Goenka et al., 2014). These nanomaterials are potential candidates for innovative applications, such as sensors, transistors, transparent and conductive thin films, power and storage devices, among others.

Despite the wide range of potential applications, graphene has some disadvantages in terms of production and processing: 1) difficulty of large-scale production keeping high quality and 2) it is not dispersible in aqueous solutions and other polar organic solvents. In this context, the oxidized form of graphene (GO) and its reduced form (rGO), (see Figure I-1), have gained special attention, since the presence of functional chemical groups (oxygen containing moieties) confer hydrophilic characteristics, thus giving good dispersibility in an ubiquitous solvent such as water.

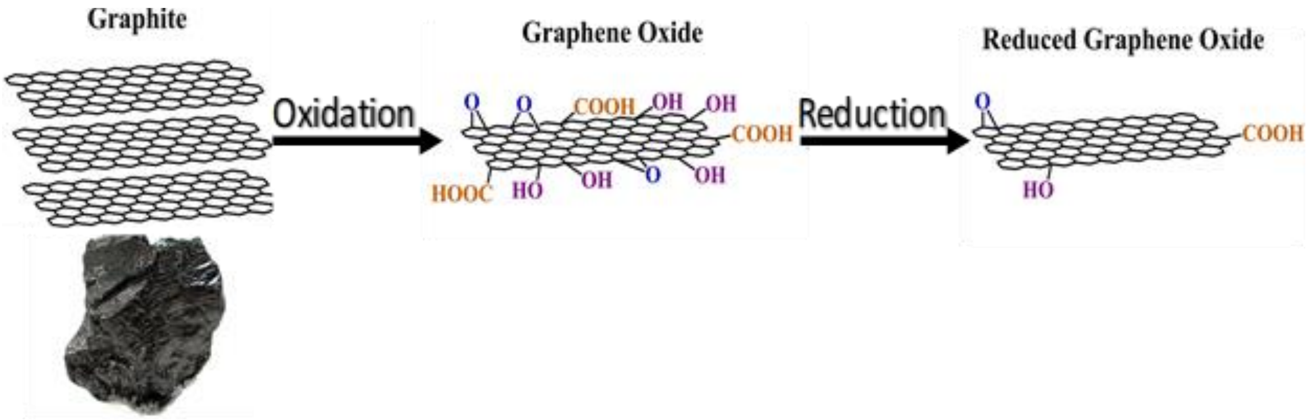


Figure I-1. Synthesis of rGO from GO which can be obtained from the exfoliation of graphite as the raw material. Adapted from (Oliveira et al., 2018).

Also, given the hydrophilic properties of oxidized graphene, as well as due to the carbon presence, several research groups began to explore these NMs in the field of neuroscience, since it shares some morphological and functional similarities with the central nervous system (CNS) (Mendonça et al., 2016a, 2016b, 2015; Monaco and Giugliano, 2014). Additionally, there are evidences that rGO contributes in inducing the temporary opening of the blood-brain barrier in rats, which might be a useful tool, with great potential for drug delivery, showing at the same time, a minimal toxicity effect (Mendonça et al., 2016, 2015).

In this sense, knowing the inexhaustible potential of carbon-base nanomaterials, this study included two nanoforms of graphene: the graphene oxide - GO and its reduced form - rGO.

Nanomaterial Characterisation

A summary of the main physico-chemical characteristics of the materials tested is presented in Table I-1, as well as the main properties of the ENMs studied (Table I-2).

Table I-1. Summary of characteristics of the tested materials, divided in silver and graphene, including supplier, state, solubility/dispersability, coating, size (nominal and Transmission/Scanning Electron Microscopy –TEM/SEM-based), purity and morphology

Characteristic	Silver						Graphene	
	polyvinylpyrrolidone PVP -AgNMs	non-coated NC - AgNMs	Ag NM300K	$AgNO_3$	Dispersant	N-acetylcysteine (NAC)	graphene oxide - GO	reduced graphene oxide - rGO
Supplier	American Elements	American Elements	JRC Repository	Sigma Aldrich	JRC Repository	Sigma Aldrich	Sigma Aldrich	Sigma Aldrich
State	Powder	Powder	Suspension	Powder	Suspension	Powder	Flake	Powder
Solubility/ dispersability	Not dispersed	Not dispersed	Dispersible	Water soluble	Soluble	Soluble	Not dispersed	Not dispersed
Coating	0.2%w/w PVP	-	-	-	-	-	-	-
nominal size (nm)	20–30	20–30	15	-	-	-	layer thickness 0.6 and average size 219	layer thickness 0.9 and average size 122.4
Purity	99%	99%	10.2% w/w Ag	>99%	-	≥99%	-	-
Morphology	Spherical *	Spherical *	Spherical *	-	-	-	Nanosheets	Nanosheets

Table I-2. Nanoparticle properties and their importance for measurement (adapted from (Tiede et al. 2008)

Property	Importance of measurement
Aggregation state	Nanoparticles that tend to aggregate may keep their functionality, however the increase in size could lead to decrease in uptake (e.g. Adult´s Survival Test in Water with AgNPs, Chapter II, see supplemental material, Figure II-S1)
Elemental composition	Different particle composition leads to different behaviour/impact. Normally increased contaminant concentration leads to increase in toxicity/impact, not always the case for nanoparticles (e.g. avoidance test for PVP-AgNM, Chapter II and rFLC for rGO, Chapter IV)
Shape	Different particle shapes can take different bioavailability and uptake (e.g. Cu-nanowires, Chapter VI, Annex, II)
Size and particle size distribution	Nanoparticles are defined and classed by their size, and size is one of the primary properties describing transport behaviour
Solubility	Soluble nanoparticles; their ionic form can be harmful or toxic (e.g. Ag NM300K, Chapter II and III)
Speciation	Different species can have different behaviour, toxicity, impact (e.g. GO versus rGO, complexes with natural organic matter or oxidation state, Chapter IV)
Structure	The structure can influence stability or behaviour (e.g. GO & rGO, two-dimensional hexagonal structure, Chapter IV)
Surface chemistry	Coatings can consist of different chemical compositions and influence particle behaviour or toxicity (e.g. PVP – AgNM, Chapter II)

The physical and chemical characterisation of nanomaterials is an essential aspect for any study regarding environmental or health impact. Although defining particle size is very important to identify a nanomaterial, the physical and chemical properties that result from size and surface effects are also relevant to characterise the system, such as surface charge, surface area to volume ratio, particle shape, crystal structure, coating/corona, aggregation / agglomeration and solubility / dissolution, amongst others have been shown to be relevant to NM characterisation (Gatoo et al., 2014).

The need for proper NM characterisation is an important aspect when assessing the NMs hazard / risk level (Hristozov et al., 2016; Nowack, 2017). Although there has been an increase in the number of methods that allow NM characterisation (for more details see Table I-3), there is still a lack of adequate quantitative methods. Currently, the most appropriate seems to be the coupling of several techniques, since there is no instrument capable of transmitting all the information at once. For now, the standardization of NMs characterisation methods is under discussion (Tämm et al., 2016).

Table I-3. Main physical and chemical properties of NMs and respective associated instruments/methods of characterisation (adapted from (Hassellöv et al., 2008; Contado, 2015; Laborda et al., 2016)).

Physical properties/metrics	***Instruments and methods***
Diameter	TEM, FESEM, ESEM, AFM, FFF, DLS
Volume	Sd-FFF
Area	TEM, ESEM, AFM
Mass	LC-ESMS, ICP-MS, SP-ICP-MS
Surface charge	Zeta-Potential, Electrophoretic Mobility
Crystal structure, Aspect ratio or other shape factor	TEM, STEM, FESEM, ESEM, XRD, TEM-XRD-SAED, SP-ICP-MS,
Chemical composition/analytes	
Elemental composition	Bulk: ICP-MS, ICP-OES, AAS-GF, XAS Single nanoparticle: TEM-EDX, SP-ICP-MS Particle population: FFF-ICP-MS
Fluorophores	Fluorescence Spectroscopy
Fullerene (‘‘molecules’’)	UV-vis, HPLC
Other properties not falling within the above classes	
Aggregation state	DLS, AFM, SP-ICP-MS, ESEM
Surface chemistry, coating composition, # of proton exchanging surface sites	TEM, SP-ICP-MS, XAS, Acid–Base Titrations

AAS-GF - Graphite Furnace Atomic Absorption Spectroscopy; AFM - Atomic Force Microscopy; DLS - Dynamic Light Scattering; EDX - Energy Dispersive X-Ray; ESEM - Environmental Scanning Electron Microscopy; FESEM - Field-Emission Scanning Electron Microscopy; FFF – Field Flow Fractionation; FFF-ICP-MS - Flow-Field-Fractionation ICP-MS; HPLC - High Performance Liquid Chromatography; ICP-MS - Inductively Coupled Plasma-Mass Spectrometry; ICP-OES - Inductively Coupled Plasma-Optical Emission Spectrometry; MS – Mass Spectrometry; Selected area electron diffraction; Sd-FFF - Sedimentation-FFF; SP-ICP-MS - Single Particle ICP-MS; STEM – Scanning Transmission Electron Microscopy; TEM - Transmission Electron Microscopy; UV-vis - Ultraviolet–Visible Spectroscopy; XAS - X-Ray Absorption Spectroscopy; XRD - X-Ray Diffraction

4. Test organism

Experiments using vertebrate animals have been broadly used for toxicological screening tests of NMs (Mendonça et al. 2015, 2016a, 2016b, 2019). *In vivo* studies are adopted since they provide closer results for determining therapeutic equivalence in humans, whereas *in vitro* tests do not address important aspects such as biodistribution, metabolism, excretion and the influence of NMs on the immune system (Nehoff et al., 2014). However, vertebrate animals, in many cases, are not necessary. It not just an ethic aspect, but also due economic, time-consuming or, simply due its use does not work as expected, i.e., as bridge between animals and humans (Collins et al., 2008; Pachapur et al., 2015). These arguments gained steam with the development of Toxicology 21st century (National Research Council, 2007), which is envisaged as "*a toxicity testing approach, using primarily cellular and in silico methods as well as lower organisms*" (Hartung, 2010). Invertebrates can overcome some of these barriers and, at the same time, can serve for the same purpose.

Enchytraeidae is a family of annelids oligochaetes, represented by soil species living in highly organic environments (Westheide and Graefe, 1992). In this work, the enchytraeid *Enchytraeus crypticus* was used (Westheide and Graefe, 1992), since they are a relevant model species in soil ecotoxicology with established guidelines (ISO - 16387, 2014; OECD - No.220, 2016). Moreover, there is already quite a lot and highly branched information about this species, as well several molecular/cellular level endpoints such as transcriptomics (Castro-Ferreira et al., 2014); oxidative stress (Ribeiro et al., 2015); metabolomics (Maria et al., 2018a); genotoxicity (Maria et al., 2018b) and epigenetics (Bicho et al., 2020). Also, at organism and population level, there is the full life cycle (Bicho et al., 2015) and multigenerational test (Bicho et al., 2017a).

The different NMs´results from these several biological organization levels/endpoints, are fundamental to deeply understand the mechanisms of *Enchytraeus crypticus*, being a very promising organism for nanomaterial toxicity screening and risk assessment of NMs.

5. Current guidelines – soil ecotoxicology

Without much doubt, the volume and range of NMs released into the environment will increase according to their predictable high demand. The primary sink for these materials are the soil (Cornelis et al., 2014; Peijnenburg et al., 2016) Knowing that the determination of nanomaterial toxicity is dependent on its dose, administration rout, physicochemical properties, exposure time and also the interaction with the destination matrix, the challenge to draw basic guidelines that encompasses all these factors, is quite complex. At the same time, as long as non-standardized (eco)toxicity methods for NMs are used, non-comparable results will occur.

In June 2006, the Organization for Economic Co-operation and Development (OECD) Working Party on Manufactured Nanomaterials (WPMN) launched the Sponsorship Programme for the Testing of Manufactured Nanomaterials (Testing Programme) to begin identifying human health and environmental safety of NMs (OECD - No.2, 2006). Between 2006 and 2019, the program wrote about 91 reports (OECD - No.91, 2019). However, only in 2015, they finally started to publish the findings of its seven years testing NMs. According to this press release, the key finding is that "test guidelines used for regular chemical substances are, in the most cases, suitable for use on nanomaterials..." (OECD, 2016b; Rasmussen et al., 2016b). Nonetheless, until now, only three test guidelines (TG) are currently published: 1) Dispersion Stability of Nanomaterials in Simulated Environmental Media (OECD - No.318, 2017), 2) 28 days (Subacute) Inhalation Toxicity Study (OECD - No.412, 2018) , and 3) 90 days (Subchronic) Inhalation Toxicity Study (OECD - No.413, 2018), for the last two studies, using rodents.

International Standard Organization (ISO) has also published a wide range of reports (over 20 pages) addressing the same theme, although most of them are still under development, as (ISO - AWI TS 21633; ISO - TS 22082) and those already published are not freely available to the community, as (ISO - TR 13014, 2012; ISO - TR 21386, 2019; ISO - TR 22019, 2019). Among them, only one TG, involving a standardized organism *Artemia sp*. Nauplius in artificial saltwater lakes to assess effects of NMs, is available (ISO - TS 20787, 2017). In this case, the NMs applicable consist of nano-objects such as nanoparticles, nanopowders, nanofibres, nanotubes, nanowires, as well as aggregates and agglomerates of such NMs.

To summarize, there is a lot of information, not only random publications across different labs, but also diverse reports from international organizations such as OECD and ISO. In this vast literature there is still no defined protocols for risk assessment (RA) of NMs for soil invertebrates. Although there is a strong trend to conduct research on NMs using traditional protocols (OECD, 2016b), our experience based in tests with soil invertebrates, shows us that we have to be cautious when using standardized tests for chemicals other than nanomaterials, for example, (Bicho et al., 2017b, 2016) showed that the current standard test under-estimated the effects of Cu(ONMs) and Ag (NM) when compared with full life cycle test (FLCt). Santos et al., 2017 and Gomes et al., 2019 also showed that the performance of the FLCt is preferable than standardized Enchytraeid Reproduction Test (ERT) using Ni(NPs) and nanopesticide, respectively. Further, there is currently a global concern on long-term effects of NMs and recommendations in this sense to assess the (eco)toxicological effects of ENMs (Amorim, 2016; OECD - No.184, 2017).

6. Improving Standard Methods – new endpoints

As already mentioned, there are so many NMs and possible combinations among them, that it would be impossible to cover all the materials/products generated. This is one of the main reasons why standardization is important, since they are instrumental in facilitating international research and trade. However, current standardized tests use one test organism within a test period, with dose–effect response for a specific endpoint (like reproduction or survival) (Løkke and Gestel, 1998). This procedure simply seems to not be enough to investigate the potential toxicity of the (all) NMs. Thus, screening of NMs should involve an in-depth study of an organism, i.e., variation of endpoints, sampling times, dose range, among others, to allow better understanding of the (eco)toxicity NM profile in soil invertebrates and to promote better choices for NMs risk assessment.

In this work, the tests performed taking into consideration the integration of various methods, combining traditional and updated/improved/modified endpoints:

1) hatching test was extended from 11 to 17 days, making it possible to differentiate a delay from total impairment; 2) survival test using organisms in its early life stage, allowing to assess juveniles ‘sensitivity compared to adults (standard test); 3) ISO water (reconstituted water) test followed by post exposure in clean soil using ERT, showing that ISO water, allowing daily assessment and record photographic; 4) for avoidance behaviour test, three more sampling time were added (24 – 72 and 96h), besides the standard 48h, allowing to detect different behaviors over time, which after integrated with 5) gene expression, enabling to understand the mechanisms behind the behavior observed in the avoidance test (Chapter II); 6) integration of standardized tests, combining ERT and avoidance behaviour, with different kind of combinations between the treated versus the control soil (Chapter III); 7) additional endpoints were added to ERT, using a reduced version of the FLCt, as hatching and size, as well as the assessment of the area instead of length, as a representative measure of adverse effects (Chapter IV).

7. Aim and outline of the thesis

The overall aim refers to assessing hazards of nanomaterials (i.e., Ag and GO) focusing on progressing from standard methods to additional details and mechanistic understanding. Hence, we hereby assessed multiple endpoints (avoidance, hatching, survival, reproduction, size and gene expression) and different exposure media (water and soil) using *Enchytraeus crypticus*.

The results obtained are organised in chapters presented in the format of journal publications (submitted or published), followed by general discussion, conclusion and last, the supplementary information regarding collaboration studies as detailed in each.

Chapter II: Natália P. Rodrigues, Janeck J. Scott-Fordsmand and Mónica J.B. Amorim (2020). “Novel understanding of toxicity in a life cycle perspective – the mechanisms that lead to population effect – the case of Ag (nano)materials" – Environmental Pollution, 262 114277.

Chapter III: Monique C. P. Mendonça*, Natália P. Rodrigues*, Janeck J. Scott-Fordsmand, Marcelo B. de Jesus and Mónica J.B. Amorim (2020) (*these authors contributed equally). "The toxicity of silver nanomaterials (NM 300K) is reduced when combined with N-Acetylcysteine: Hazard assessment on *Enchytraeus crypticus*" – Environmental Pollution, 256 113484.

Chapter IV: Monique C. P. Mendonça*, Natália P. Rodrigues*, Marcelo B. de Jesus and Mónica J.B. Amorim (2019) (*these authors contributed equally). "Graphene-based nanomaterials in soil: ecotoxicity assessment using *Enchytraeus crypticus* reduced full life cycle" – Nanomaterials, 9, (6), 858.

Chapter V: General Discussion within an integrated perspective and Final Remarks

Chapter VI: Supplementary Research

Annex I: Bicho R.C., Ribeiro T., Rodrigues N.P., Scott-Fordsmand J.J., Amorim M.J.B (2016). "Effects of Ag nanomaterials (NM300K) and Ag salt ($AgNO_3$) can be discriminated in a full life cycle long term test with *Enchytraeus crypticus*" – International Journal of Hazardous Materials, 318 608-614.

Participation in this work involved laboratory work (standard tests) and writing - review of materials and methods.

Annex II: Gomes S.I.L., Roca C.P., Pegoraro N., Trindade T., Scott-Fordsmand J.J., Amorim M.J.B (2018). "High-throughput tool to discriminate effects of NMs (Cu-NPs, Cu-nanowires, $CuNO_3$, and Cu salt aged): transcriptomics in *Enchytraeus crypticus*" – Nanotoxicology, 12, (4), 325-340.

Participation in this work involved laboratory work (synthesis and characterisation of copper nanowires) - writing and editing materials and methods

References

Alexander, J.W., 2009. History of the medical use of silver. Surg. Infect. (Larchmt). https://doi.org/10.1089/sur.2008.9941

Amorim, M.J.B., 2016. The daunting challenge of ensuring sustainable development of nanomaterials. International Journal of Environmental Research and Public Health 13, (2), 245. https://doi.org/10.3390/ijerph13020245

Amorim, M.J.B., Roca, C.P., Scott-Fordsmand, J.J., 2016. Effect assessment of engineered nanoparticles in solid media – Current insight and the way forward. Environmental Pollution 218, 1370–1375. https://doi.org/10.1016/j.envpol.2015.08.048

Binnig, G., 1988. Atomic force microscope and method for imaging surfaces with atomic resolution. US Pat. 4,724,318 1–8.

Bicho, R., Ribeiro, T., Rodrigues, N., 2016. Effects of Ag nanomaterials (NM300K) and Ag salt ($AgNO_3$) can be discriminated in a full life cycle long term test with *Enchytraeus crypticus*. Journal of Hazardous Materials, 318 608-614. https://doi.org/ 10.1016/j.jhazmat.2016.07.040

Bicho, R., Roelofs, D., Scott-Fordsmand, J., Amorim, M., 2020. Epigenetic effects of (nano) materials in environmental species–Cu case study in *Enchytraeus crypticus*. Environment International 136, (105447). https://doi.org/10.1016/j.envint.2019.105447

Bicho, R., Santos, F., Scott-Fordsmand, J., 2017a. Multigenerational effects of copper nanomaterials (CuONMs) are different of those of CuCl2: exposure in the soil invertebrate *Enchytraeus crypticus*. Scientific Report, 7. https://doi.org/10.1038/s41598-017-08911-0

Bicho, R., Santos, F., Scott-Fordsmand, J., 2017b. Effects of copper oxide nanomaterials (CuONMs) are life stage dependent–full life cycle in *Enchytraeus crypticus*. Environmental Pollution, 224 117-124. https://doi.org/10.1016/j.envpol.2017.01.067

Bicho, R.C., Santos, F.C.F., Gonçalves, M.F.M., Soares, A.M.V.M., Amorim, M.J.B., 2015. Enchytraeid Reproduction TestPLUS: hatching, growth and full life cycle test—an optional multi-endpoint test with *Enchytraeus crypticus*. Ecotoxicology 24, 1053–1063. https://doi.org/10.1007/s10646-015-1445-5

Bradford, A., Handy, R.D., Readman, J.W., Atfield, A., Muehling, M., 2009. Impact of Silver Nanoparticle Contamination on the Genetic Diversity of Natural Bacterial Assemblages in Estuarine Sediments. Environment Science Technology 43, 4530–4536. https://doi.org/10.1021/es9001949

Bhushan, B., 2017. Introduction to nanotechnology. Springer Handbooks. https://doi.org/10.1007/978-3-662-54357-3_1

Castro-Ferreira, M.P., de Boer, T.E., Colbourne, J.K., Vooijs, R., van Gestel, C.A.M., van Straalen, N.M., Soares, A.M.V.M., Amorim, M.J.B., Roelofs, D., 2014. Transcriptome assembly and microarray construction for *Enchytraeus crypticus*, a model oligochaete to assess stress response mechanisms derived from soil conditions. BMC Genomics 15, 302. https://doi.org/10.1186/1471-2164-15-302

Cha, C., Shin, S.R., Annabi, N., Dokmeci, M.R., Khademhosseini, A., 2013. Carbon-based nanomaterials: Multifunctional materials for biomedical engineering. ACS Nano. https://doi.org/10.1021/nn401196a

Chernousova, S., Epple, M., 2013. Silver as antibacterial agent: Ion, nanoparticle, and metal. Angew. Chemie - Int. Ed. https://doi.org/10.1002/anie.201205923

Collins, F.S., Gray, G.M., Bucher, J.R., 2008. Transforming environmental health protection. Science (80). https://doi.org/10.1126/science.1154619

Colvin, V.L., 2003. The potential environmental impact of engineered nanomaterials. Nature biotechnology, 21, 1166–1170. https://doi.org/10.1038/nbt875

Contado, C., 2015. Nanomaterials in consumer products: A challenging analytical problem. Front. Chem. https://doi.org/10.3389/fchem.2015.00048

Cornelis, G., Hund-Rinke, K., Kuhlbusch, T., Van Den Brink, N., Nickel, C., 2014. Fate and Bioavailability of Engineered Nanoparticles in Soils: A Review. Critical Reviews in

Environmental Science and Technology. 44, 2720–2764. https://doi.org/10.1080/10643389.2013.829767

Deguchi, S., Mukai, S.A., Yamazaki, T., Tsudome, M., Horikoshi, K., 2010. Nanoparticles of fullerene C60 from engineering of antiquity. J. Phys. Chem. C 114, 849–856. https://doi.org/10.1021/jp909331n

Edward Davidson, H., 2012. When enough is enough. Nat. Nanotechnol. 409–411. https://doi.org/10.4140/TCP.n.2017.425

EUROPEAN COMMISSION, 2011. Definition - Nanomaterials - Environment - European Commission. https://ec.europa.eu/environment/chemicals/nanotech/faq/definition_en.htm (accessed 1.12.20).

Feynman, R., 1960. There’s plenty of room at the bottom, in: Institute of Technology, Engineering and Science. pp. 77–92. https://doi.org/10.1201/9780429500459.

Fung, M.C., Bowen, D.L., 1996. Silver products for medical indications: Risk-benefit assessment. J. Toxicol. - Clin. Toxicol. https://doi.org/10.3109/15563659609020246

Gatoo, M.A., Naseem, S., Arfat, M.Y., Mahmood Dar, A., Qasim, K., Zubair, S., 2014. Physicochemical properties of nanomaterials: Implication in associated toxic manifestations. Biomed Res. Int. https://doi.org/10.1155/2014/498420

Geim, A.K., Novoselov, K.S., 2009. The rise of graphene, in: Nanoscience and Technology: A Collection of Reviews from Nature Journals. World Scientific Publishing Co., 11–19. https://doi.org/10.1142/9789814287005_0002

Giese, B., Klaessig, F., Park, B., Kaegi, R., Steinfeldt, M., Wigger, H., Von Gleich, A., Gottschalk, F., 2018. Risks, Release and Concentrations of Engineered Nanomaterial in the Environment Science Reports. 8, 1565. https://doi.org/10.1038/s41598-018-19275-4

Goenka, S., Sant, V., Sant, S., 2014. Graphene-based nanomaterials for drug delivery and tissue engineering. Journal of Controlled Release. https://doi.org/10.1016/j.jconrel.2013.10.017

Gomes, S.I.L., Scott-Fordsmand, J.J., Campos, E.V.R., Grillo, R., Fraceto, L.F., Amorim, M.J.B., 2019. On the safety of nanoformulations to non-target soil invertebrates-an atrazine case study. Environmental Science: Nano 6, 1950–1958. https://doi.org/10.1039/c9en00242a

Hartung, T., 2010. Evidence-Based Toxicology - The Toolbox of Validation for the 21st Century? ALTEX. https://doi.org/10.14573/altex.2010.4.253

Hill, W., Pillsbury, D., 1940. Argyria, The Pharmacology of Silver. Arch. Otolaryngol. Head Neck Surg. 31, 1036–1037. https://doi.org/10.1001/archotol.1940.00660011051016

Hassellöv, M., Readman, J.W., Ranville, J.F., Tiede, K., 2008. Nanoparticle analysis and characterisation methodologies in environmental risk assessment of engineered nanoparticles. Ecotoxicology. https://doi.org/10.1007/s10646-008-0225-x

Hristozov, D., Gottardo, S., Semenzin, E., Oomen, A., Bos, P., Peijnenburg, W., van Tongeren, M., Nowack, B., Hunt, N., Brunelli, A., Scott-Fordsmand, J.J., Tran, L., Marcomini, A., 2016. Frameworks and tools for risk assessment of manufactured nanomaterials. Environ. Int. https://doi.org/10.1016/j.envint.2016.07.016

ISO - 16387, 2014. Soil quality — Effects of contaminants on Enchytraeidae (*Enchytraeus* sp.) — Determination of effects on reproduction, Geneva.

ISO - AWI TS 21633, Under development - Label-free impedance technology to assess the toxicity of nanomaterials *in vitro*.

ISO - TR 13014, 2012. Nanotechnologies — Guidance on physico-chemical characterisation of engineered nanoscale materials for toxicologic assessment [WWW Document]. URL https://www.iso.org/standard/52334.html (accessed 1.14.20).

ISO - TR 21386, 2019. Nanotechnologies — Considerations for the measurement of nano-objects and their aggregates and agglomerates (NOAA) in environmental matrices [WWW Document]. URL https://www.iso.org/standard/70848.html (accessed 1.14.20).

ISO - TR 22019, 2019. Nanotechnologies — Considerations for performing toxicokinetic studies with nanomaterials [WWW Document]. URL https://www.iso.org/standard/72381.html (accessed 1.14.20).

ISO - TS 20787, 2017. Nanotechnologies - Aquatic toxicity assessment of manufactured nanomaterials in saltwater lakes using *Artemia* sp. Nauplii [WWW Document]. URL https://www.iso.org/standard/69087.html (accessed 1.14.20).

ISO - TS 22082, Under development - Nanotechnologies — Toxicity assessment of nanomaterials using dechorionated zebrafish embryo [WWW Document]. URL https://www.iso.org/standard/72516.html (accessed 1.14.20)

Kessler, R., 2011. Engineered nanoparticles in consumer products: understanding a new ingredient. Environmental Health Perspect. https://doi.org/10.1289/ehp.119-a120

Kittler, S., Greulich, C., Diendorf, J., Köller, M., Epple, M., 2010. Toxicity of silver nanoparticles increases during storage because of slow dissolution under release of silver ions. Chem. Mater. 22, 4548–4554. https://doi.org/10.1021/cm100023p

Krug, H.F., Wick, P., 2011. Nanotoxicology: An interdisciplinary challenge. Angewandte Chemie International Edition. 50, 1260–1278. https://doi.org/10.1002/anie.201001037

Laborda, F., Bolea, E., Cepriá, G., Gómez, M.T., Jiménez, M.S., Pérez-Arantegui, J., Castillo, J.R., 2016. Detection, characterisation and quantification of inorganic engineered nanomaterials: A review of techniques and methodological approaches for the analysis of complex samples. Anal. Chim. Acta. https://doi.org/10.1016/j.aca.2015.11.008

Løkke, H., Gestel, C.A.M. van., 1998. Handbook of soil invertebrate toxicity tests.

Lopes, S., Ribeiro, F., Wojnarowicz, J., Łojkowski, W., Jurkschat, K., Crossley, A., Soares, A.M.V.M., Loureiro, S., 2014. Zinc oxide nanoparticles toxicity to *Daphnia magna*: size-dependent effects and dissolution. Environ. Toxicol. Chem. 33, 190–8. https://doi.org/10.1002/etc.2413

Maria, V.L., Licha, D., Ranninger, C., Scott-Fordsmand, J.J., Huber, C.G., Amorim, M.J.B., 2018a. The *Enchytraeus crypticus* stress metabolome–CuO NM case study. Nanotoxicology 12, 766–780. https://doi.org/10.1080/17435390.2018.1481237

Maria, V.L., Ribeiro, M.J., Guilherme, S., Soares, A.M.V.M., Scott-Fordsmand, J.J., Amorim, M.J.B., 2018b. Silver (nano)materials cause genotoxicity in *Enchytraeus crypticus*, as determined by the comet assay. Environmental Toxicololy and Chemistry. 37, 184–191. https://doi.org/10.1002/etc.3944

Martins, M.A., Trindade, T., 2012. Os nanomateriais e a descoberta de novos mundos na bancada do químico. Quim. Nova 35, 1434–1446. https://doi.org/10.1590/S0100-40422012000700026

Maynard, A.D., 2011. Don't define nanomaterials. Nature 475, 31. https://doi.org/10.1038/475031a

Mendonça, M.C.P., Soares, E.S., de Jesus, M.B., Ceragioli, H.J., Ferreira, M.S., Catharino, R.R., da Cruz-Höfling, M.A., 2015. Reduced graphene oxide induces transient blood-brain barrier opening: An *in vivo* study. J. Nanobiotechnology 13. https://doi.org/10.1186/s12951-015-0143-z

Mendonça, M.C.P., Soares, E.S., De Jesus, M.B., Ceragioli, H.J., Batista, Â.G., Nyúl-Tóth, Á., Molnár, J., Wilhelm, I., Maróstica, M.R., Krizbai, I., Da Cruz-Höfling, M.A., 2016a. PEGylation of reduced graphene oxide induces toxicity in cells of the blood-brain barrier: An *in vitro* and *in vivo* study. Molecular Pharmaceutics 13, 3913–3924. https://doi.org/10.1021/acs.molpharmaceut.6b00696

Mendonça, M.C.P., Soares, E.S., de Jesus, M.B., Ceragioli, H.J., Irazusta, S.P., Batista, Â.G., Vinolo, M.A.R., Maróstica Júnior, M.R., Cruz-Höfling, M.A., 2016b. Reduced graphene oxide: Nanotoxicological profile in rats. J. Nanobiotechnology 14. https://doi.org/10.1186/s12951-016-0206-9

Mendonça, Monique Culturato Padilha, Luiz Bandeira Ferreira, Cintia Rizoli, Ângela Giovana Batista, Mário Roberto Maróstica Júnior, Emanueli do Nascimento da Silva, Solange Cadore, Nelson Durán, Maria Alice da Cruz-Höfling, and Marcelo Bispo de Jesus. 2019. "N-Acetylcysteine Reverses Silver Nanoparticle Intoxication in Rats." Nanotoxicology 13(3):326–338. https://doi.org/10.1080/17435390.2018.1544302

Monaco, A.M., Giugliano, M., 2014. Carbon-based smart nanomaterials in biomedicine and neuroengineering. Beilstein Journal of Nanotechnoly https://doi.org/10.3762/bjnano.5.196

Murphy, C.J., Buriak, J.M., 2015. Best Practices for the Reporting of Colloidal Inorganic Nanomaterials. Chem. Mater. 27, 4911–4913. https://doi.org/10.1021/acs.chemmater.5b02323

National Nanotechnology Initiative website, Benefits and Applications, http://www.nano.gov/you/nanotechnology- benefits (accessed 2.06.20).

National Research Council, N.R., 2007. Toxicity Testing in the 21st Century: A Vision and a Strategy.

Navratilova, J., Praetorius, A., Gondikas, A., Fabienke, W., von der Kammer, F., Hofmann, T., 2015. Detection of engineered copper nanoparticles in soil using single particle ICP-MS. Internation Journal of Environmental Research and Public Health 12, 15756–15768. https://doi.org/10.3390/ijerph121215020

Nehoff, H., Parayath, N.N., Taurin, S., Greish, K., 2014. *In Vivo* Evaluation of Acute and Chronic Nanotoxicity, in: Handbook of Nanotoxicology, Nanomedicine and Stem Cell Use in Toxicology. 65–86. https://doi.org/10.1002/9781118856017.ch3

Nowack, B., 2017. Evaluation of environmental exposure models for engineered nanomaterials in a regulatory context. NanoImpact. https://doi.org/10.1016/j.impact.2017.06.005

OECD, 2016a. No. 83: Series on the Safety of Manufactured Nanomaterials - Silver Nanoparticles: Summary of the Dossier. Paris. https://doi.org/ENV/JM/MONO(2007)10

OECD, 2016b. OECD chemical studies show way forward for nanomaterial safety [WWW Document]. URL http://www.oecd.org/chemicalsafety/nanosafety/news-nanomaterial-safety.htm (accessed 10.30.19).

OECD - No.184, 2017. Environment, Health and Safety Publications Series on Testing and Assessment No 184. Revised Guidance Document on Developing and Assessing Adverse Outcome Pathways. Organization for Economic Cooperation and Development.

OECD - No.2, 2006. Series on the Safety of Manufactured Nanomaterials - Current Developments/Activities on the safety of Manufactured Nanomaterials. London. https://doi.org/10.1787/oecd_papers-v7-art36-en

OECD - No.220, 2016. Guidelines for testing of chemicals— Enchytraeid reproduction test, Paris.

OECD - No.318, 2017. Dispersion Stability of Nanomaterials in Simulated Environmental Media 1–4. https://doi.org/10.1787/9789264067394-eng

OECD - No.412, 2018. Subacute Inhalation Toxicity: 28-Day Study, OECD Guidelines for the Testing of Chemicals, Section 4. Paris. https://doi.org/10.1787/9789264070783-en

OECD - No.413, 2018. Subchronic Inhalation Toxicity: 90-day Study, OECD Guidelines for the Testing of Chemicals, Section 4. Paris. https://doi.org/10.1787/9789264070806-en

OECD - No.91, 2019. Series on the Safety of Manufactured Nanomaterials - Guiding Principles for Measurements and Reporting for Nanomaterials: Physical Chemical Parameters. Paris.

Oliveira, A.E.F., Braga, G.B., Tarley, C.R.T., Pereira, A.C., 2018. Thermally reduced graphene oxide: synthesis, studies and characterisation. J. Mater. Sci. 53, 12005–12015. https://doi.org/10.1007/s10853-018-2473-3

Pachapur, V., Brar, S.K., Verma, M., Surampalli, R.Y., 2015. Nano-ecotoxicology of natural and engineered nanomaterials for animals and humans, in: Nanomaterials in the

Environment. American Society of Civil Engineers (ASCE), 421–438. https://doi.org/10.1061/9780784414088.ch16

Parada, J., Rubilar, O., Fernández-Baldo, M.A., Bertolino, F.A., Durán, N., Seabra, A.B., Tortella, G.R., 2019. The nanotechnology among US: are metal and metal oxides nanoparticles a nano or mega risk for soil microbial communities? Critical Reviews in Biotechnology. https://doi.org/10.1080/07388551.2018.1523865

Peijnenburg, W., Praetorius, A., Scott-Fordsmand, J.J., Cornelis, G., 2016. Fate assessment of ENPs in solid media – current insights and the way forward. Environmental Pollution 218, 1365–1369.

Piccinno, F., Gottschalk, F., Seeger, S., Nowack, B., 2012. Industrial production quantities and uses of ten engineered nanomaterials in Europe and the world. Journal of Nanoparticle Research. 14, 1109. https://doi.org/10.1007/s11051-012-1109-9

Pulit-Prociak, J., Banach, M., 2016. Silver nanoparticles - A material of the future...? Open Chemistry. 14, 76–91. https://doi.org/10.1515/chem-2016-0005

Rahman, M.F., Wang, J., Patterson, T. a., Saini, U.T., Robinson, B.L., Newport, G.D., Murdock, R.C., Schlager, J.J., Hussain, S.M., Ali, S.F., 2009. Expression of genes related to oxidative stress in the mouse brain after exposure to silver-25 nanoparticles. Toxicology Letters 187, 15–21. https://doi.org/10.1016/j.toxlet.2009.01.020

Rasmussen, K., González, M., Kearns, P., Sintes, J.R., Rossi, F., Sayre, P., 2016a. Review of achievements of the OECD Working Party on Manufactured Nanomaterials' Testing and Assessment Programme. From exploratory testing to test guidelines. Regulatory Toxicology and Pharmacoly 74, 147–160. https://doi.org/10.1016/j.yrtph.2015.11.004

Rasmussen, K., González, M., Kearns, P., Sintes, J.R., Rossi, F., Sayre, P., 2016b. Review of achievements of the OECD Working Party on Manufactured Nanomaterials' Testing and Assessment Programme. From exploratory testing to test guidelines. Regulatory Toxicology and Pharmacology. 74, 147–160. https://doi.org/10.1016/j.yrtph.2015.11.004

Reidy, B., Haase, A., Luch, A., Dawson, K.A., Lynch, I., 2013. Mechanisms of silver nanoparticle release, transformation and toxicity: A critical review of current knowledge and recommendations for future studies and applications. Materials (Basel). 6, 2295–2350. https://doi.org/10.3390/ma6062295

Ribeiro, M.J., Maria, V.L., Scott-Fordsmand, J.J., Amorim, M.J.B., 2015. Oxidative Stress Mechanisms Caused by Ag Nanoparticles (NM300K) are Different from Those of $AgNO_3$: Effects in the Soil Invertebrate *Enchytraeus crypticus*. International Journal of Environmental Research of Public Health 12, 9589–9602. https://doi.org/10.3390/ijerph120809589

Santos, F.C.F., Gomes, S.I.L., Scott-Fordsmand, J.J., Amorim, M.J.B., 2017. Hazard assessment of nickel nanoparticles in soil-The use of a full life cycle test with *Enchytraeus crypticus*. Environmental Toxicology and Chemistry. 9999, 1–8. https://doi.org/10.1002/etc.3853

Sargent, J.F., 2016. Nanotechnology: A policy primer, in: Advances in Nanotechnology. pp. 123–136. www.crs.gov

Smalley, R.E., 1997. Discovering the fullerenes. Rev. Mod. Phys. 69, 723–730. https://doi.org/10.1103/revmodphys.69.723

Schlich, K., Klawonn, T., Terytze, K., Hund-Rinke, K., 2013. Hazard assessment of a silver nanoparticle in soil applied via sewage sludge. Environmental Sciences Europe 25, 17. https://doi.org/10.1186/2190-4715-25-17

Scott-Fordsmand, J.J., Peijnenburg, W., Amorim, M.J.B., Landsiedel, R., Oorts, K., 2016. The way forward for risk assessment of nanomaterials in solid media. Environmental Pollution 218, 1363–1364. https://doi.org/10.1016/j.envpol.2015.11.048

Shoults-Wilson, W., Reinsch, B., 2011. Role of particle size and soil type in toxicity of silver nanoparticles to earthworms. Soil Science Society of America Journal 75, (2). 365-377. https://doi:10.2136/sssaj2010.0127nps

Tämm, K., Sikk, L., Burk, J., Rallo, R., Pokhrel, S., Mädler, L., Scott-Fordsmand, J.J., Burk, P., Tamm, T., 2016. Parametrization of nanoparticles: Development of full-particle nanodescriptors. Nanoscale 8, 16243–16250. https://doi.org/10.1039/c6nr04376c

Trindade, T., Thomas, P.J., 2013. Defining and Using Very Small Crystals, in: Comprehensive Inorganic Chemistry II (Second Edition): From Elements to Applications. pp. 343–369. https://doi.org/10.1016/B978-0-08-097774-4.00416-2

Valiev, R., 2002. Materials science: Nanomaterial advantage. Nature. https://doi.org/10.1038/419887a

Van der Ploeg, M.J.C., Handy, R.D., Waalewijn-Kool, P.L., van den Berg, J.H.J., Herrera Rivera, Z.E., Bovenschen, J., Molleman, B., Baveco, J.M., Tromp, P., Peters, R.J.B., Koopmans, G.F., Rietjens, I.M.C.M., Van den Brink, N.W., 2014. Effects of silver nanoparticles (NM-300K) on *Lumbricus rubellus* earthworms and particle characterisation in relevant test matrices including soil. Environ. Toxicol. Chem. 33, 743–752. https://doi.org/10.1002/etc.2487

Wagner, S., Gondikas, A., Neubauer, E., Hofmann, T., Von Der Kammer, F., 2014. Spot the difference: Engineered and natural nanoparticles in the environment-release, behavior, and fate. Angew. Chemie - Int. Ed. https://doi.org/10.1002/anie.201405050

Westheide, W., Graefe, U., 1992a. Two new terrestrial Enchytraeus species (Oligochaeta, Annelida). Journal of Nature History 26, 479–488. https://doi.org/10.1080/00222939200770311

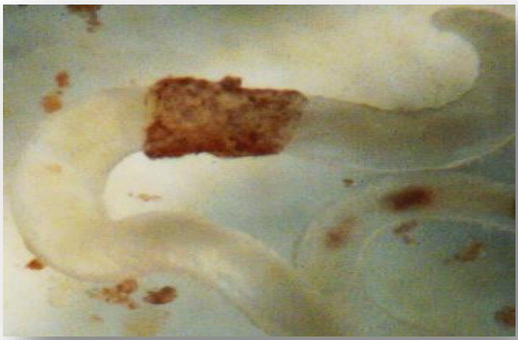


Photo by Natália Rodrigues

Chapter II

Novel understanding of toxicity in a life cycle perspective – the mechanisms that lead to population effect – the case of Ag (nano)materials

Novel understanding of toxicity in a life cycle perspective – the mechanisms that lead to population effect – the case of Ag (nano)materials

Natália P. Rodrigues[1], Janeck J. Scott-Fordsmand[2], Mónica J.B. Amorim[1]

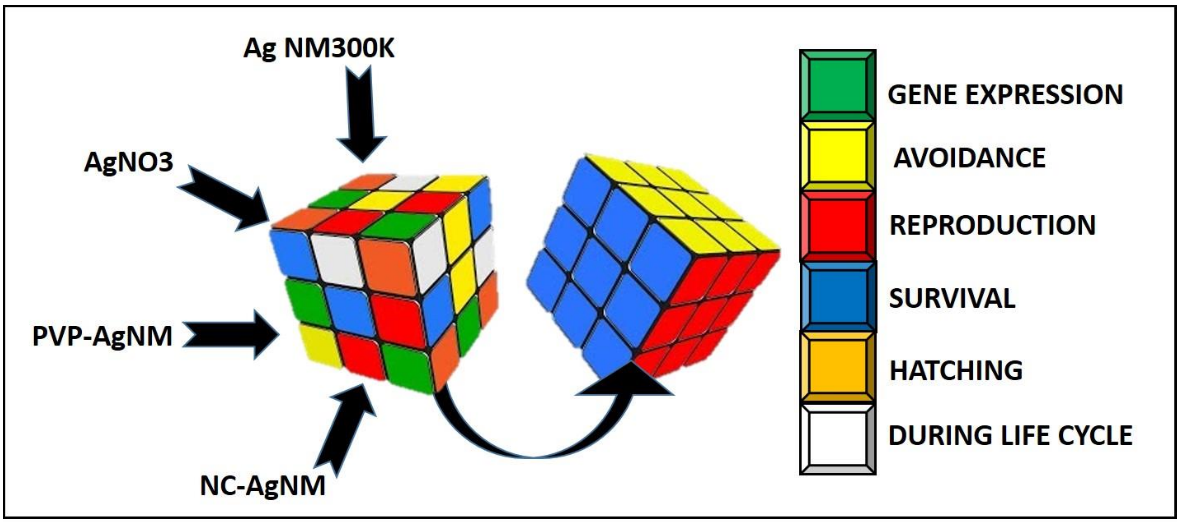
[1]Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal.

[2]Department of Bioscience, Aarhus University, Vejlsovej 25, PO BOX 314, DK-8600 Silkeborg, Denmark

Published in Environmental Pollution (2020)

DOI: https://doi.org/10.1016/j.envpol.2020.114277

TOC



Highlights

- Novel multi-endpoint approach for mechanistic based hazard assessment in *E. crypticus*
- Ag exposed organisms cannot avoid Ag after 24 hours.
- Ag affects the GABBAergic correlating to avoidance.
- Ag affects primarily embryo development and juvenile stages.
- High ecological impact of Ag long term exposure expected.

Abstract

Silver (Ag) is amongst the most well studied nanomaterials (NMs), although most studies have only dealt with a single AgNM at a time and one biological endpoint. We here integrate the results of various testing-tools (endpoints) using a terrestrial worm, the standard ecotoxicological model organism *Enchytraeus crypticus*. Exposure spanned both water and soil exposure, it covered all life stages (cocoons, juveniles and adults), varying exposure durations (1-2-3-4-5-21 days), and covered 5 biological end- points: hatching success, survival, reproduction, avoidance and gene expression (qPCR target genes GABA and Acetyl cholinesterase). We tested 4 Ag materials: PVP coated (PVP-AgNM), non-coated (NC-AgNM), the JRC reference Ag NM300K and $AgNO_3$. Results showed that short-term exposure via water to assess impact on cocoons' hatching predicted longer term effects such as survival and reproduction. Moreover, if we extended the exposure from 11 to 17 day this allowed discrimination between hatch delay and impairment. Exposure of juveniles and adults via water showed that juveniles were most sensitive with survival affected. Across materials the following toxic ranking was observed: $AgNO_3 \geq$ AgNM300K $\gg$ NC-AgNM $\geq$ PVP-AgNM. *E. crypticus* avoided $AgNO_3$ in a dose-response manner, avoiding most during the first 24 h. Avoidance of Ag NM300K and NC-AgNM only occurred during the first 24 hand the PVP coated AgNM were not avoided at all. The up-regulation of the GABA triggering anesthetic effects, indicated the high ecological impact of Ag materials in soil: Ag affects the GABAergic system hence organisms were not able to efficiently avoid and became intoxicated, this caused impacts in terms of survival and reproduction.

Keywords: avoidance; reproduction; survival; hatching; gene expression; safety-by-design

1. Introduction

Silver (Ag), in line with TiO_2, is among the most studied nano-materials (NMs) (Hansen and Baun, 2012), yet most studies have only dealt with a single AgNM at a time and one testing approach (tool). In brief, silver nanomaterials (AgNMs) are widely used worldwide, for example in the mean total productions of nano-Ag estimated for EU in 2014 was 50 tons (Sun et al., 2016). Depending on the products applying nano-Ag, different shares of amounts currently produced are entering into in-use stock or are released into technical and environmental compartments. Predictions indicated an annual increase of 1 µg/kg dry soil for AgNMs (Schlich et al., 2013) and predicted environmental concentrations (PECs) of 1.5 - 1.6 µg/kg for soil receiving sewage sludge (Mueller and Nowack, 2008; Schlich et al., 2013); for the U.S. predictions reach up to 13 µg/kg in the soil via sewage sludge (Gottschalk et al., 2009). The terrestrial environment is one of the primary receivers of NMs (Gottschalk et al., 2015) through the application of sewage sludge as fertilizer (Del Real et al., 2016) and is also the major sink among all environmental compartments. The latest soil PEC from accumulation of nano-Ag in sludge treated soil is 13.7 µg/kg in the EU in 2014 (Sun et al., 2016).

Studies in terrestrial compartment are much fewer when compared to aquatic compartment, and the studies are of increased complexity due to the soil multifaceted matrix, let aside the range of different soils available (Topuz and van Gestel, 2017). Silver nanomaterials have been studied in detail for certain model species like Enchytraeids, and in this case various levels, e.g. organismal survival and reproduction (Bicho et al., 2016a; Gomes et al., 2013), transcriptomics (Gomes et al., 2017), biomarkers (Ribeiro et al., 2015), or cellular energy allocation (S. Gomes et al., 2015), and even using lower complexity media like a mixture of sand and water (Topuz and van Gestel, 2015). Because characterisation of NMs suffers from lack of sufficient instruments and bias, the use of very well characterised reference materials such as the JRC repository Ag NM300K (Klein et al., 2011) is highly beneficial for comparison purposes and because it represents a good case for proof-of-concept. The best interpretation of results will benefit from a range of combined endpoints, e.g. various levels of biological organization and refined/detailed test designs (Amorim et al., 2016), this has been long argued and represents still a way forward. We here combine a range tools to study the biological effect at all life stages, linking effects between novel and traditional ecotoxicological measures. We used the standard ecotoxicological model species *Enchytraeus crypticus* (ISO, 2003; OECD, 2004a). In summary, with regard to exposure pathway we used both water and/or soil, with an exposure duration up to 21 days (i.e. 1-2-3-4-5- 21 days), depending on the life-stage studied. With regard to hazard, we tested all life stages (cocoons, juveniles, adults), with a total of five endpoints: hatching success, survival, reproduction, avoidance and gene expression (qPCR target genes GABA and Acetyl cholinesterase). The testing was done using 4 Ag materials: PVP coated (PVP-

AgNM), non-coated (NC AgNM), the JRC reference Ag NM300K and $AgNO_3$, a total of 25 tests.

2. Materials and Methods

2.1 Test species

Enchytraeus crypticus (Oligochaeta: Enchytraeidae) was used. Cultures are kept in agar plates prepared with a salt solution, fed with autoclaved grinded oats ad libitum and kept in the laboratory at 18 ºC and a 16:8 (light: dark) photoperiod. Synchronized cultures are prepared by transferring adults with well-developed clitellum into fresh agar plates to lay cocoons. These synchronized 1e2 days old cocoons are used for hatching test. Organisms of older life stages are obtained after, leaving the cultures to grow until 11 (juveniles) and ≥ 25 (mature adults) days old organisms. For further details please see (Bicho et al., 2015).

2.2 Test Materials and characterisation

Silver nitrate (AgNO3 > 99%, Sigma Aldrich) and three Ag nano-materials (NMs) were used. The AgNMs were the non-coated AgNMs (NC-AgNMs) (99%, 20-30 nm, American Elements), poly- vinylpyrrolidone (PVP)-coated AgNMs (PVP- AgNMs) (99%, 20-30 nm, American Elements) and Ag NM300K (10.2% w/w Ag, 15 nm; JRC Repository, (Klein et al., 2011)). The Ag NM300K is a dispersion in 4% polyoxyethylene glycerol trioleate and polyoxyethylene (20) orbitan mono-Laurat (Tween 20, also obtained from the Joint Research Centre, EU (Klein et al., 2011)); the dispersant was also tested alone. The characteristics of the materials are summarized in Table II-S1 (adapted from (Gomes et al., 2017)).

2.3 Test Media

2.3.1 Water

Standard ISO (International Organization for Standardization) reconstituted water was used (OECD, 2004a).

2.3.2 Soil

Natural standard soil (Speyer, Germany), the LUFA 2.2 soil, was used. This soil is well characterised and commercially available. Main properties are pH (0.01 M CaCl2) of 5.5, 1.61 % organic matter, 10.0 meq/100 g CEC (cation exchange capacity), 43.3 % WHC (water holding capacity), grain size distribution of 7.9 % clay, 16.3 % silt, and 75.8 % sand.

2.4 Spiking procedures, test concentrations and characterisation

An overview of all tests performed can be depicted in Table II-S2. For the water exposure, the suspensions with powder materials (PVP-AgNM and NC-AgNM) were prepared using ultrapure water and sonicated for 30 min (JP Selecta Ultrasonic Bath; 150 W). After sonication, 0.5mL of the suspensions were added to each well of the test plates containing 0.5mL of the test medium (ISO water). For water soluble $AgNO_3$ and dispersed Ag NM300K, stock solution/dispersion were prepared with water and serially diluted to obtain the corresponding concentration range, adding 0.5mL of stock solution/dispersion to each well of the test plates containing 0.5mL of the test medium. The exposure regime aimed at sub-lethal concentration range.

For the soil exposure, the dry materials PVP-AgNM and NC-AgNM were added as powder to the dry soil following the recommendations (OECD, 2012). To reach 50% of the soil WHC, deionised water was added and well mixed, done individually for each replicate. The aqueous materials solutions, $AgNO_3$, Ag NM300K and the dispersant, were added to the pre-moistened soil. The control dispersant was made adding the equivalent volume used with the highest Ag NM300K concentration. Four replicates per treatment were performed.

The soil Ag spiking was confirmed with flame Atomics Absorption Analysis (AAS) measurement. The soil for the analysis (1 g dry weight) was digested using 7N HNO_3 (nitric acid) and heating up to 110ºC until all brown fumes were gone (for AAS details see (Mariyadas et al., 2018)). In a concurrent experiment the Ag^+ present in the water was measured with an ion-selective electrode (ISE 25S, Radiometer) in combination with the reference electrode (REF251, radiometer) equipped with a double salt bridge. The inner salt bridge contained saturated KCl and the outer 0.1 M KNO_3 (Gomes et al., 2013). The ISE was calibrated with Ag^+ standards made by dissolution from an $AgNO_3$ stock solution in 0.1 M KNO_3, detection limit 10-6.

2.5 Tests performance

2.5.1 Exposure via ISO water

The test conditions were done based on the *Daphnia* acute immobilization test (OECD, 2004a) as described by Römbke and Knacker (Rombke and Kcnacker, 1989) for *Enchytraeus albidus*, and as reproduced in (Bicho et al., 2016b; S. I. L. Gomes et al., 2015). For each test condition, 5 organisms were selected per replicate. The exposure was performed in 24-well plates, in which each well corresponded to a replicate and contained 0.5mL of ISO water, 0.5 mL of spiked solution, plus 5 organisms. The test conditions were 20±1 ºC with a 16:8-h photoperiod. Test was performed using 3 different life stages, 1-2 days' cocoons, 10-11 days' juveniles and matured adults with clitellum and of similar size and the test duration and measured endpoints varied accordingly, i.e. no. of hatched juveniles and survival. For the hatching, exposure lasted up to 17 days, monitoring daily the number of hatched juveniles (via observation under the binocular). For juveniles and adults, survival was evaluated every 24h, for 5 days and organisms were considered dead when not responding to any mechanical stimulus. Observations were made using a stereo microscope and it was captured in photographs (Dinocapture 2.0) (Figure II-S1).

2.5.2 Post-exposure in clean soil

Organisms exposed to ISO water were transferred to control (non-spiked) LUFA 2.2 soil. The surviving organisms from each test condition were pooled in groups of 10 to be introduced on test vessels with soil. The procedure followed the Enchytraeid test guideline (ISO, 2003; OECD, 2004b) during 21 days' exposure. Four replicates per treatment were done.

2.5.3 Exposure via soil

2.5.3.1 Enchytraeid Reproduction Test (ERT)

The procedures followed the standard guideline (ISO, 2003; OECD, 2004b). Adults (10) with well-developed clitellum were selected from cultures and introduced in each test vessel containing 20 g of moist soil and food supply. Test run at 20ºC and 16:8 h photoperiod for 21 days. Four replicates per treatment were used. Food and water were replenished weekly. To extract organisms from soil and counting, replicates were fixated with 96 % ethanol and Bengal red (1 % solution in ethanol). After 2 h, soil samples were sieved through 3 meshes (1.6, 0.5, 0.2 mm) to separate individuals from most of the soil and facilitate counting using the stereo microscope.

2.5.4 Avoidance test

The avoidance test was performed following the earthworm avoidance test guideline with adaptations (Amorim and Scott-Fordsmand, 2012), using the two-chamber design. In short, plastic containers (7.8×4.3ø cm) with one removable plastic divider were used; each replicate contained 40 g of soil (20 g each side), this being control (dispersant control in the case of Ag NM300K testing and water control for the other) and spiked soil. After this, the wall was gently removed and ten adults with well-developed clitellum were placed on the contact line of the soils. Boxes were covered with lids (containing small holes) and were incubated at 16:8 h light/dark photoperiod cycle and temperature of 20±1°C. At the end of the test period, the divider was inserted in the separation line between the two soils with each side of the box independently searched for worms. Additional exposure times were done to the standard 48h test, i.e., 24, 48, 72 and 96h of exposure. Five replicates per treatment were used.

2.6 Gene expression – quantitative real-time PCR

Test procedures followed the detailed in (Bicho et al., 2014). Three replicates were used per treatment, which consisted of a glass vessel (8×4ø cm) containing 20 g of soil and 20 adults per replicate. The organisms were exposed to 24 and 96h under controlled standard test conditions. RNA was extracted from the pool of organisms per replicate, its quantity and purity were measured, and the integrity checked. Gene targets included the homologous to AChE and GABA-associated protein receptor (GABAAR), retrieved from the *E. crypticus* library (Castro-Ferreira et al., 2014). Actin was used as housekeeping gene being its suitability tested in all samples. Designed primers (Oligo ExplorerTM, version 1.1.2) efficiency and specificity were determined by observing the standard and melting curves for all primer sets.

The exposure design targeted the response shift between avoidance to no avoidance at a given concentration and time to observe effect (24h and 96h for 640 mg NC-AgNM /kg and for 20 mg $AgNO_3$/kg).

2.7. Data analysis

ANOVA (Analysis of variance) followed by Dunnett´s comparison post-hoc test ($p \leq 0.05$) was done to assess differences between control and treatments (SigmaPlot 11.0). Effect Concentrations (ECx) estimates were performed for the various endpoints modelling data, using Toxicity Relationship Analysis Program (TRAP v1.22). Effect Time (ETx) calculations were performed by using the time instead of concentration as a variable and inverting the y (effect) data to fit a normal dose response model, being inverted again for the equivalent estimation.

Avoidance was calculated as the mean percentage of net responses (NR) as follows: NR=((C−T)/N)*100, where C is the number of organisms observed in the control soil, T is the number of organisms observed in test soil and N is the total number of organisms per replicate. Positive (+) NR indicates avoidance and negative (−) NR indicates non-response (or attraction) to the treatment.

The Relative Expression Software Tool (REST-MSC) was used for qPCR data, where the mean normalized expression was calculated from the Ct values of the test genes. Differences between treated groups versus control were assess ($p<0.05$ based on pairwise fixed real- location randomization test).

3. Results

3.1 Characterisation

The soil Ag spiking measurement was confirmed (AAS measurement method), being between 95-109% Ag recovery (n = 1 per concentration), with no concentration dependent pattern.

3.2 Exposure via ISO water

Overall, no significant changes occurred for pH in all tests, being ca. 6.5±0.5, for all treatments and sampling times. Hence, although it may be inferred that the Ag salt may affect the pH, this was not the case. Ag NM300K was the only material observed to dissolve, this up to 15% in the first day but decreased rapidly in release to near zero after 3 - 4days (Fig. II-S2).

Results in terms of hatching success of the cocoons exposed via water up to 17 days can be observed in Figure II-1.

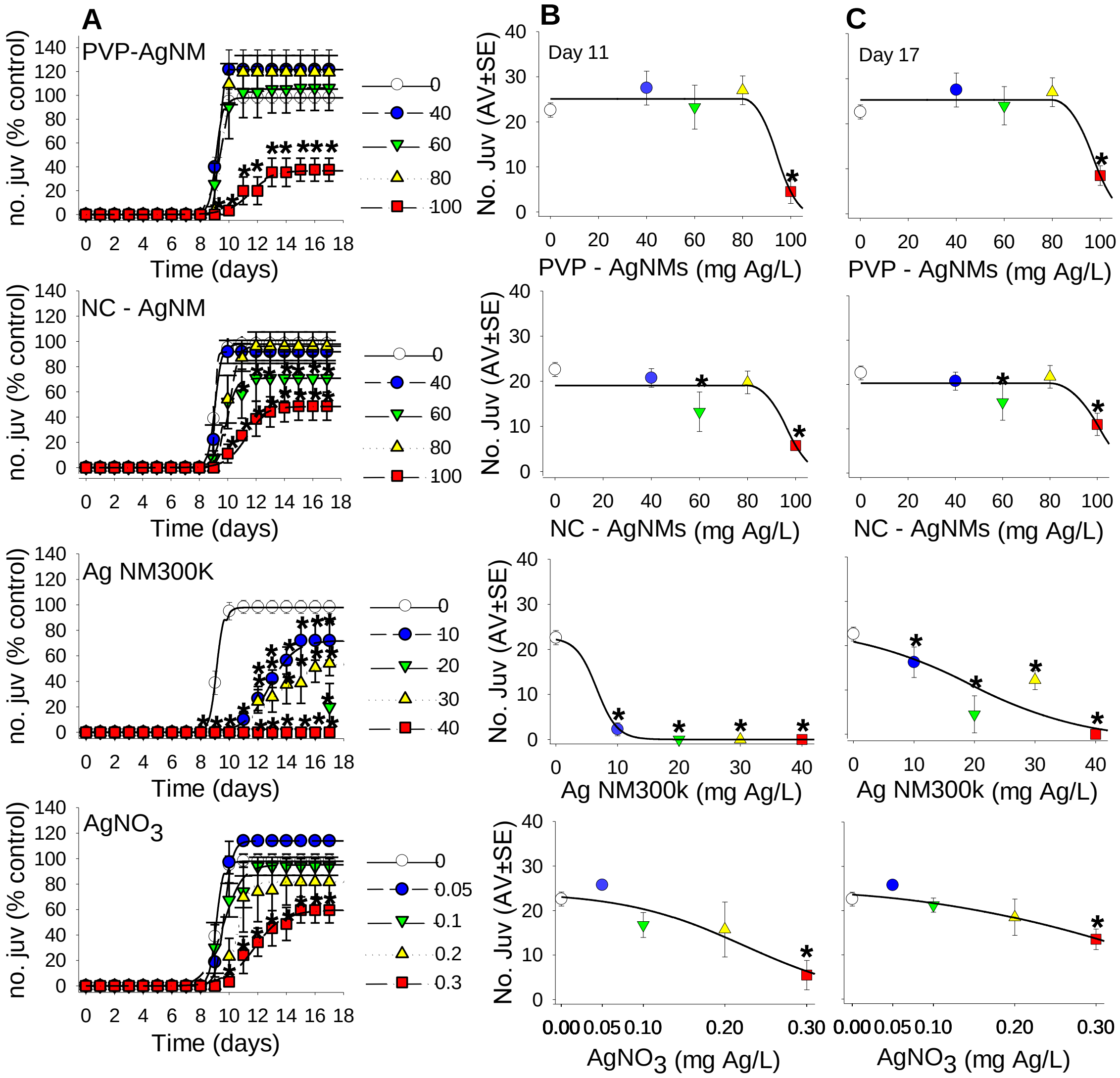


Figure II-1. Results in terms of hatching from the 1-2d old cocoons of *Enchytraeus crypticus*, when exposed to PVP-AgNM, NC-AgNM, Ag NM300K and $AgNO_3$ via ISO water media. A) Daily monitoring during 17 days. B) hatching dose response at day 11 and C) day 17. All values are expressed as average ± standard error (Av±SE). The lines represent the model fit to data. * p<0.05, Dunnets'.

By day 11 the maximum number of juveniles hatched in controls and there were clearly delays and/or reductions in relation to increasing Ag concentrations. The estimates for effect on time to hatch (ETx) per treatment can be found in Table II-1. Ag NM300K was the most toxic among the nanomaterials, both a delay (e.g. at 10 mg Ag NM300K, 10% hatch (day 11) to 80% hatch (day 15), see Figure II-1A) and a reduction, i.e. hatching impairment (e.g. 40 mg Ag NM300K, 0% hatching (11-17 days), see Figure II-1B). A note, for Ag NM300K the 20 mg exposure deviated from the dose-response pattern, showing a higher toxicity than expected based on concentration-response (especially obvious at day 17). This could be random variation, but has been noted in previous studies.

Table II-1. Summary of the time to hatch (ET: Effect Time), in days for cocoons of *Enchytraeus crypticus* when exposed to PVP-AgNM, NC-AgNM, Ag NM300K and $AgNO_3$ in ISO water up to 17 days. CI: 95% confidence interval. S: slope; Y0: intercept. n.d.: not determined; Log 2 par: Logistic 2 parameters; Thresh.2 par.: Threshold sigmoid 2 parameters.

Material/ Concentration	**ET10** (days)	**ET50** (days)	**ET80** (days)	**Model & parameters**
Control				
0	9.1 (8.9-9.3)	9.6 (9.1-10.2)	10 (9.3-10.5)	Log 2 par (S: 0.9; Y0: 98)
PVP-AgNM				
40	9.3 (n.d.)	9.6 (n.d.)	9.8 (n.d.)	Threshold 2 par (S: 1; Y0: 121.6)
60	9.3 (8.9-10)	10.2 (9.3-11.1)	10.5 (9.5-11.5)	Log 2 par (S: 0.7; Y0: 105)
80	9.6 (9.1-10.2)	10.2 (9.8-10.5)	10.4 (9.8-10.7)	Log 2 par (S: 0.7; Y0: 119.4)
100	11.5 (10.7-12.4)	13.1 (11.6-14.7)	n.d.	Log 2 par (S: 0.4; Y0: 36.7)
NC-AgNM				
40	9.1 (-28-42.7)	9.3 (-97.3-105)	9.5 (-121-127)	Log 2 par (S: 1; Y0: 91.8)
60	9.6 (9.3-10)	10.7 (9.8-11.6)	10.9 (9.8-12.2)	Log 2 par (S: 0.5; Y0: 70.7)
80	10.2 (10-10.4)	10.9 (10.2-11.5)	11.1 (10.4-11.8)	Log 2 par (S: 0.6; Y0: 96.2)
100	11.3 (10.7-11.6)	12.7 (11.5-13.6)	n.d.	Log 2 par (S: 0.4; Y0: 48.4)
Ag NM300K				
10	12.7 (12.5-13.3)	14.7 (13.8-15.6)	15.3 (14.2-16.5)	Log 2 par (S: 0.4; Y0: 72)
20	n.d.	n.d.	n.d.	-
30	13.1 (12.4-13.6)	15.8 (14.4-17.1)	n.d.	Log 2 par (S: 0.3; Y0: 54.2)
40	n.d.	n.d.	n.d.	-
$AgNO_3$				
0.05	9.6 (9.3-9.6)	10.2 (10-10.5)	10.4 (10.4-10.9)	Log 2 par (S: 0.7; Y0: 114)
0.1	9.6 (9.3-9.8)	11.1 (10.4-11.8)	11.6 (10.7-12.4)	Log 2 par (S: 0.4; Y0: 95.1)
0.2	10.5 (10-10.9)	11.3 (10.4-12.4)	11.6 (10.4-12.7)	Log 2 par (S: 0.3; Y0: 83.3)
0.3	11.8 (11.1-12.2)	14 (12.7-15.3)	n.d.	Log 2 par (S: 0.3; Y0: 59.7)

Monitoring for hatching and number of juveniles daily allowed to visually observe the impacts of the Ag materials at the macroscopic level (Figure II-S1), e.g. compared to controls, exposure to Ag NM300K particles agglomerated on the surface of the cocoons, transparency of the cocoon membrane was reduced, and with apparent increased hardness.

For juveniles, exposure via water caused mortality, e.g. in a concentration response manner for Ag NM300K (Figure II-2).

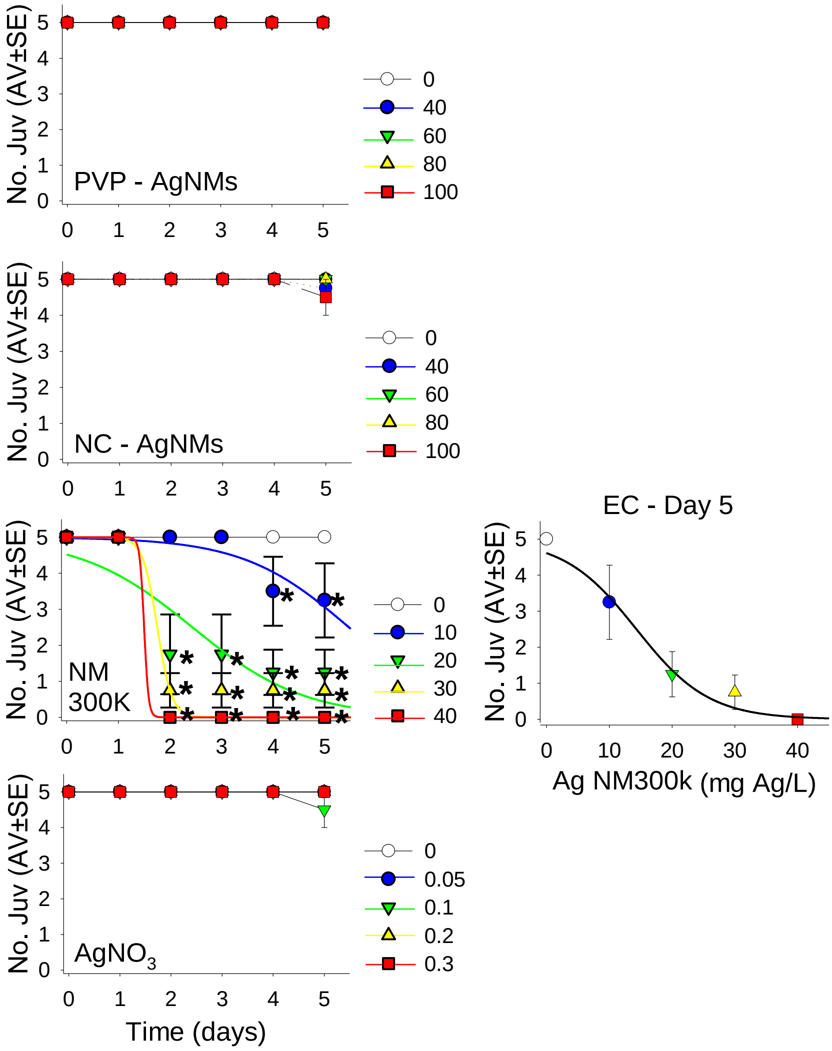


Figure II-2. Results in terms of survival of juveniles (10-11 days old) of Enchytraeus crypticus when exposed to PVP-AgNM, NC-AgNM, Ag NM300K and AgNO3 (mg Ag/L) in ISO water media for 5 days. Right panel shows the plot of effect at day 5 and the dose-response model line. * $p < 0.05$ Dunnets'. All values are expressed as average ± standard error (Av ± SE).

Regarding the exposure of adults via water for 5 days, no mortality occurred (Figure II-3) although the macroscopic visualization showed what seemed to be an adsorption of particles to the clitellum area (Figure II-S3).

3.3 Post-exposure in clean soil

The transfer to clean soil of the pre exposed adults via ISO water did not cause significant impact in survival and reproduction (Figure II-3A).

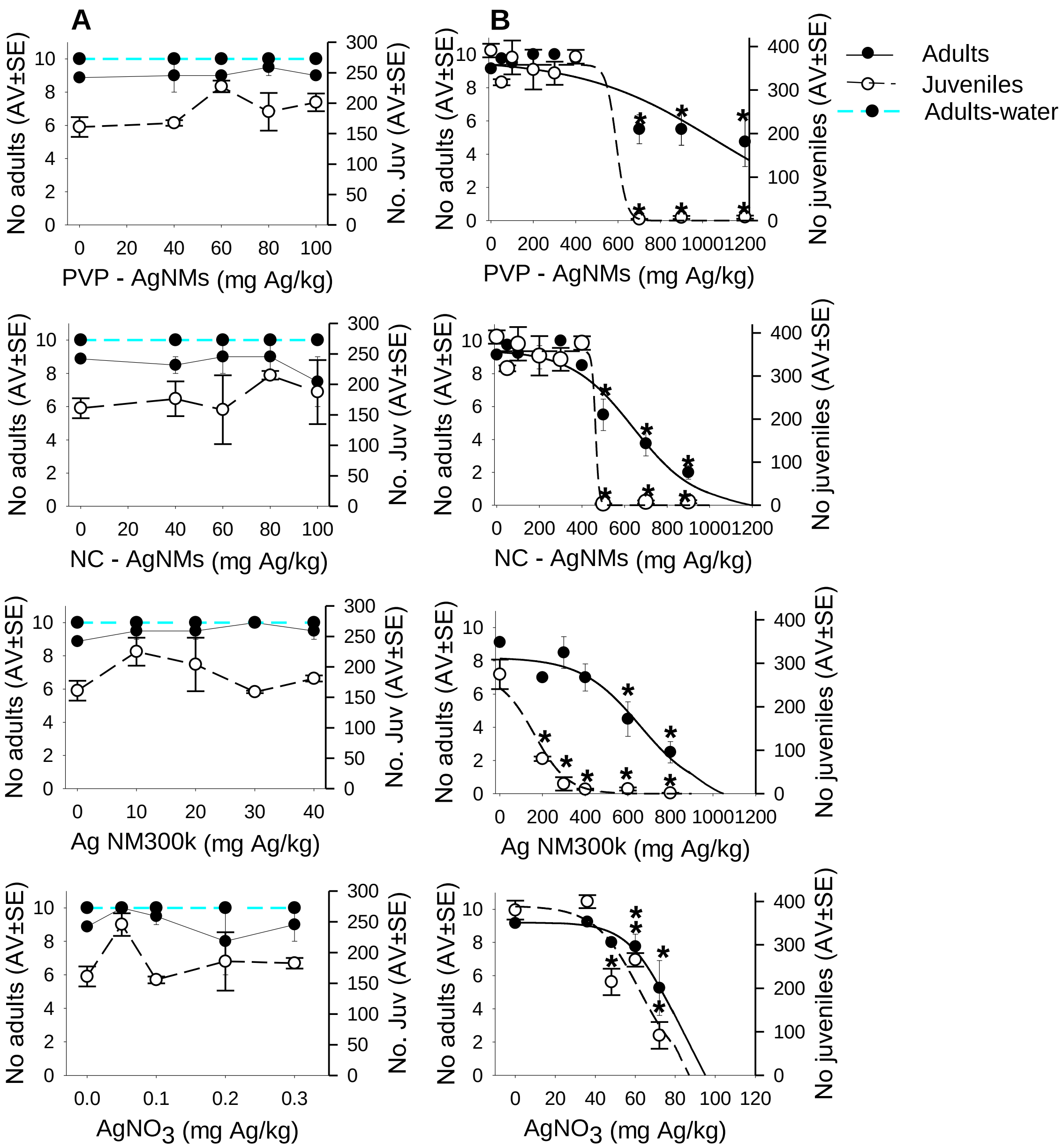


Figure II-3. Results in terms of the survival and reproduction of *Enchytraeus crypticus*, when exposed to PVP-AgNM, NC-AgNM, Ag NM300K and $AgNO_3$ (mg Ag/ L). A) Post exposure in LUFA 2.2 clean soil after 5 days in ISO water media (dashed line, 100% survival). B) in LUFA 2.2 soil in standard test, the lines represent the model fit to data. All values are expressed as average ± standard error (Av ± SE). $AgNO_3$ and Ag NM300K from (Bicho et al., 2016a).

3.4 Exposure via soil

3.4.1 Enchytraeid Reproduction Test (ERT)

All tests fulfilled the validity criteria as within guideline (ISO, 2014; OECD, 2016) and pH did not vary significantly throughout the test being 5.4 ± 0.05 and 5.3 ± 0.06 at start and end respectively. No significant differences occurred between water control and the dispersant tested alone, hence the plots show a pool of both.

Results showed a dose response effect for both survival and reproduction within the tested range (Figure II-4B). Effect concentrations estimates can be found on Table II-2; PVP-AgNM was the least toxic, followed by NC-AgNM and Ag NM300K, with $AgNO_3$ being the most toxic.

Further detailed observations at test end showed the presence of unhatched cocoons (Figure II-S4). This seems to be in line with the potential delay observed in terms of time to hatch.

Table II-2. Effect concentrations (mg/kg) estimate for *Enchytraeus crypticus* when exposed to PVP-AgNM, NC-AgNM, Ag NM300K and $AgNO_3$ in LUFA 2.2 soil. Results show the 95 % confidence intervals (in brackets). S: slope; Y0: intercept. n.d.: not determined.

Endpoint	EC10	EC20	EC50	EC80	Model parameters
Survival					
PVP-AgNM	372 (164-579)	626 (485-767)	1060 (929-1192)	1495 (1246-1743)	Log 2 par S: 0.0008; Y0: 9.7
NC-AgNP	321 (235-407)	439 (377-501)	641 (593-689)	843 (762-923)	Log 2 par S: 0.0017; Y0: 9.4
Ag NM300K	356 (195-517)	467 (353-580)	657 (578-735)	846 (709-984)	Log 2 par S: 0.0018; Y0: 8.2
$AgNO_3$	52 (38-67)	61 (52-69)	75 (66-84)	90 (70-110)	Log 2 par S: 0.02; Y0: 9.2
Reproduction					
PVP-AgNM	539	558	592	625	Log 2 par S: 0.01; Y0: 358
NC-AgNM	446	453	464	475	Log 2 par S: 0.031; Y0: 357.6
Ag NM300K	n.d.	52	161 (102-200?)	270 (192-348)	Log 2 par S: 0.003; Y0: 274.5
$AgNO_3$	38 (24-51)	47 (37-56)	62 (57-68)	78 (67-88)	Log 2 par S: 0.02; Y0: 389.8

For an LC/EC comparison, an overview of the linear part of the concentration response curve is provided in Figure II-S5; Ag NM300K showed similar chronic (reproduction effect) toxicity to $AgNO_3$. The best comparison is probably to compare both the general concentration level and the steepness. With regard to general concentration level, it is for example clear that for AgNM300K reproduction is affected at lower exposure concentrations than survival. With regard to steepness, it is for example clear that for PVP-AgNM a "small" change in exposure concentration (539-625 mg Ag/kg, 1.2-fold change) caused a dramatic change in the reproductive success, whereas for mortality the same relative change is only obtained by a larger change in the exposure concentration from (372-1495 mg Ag/kg, 4-fold change). Steepness in Figure II-S5 is the inverse of "S" in Table II-2.

3.4.2 Avoidance test

Results from avoidance test were within validity criteria, i.e. survival ≥90%. The avoidance behavior response can be observed in Figure II-4.

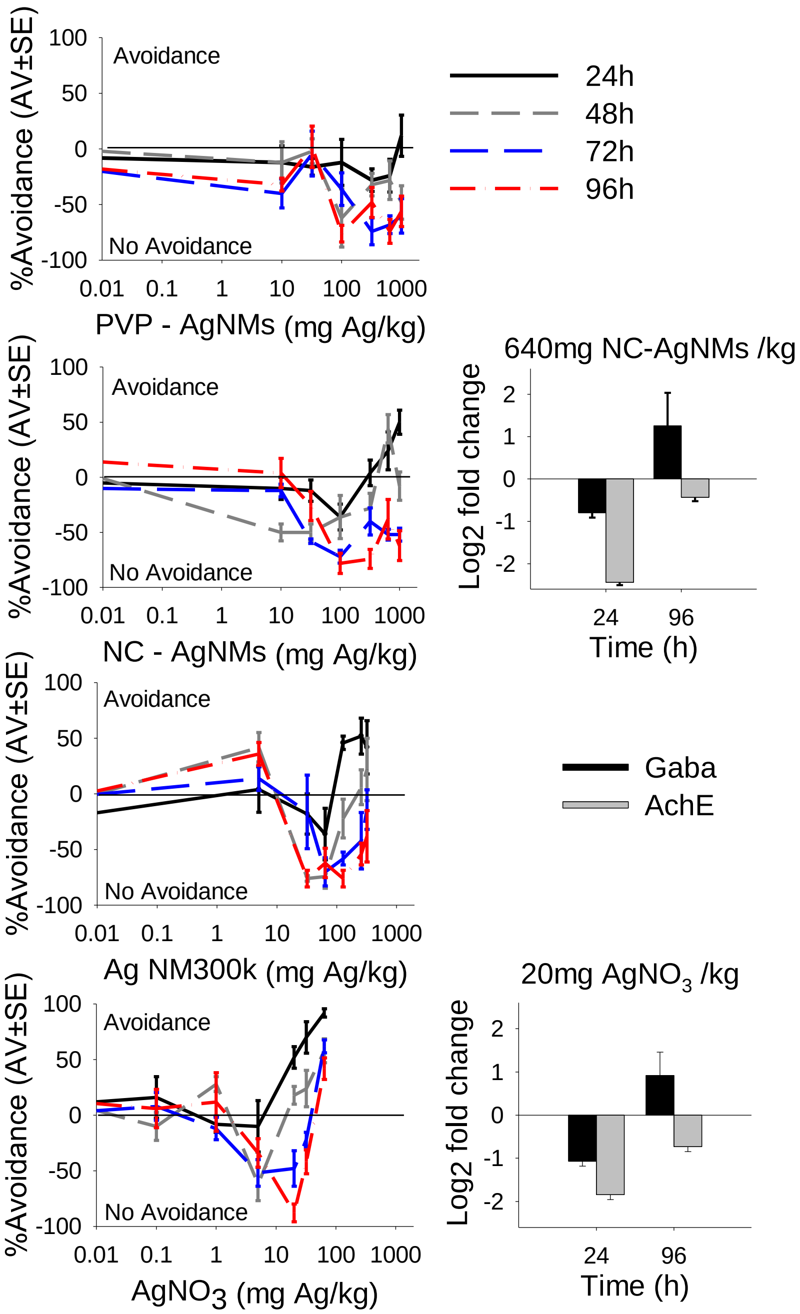


Figure II-4. Left panel: Results of the avoidance test in *Enchytraeus crypticus* when exposed to PVP-AgNM, NC-AgNM, Ag NM300K and $AgNO_3$ (mg Ag/Kg DW soil) in

LUFA 2.2 soil. Right panel: Quantitative gene expression (qPCR) for acetylcholinesterase (AChE) and gamma-aminobutyric acid receptor-associated protein ($GABA_AR$) codifying genes from *Enchytraeus crypticus* when exposed to 640 mg NC-AgNM/kg and 20 mg $AgNO_3$/kg in LUFA 2.2 soil during 24 and 96h. All values are expressed as average ± standard error (Av ± SE).

Results showed that no avoidance occurred for PVP-AgNMs, while the NC-AgNMs caused avoidance during the first 24h of exposure at highest concentrations. The extended exposure time from 24h onwards (48h-96h) showed a clear reduction of the ability to avoid and the apparent attraction behaviour when worms have their sensory organs affected. Ag NM300K triggered avoidance at 128, 256 and 320mg/kg during the first 24h after which (48-96h) less to no avoidance was observed. Hence, a similar pattern was observed across the materials.

An inspection of the gene regulation for acetylcholinesterase (AChE) and gamma-aminobutyric acid receptor-associated protein (GABAAR) at 640 mg NC-AgNM /kg, a concentration where avoidance was observed at 24h and not at 96h, showed both an up-regulation of GABA and reduction of AChE. For $AgNO_3$ avoidance occurs in a dose response manner although the trend to decrease the ability to avoid with time is apparent from 24h to 96h. Again, here an inspection of the gene expression at 20 mg $AgNO_3$/kg, a concentration where avoidance was observed at 24h and not at 96h, showed up-regulation of GABA and reduction of AChE (Figure II-4).

4. Discussion

Initial exposed via water, with later transfer to soil, made it feasible to monitor and assess the hatching success, considered the first step in the life cycle of the organisms. In previous studies, a short term exposure (up to 11 days) has been aimed to and recommended for the hatching endpoint, but we here extended the exposure period to 17 days and monitored effects daily. This extension (11 to 17 days) further allowed discrimination between hatch delay and impairment, hence it represents a possible alternative for testing of nanomaterials. Most important to note is that we gained an additional endpoint: time to hatch. The duration of time to hatch in treatments compared to control is of high ecological relevance and a delay (if not impairment) will influence population dynamics. For example, if we are observing the response to toxicity as expressed by a delay in hatching, this means that the population numbers will be lower at a certain time frame but one could argue that with time such population numbers can catch up. On the other hand, one should not forget that there are innumerous interactions with other organisms and feedback loops between these that will be changed too, this with carry unpredictable level of impact (Mendes et al., 2019, 2018; Menezes-Oliveira et al., 2014).

Results confirmed the potential for hatching effects to predict longer term effects such as survival and reproduction (Bicho et al., 2016a). This is most valid of course if the mode of action of the material occurs in the developmental or early life stages, since exposure will be via the 1-2 days old cocoons membrane. It is not always the case that the nano form affects hatching, for instance Cu salt affected hatching success of *E. crypticus* but CuNMs did not (Bicho et al., 2017). The sources for the variation between materials can be diverse, the size and behaviour of materials (smaller less aggregated particles may enter the membrane), the mechanism of toxicity and exposure route. For $AgNO_3$ the correlation between survival and reproduction is ca. 1:1 and hence Ag ions seem to affect primarily survival, whereas for Ag NM300K the effect occurs both in terms of survival and reproduction (Figure II-S5).

Regarding hatching, the deviation from the dose-response pattern for the 20mg Ag NM300K/kg exposure, i.e. showing a higher than expected toxicity, supports previous similar observation by (Bicho et al., 2016a) where the same concentration caused a severe decrease in reproduction. Again, although this here may be random variation, the most logical explanation is the concentration dependent agglomeration, i.e. the higher the concentration the higher the agglomeration (Stebounova et al., 2011). Hence, it appears from the biological results that there will be an “optimum” lower concentration for maximum dispersion/toxicity, between 20-40mg for Ag NM300K in ISO water and LUFA 2.2 soil. Similar observations have been reported before (van der Ploeg et al., 2014). This suggests that for nanomaterials we can expect an inverse-hormesis (inverted bell or J-shape) at low-intermediate concentration. This obviously poses similar or even bigger challenges in relation to modelling the concentration-response curves, especially in regard to defining when negative effect starts, as also discussed by (Chevillotte et al., 2017a, 2017b). It should be noted that form an ecological point of view both positive and negative changes in a concentration-response curve can have severe community impacts.

Regarding the exposure via water of other life stages like adults and juveniles, no effects on survival occurred for adults, and for juveniles to a low extent (for Ag NM300K). This again confirms that Ag affects mostly earlier life stages (compared to later) and that the mode of action targets the organism development. The observed effect on juvenile survival by NM300K point that the effect here is driven (to some extent at least) by free ions. Although, it should be considered that when organisms are pre- sent in the ISO water there will also be organic matter present from feces or mucus from the organism to which the ions will be bound and hence not free. However, it does point at the dissolution scenario of considerable environmental importance both in relation to amount and timing.

In exposure via soil, when focusing on the comparison between PVP coated AgNMs and non-coated AgNMs, results showed lower toxicity of the PVP coated for all endpoints, i.e., PVP-AgNM represented a safer by design option. This however becomes less straight forward when we consider that PVP coated AgNMs where less avoided than non-coated NMs, hence PVP-AgNMs exposure is likely higher under realistic environmental conditions and may lead to bioaccumulation. Differences in coatings have shown that differences in dissolution rates may explain the different uptake kinetics and consequent toxicity, e.g. lower for citrate coated AgNP (Topuz and van Gestel, 2015). Authors also argue that the toxicity of AgNPs is probably mainly attributable to the release of Ag ions (Topuz and van Gestel, 2015) but do not disregard the role of nanoparticulate Ag (Topuz and van Gestel, 2017). Characterisation of the materials in OECD soil (Mariyadas et al., 2018) at 24, 48, 72 and 96h after spiking showed total Ag soil concentration in the soil and in soil:water solution (flame atomic absorption spectroscopy (AAS). Two aspects were noticeable, for the soil solution the 200 KDa filtered (30 – 200 KDa) fraction had a continuous increase over time for the nano-forms but not for the AgNO3, whereas the 3 KDa filtered fractions had a continuous decrease. None of this corre- lates with changes in EC50.

For the Ag in soil-solution the analysis included a size filtering (Minisart-plus®, Microsep Advacne®) step using four fraction size filters (Kilodalton): >200 KDa, 30 – 200 KDa, 3 –

30 KDa and <3 KDa, which should in principle correspond to smaller agglomerated (i.e. >200 Kda), discriminate free particles (i.e. below 30 KDa) and free ions (i.e. below 3 KDa). Overall, initially (24h) the majority of Ag was in the >200 KDa fraction and as time progressed the size fraction of the Ag changed to the 30 – 200 KDa, with very little Ag present in the <3 KDa and 3 – 30 KDa samples (except for $AgNO_3$). For the $AgNO_3$ samples there was a more even distribution, with almost 60% Ag present in the <3 KDa fraction at 48 h and then evenly distributed between the <3 KDa, 30 – 200 KDa and >200 KDa at 72 – 96h. Although there are obvious differences between the soils the relative pattern can be assumed similar and hence we could not find the explanation for the changes in the ECs, by observing the soil solution concentrations. There are evidences that for NMs equilibrium is not reached (Peijnenburg et al., 2016), and so changes of effects over time should be expected. As observed, $AgNO_3$ was avoided in a dose-response manner, avoiding best during the first 24h; avoidance of Ag NM300K and NC-AgNM only occurred during the first 24h and the PVP coated AgNM was not avoided. Hence, an interesting observation is that avoidance was always larger during the first 24h, after which it kept decreasing with time (up to 96h). This differs from observations in *Eisenia fetida*, also a terrestrial oligochaete, where the avoidance behaviour was more consistent and effective throughout time and dose-response (Mariyadas et al., 2018). The inability of organisms to avoid certain chemical substances has been previously described (Bicho et al., 2014; Pereira et al., 2013) and also found associated with the GABAergic system in *E. crypticus* (Bicho et al., 2014). The up-regulation of the GABA is known to trigger anesthetic effects, hence this may at least partially explain the observations: GABA was upregulated after Ag exposure being further expressed from 24h to 96h, hence reduced ability to avoid. Overall, this indicated the high ecological impact of Ag materials in soil: Ag activates the GABAergic system hence organisms are not able to efficiently avoid contaminated patches and escape to cleaner surroundings, become intoxicated, with survival and reproduction being further impacted in the longer term. So, in principle, GABA could be a biomarker of AgNM exposure that links to avoidance effects.

In summary, the above show that risk assessment of (nano)materials should be more inclusive of the wide variety of hazard measurements, beyond OECD standard tests produced data at the moment, otherwise real life effects are not identified. Standardization organizations are aware of the need for steps towards more timely procedures, but it is obviously time to further promote and ensure such inclusions. This will also promote a movement towards understanding the risk, rather than assessing it - mechanistic based risk assessment - which carries immense potential to progress in terms of possibilities for protection and mitigation strategies.

5. Conclusions

This study showed that the detailed inspection via 1) increasing complexity of exposure media (water and soil), 2) wide range of endpoints (hatching success, time to hatch (novel), survival, reproduction, avoidance behaviour, targeted gene expression), 3) increased time series (24-48-72-96h' avoidance, 1-2-3-4-…-17 days hatching success) and 4) a range of Ag (nano)materials (PVP-AgNM, NC-AgNM, Ag NM300K, $AgNO_3$), allowed us to identify the key initiating events that caused long term effects. To summarise, the key event was an Ag targeting of embryo stages development, i.e. lower exposure concentrations cause a delay in hatching and higher stopped differentiation processes and impaired it totally. When exposed as adults *E. crypticus* were much less affected by Ag even at higher doses. For both the key event and the stage, the biological effect differed depending on the NM, with the PVP coated showing in general least toxicity. It was shown that Ag interferes with the GABAergic mechanism, causing paralysis due to GABA up-regulation (effects get more pronounced from 24h after exposure), probably affecting the organisms' ability to avoid silver. Last, a correlation was found between short and longer term effects, i.e. survival and decrease in hatching to Ag, supporting the role of hatching impairment as a key event.

6. Acknowledgements

This study was supported by the European Commission Projects FP7–MARINA (G.A. no. 263215) and H2020-NMBP-2017 BIORIMA (GA No. 760928) and H2020-NMBP-14-2018 NanoInformaTIX (GA No. 814426). Further support within CESAM [UID/AMB/50017/2019], to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020, and by FCT-

Fundação para a Ciência e Tecnologia via the individual PhD grant to Natália Rodrigues (SFRH/BD/87787/2012).

References

Amorim, M.J.B., Roca, C.P., Scott-Fordsmand, J.J., 2016. Effect assessment of engineered nanoparticles in solid media - Current insight and the way forward. Environ. Pollut. 218, 1370–1375. doi:10.1016/j.envpol.2015.08.048

Amorim, M.J.B., Scott-Fordsmand, J.J., 2012. “Toxicity of copper nanoparticles and CuCl2 salt to *Enchytraeus albidus* worms: survival, reproduction and avoidance responses”. Environ. Pollut. 164, 164–8. doi:10.1016/j.envpol.2012.01.015.

Bicho, R., Santos, F., Goncalves, M., Soares, A., Amorim, M., 2015. “Enchytraeid Reproduction Test(PLUS): hatching, growth and full life cycle test--an optional multi-endpoint test with *Enchytraeus crypticus*”. Ecotoxicology 24, 1053–1063. doi:10.1007/s10646-015-1445-5.

Bicho, R., Santos, F., Scott-Fordsmand, J., Amorim, M., 2017. “Effects of copper oxide nanomaterials (CuONMs) are life stage dependent – full life cycle in *Enchytraeus crypticus”*. Environ. Pollut. 224, 117–124. doi:10.1016/j.envpol. 2017.01.067.

Bicho, R.C., Gomes, S.I.L., Soares, A.M.V.M., Amorim, M.J.B., 2014. “Non-avoidance behaviour in enchytraeids to boric acid is related to the GABAergic mechanism”. Environ. Sci. Pollut. Res. 22, 6898–6903. doi:10.1007/s11356-014-3921-5.

Bicho, R.C., Ribeiro, T., Rodrigues, N.P., Scott-Fordsmand, J.J., Amorim, M.J.B.B., 2016a. “Effects of Ag nanomaterials (NM300K) and Ag salt ($AgNO_3$) can be discriminated in a full life cycle long term test with *Enchytraeus crypticus”*. J. Hazard. Mater. 318, 608–614. doi:10.1016/j.jhazmat.2016.07.040.

Bicho, R.C., Soares, A.M.V.M., Nogueira, H.I.S., Amorim, M.J.B., 2016b. “Effects of europium polyoxometalate encapsulated in silica nanoparticles (nanocarriers) in soil invertebrates”. J. Nanoparticle Res. 18, 360. doi:10.1007/s11051-016-3662-0.

Castro-Ferreira, M.P., de Boer, T.E., Colbourne, J.K., Vooijs, R., van Gestel, C. a M., van Straalen, N.M., Soares, A.M.V.M., Amorim, M.J.B., Roelofs, D., 2014. “Transcriptome assembly and microarray construction for *Enchytraeus crypticus*, a model oligochaete to assess stress response mechanisms derived from soil conditions”. BMC Genomics 15, 302. doi:10.1186/1471-2164-15-302.

Chevillotte, G., Bernard, A., Varret, C., Ballet, P., Bodin, L., Roudot, A.C., 2017a. “Probabilistic assessment method of the non-monotonic dose-responses-Part II: Robustness assessment”. Food Chem. Toxicol. 110, 214–228. doi:10.1016/j.fct.2017.10.030.

Chevillotte, G., Bernard, A., Varret, C., Ballet, P., Bodin, L., Roudot, A.C., 2017b. “Probabilistic assessment method of the non-monotonic dose-responses-Part I: Methodological approach”. Food Chem. Toxicol. 106, 376–385. doi:10.1016/j.fct.2017.05.070.

Del Real, A.E.P., Castillo-Michel, H., Kaegi, R., Sinnet, B., Magnin, V., Findling, N., Villanova, J., Carriére, M., Santaella, C., Fernández-Martínez, A., Levard, C., Sarret, G., 2016. “Fate of Ag-NPs in Sewage Sludge after Application on Agricultural Soils”. Environ. Sci. Technol. 50, 1759–1768. doi:10.1021/acs.est.5b04550.

Gomes, S., Scott-Fordsmand, J., Amorim, M., 2015. “Cellular Energy Allocation to Assess the Impact of Nanomaterials on Soil Invertebrates (Enchytraeids): The Effect of Cu and Ag”. Int. J. Environ. Res. Public Health 12, 6858–6878. doi:10.3390/ijerph120606858.

Gomes, S.I.L., Caputo, G., Pinna, N., Scott-Fordsmand, J.J., Amorim, M.J.B., 2015. “Effect of 10 different TiO 2 and ZrO 2 (nano)materials on the soil invertebrate *Enchytraeus crypticus”*. Environ. Toxicol. Chem. 34, 2409–2416. doi:10.1002/etc.3080.

Gomes, S.I.L., Roca, C.P.P., Scott-Fordsmand, J.J., Amorim, M.J.B., 2017. “High-throughput transcriptomics reveals uniquely affected pathways: AgNPs, PVP-coated AgNPs and Ag NM300K case studies”. Environ. Sci. Nano 4, 929–937. doi:10.1039/C6EN00652C.

Gomes, S.I.L., Soares, A.M.V.M., Scott-Fordsmand, J.J., Amorim, M.J.B., 2013. “Mechanisms of response to silver nanoparticles on *Enchytraeus albidus* (Oligochaeta):

Survival, reproduction and gene expression profile”. J. Hazard. Mater. 254–255, 336–344. doi:10.1016/j.jhazmat.2013.04.005.

Gottschalk, F., Lassen, C., Kjoelholt, J., Christensen, F., Nowack, B., 2015. “Modeling flows and concentrations of nine engineered nanomaterials in the Danish environment”. Int. J. Environ. Res. Public Health 12, 5581–602. doi:10.3390/ijerph120505581.

Gottschalk, F., Sonderer, T., Scholz, R.W., Nowack, B., 2009. “Modeled environmental concentrations of engineered nanomaterials (TiO2, ZnO, Ag, CNT, fullerenes) for different regions”. Environ. Sci. Technol. 43, 9216–9222. doi:10.1021/es9015553.

Hansen, S.F., Baun, A., 2012. “When enough is enough”. Nat. Nanotechnol. 7, 409–411. doi:10.4140/TCP.n.2017.425.

ISO, 2014. 16387 Soil quality — Effects of contaminants on Enchytraeidae (*Enchytraeus* sp.) — Determination of effects on reproduction. ISO (International Organization for Standardization), Geneva.

ISO, 2003. Soil quality—Effects of pollutants on Enchytraeidae (*Enchytraeus* sp.). Determination of effects on reproduction. ISO 16387. Geneva, Switzerland.

Klein, C.L., Comero, S., Stahlmecke, B., Romazanov, J., Kuhlbusch, T.A.J., Doren, E. Van, Mast, P.-J.D.T.J., Wick, P., Krug, H., Locoro, G., Hund-Rinke, K., Kördel, W., Friedrichs, S., Maier, G., Werner, J., Linsinger, T., Gawlik, B.M., 2011. “NM-Series of Representative Manufactured Nanomaterials, NM-300 Silver Characterisation, Stability, Homogeneity”. Luxembourg: Publications Office of the European Union. doi:10.2788/23079.

Mariyadas, J., Amorim, M.J.B., Jensen, J., Scott-Fordsmand, J.J., 2018. “Earthworm avoidance of silver nanomaterials over time”. Environ. Pollut. 239, 751–756. doi:10.1016/j.envpol.2018.04.059.

Mendes, L.A., Amorim, M.J.B., Scott-Fordsmand, J.J., 2019. Assessing the toxicity of safer by design CuO surface-modifications using terrestrial multispecies assays. Sci. Total Environ. 678, 457–465. doi:10.1016/j.scitotenv.2019.04.444

Mendes, L.A., Amorim, M.J.B., Scott-Fordsmand, J.J., 2018. Interactions of Soil Species Exposed to CuO NMs are Different from Cu Salt: A Multispecies Test. Environ. Sci. Technol. 52, 4413–4421. doi:10.1021/acs.est.8b00535

Menezes-Oliveira, V.B.B., Scott-Fordsmand, J.J.J., Soares, A.M.V.M., Amorim, M.J.B.J.B., 2014. Development of ecosystems to climate change and the interaction with pollution—Unpredictable changes in community structures. Appl. Soil Ecol. 75, 24–32. doi:10.1016/j.apsoil.2013.10.004

Mueller, N.C., Nowack, B., 2008. "Exposure Modeling of Engineered Nanoparticles in the Environment". Environ. Sci. Technol. 42, 4447–4453. doi:10.1021/es7029637.

OECD, 2016. Test No. 220: Guidelines for testing of chemicals— Enchytraeid reproduction test. OECD (Organization for Economic Cooperation and Development). OECD Publishing, Paris.

OECD, 2012. Organisation for Economic Cooperation and Development. Guidance on sample preparation and dosimetry for the safety testing of manufactured nanomaterials. Series on the Safety of Manufactured Nanomaterials No. 36. doi:ENV/JM/MONO(2007)10.

OECD, 2004a. Test No. 202: OECD Guidelines for testing of Chemicals — Daphnia sp., Acute Immobilisation Test. OECD (Organization for Economic Cooperation and Development). OECD Publishing, Paris. Paris, France. doi:10.1787/9789264069947-en.

OECD, 2004b. OECD guidelines for the testing of chemicals 220. Enchytraeid Reproduction Test.

Peijnenburg, W., Praetorius, A., Scott-Fordsmand, J.J., Cornelis, G., 2016. Fate assessment of ENPs in solid media – current insights and the way forward. Environ. Pollut. 218, 1365–1369.

Pereira, C.M.S., Novais, S.C., Soares, A.M.V.M., Amorim, M.J.B., 2013. Dimethoate affects cholinesterases in *Folsomia candida* and their locomotion--false negative results of an avoidance behaviour test. Sci. Total Environ. 443, 821–7. doi: 10.1016/j.scitotenv.2012.11.044.

Ribeiro, M.J., Maria, V.L., Scott-Fordsmand, J.J., Amorim, M.J.B., 2015. “Oxidative Stress Mechanisms Caused by Ag Nanoparticles (NM300K) are Different from Those of $AgNO_3$: Effects in the Soil Invertebrate *Enchytraeus crypticus”*. Int. J. Environ. Res. Public Health 12, 9589–9602. doi:10.3390/ijerph120809589.

Rombke, J., Kcnacker, T., 1989. “Aquatic toxicty test for enchytraeids”. Hydrobiologia 180, 235–242.

Schlich, K., Klawonn, T., Terytze, K., Hund-Rinke, K., 2013. “Hazard assessment of a silver nanoparticle in soil applied via sewage sludge”. Environ. Sci. Eur. 25, 17.

Stebounova, L.V., Guio, E., Grassian, V.H., 2011. Silver nanoparticles in simulated biological media: a study of aggregation, sedimentation, and dissolution. J Nanopart Res 13, 233–244. doi:https://doi.org/10.1007/s11051-010-0022-3

Sun, T.Y., Bornhöft, N.A., Hungerbühler, K., Nowack, B., 2016. “Dynamic Probabilistic Modeling of Environmental Emissions of Engineered Nanomaterials”. Environ. Sci. Technol. 50, 4701–4711. doi:10.1021/acs.est.5b05828.

Topuz, E., van Gestel, C.A.M., 2017. The effect of soil properties on the toxicity and bioaccumulation of Ag nanoparticles and Ag ions in Enchytraeus crypticus. Ecotoxicol. Environ. Saf. 144, 330–337. doi:10.1016/j.ecoenv.2017.06.037

Topuz, E., van Gestel, C.A.M., 2015. Toxicokinetics and toxicodynamics of differently coated silver nanoparticles and silver nitrate in Enchytraeus crypticus upon aqueous exposure in an inert sand medium. Environ. Toxicol. Chem. 34, 2816–2823. doi:10.1002/etc.3123

van der Ploeg, M.J.C., Handy, R.D., Waalewijn-Kool, P.L., van den Berg, J.H.J., Herrera Rivera, Z.E., Bovenschen, J., Molleman, B., Baveco, J.M., Tromp, P., Peters, R.J.B., Koopmans, G.F., Rietjens, I.M.C.M., Van den Brink, N.W., 2014. “Effects of silver nanoparticles (NM-300K) on Lumbricus rubellus earthworms and particle characterisation in relevant test matrices including soil”. Environ. Toxicol. Chem. 33, 743–752. doi:10.1002/etc.2487.

Supplementary materials

Table II-S1. Summary information of the characteristics of the tested materials in terms of supplier, state, solubility, coating, nominal size (according to the supplier), size measured via TEM or SEM (Transmission or Scanning Electron Microscopy) measurements, purity and morphology. PVP: polyvinylpyrrolidone, w/w: wet weight.

	$AgNO_3$	**NC-AgNMs**	**PVP-AgNMs**	**Ag NM300K**	**Dispersant (Tween 20)**
Supplier	Sigma Aldrich	AG-M-03M-NP.020N (American Elements)	AG-M-03M-NPCP.020N (American Elements)	JRC repository	
State	Powder	Powder	Powder	Suspension	Suspension
Solubility	Water soluble	Not dispersed	Not dispersed	Dispersible	Soluble
Coating	-	-	0.2%w/w PVP	-	-
Nominal size (nm)	Not relevant	20-30	20-30	15	Not relevant
TEM/SEM (nm)	-	26±4	25±5	17±8	-
Purity	> 99%	99%	99%	10.2% w/w Ag	-
Morphology		Spherical*	Spherical*	Spherical	-

*Agglomerates observed

Table II-S2. Overview of the test designs performed with *Enchytraeus crypticus*, indicating the life stage, endpoints, exposure time (sampling dates), test material and concentrations.

no.	Test material	Concentration (mg Ag/L; mg Ag/kg)	Life stage	Endpoint	Time (days)
Water					
1	PVP-AgNM	0-40-60-80-100	Cocoons (1-2d)	Hatching	17 (1-17)
2	NC-AgNP	0-40-60-80-100		Hatching	17 (1-17)
3	Ag NM300K	0-10-20-30-40		Hatching	17 (1-17)
4	$AgNO_3$	0-0.05-0.1-0.2-0.3		Hatching	17 (1-17)
5	PVP-AgNM	0-40-60-80-100	Juveniles (10–11d)	Survival	1-2-3-4-5
6	NC-AgNM	0-40-60-80-100		Survival	1-2-3-4-5
7	Ag NM300K	0-10-20-30-40		Survival	1-2-3-4-5
8	$AgNO_3$	0-0.05-0.1-0.2-0.3		Survival	1-2-3-4-5
9	PVP-AgNM	0-40-60-80-100	Adults (mature)	Survival	1-2-3-4-5
10	NC-AgNM	0-40-60-80-100		Survival	1-2-3-4-5
11	Ag NM300K	0-10-20-30-40		Survival	1-2-3-4-5
12	$AgNO_3$	0-0.05-0.1-0.2-0.3		Survival	1-2-3-4-5
Soil					
12	PVP-AgNM	0 (post exposure of no. 9)	Adults (mature) & pre-exposed	Survival; Reproduction	21
13	NC-AgNM	0 (post exposure of no.10)		Survival; Reproduction	21
14	Ag NM300K	0 (post exposure of no.11)		Survival; Reproduction	21
15	$AgNO_3$	0 (post exposure of no.12)		Survival; Reproduction	21
16	PVP-AgNM	0-50-100-200-300-400-700-900-1200	Adults (mature)	Survival; Reproduction	21
17	NC-AgNM	0-50-100-200-300-400-500-700-900		Survival; Reproduction	21
18	Ag NM300K	0-disp800-200-400-600-800		Survival; Reproduction	21
19	$AgNO_3$	0-36-48-60-72		Survival; Reproduction	21
20	PVP-AgNM	0 vs: 10-32-100-320-640-1000	Adults (mature)	Avoidance behaviour	1-2-3-4
21	NC-AgNM	0 vs: 0-32-100-320-640-1000		Avoidance behaviour	1-2-3-4
22	Ag NM300K	disp320 vs: 5-32-64-128-256-320		Avoidance behaviour	1-2-3-4
23	$AgNO_3$	0 vs: 0.1-1-5-20-32-64		Avoidance behaviour	1-2-3-4
24	NC-AgNM	0-640	Adults (mature)	AChE & GABBA Gene exp	1 & 4
25	$AgNO_3$	0-20		AChE & GABBA Gene exp	1 & 4

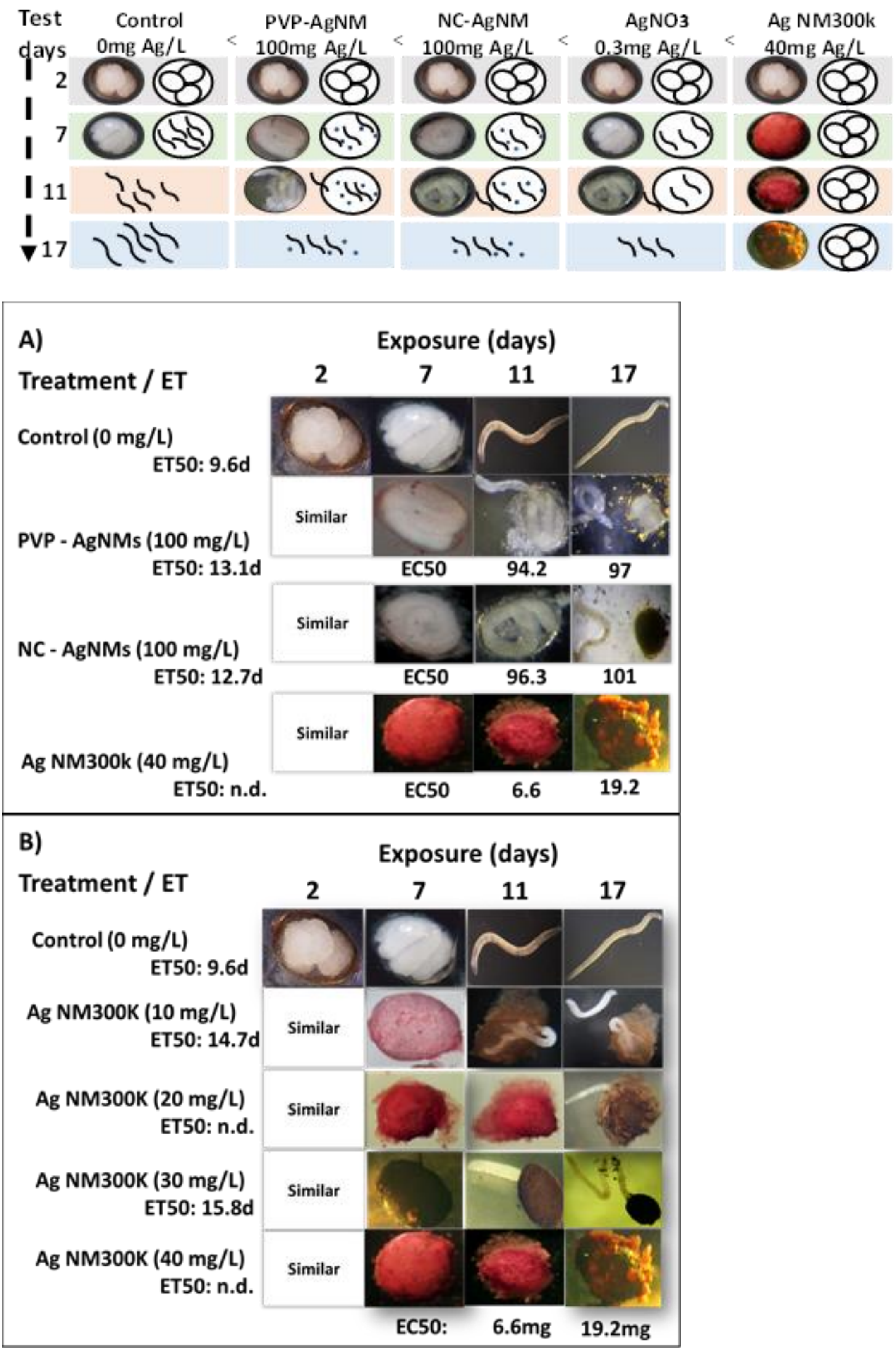


Figure II-S1. Schematic and macroscopic visualization of embryo development of *Enchytraeus crypticus* in ISO water medium along time (2-7-11-17 days), when exposed to

A) 100 mg PVP-AgNM/L, 100mg NC-AgNM/L and 40 mg Ag NM300K/L and B) 0, 10, 20, 30, 40 mg Ag NM300K/L.

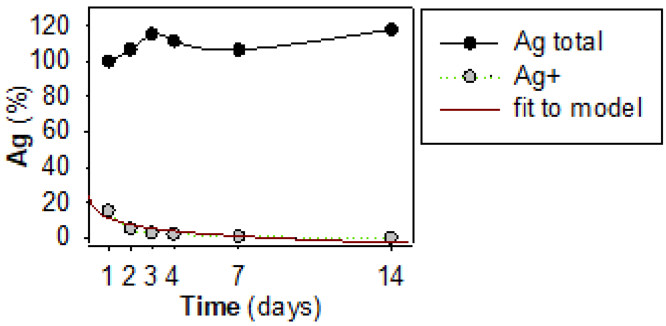


Figure II-S2. Free Ag ions (%) from Ag NM300K as measured by Ion Selective Electrode over 14 days in water. The red line represents the fit to model (logarithmic).

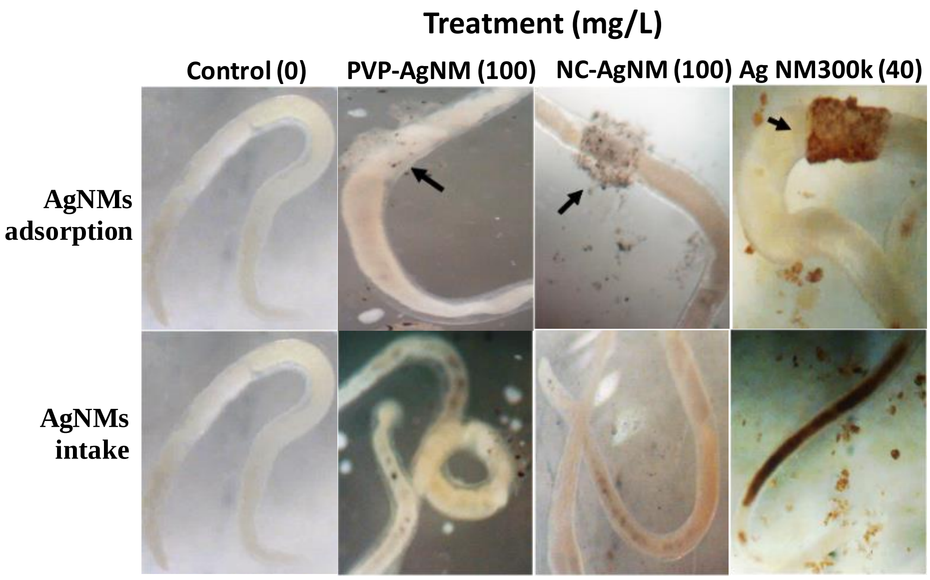


Figure II-S3. Macroscopic visualization of *Enchytraeus crypticus* adults when exposed during 5 days in ISO water to the highest tested concentrations (mg Ag/L) of PVP-AgNM, NC-AgNM, Ag NM300K. Black arrows indicate AgNMs agglomeration or adsorption in the clitellum area.

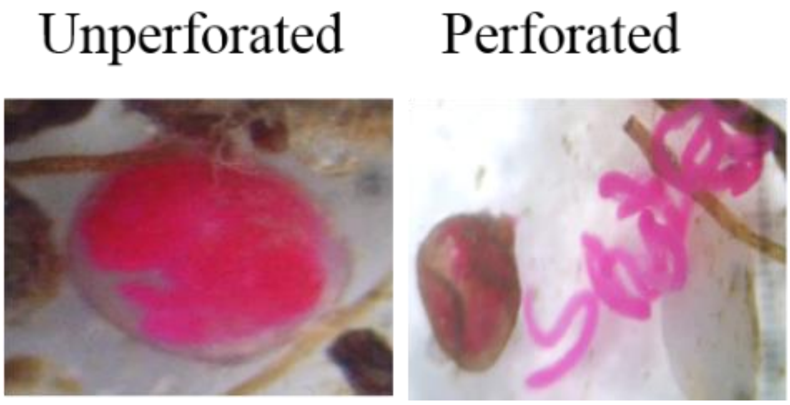


Figure II-S4. Macroscopic visualization of unhatched cocoons found at test end, after 21 days of exposure (Enchytraeidae Reproduction Test) in LUFA 2.2 soil to ≥ 500 mg PVP-AgNM /Kg and ≥ 700 mg NC-AgNM /Kg treatments. A) cocoon with juveniles inside Bengal red stained. B) cocoon after perforated membrane and juveniles outside.

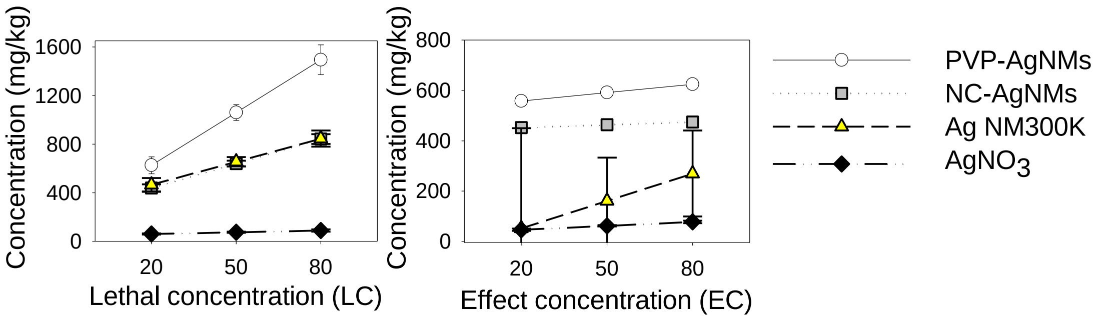


Figure II-S5. Survival (left side) and reproduction (right side) estimated Effect Concentration values for *Enchytraeus crypticus* when exposed in LUFA 2.2 soil spiked to PVP-AgNM, NC-AgNM, Ag NM300K and $AgNO_3$. Values are expressed as EC ± confidence intervals (± CI).

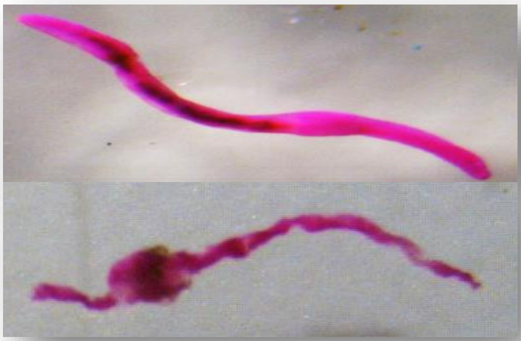


Photo by Natália Rodrigues

Chapter III

The toxicity of silver nanomaterials (NM 300K) is reduced when combined with N-Acetylcysteine: Hazard assessment on Enchytraeus crypticus

The toxicity of silver nanomaterials (NM 300K) is reduced when combined with N-Acetylcysteine: Hazard assessment on Enchytraeus crypticus

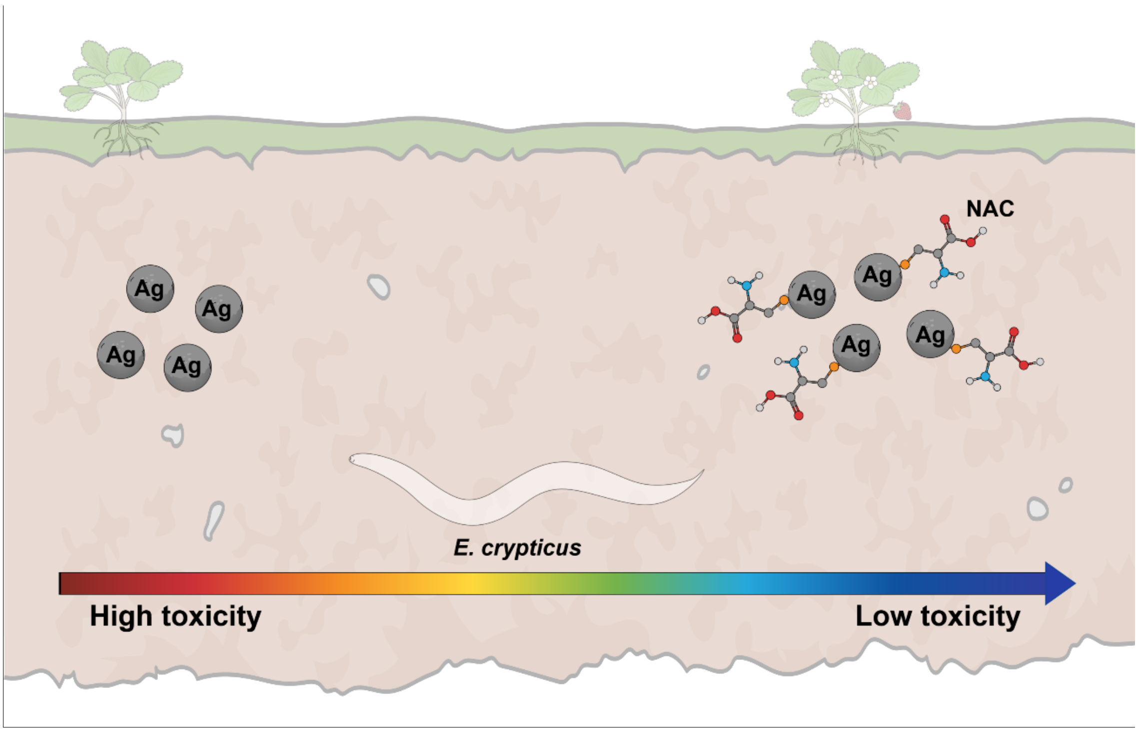
Monique C. P. Mendonça[1,2*δ], Natália P. Rodrigues[2,δ], Marcelo B. de Jesus[1], Janeck J. Scott-Fordsmand[3] and Mónica J.B. Amorim[2*]

[1]Departament of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas, Campinas, São Paulo, 13083-970, Brazil. [2]Department of Biology, CESAM, University of Aveiro, Aveiro, 3810-193, Portugal. [3]Department of Bioscience, Aarhus University, Silkborg, DK-8600, Denmark δ: these authors contributed equally

Published in Environmental Pollution (2019)

DOI: 10.1016/j.envpol.2019.113484

Graphical Abstract



Highlights

- thiol antioxidants such as N-acetylcysteine (NAC) and reduced glutathione (GSH) reduce toxicity of Ag

- *Enchytraeus crypticus* were exposed to Ag materials after spiking soil with NAC (and GSH).

- Added NAC remarkably reversed the toxic effects caused by Ag materials.

- Added GSH corroborates that thiol groups must play a key role in reducing Ag toxicity.

Abstract

The widespread production and use of silver nanomaterials (AgNMs) in consumer and medical products have been raising environmental concerns. Once in the environment, the soil is one of the major sinks of AgNMs due to e.g. sewage sludge applications, and invertebrates are directly exposed. In this study, we investigate the potential of N-acetylcysteine (NAC) to reduce the toxic effects of AgNM 300K (and $AgNO_3$) on the soil invertebrate *Enchytraeus crypticus*. AgNM 300K induces mortality, reproduction impairment, and avoidance. The addition of NAC to the soil showed a remarkable reduction in the toxicity of Ag, indicating that NAC can act as a detoxifying agent for terrestrial organisms exposed to Ag materials. That the reduction in toxicity likely is caused by thiol groups, was confirmed by GSH and GSSH studies. Identifying the mechanisms and hence alternatives that allow the recovery of contaminated soils is an important mitigation measure to promote environmental safety and reduce the associated risks to human health. Further, it may inform on strategies to implement in safe-by-design industry development.

Keywords: invertebrates, nanomaterials, thiol compounds, avoidance behavior, safe-by-design.

1. Introduction

During the past two decades, nanomaterials (NMs) became part of many products, being applied in numerous sectors including medical, industrial, environmental, and agricultural industries due to their unique physicochemical properties (Khan et al., 2017; Purohit et al., 2017; Stark et al., 2015). Silver NMs are among the most widely used NMs in consumer products. The global production of AgNMs is estimated to be between 500 tons and 1,000 tons per year (Calderón-Jiménez et al., 2017; Giese et al., 2018). This widespread production and use are known to have potential risks for organisms and ecosystems, with the terrestrial compartment as a major sink (Cornelis et al., 2014).

In the soil, AgNMs exposure may disrupt the metabolic activities and the diversity of soil microbial populations (i.e., bacteria, archaea, or eukarya) and can further negatively impacts the plant-growth, development, and productivity (Anjum et al., 2013; McKee and Filser, 2016). Regarding terrestrial invertebrates, many studies have shown that AgNMs have harmful effects on e.g. enchytraeids in terms of survival, growth, reproduction (Bicho et al., 2016; Gomes et al., 2013), avoidance behaviour (Lobe et al., 2018), generation of oxidative stress (Ribeiro et al., 2015) and genotoxicity (Maria et al., 2018).

A possibility to reduce or remediate the effects of contaminated sites would offer an important value to the environmental safety, and in designing novel safer materials. It has been previously shown that thiol antioxidants such as N-acetylcysteine (NAC) and reduced glutathione (GSH) inhibited the cytotoxic effects of AgNMs in Huh-7 hepatocarcinoma cells (Ferreira et al., 2019) and significantly attenuated all toxic effects induced by AgNMs in Wistar rats exposed to a sublethal intravenous dose (Mendonça et al., 2019). To further substantiate the findings shown in cells and vertebrates, we here assessed the effect of added NAC for invertebrates. In this study we measured the survival, reproduction, and avoidance behaviour in *Enchytraeus crypticus,* a standard species for evaluation of soil ecotoxicology, when exposed to soil spiked with AgNM 300K (and silver nitrate ($AgNO_3$)) combined with and without NAC. To confirm the results, we studied also the potentially mitigating effects if the GSH (reduced glutathione) and GSSG (oxidized glutathione), keeping in mind that NAC is a precursor for GSH.

2. Materials and Methods

2.1 Test organisms

The standard test organism *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. The cultures were kept in Petri dishes with agar consisting of Bacti-Agar medium (Oxoid, Agar No. 1) and a mixture of salt solutions at the final concentrations of 2 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4$, 0.08 mM KCl, and 0.75 mM $NaHCO_3$. The organisms were maintained in a 16:8 h light/dark cycle, in a temperature-controlled room (20 ± 1 °C) and fed *ad libitum* with ground autoclaved oats twice a week. Adults with a well-developed clitellum and similar size were used for the tests.

2.2 Test Materials

The AgNMs used in this study was the fully characterised standard reference material AgNM 300K from the European Commission Joint Research Centre (JRC). The material is an aqueous dispersion of nano-Silver (< 20 nm) with stabilizing agents, consisting of 4% w/w% each of Polyoxyethylene Glycerol Trioleate and Polyoxyethylene (20) Sorbitan mono-Laurat (Tween 20) (Klein et al. 2011). N-acetylcysteine (catalog #A9159, CAS 616-91-1) was purchased from Sigma Aldrich (St. Louis, MO, USA). GSH (catalog #A2084, CAS 70-18-8) and GSSG (catalog #A2243, CAS 27025-41-8) were purchased from PanReac (Barcelona, Spain).

2.3 Test soil and spiking procedure

The standard LUFA 2.2 natural soil (Speyer, Germany), was used. In short the main characteristics were 1.77 % organic matter, pH (0.01 M $CaCl_2$) 5.5, 10.1 meq/100 g cation exchange capacity, 45.8 % maximum water holding capacity, grain size distribution of 7.3% clay (< 0.002 mm), 13.8 % silt (0.002 to 0.05 mm), and 78.9 % sand (0.05 to 2.0 mm). The soil was dried at 80°C for 48 h before use.

Prior spiking with Ag materials (described ahead), the soil was spiked with NAC (600 mg/Kg), GSH (600 mg/Kg) and oxidized glutathione (GSSG) (600 mg/Kg) added as a dry powder to the soil (OECD, 2012). The effects of GSH and GSSG were investigated to assess whether the thiol groups play a key role in the reduction of Ag contaminated soil toxicity. The concentration of NAC was selected based on the results of a pilot study in which organisms were exposed to 600, 1200 and 1800 mg NAC/Kg, showing that 600 mg NAC/Kg seemed the best compromise, i.e., the least added without adverse effects when administered alone (Figure III-S1, Supplementary information), e.g. as can be observed, 1800 mg NAC/Kg caused 100% mortality.

Spiking of AgNM 300K (0, 20, 60, and 170 mg Ag/kg soil DW) and $AgNO_3$ (0, 45, 60 and 72 mg Ag/Kg soil DW) was done as an aqueous solution and added onto pre-moistened soil (25% w/w). For the avoidance behaviour test, the concentrations of 20 mg Ag/kg for $AgNO_3$ and 320 mg Ag/Kg for AgNMs were also included. For the survival and reproduction tests, a control dispersant was performed by adding the same volume of dispersant as present in AgNM 300K at the highest concentration for the avoidance test, the control dispersant was always added in the equivalent to each Ag concentration. Silver nitrate was used as reference material to assess the effects of non-particulate silver.

After thoroughly mixed the compounds in the soil, demineralized water was added to reach 50% of soil water holding capacity. The spiked soil was allowed to equilibrate for 3 days at 20 °± 1 °C, 16:8 h light:dark before starting the exposure.

2.4 Test Procedures

2.4.1 Avoidance behaviour

Testing was carried out following the ISO 17512-1 (ISO, 2008) guideline. Five replicates were used for each treatment in circular plastic boxes divided into two equal compartments with a plastic barrier. To each chamber 20 g of soil was added, one compartment was filled with the spiked soil and the other was filled with control soil. After the soil distribution, the plastic barrier was gently removed, and ten adult organisms were placed in the middle line between the two compartments. After 48 h test period in a controlled environment (20 ± 1°C with a 16 h light/8 h dark regime), the plastic barrier was inserted again, the soil removed and the number of organisms present in each compartment counted. The percentage of avoidance behaviour was calculated using the following equation: % avoidance = C-T/N ×100, where C, T, and N represent the number of organisms in control soil (C), contaminated treatment soil (T) and the total number of organisms per replicate (N), respectively. Positive percentages indicate avoidance of the treated soil, while negative percentages indicate an attraction for $AgNO_3$ or AgNMs-treated soils. A control with clean soil, dispersant-treated soil, and NAC-treated soil on both sides of the units was also carried out in the test.

2.4.2 Survival and reproduction

The survival and reproduction test were performed following the standard OECD guideline 220 (OECD - No. 220 2016) and ISO 16387:2014 (ISO - 16387 2014), over an exposure period of 21 days. Four replicates were used per treatment. For each replicate, ten adult organisms were introduced in a test vessel containing 20 g of moist soil and 2 mg of food. Weekly, water content and food were replenished. After 21 days, all organisms were fixated with 96 % ethanol and stained with 1% Bengal rose. Soil samples were then incubated overnight at 4 °C to obtain optimal staining. The pink-stained organisms were separated from soil particles through a 1.6, 0.5, and 0.3 mm sieve, transferred into a plastic box and manually counted using a stereomicroscope (Zeiss Stemi 2000-C).

3. Data Analysis

Differences between treatments were assessed using one-way analysis of variance (ANOVA) followed by Dunnets' test ($p < 0.05$) (SigmaPlot 1997). Effect Concentrations (ECx) were estimated modelling data to a logistic 2 parameters regression model using Toxicity Relationship Analysis Program software (TRAP 1.30a) (Erickson 2015).

4. Results

Soil pH did not vary significantly throughout the concentration range and exposure period.

4.1 Avoidance behavior

Validity criteria were fulfilled, i.e., less than 20% of mortality and random distribution between the two sides of the test box (control-control).

E. crypticus avoided the soil spiked with Ag, both AgNM 300K and $AgNO_3$, in a dose-response manner (Fig III-1), for AgNM 300K from 60 mg /Kg and for $AgNO_3$ from 20mg/Kg. Further, when the soil was spiked with NAC + Ag (blue lines), the avoidance response was similar to the vs dispersant (black lines), although to a slightly lower extent. Exposure of Ag versus NAC showed that for low Ag concentrations (20-60mg for AgNM 300K and 20mg $AgNO_3$) there was no avoidance of Ag (red lines).

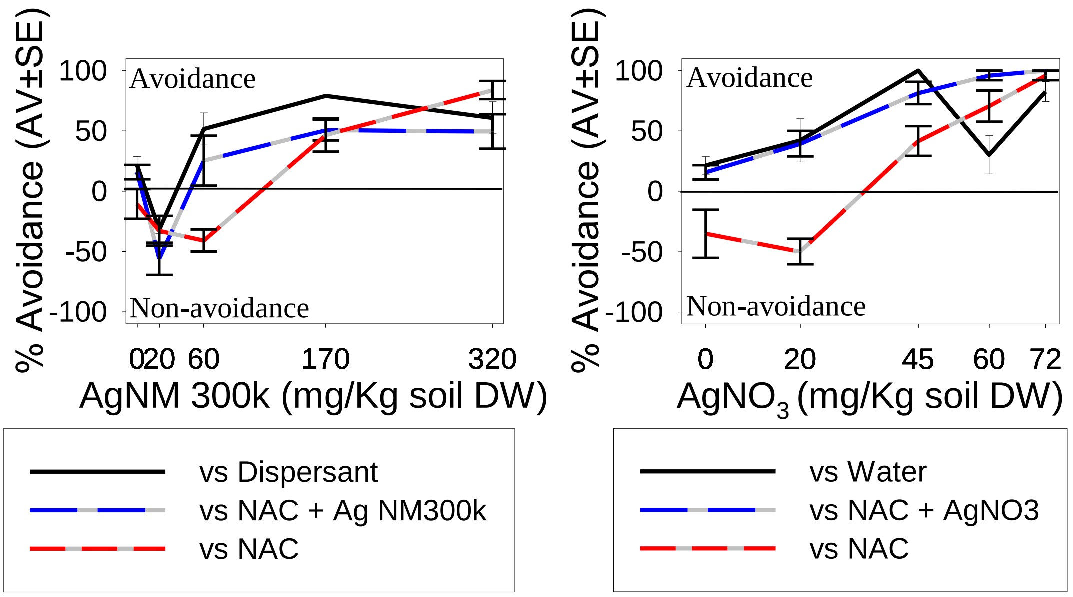


Figure III-1. Effects of 600 mg N-acetylcysteine (NAC)/Kg soil D.W. on avoidance behaviour of *Enchytraeus crypticus* when exposed in LUFA 2.2 soil spiked with AgNM 300K and $AgNO_3$. The dual combinations varied to assess Ag forms versus control (dispersant or water respectively), NAC and Ag+NAC. Values expressed as mean ± SE (n=5).

Despite 100% survival at the end of the test, there were visible morphological changes in some organisms exposed to 72 mg Ag/kg for $AgNO_3$, indicating a deteriorated health status (reduced biomass, scarred clitellum) (Fig III-S2, Supplementary information).

4.2 Survival and Reproduction

The validity criteria were fulfilled, i.e., the number of juveniles ≥ 25 per test vessel, adults mortality ≤ 20%, and coefficient of variation for reproduction ≤ 50 %, in control replicates.

Results of exposure to AgNM 300K and $AgNO_3$ confirmed the expected dose-response effect (Figure III-2). When the soil was pre-spiked with NAC, no effects of either Ag material were observed in terms of survival or reproduction.

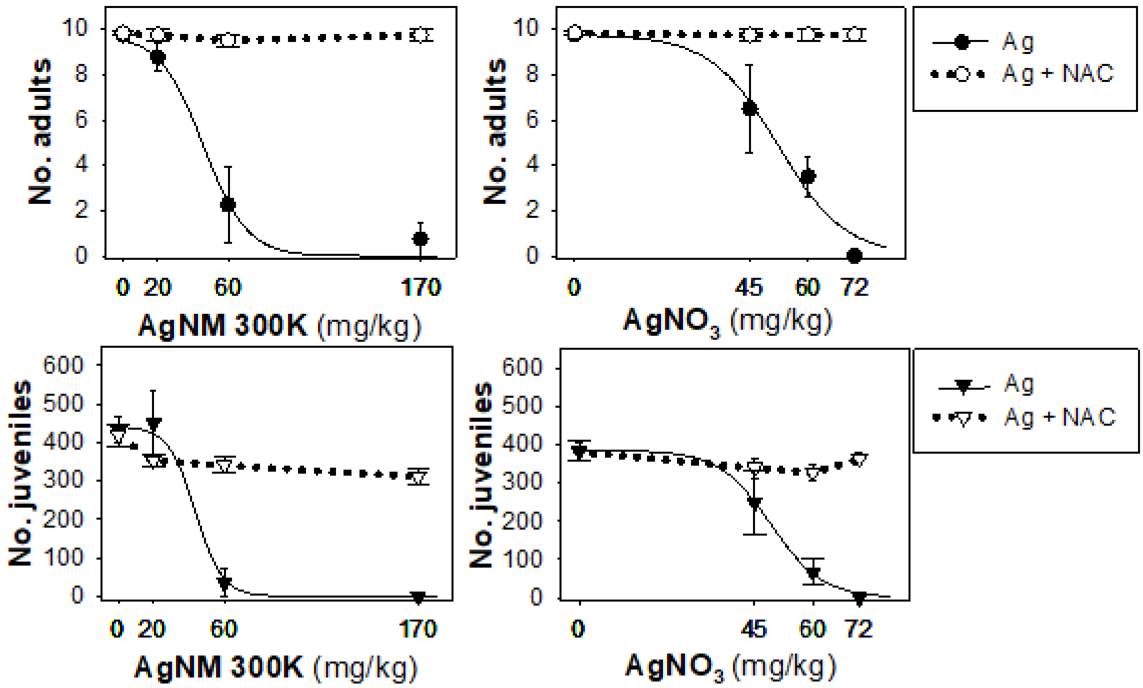


Figure III-2. Effects of AgNM 300K and $AgNO_3$ spiked LUFA 2.2 soil on survival and reproduction of *Enchytraeus crypticus* with and without 600mg N-acetylcysteine (NAC)/kg soil D.W. Values are expressed as average ± standard error. The straight lines represent the model fit to the data.

Effect Concentrations (often also termed Bench Mark Concentrations) were estimated and can be seen in full detail in Table III-1.

Table III-1. Estimated Effect Concentrations (EC) for *Enchytraeus crypticus* when exposed to AgNM 300K and $AgNO_3$ in the presence and absence of NAC. Logistic 2 parameters model was used. Confidence intervals (95% CI) are shown in brackets. n. e.: no effect; S: Slope; Y0: interception

Material / Endpoint	**EC_{10} (mg/kg)**	**EC_{20} (mg/kg)**	**EC_{50} (mg/kg)**	**EC_{80} (mg/kg)**	**Model Parameters**
Survival					
AgNM 300K	19 (1-36)	29 (15-43)	46 (37-55)	63 52-75)	S: 0.006 Y0: 9.75
$AgNO_3$	33 (19-47)	40 (30-50)	52 (47-58)	64 (56-73)	S: 0.042 Y0:9.75
AgNM 300K+NAC	n.e.	n.e.	n.e.	n.e.	-
$AgNO_3$+NAC	n.e.	n.e.	n.e.	n.e.	-
Reproduction					
AgNM 300K	27 (-12-66)	33 (1-65)	44 (21-67)	54 (35-74)	S: 0.011 Y0: 441.67
$AgNO_3$	35 (21-48)	40 (30-50)	49 (43-55)	58 (49-68)	S: 0.041 Y0: 386.25
AgNM 300K+NAC	n.e.	n.e.	n.e.	n.e.	-
$AgNO_3$+NAC	n.e.	n.e.	n.e.	n.e.	-

Results from the experiment with added GSH and GSSH (Figure III-3) showed that again a compensatory effect was measured, where the reduced glutathione (GSH) had the largest impact on the reduction of Ag toxicity, followed by the oxidized form (GSSH) with a small positive effect, but still reduced compared to the exposure to Ag forms alone.

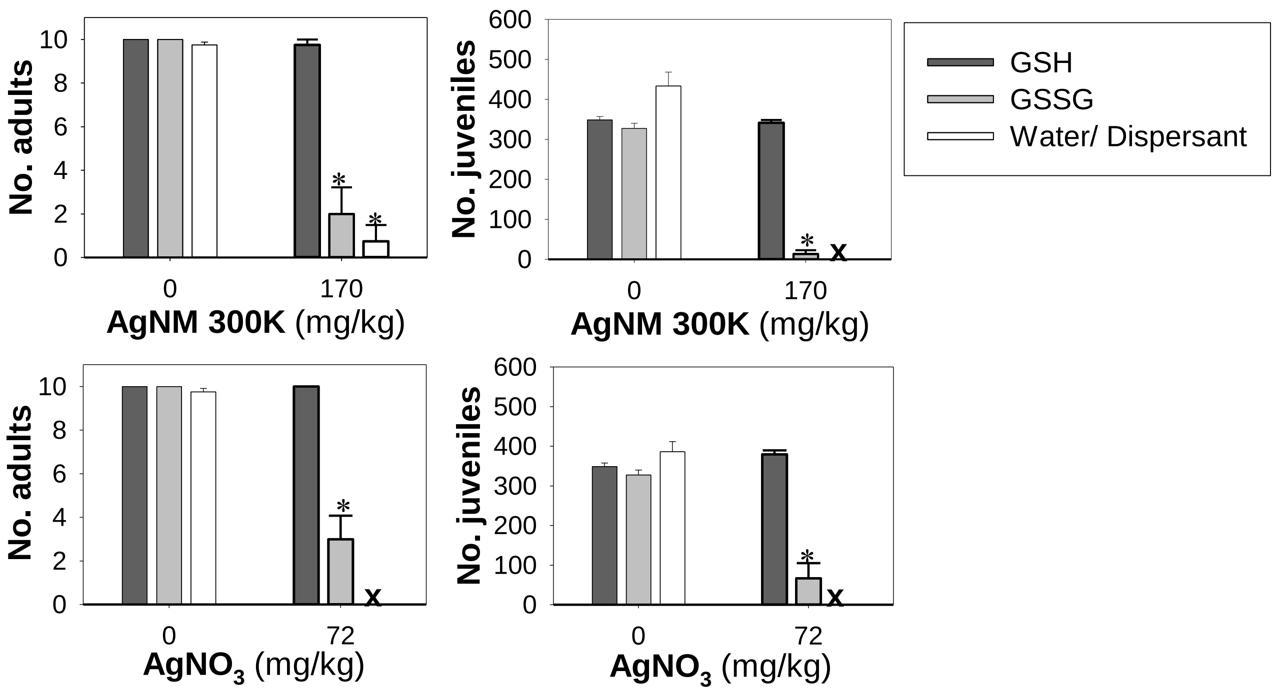


Figure III-3. Effects of AgNM 300K (170 mg/Kg) and $AgNO_3$ (72 mg/Kg) spiked LUFA 2.2 soil on survival and reproduction of *Enchytraeus crypticus* without (water/Dispersant) and with pre-added 600 mg/Kg oxidized glutathione (GSSG) and 600 mg/Kg reduced glutathione (GSH). Values are expressed as average ± standard error. One-way ANOVA, Dunnets' test: *: $p<0.05$ control vs treatment. X: total mortality and lack of reproduction.

5. Discussion

The present study confirmed the previously assessed Ag toxicity for both the soluble salt and nano-form to *E. crypticus* (R. Bicho et al. 2016). Several studies have been performed on enchytraeids (R. Bicho et al. 2016; S. Gomes, Soares, and Scott-Fordsmand 2013; Ribeiro et al. 2018). The toxic effects of AgNMs have been described to be partially attributed to the released Ag ions (Ag^+) (Le Ouay and Stellacci, 2015). The accumulation of Ag^+ inside the cell, can induce the generation of oxidative stress leading to, among others, the activation of antioxidant enzymes, depletion of antioxidant molecules (e.g. glutathione), and (dis)binding of proteins (Durán et al. 2016).

The toxic effects of AgNMs in the environment will be strongly influenced by the environmental media, e.g. in terrestrial, some of the key players will involve the type and amount of organic matter, dissolved oxygen, chloride (Cl-) and sulfide (S2−) ions, biomacromolecules (DNA and protein), and other compounds that have high affinity for silver such as sulfur-containing compounds (thiols -SH) (Levard et al. 2013; McShan, Ray, and Yu 2014). It has been shown that sulfidation decreased AgNMs toxicity to aquatic (Devi et al. 2015; Levard et al. 2013; Pokhrel, Dubey, and Scheuerman 2013) and to terrestrial organisms (Levard et al. 2013; Starnes et al. 2015; Yang et al. 2012). The added compound in our experiment, NAC, is a natural thiol-containing antioxidant and an established mucolytic agent and antidote for acetaminophen and heavy metal (Samuni et al. 2013). The addition of NAC to the soil acted as a potential detoxifying agent for Ag contaminated soil, causing a significant decrease in the toxicity of AgNM 300K and $AgNO_3$ to the survival and reproduction of *E. crypticus*. A simple molar ratio (NAC and Ag) calculation indicated that at the highest $AgNO_3$ concentration (72 mg Ag/kg), approximately 18% of the 600 mg NAC/kg would be used to bind all the Ag ions. The equivalent calculation for AgNM 300K (170 mg Ag/kg) means 43% used NAC (and 81% NAC used for the 320 mg Ag/kg in the avoidance test). Our findings are consistent with the observation Yang et al. (2012) (Yang et al. 2012), in which NAC completely rescued the growth inhibition of *Caenorhabditis elegans* induced by AgNPs and $AgNO_3$. Recent studies using NAC also indicate their positive effects on lifespan extension under oxidative stress conditions and increased resistance to the environmental stressor paraquat in *Caenorhabditis elegans* (De Magalhaes Filho et al. 2018; Oh, Park, and Park 2015) and Drosophila (Niraula and Kim 2019). The reduction of Ag toxicity when combined with NAC in *E. crypticus* must be due to the complexation of the free toxic Ag+ by thiol groups (-SH). It is not known, whether this anti-toxicity effect was caused by NAC-Ag binding in the soil core or inside the organism, as NAC can potentially cross cell membranes, which GSH does not seem to be able to do due to its negative charge at physiological pH (Tardiolo, Bramanti, and Mazzon 2018), given the experimental design (adding NAC to soil) it seem most plausible that NAC-Ag binding was in the soil. The equivalent exposure using GSH, another thiol compound, and GSSG, a non-thiol compound showed that GSH induced a reduction of the effect similar to NAC, whereas GSSG nearly did not change the Ag toxicity impact. Obviously since the molar weight of the GSH is almost twice as high

(1.9 times higher) than for NAC, then more of the GSH (weight wise) was involved in the detoxification, i.e., twice as much GSH compared to NAC would be required to perform the same Ag detoxification level. For the 72 mg Ag/kg exposure ($AgNO_3$) 34 % of the GSH would be involved, for the 170 mg Ag/Kg exposure (AgNM 300K) 81% of the GSH would be involved, and finally (although not performed) for the 320 mg Ag/Kg exposure (avoidance) 50% more GSH would be required to be added to mitigate the effects of the ions. Obviously, for the nanomaterials the GSH will bind only to the particle surface (and not to the inner), hence a relatively lower amount of binding sites is present (Ma et al. 2019).

Avoidance behavior tests confirmed the ability of *E. crypticus* to avoid Ag and in a dose-response manner. Similar avoidance behavior for $AgNO_3$ and AgNM 300K has been reported before for the earthworms *Eisenia fetida* (Mariyadas et al. 2018), *Allolobophora chlorotica* (Brami et al. 2017) and the isopod *Porcellionides pruinosus* (Tourinho et al. 2015). Interestingly, although survival and reproduction were virtually not affected when Ag materials were spiked in soil containing NAC, *E. crypticus* still expressed avoidance behavior for those soils and preferred a non-spiked soil. It could be that the period of the test, 48 hours, was too short for the Ag binding to thiol groups and hence the worms still avoid it and sense it. This avoidance was not due to the NAC itself since the result of the control (water or dispersant) “versus NAC” alone was a random distribution. This is in principle always a good trait, to be able to prevent and thus have the opportunity to escape to cleaner environment patches if these exist in the real scenarios.

To conclude, this study presents novel evidence suggesting that NAC (or GSH) could be used to reduce the toxic effects of Ag materials on soil invertebrates such as enchytraeids. It would be of interest to study its applicability in safe-by-design developments (e.g. by surface coating the NMs with NAC as performed by (Zhang et al. 2018) and (Li et al. 2019; Ma et al. 2019), or with GSH by (Ma et al. 2019). Surface coating of AgNM can reduce the AgNM related reactive oxygen species (ROS) production while containing the antimicrobial effect (Zhang et al. 2018); it further allow for a diverse surface-functionalization of the AgNM, e.g. with various proteins or anticancer compounds (Li et al. 2019).

6. Acknowledgments

This study was financially supported by São Paulo Research Foundation (FAPESP) (grant #2017/18867-1 and #2014/03002-7). Further support was provided by BIORIMA H2020-NMBP-2017 (GA No. 760928). Thanks are due to FCT (Fundação para a Ciência e Tecnologia) / MCTES (Ministério da Ciência e Tecnologia do Ensino Superior) for the financial support to CESAM (UID/AMB/50017/2019) and a PhD grant to Natália P. Rodrigues (SFRH/BD/87787/2012).

Conflicts of interest

No conflicts of interest to declare.

References

Anjum, N.A., Gill, S.S., Duarte, A.C., Pereira, E., Ahmad, I., 2013. "Silver nanoparticles in soil-plant" systems. J. Nanoparticle Res. 15:1896. https://doi.org/10.1007/s11051-013-1896-7.

Bicho, R.C., Ribeiro, T., Rodrigues, N.P., Scott-Fordsmand, J.J., Amorim, M.J.B., 2016. "Effects of Ag nanomaterials (NM300K) and Ag salt ($AgNO_3$) can be discriminated in a full life cycle long term test with *Enchytraeus crypticus"*. J. Hazard. Mater. 318, 608–614. https://doi.org/10.1016/j.jhazmat.2016.07.040.

Brami, C., Glover, A.R., Butt, K.R., Lowe, C.N., 2017. "Effects of silver nanoparticles on survival, biomass change and avoidance behaviour of the endogeic earthworm *Allolobophora chlorotica*". Ecotoxicol. Environ. Saf. 141, 64–69. https://doi.org/10.1016/j.ecoenv.2017.03.015.

Calderón-Jiménez, B., Johnson, M.E., Montoro Bustos, A.R., Murphy, K.E., Winchester, M.R., Vega Baudrit, J.R., 2017. "Silver Nanoparticles: Technological advances, societal impacts, and metrological challenges”. Front. Chem. 5, 1–26. https://doi.org/10.3389/fchem.2017.00006.

Cornelis, G., Hund-Rinke, K., Kuhlbusch, T., Van Den Brink, N., Nickel, C., 2014. “Fate and bioavailability of engineered nanoparticles in soils: A review”. Crit. Rev. Environ. Sci. Technol. 44, 2720–2764. https://doi.org/10.1080/10643389.2013.829767.

De Magalhaes Filho, C.D., Henriquez, B., Seah, N.E., Evans, R.M., Lapierre, L.R., Dillin, A., 2018. “Visible light reduces C. elegans longevity”. Nat. Commun. 9. https://doi.org/10.1038/s41467-018-02934-5.

Devi, G.P., Ahmed, K.B.A., Varsha, M.K.N.S., Shrijha, B.S., Lal, K.K.S., Anbazhagan, V., Thiagarajan, R., 2015. “Sulfidation of silver nanoparticle reduces its toxicity in zebrafish”. Aquat. Toxicol. 158, 149–156. https://doi.org/10.1016/j.aquatox.2014.11.007.

Durán, N., Durán, M., de Jesus, M.B., Seabra, A.B., Fávaro, W.J., Nakazato, G., 2016. “Silver nanoparticles: A new view on mechanistic aspects on antimicrobial activity”. Nanomedicine Nanotechnology, Biol. Med. 12, 789–799. https://doi.org/10.1016/j.nano.2015.11.016.

Erickson, R., 2015. Toxicity Relationship Analysis Program (TRAP), Ver 1.30a. EPA 600/C-11/002.

Ferreira, L.A.B., Bernardes, J.S., Durán, N.C., de Jesus, M.B., 2018. “Thiol antioxidants interfere with assessing silver nanoparticle cytotoxicity”. Submitted.

Giese, B., Klaessig, F., Park, B., Kaegi, R., Steinfeldt, M., Wigger, H., Von Gleich, A., Gottschalk, F., 2018. “Risks, release and concentrations of engineered nanomaterial in the environment”. Sci. Rep. 8, 1–18. https://doi.org/10.1038/s41598-018-19275-4.

Gomes, S.I.L., Soares, A.M.V.M., Scott-Fordsmand, J.J., Amorim, M.J.B., 2013. “Mechanisms of response to silver nanoparticles on *Enchytraeus albidus* (Oligochaeta): Survival, reproduction and gene expression profile”. J. Hazard. Mater. 254–255, 336–344. https://doi.org/10.1016/J.JHAZMAT.2013.04.005.

ISO, 2014. ISO 16387:2014. Soil quality — Effects of contaminants on Enchytraeidae (*Enchytraeus* sp.) — Determination of effects on reproduction.

ISO, 2008. ISO 17512-1:2008(en). Soil quality — Avoidance test for determining the quality of soils and effects of chemicals on behaviour — Part 1: Test with earthworms (*Eisenia fetida* and *Eisenia andrei*).

Khan, Ibrahim, Saeed, K., Khan, Idrees, 2017. "Nanoparticles: Properties, applications and toxicities". Arab. J. Chem. https://doi.org/10.1016/J.ARABJC.2017.05.011.

Klein, C.L., Comero, S., Stahlmecke, B., Romazanov, J., Kuhlbusch, T.A.J., Doren, E. Van, Mast, P.D.T.J., Wick, P., Krug, H., Locoro, G., Kördel, W., Friedrichs, S., Maier, G., Werner, J., Linsinger, T., Gawlik, B.M., 2011. "NM-Series of representative manufactured nanomaterials nm-300 silver characterisation, stability, homogeneity". https://doi.org/10.2788/23079.

Le Ouay, B., Stellacci, F., 2015. "Antibacterial activity of silver nanoparticles: A surface science insight". Nano Today 10, 339–354. https://doi.org/10.1016/j.nantod.2015.04.002.

Levard, C., Hotze, E.M., Colman, B.P., Dale, A.L., Truong, L., Yang, X.Y., Bone, A.J., Brown, G.E., Tanguay, R.L., Di Giulio, R.T., Bernhardt, E.S., Meyer, J.N., Wiesner, M.R., Lowry, G. V., 2013. "Sulfidation of silver nanoparticles: Natural antidote to their toxicity". Environ. Sci. Technol. 47, 13440–13448. https://doi.org/10.1021/es403527n.

Li, C., Zhang, H., Gong, X., Li, Q., Zhao, X., 2019. "Synthesis, characterisation, and cytotoxicity assessment of N-acetyl-L-cysteine capped ZnO nanoparticles as camptothecin delivery system". Colloids Surfaces B Biointerfaces 174, 476–482. doi:10.1016/j.colsurfb.2018.11.043.

Lobe, P.D.D., Filser, J., Otomo, P.V., 2018. "Avoidance behaviour of *Enchytraeus albidus* (Oligochaeta) after exposure to AgNPs and $AgNO_3$ at constant and fluctuating temperature". Eur. J. Soil Biol. 87, 40–45. https://doi.org/10.1016/j.ejsobi.2018.05.002.

Ma, Y.F., Wang, L.J., Zhou, Y.L., Zhang, X.X., 2019. "A facilely synthesized glutathione-functionalized silver nanoparticle-grafted covalent organic framework for rapid and highly efficient enrichment of N-linked glycopeptides”. Nanoscale 11, 5526–5534. https://doi.org/10.1039/c9nr00392d.

Maria, V.L., Ribeiro, M.J., Guilherme, S., Soares, A.M.V.M., Scott-Fordsmand, J.J., Amorim, M.J.B., 2018. “Silver (nano)materials cause genotoxicity in *Enchytraeus crypticus*, as determined by the comet assay”. Environ. Toxicol. Chem. 37, 184–191. https://doi.org/10.1002/etc.3944.

Mariyadas, J., Amorim, M.J.B., Jensen, J., Scott-Fordsmand, J.J., 2018. “Earthworm avoidance of silver nanomaterials over time”. Environ. Pollut. 239, 751–756. https://doi.org/10.1016/j.envpol.2018.04.059.

McKee, M.S., Filser, J., 2016. “Impacts of metal-based engineered nanomaterials on soil communities”. Environ. Sci. Nano 3, 506–533. https://doi.org/10.1039/c6en00007j.

McShan, D., Ray, P.C., Yu, H., 2014. “Molecular toxicity mechanism of nanosilver”. J. Food Drug Anal. 22, 116–127. https://doi.org/10.1016/j.jfda.2014.01.010.

Mendonça, M.C.P., Ferreira, L.B., Rizoli, C., Batista, Â.G., Maróstica Júnior, M.R., da Silva, E. do N., Cadore, S., Durán, N., Cruz-Höfling, M.A. da, de Jesus, M.B., 2019. N-Acetylcysteine reverses silver nanoparticle intoxication in rats”. Nanotoxicology 13, 326–338. https://doi.org/10.1080/17435390.2018.1544302.

Niraula, P., Kim, M.S., 2019. “N-Acetylcysteine extends lifespan of Drosophila via modulating ROS scavenger gene expression”. Biogerontology 4. https://doi.org/10.1007/s10522-019-09815-4.

OECD, 2015. Guidelines for the Testing of Chemicals. No. 220 - Enchytraeid Reproduction Test. https://doi.org/10.1787/9789264203785-en.

Oh, S., Park, J., Park, S., 2015. “Lifespan extension and increased resistance to environmental stressors by N-Acetyl-L-Cysteine in *Caenorhabditis elegans*”. Clinics 70, 380–386. https://doi.org/10.6061/clinics/2015(05)13.

Pokhrel, L.R., Dubey, B., Scheuerman, P.R., 2013. “Impacts of select organic ligands on the colloidal stability, dissolution dynamics, and toxicity of silver nanoparticles”. Environ. Sci. Technol. 47, 12877–12885. https://doi.org/10.1021/es403462j.

Purohit, R., Mittal, A., Dalela, S., Warudkar, V., Purohit, K., Purohit, S., 2017. Social, “Environmental and Ethical Impacts of Nanotechnology” 4, 5461–5467. https://doi.org/10.1016/j.matpr.2017.05.058.

Ribeiro, M.J., Maria, V.L., Scott-Fordsmand, J.J., Amorim, M.J.B., 2015. “Oxidative stress mechanisms caused by Ag nanoparticles (NM300K) are different from those of $AgNO_3$: Effects in the soil invertebrate *Enchytraeus crypticus”*. Int. J. Environ. Res. Public Health 12, 9589–9602. https://doi.org/10.3390/ijerph120809589.

Samuni, Y., Goldstein, S., Dean, O.M., Berk, M., 2013. “The chemistry and biological activities of N-acetylcysteine”. Biochim. Biophys. Acta - Gen. Subj. 1830, 4117–4129. https://doi.org/10.1016/j.bbagen.2013.04.016.

SigmaPlot, 1997. Statistical Package for the Social Sciences, 11 ed.

Stark, W.J., Stoessel, P.R., Wohlleben, W., Hafner, A., 2015. “Industrial applications of nanoparticles”. Chem. Soc. Rev. 44, 5793–5805. https://doi.org/10.1039/c4cs00362d.

Starnes, D.L., Unrine, J.M., Starnes, C.P., Collin, B.E., Oostveen, E.K., Ma, R., Lowry, G. V., Bertsch, P.M., Tsyusko, O. V., 2015. “Impact of sulfidation on the bioavailability and toxicity of silver nanoparticles to Caenorhabditis elegans”. Environ. Pollut. 196, 239–246. https://doi.org/10.1016/j.envpol.2014.10.009.

Tardiolo, G., Bramanti, P., Mazzon, E., 2018. “Overview on the Effects of N - Acetylcysteine in Neurodegenerative Diseases”. Molecules 23, 3305 https://doi.org/10.3390/molecules23123305.

Tourinho, P.S., Van Gestel, C.A.M., Jurkschat, K., Soares, A.M.V.M., Loureiro, S., 2015. “Effects of soil and dietary exposures to Ag nanoparticles and $AgNO_3$ in the terrestrial isopod *Porcellionides pruinosus”*. Environ. Pollut. 205, 170–177. https://doi.org/10.1016/j.envpol.2015.05.044.

Vance, M.E., Kuiken, T., Vejerano, E.P., McGinnis, S.P., Hochella, M.F., Rejeski, D., Hull, M.S., 2015. “Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory”. Beilstein J. Nanotechnol. 6, 1769–1780. https://doi.org/10.3762/bjnano.6.181.

Yang, X., Gondikas, A.P., Marinakos, S.M., Auffan, M., Liu, J., Hsu-Kim, H., Meyer, J.N., 2012. “Mechanism of silver nanoparticle toxicity is dependent on dissolved silver and surface coating in *Caenorhabditis elegans*”. Environ. Sci. Technol. 46, 1119–1127. https://doi.org/10.1021/es202417t.

Zhang, H., Hatoko, M., Yin, D., Yang, Y., Zeng, Y., Komasa, S., Kusumoto, T., Nishizaki, H., Shimizu, H., Zhao, W., Okazaki, J., 2018. “Antibacterial activity and biocompatibility of nanoporous titanium doped with silver nanoparticles and coated with n-acetyl cysteine”. J. Hard Tissue Biol. 27, 351–358. doi:10.2485/jhtb.27.351.

Supplementary Materials

Figure III-S1 and S2 are provided.

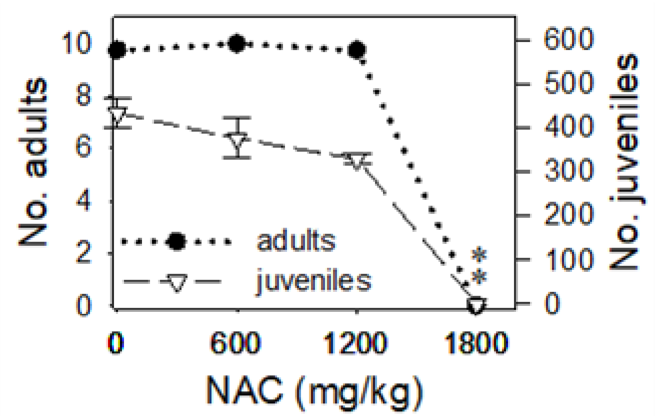


Figure III-S1. Effects of NAC spiked LUFA 2.2 soil on survival and reproduction of *Enchytraeus crypticus*. Values are expressed as average ± standard error. One-way ANOVA, Dunnets’ test: *: $p<0.05$ control *vs* treatment.

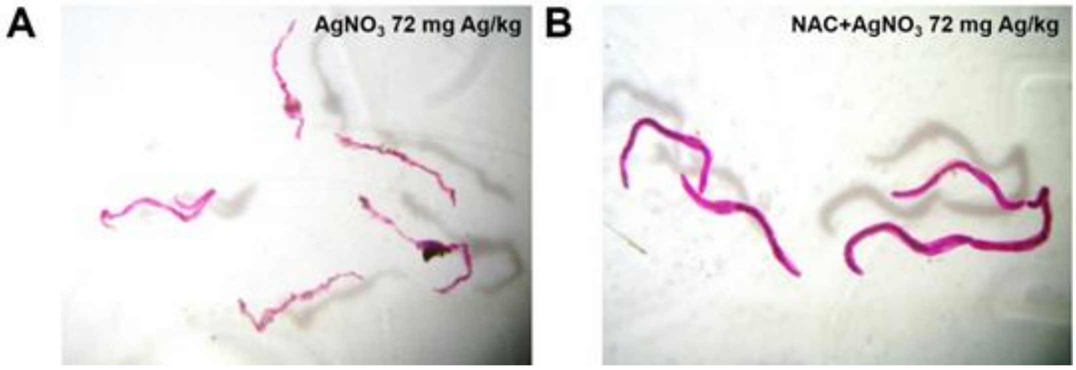


Figure III-S2. Representative images of Bengal rose-stained *Enchytraeus crypticus* exposed in an avoidance test to (a) $AgNO_3$ 72 mg Ag/Kg and (b) NAC+$AgNO_3$ 72 mg Ag/Kg for 48 hours. Light microscopy, 0.6 X.

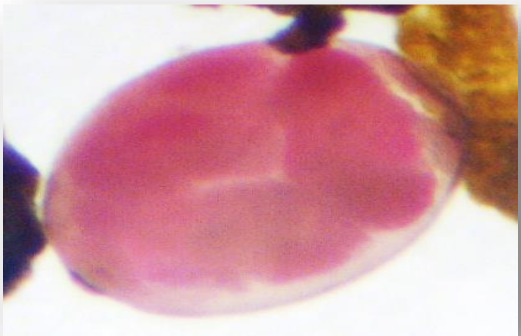


Photo by Natália Rodrigues

Chapter IV

Graphene-based nanomaterials in soil: ecotoxicity assessment using Enchytraeus crypticus reduced full life cycle

Graphene-based nanomaterials in soil: ecotoxicity assessment using Enchytraeus crypticus reduced full life cycle

Monique C. P. Mendonça[1,2*δ], Natália P. Rodrigues[2,δ], Marcelo B. de Jesus[1] and Mónica J.B. Amorim[2*]

[1] Department of Biochemistry and Tissue Biology, Institute of Biology, University of Campinas, Campinas, São Paulo, 13083-970, Brazil. [2] Department of Biology, CESAM, University of Aveiro, Aveiro, 3810-193, Portugal.[δ]: these authors contributed equally

Published in Nanomaterials (2019)
DOI: 10.3390/nano9060858

Abstract

Graphene-based nanomaterials (GBNs) possess unique physicochemical properties, allowing a wide range of applications in physical, chemical, and biomedical fields. Although GBNs are broadly used, information about their adverse effects on ecosystem health, especially in the terrestrial environment, is limited. Therefore, this study aims to assess the toxicity of two commonly used derivatives of GBNs, graphene oxide (GO) and reduced graphene oxide (rGO), in the soil invertebrate *Enchytraeus crypticus* using a reduced full life cycle test. At higher exposure concentrations, GO induced high mortality and severe impairment in the reproduction rate, while rGO showed little adverse effect up to 1000 mg/kg. Collectively, our body of results suggests that the degree of oxidation of GO correlates with their toxic effects on *E. crypticus*, which argues against generalization on GBNs ecotoxicity. Identifying the key factors affecting the toxicity of GBNs, including ecotoxicity, is urgent for the design of safe GBNs for commercial purposes.

Keywords: graphene oxide; reduced graphene oxide; terrestrial environment; survival; reproduction; hatching success

1. Introduction

Over the last decade, the production and use of nanomaterials (NMs) have been rapidly expanding due to a wide range of applications in many industrial sectors. Among them, graphene-based nanomaterials (GBNs) have attracted considerable interest because of their fascinating optical, electrical, mechanical, and thermal properties. These properties allowed the GBNs application in electronic devices, energy storage, biosensors, semiconductors, water purification (filter), biomedicine, tissue engineering, and drug/gene delivery (Qu et al. 2018; Ray 2015). From 2010 to 2017, the compound annual growth rate (CAGR) of graphene composites worldwide was estimated on 61% (ReportLinker 2018). By 2023, the global market volume of graphene composites may reach 1521 tons (Statista 2018).

Graphene nanomaterials consisting solely of carbon are known to be non-toxic; however, graphene derivatives like graphene oxide (GO) and reduced graphene oxide (rGO) can contain residual metals and impurities from the chemicals used in the treatment process for oxidation and reduction (Nurunnabi et al. 2015). Moreover, GO and rGO can be functionalized with a wide variety of compounds, such as polymers and biomolecules, that can alter their structure and, consequently, their toxicity (Mendonça et al. 2016; Zare-Zardini et al. 2018). Thus, with the increasing use and production of GBNs, it is expected that large quantities of industrial waste will end up in the environment, representing a risk for human health and living organisms. Thus, systematic investigation of any potential toxic effects of GO/rGO is essential to ensure assessment of safety at biological and environmental levels.

The terrestrial environment is one of the primary sinks of NMs, and soil invertebrates are directly affected by these compounds. Hence, such organisms can serve as soil sentinels of quality disturbances, where several are used as surrogates, like enchytraeids (Enchytraeidae, Oligochaeta, Annelida). Enchytraeids are essential for organic matter decomposition and live in close contact with the soil pore water. Therefore, they are exposed to soil contamination via dermal, gastrointestinal, and respiratory routes, being often very sensitive to a broad range of stressors (Castro-Ferreira et al. 2012). Furthermore, its use has been supported by the internationally accepted guidelines designed to assess the effects of substances on the survival and reproductive output of *Enchytraeus* sp.(ISO -

16387 2014; OECD 2002) The evaluation of additional endpoints, e.g., hatching success, maturity, and growth, was proposed by Bicho et al. (2015) in a full life cycle test to complement the hazard assessment in different life cycle stages (R. C. Bicho, Santos, et al. 2015).

Currently, little is known about the impacts of GBNs on the terrestrial environment, and their effects on the life cycle of soil invertebrates remain largely unaddressed. Hence, we here evaluate the environmental impact of GBNs using *Enchytraeus crypticus.* The effects of the two commonly used derivatives of GBNs, GO, and rGO were investigated, using *Enchytraeus crypticus* in a reduced full life cycle test, measuring cocoons hatching and size, adult survival, and reproduction rate (number of juveniles).

2. Materials and Methods

2.1 Test Nanomaterials and Characterisation

Commercial GO (catalog #763713**,** Sigma-Aldrich, St. Louis, MO, USA**)** were purchased in flakes and rGO **(**catalog #777684**,** Sigma-Aldrich**)** in powder. The GO flakes were suspended in deionized water (10 mg/mL) and sonicated in an ultrasonic bath for 30 min immediately prior to use. The characterisation data of the commercial GBNs are available on the supplier's webpage and have also been previously assessed (Kang et al. 2017; Seifati, Nasirizadeh, and Azimzadeh 2018). Briefly, the tested GO was composed of carbon **(**42–52 wt %) and oxygen (≥36 wt %) and exhibited the size of 219 nm (performed using dynamic light scattering and zeta potential of −14.13 ± 11.1**.** Reduced GO was composed of carbon (≥75 wt %), nitrogen (>5 wt %) and oxygen (≤22 wt %), and exhibited a BET surface area of 450 m^2/g. Further characterisation of the size and zeta potential of rGO was not possible as it was added to the soil as a dry powder (not water-dispersed).

2.2 Test organism

*C*ultures of test species *Enchytraeus crypticus* (Enchytraeidae, Oligochaeta) were maintained in Petri dishes filled with agar, consisting of Bacti-Agar medium (Oxoid, Agar No. 1) and a mixture of salt solutions at the final concentrations of 2 mM $CaCl_2 \cdot 2H_2O$, 1 mM $MgSO_4$, 0.08 mM KCl, and 0.75 mM $NaHCO_3$. The organisms were cultivated with a photoperiod of 16:8 h light/dark, temperature of 20 ± 1 °C, and fed autoclaved ground oats twice per week.

2.3 Test Soil and Spiking Procedure

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The composition is as follows: 1.77% organic matter, pH (0.01 M $CaCl_2$) 5.5, 10.1 meq/100 g cation exchange capacity, 45.8% maximum water-holding capacity (WHC), grain size distribution of 7.3% clay (<0.002 mm), 13.8% silt (0.002 to 0.05 mm), and 78.9% sand (0.05 to 2.0 mm). Soil samples were oven-dried at 80 °C for 48 h before use. Spiking was performed according to the recommendations for nanomaterials by the Organization for Economic Co-operation and Development (OECD) (OECD 2012). GO was added as aqueous solution to pre-moistened soil (25% (w/w), whereas rGO was added as a dry powder to the soil as recommended for poorly dispersed materials. Since no toxicity data were available for soil invertebrates, GO and rGO were tested using a wide range of concentrations (0, 5, 250, and 1000 mg GO or rGO/kg dry soil.) Soil moisture was adjusted to 50% of the WHC and allowed to equilibrate for 24 h before starting exposure.

2.4 Exposure Experimental Design

An adapted version of the full life cycle test described in Bicho et al. (2015) was conducted (R. C. Bicho, Santos, et al. 2015). Briefly, age-synchronized 1- to 2-day-old cocoons (n = 10 per replicate) were introduced in each test vessel containing 10 g of soil. Exposure period was 11 days (hatching success and size (area in mm^2) endpoints) and 46 days (survival and reproduction endpoints) at a constant temperature of 20 ± 1 °C with a photoperiod of 16:8 h light/dark. Four replicates per treatment were used. After hatching, the test organisms were fed once a week with autoclaved ground oats, and the water loss was replenished.

To extract organisms from the soil for counting, replicates were fixated with 96% ethanol and stained with 1% Bengal rose. After incubation overnight at 4 °C, soil samples were sieved in a 1.6, 0.5, and 0.3 mm mesh and the collected adults and juveniles were manually counted using a stereomicroscope (Zeiss Stemi 2000-C) to determine effects on survival and reproduction. The survival was determined on the adult survival, whereas reproduction was based on the number of juveniles produced. After counting, sizes were recorded for 11-day-old juveniles. The procedure consisted of placing each organism in a drop of water and photograph using a Dino-Eye camera and Dino-Lite software (Dino-Lite Digital Microscope, AnMo Electronics Corporation, New Taipei City, Taiwan). Size was measured by evaluating the area (mm^2) from the object contour delimitation function using ImageJ software (NIH, Bethesda, MD, USA) after calibration, with the metric ruler, of the pixel/millimeter ratio (Figure IV-1).

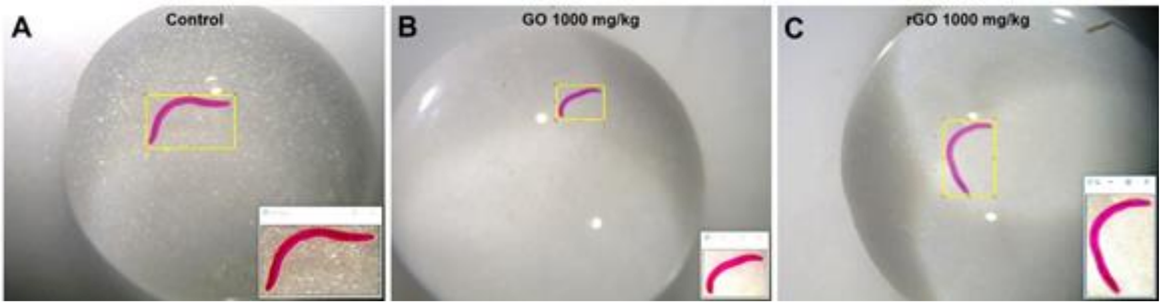


Figure IV-1. Illustration of the methodology used for quantitative analysis of *E. crypticus* size (in mm^2). Representative images of (A) control, organisms exposed to either (B) 1000 mg graphene oxide (GO)/kg or (C) 1000 mg reduced graphene oxide (rGO)/kg. Insets show the same animals after contour delimitation using ImageJ software.

3. Data Analysis

Results are presented as means ± standard error of the means (SEM) and analyzed for statistical significance by one-way analysis of variance (ANOVA), followed by Dunnett's multiple comparison tests using GraphPad Prism 5.0 (GraphPad Software, San Diego, CA, USA). Student's *t*-test (two-tailed, unpaired) was used to compare the treatment effects (GO vs. rGO). The effect of treatment and concentration, as well as their interaction, was further determined using two-way ANOVA. p value < 0.05 was considered for statistical significance. Effect concentrations (ECx) were estimated using the Toxicity Relationship Analysis Program (TRAP 1.30a) applying the best-fitting regression model (logistic 2 parameters or threshold sigmoid 2 parameters).

4. Results

No significant changes occurred in the soil pH (5.7 ± 0.06) during the experimental period regardless of the treatment condition. Results of the exposure of the soil invertebrate *E. crypticus* to GBNs (GO and rGO) using the reduced full life cycle test (cocoon hatching, size, survival, and reproduction) can be observed in Figure IV-2.

The dose response effect concentrations were estimated (Table IV-1) and predictive models and parameters are given.

Table IV-1. Estimated effect concentrations (EC) for *Enchytraeus crypticus* when exposed to GO and rGO in a full life cycle test, in terms of hatching and size (area, in mm^2) at day 11, survival, and reproduction at day 46. Logistic 2 parameters and threshold sigmoid 2 parameters models were used. Confidence intervals (95% CI) are shown in brackets. n.e.: no effect; n.d.: not determined/out of range; S: Slope; Y0: interception.

Nanomaterial/Endpoint	EC_{10} (mg/kg)	EC_{20} (mg/kg)	EC_{50} (mg/kg)	EC_{80} (mg/kg)	Model and Parameters
Hatching					
GO	129 (−36–294)	202 (123–282)	329 (197–460)	455 (159–751)	Log 2 par S: 0.0009 Y0: 33; R^2 = 0.9
rGO	753 (n.d.)	834 (n.d.)	973 (n.d.)	1111 (n.d.)	Log 2 par S: 0.0008; Y0: 23.2; R^2 = 0.3
Size					
GO	490 (82–898)	685 (385–984)	1017 (725–1310)	1349 (858–1841)	Log 2 par S: 0.00092; Y0: 0.06; R^2 = 0.2
rGO	n.e.	n.e.	n.e.	n.e.	-
Reproduction					
GO	650 (n.d.)	683 (n.d.)	740 (n.d.)	798 (n.d.)	Log 2 par S:0.006; Y0: 1658; R^2 = 0.6
rGO	860 (n.d.)	925 (n.d.)	1034 (n.d.)	1144 (n.d.)	Log 2 par S: 0.003; Y0: 1530; R^2 = 0.2
Survival					
GO	353 (n.d.)	384 (n.d.)	447 (n.d.)	492 (n.d.)	Thresh 2 par S: 0.002; Y0: 16.9; R^2 = 0.5
rGO	696 (n.d.)	881 (n.d.)	1248 (n.d.)	1512 (n.d.)	Thresh 2 par S: 0.00099; Y0: 15.3; R^2 = 0.7

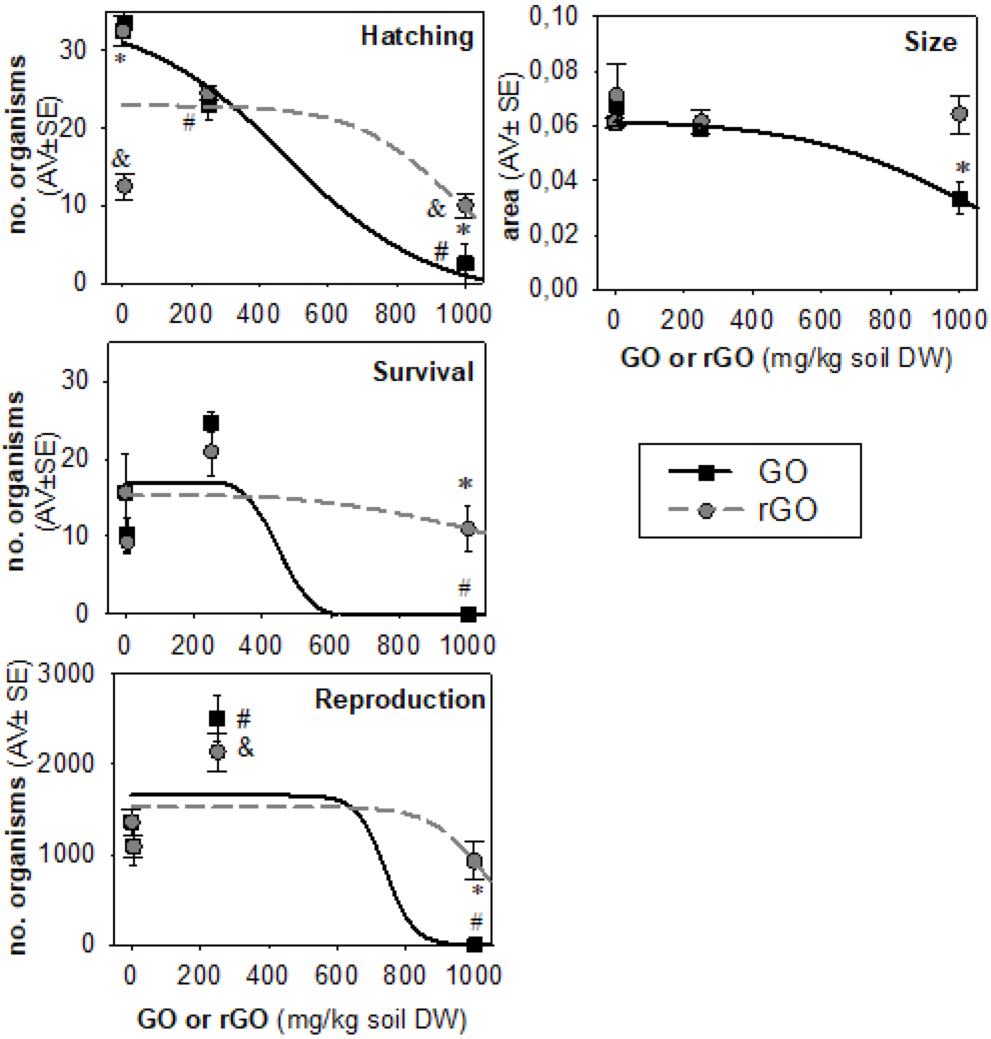


Figure IV-2. Effects of graphene oxide (GO) and reduced graphene oxide (rGO) in *Enchytraeus crypticus* after 11 days (hatching and size (area in mm^2) and 46 days of exposure (reproduction and survival). All values are expressed in average ± standard error of the means (AV ± SEM). Lines represent the data fit to model. #: $p < 0.05$ GO vs. 0 (control group); & $p < 0.05$ rGO vs. 0 (control group) following one-way ANOVA plus Dunnett's post hoc test. *: $p < 0.05$ GO vs. rGO compared to the corresponding concentration following Student's *t*-test.

Exposure to GO caused a clear dose–response effect on hatching success. Significant decrease upon hatching was observed at 250 mg GO/kg ($p < 0.05$), and even higher at 1000 mg GO/kg ($p < 0.001$). For rGO-exposed organisms, hatching was significantly lower in cocoons treated with the lowest concentration of 5 mg rGO/kg ($p < 0.001$) similar to the observed in the highest concentration of 1000 mg rGO/kg ($p < 0.05$). Comparing GO and rGO, we observe differences in the hatchability of cocoons at the concentrations of 5 and 1000 mg/kg. Interestingly, in terms of size of the hatched organisms, it decreased only for GO exposure (1000 mg GO/kg, $p < 0.05$).

Survival and reproduction were significantly affected by GO at the highest exposure concentration tested ($p < 0.001$), showing 100% mortality. On the other hand, 1000 mg rGO/kg showed little to no toxicity. Comparing GO and rGO, GO was significantly more toxic towards *E. crypticus* as shown by the decrease in the survival ($p < 0.05$) and reproduction rate ($p < 0.001$).

Additionally, two-way ANOVA was conducted to compare differences between GO and rGO treatment on different concentrations and the interaction between the variables (treatment and concentration) (Table IV-S1). The analysis revealed that treatment significantly affected the reproduction ($p < 0.01$), while the concentration affected all life cycle parameters (hatching, survival, and reproduction, $p < 0.0001$), except for the size ($p = 0.0896$). There was an interaction between treatment and concentration in relation to hatching ($p < 0.001$) and reproduction ($p < 0.01$).

5. Discussion

Comparison between GO and rGO showed higher toxicity of GO, which could be associated with higher oxidative stress, given their distinct structural and functional surface properties (Khan et al. 2015). Graphene oxide is a single-atomic-layered material comprising carbon, hydrogen, and oxygen molecules derived from the exfoliation of graphite oxide. The oxidation of graphite resulted in abundant oxygenated functional groups, such as hydroxyl and epoxides on the basal plane and carbonyl and carboxyl groups at the edges. Subsequent reduction of GO is efficient to remove some of these oxygen-containing functional groups and recovery the π-conjugated structure of graphene, leading to the formation of rGO (Ray 2015). In general, graphene materials, which contain a higher density of functional groups, have a higher chance of interacting with cells, resulting in cell deposition and increased cytotoxicity. Thus, it suggests that the higher oxygen content of GO was responsible for the increased toxicity observed compared to their reduced counterpart (rGO) (Das et al. 2013; Evariste et al. 2019; Li et al. 2018; Liu et al. 2011).

Similarly to *E. crypticus*, in other species, e.g., in the nematode *Caenorhabditis elegans*, the reproductive toxicity from GO was higher compared to rGO (Chatterjee et al., 2017). In *E. crypticus*, the higher toxicity of GO compared to rGO was also preceded by a higher size reduction of the hatched juveniles. This could well mean a correlation between size of offspring and survival levels. The relationship between organisms size and performance have been reported before (e.g.,(Amorim, Pereira, and Soares 2017; Guimarães et al. 2019a, 2019b)) and is often linked to energy allocation and trade-offs, e.g., fewer and larger animals are more fit to stress than many and smaller (K and R strategies). This was not the case here, where we observed a lower number of also smaller hatched juveniles. Effects seem additive and, hence, severely decreased survival occurred.

More of a physiological aspect of the organisms, the effects observed in terms of hatching success (11 days) were in good agreement with effects observed in survival (46 days), for both GO and rGO, indicating a good predictability of effects between early and later stages of development. This is not always the case, but it has been observed before for *E. crypticus*, e.g., when exposed to silver nitrate and silver nanoparticles (R. Bicho et al. 2016). On the other hand, exposure to copper oxide nanomaterials (R. C. Bicho et al. 2017) or nickel nanoparticles (Santos et al. 2017) showed that the observed decreased hatching was in fact a delay, and organisms recovered with time. Similarly, the observed reduction in hatching at 5 and 1000 mg rGO/kg was partly a delay and effects were diluted with time, as observed in terms of survival and reproduction.

The 250 mg GO or rGO/kg seems to cause a *hormesis*-like effect, i.e., a *stimulus* in the performance of the organisms at low doses, as observed by an increase in both survival and reproduction. This has also been observed when *E. crypticus* were exposed to multiwalled carbon nanotubes (R. C. Bicho, Ribeiro, et al. 2015), indicating that this could be the result of beneficial added carbon to the system, up to a certain maximum level.

The survival and reproduction followed a similar response pattern and, hence, effects on reproduction appear to be a direct consequence of decreased survival rather than a specific reproductive impairment. In *C. elegans*, and as opposed to this, Kim et al. (2018)

suggested that GO exposure caused reproductive toxicity by suppressing spermatogenesis of *C. elegans* hermaphrodites during development, resulting in decreased sperm numbers and progeny numbers (Kim et al. 2018).

Previous studies showed that GO is toxic to several organisms, including microbial communities, bacteria, fungi, plants, tadpoles, and rodents (An et al. 2018; Evariste et al. 2019; He et al. 2017; Jastrzębska and Olszyna 2015; Ren et al. 2018). It has been shown that the presence of GO (50 to 300 mg/L) can significantly impact bacterial metabolic activity, bacterial viability, and biological removal of nutrients, such as organics, nitrogen, and phosphorus, in the activated sludge. These effects lead to compromised wastewater treatment performance since an efficient biological wastewater treatment requires the functioning of diverse microbial species (Ahmed and Rodrigues 2013). Conversely, Esquivel-Gaon and co-workers did not observe any changes on bacterial growth, morphology, and DNA fragmentation of the common soil bacteria *Pseudomonas putida* and on nitrifying bacteria after rGO exposure (Esquivel-Gaon et al. 2018). In invertebrates, GO was reported to decrease the survival rate and inhibit the swimming behavior of the crustacean *Amphibalanus amphitrite* nauplii, to reduce the burrowing activity of the oligochaete *Tubifex tubifex*, and to induce cellular damage and reduce the metabolic capacity (higher glycogen content) of the polychaeta *Diopatra neapolitana* (De Marchi et al. 2018). Results from other studies also indicate that the reduced form of GO caused higher toxicity compared to the oxidized form (Contreras-Torres et al. 2017; Guo et al. 2017; Jaworski et al. 2015; De Marchi et al. 2018). Although most of the studies attributed these differences between GO and rGO to the differences in experimental approach, type base of material, size, thickness (number of layers), shape, and coatings, it is also necessary to consider the oxygen content and the carbon radical density of the GBN surfaces (Evariste et al. 2019; Li et al. 2018).

The potential environmental risk related to these GBN compounds are not yet clear enough and further mechanistic studies are recommended as it is very important to ensure the environmental safety of GBNs.

6. Conclusions

Chronic exposure to GO significantly impaired survival and reproduction (rGO caused nearly no toxicity up to 1000 mg/kg). The differences observed indicated that the higher oxygen content of GO must play a major role in the induction of toxicity towards *E. crypticus*. Hormesis at lower doses (250 mg/kg) was observed and could be due to the beneficial added carbon to the system. Effects of GO occurred at early life stage development with decreased hatching success (and smaller size of hatched organisms). There was good predictability of GO effects between early and later stages of development, i.e., hatching and survival. The use of a full life cycle test allowed further understanding of the mechanisms of action of GO. Further in-depth studies are recommended including the usage of more species and endpoints.

Author Contributions

Conceptualization, M.C.P.M., N.P.R., M.B.J. and M.J.B.A.; methodology, M.C.P.M. and N.P.R.; writing—original draft preparation, M.C.P.M.; writing—review and editing, M.C.P.M., N.P.R., M.B.J. and M.J.B.A..; supervision, M.B.J. and M.J.B.A.; project administration, M.B.J. and M.J.B.A.; funding acquisition, M.B.J. and M.J.B.A.

Funding

This study was financially supported by São Paulo Research Foundation (FAPESP) (grant #2017/18867-1). Further support was provided by the European Commission via H2020-NMBP-2017 BIORIMA project (GA No. 760928), by CESAM (UID/AMB/50017/2019) and a PhD grant to Natália P. Rodrigues (SFRH/BD/87787/2012) through national funds via FCT/MCTES.

References

Amorim, M.J.B.; Pereira, C.; Soares, A.M.V.M.; Scott-Fordsmand, J.J. "Does long term low impact stress cause population extinction?" Environ. Pollut. 2017, 220, 1014–1023.

Ahmed, F.; Rodrigues, D.F. "Investigation of acute effects of graphene oxide on wastewater microbial community: A case study". J. Hazard. Mater. 2013, 256, 33–39.

An, W.; Zhang, Y.; Zhang, X.; Li, K.; Kang, Y.; Akhtar, S.; Sha, X.; Gao, L. "Ocular toxicity of reduced graphene oxide or graphene oxide exposure in mouse eyes". Exp. Eye Res. 2018, 174, 59–69.

Bicho, R.C.; Santos, F.C.F.; Gonçalves, M.F.M.; Soares, A.M.V.M.; Amorim, M.J.B. Enchytraeid Reproduction TestPLUS: "Hatching, growth and full life cycle test—An optional multi-endpoint test with *Enchytraeus crypticus"*. Ecotoxicology 2015, 24, 1053–1063.

Bicho, R.C.; Ribeiro, M.J.; Scott-Fordsmand, J.J.; Amorim, M. "Tracing effects of NMs along their life cycle—Toxicity in soil (*Enchytraeus crypticus*)". Poster presentation. In Proceedings of the SETAC Europe 25th Annual Meeting, Catalonia Barcelona, Spain, 3–7 May 2015; p. 400.

Bicho, R.C.; Ribeiro, T.; Rodrigues, N.P.; Scott-Fordsmand, J.J.; Amorim, M.J.B. "Effects of Ag nanomaterials (NM300K) and Ag salt ($AgNO_3$) can be discriminated in a full life cycle long term test with *Enchytraeus crypticus"*. J. Hazard. Mater. 2016, 318, 608–614.

Bicho, R.C.; Santos, F.C.F.; Scott-Fordsmand, J.J.; Amorim, M.J.B. "Effects of copper oxide nanomaterials (CuONMs) are life stage dependent-full life cycle in *Enchytraeus crypticus"*. Environ. Pollut. 2017, 224, 117–124.

Castro-Ferreira, M.P.; Roelofs, D.; van Gestel, C.A.M.; Verweij, R.A.; Soares, A.M.V.M.; Amorim, M.J.B. "*Enchytraeus crypticus* as model species in soil ecotoxicology". Chemosphere 2012, 87, 1222–1227.

Chatterjee, N.; Kim, Y.; Yang, J.; Roca, C.P.; Joo, S.W.; Choi, J. "A systems toxicology approach reveals the Wnt-MAPK crosstalk pathway mediated reproductive failure in

Caenorhabditis elegans exposed to graphene oxide (GO) but not to reduced graphene oxide (rGO)". Nanotoxicology 2017, 11, 76–86.

Contreras-Torres, F.F.; Rodríguez-Galván, A.; Guerrero-Beltrán, C.E.; Martínez-Lorán, E.; Vázquez-Garza, E.; Ornelas-Soto, N.; García-Rivas, G. "Differential cytotoxicity and internalization of graphene family nanomaterials in myocardial cells". Mater. Sci. Eng. C 2017, 73, 633–642.

Das, S.; Singh, S.; Singh, V.; Joung, D.; Dowding, J.M.; Reid, D.; Anderson, J.; Zhai, L.; Khondaker, S.I.; Self, W.T.; et al. "Oxygenated functional group density on graphene oxide: Its effect on cell toxicity". Part. Part. Syst. Charact. 2013, 30, 148–157.

De Marchi, L.; Pretti, C.; Gabriel, B.; Marques, P.A.A.P.; Freitas, R.; Neto, V. "An overview of graphene materials: Properties, applications and toxicity on aquatic environments". Sci. Total Environ. 2018, 631, 1440–1456.

Evariste, L.; Lagier, L.; Gonzalez, P.; Mottier, A.; Mouchet, F.; Cadarsi, S.; Lonchambon, P.; Daffe, G.; Chimowa, G.; Sarrieu, C.; et al. "Thermal Reduction of Graphene Oxide Mitigates Its *In Vivo* Genotoxicity toward *Xenopus Laevis* Tadpoles". Nanomaterials 2019, 9, 584.

Esquivel-Gaon, M.; Nguyen, N.H.A.; Sgroi, M.F.; Pullini, D.; Gili, F.; Mangherini, D.; Pruna, A.I.; Rosicka, P.; Sevcu, A.; Castagnola, V. "*In Vitro* and environmental toxicity of reduced graphene oxide as an additive in automotive lubricants". Nanoscale 2018, 10, 6539–6548.

Guimarães, B.; Maria, V.L.; Römbke, J.; Amorim, M.J.B. "Exposure of *Folsomia candida* (Willem 1902) to the flubenzuron over three generations—Increase of toxicity in the third generation". Appl. Soil Ecol. 2019, 134, 8–14.

Guimarães, B.; Maria, V.L.; Römbke, J.; Amorim, M.J.B. "Multigenerational exposure of *Folsomia candida* to ivermectin—Using avoidance, survival, reproduction, size and cellular markers as endpoints". Geoderma 2019, 337, 273–279.

Guo, Z.; Xie, C.; Zhang, P.; Zhang, J.; Wang, G.; He, X.; Ma, Y.; Zhao, B.; Zhang, Z. “Toxicity and transformation of graphene oxide and reduced graphene oxide in bacteria biofilm”. Sci. Total Environ. 2017, 580, 1300–1308.

He, K.; Chen, G.; Zeng, G.; Peng, M.; Huang, Z.; Shi, J.; Huang, T. “Stability, transport and ecosystem effects of graphene in water and soil environments”. Nanoscale 2017, 9, 5370–5388.

International Organization for Standardization. Soil quality—Effects of contaminants on Enchytraeidae (*Enchytraeus* sp.)—Determination of effects on reproduction. IOS: Geneva, Switzerland, 2014.

Jastrzębska, A.M.; Olszyna, A.R. “The ecotoxicity of graphene family materials: Current status, knowledge gaps and future needs”. J. Nanopart. Res. 2015, 17, 40.

Jaworski, S.; Sawosz, E.; Kutwin, M.; Wierzbicki, M.; Hinzmann, M.; Grodzik, M.; Winnicka, A.; Lipińska, L.; Włodyga, K.; Chwalibog, “A. *In Vitro* and *In Vivo* effects of graphene oxide and reduced graphene oxide on glioblastoma”. Int. J. Nanomed. 2015, 10, 1585–1596.

Kang, Y.; Liu, J.; Wu, J.; Yin, Q.; Liang, H.; Chen, A.; Shao, L. “Graphene oxide and reduced graphene oxide induced neural pheochromocytoma-derived PC12 cell lines apoptosis and cell cycle alterations via the ERK signaling pathways”. Int. J. Nanomed. 2017, 12, 5511–5523.

Khan, M.; Tahir, M.N.; Adil, S.F.; Khan, H.U.; Siddiqui, M.R.H.; Al-Warthan, A.A.; Tremel, W. “Graphene based metal and metal oxide nanocomposites: Synthesis, properties and their applications”. J. Mater. Chem. A 2015, 3, 18753–18808.

Kim, Y.; Jeong, J.; Yang, J.; Joo, S.W.; Hong, J.; Choi, J. “Graphene oxide nano-bio interaction induces inhibition of spermatogenesis and disturbance of fatty acid metabolism in the nematode *Caenorhabditis elegans*”. Toxicology 2018, 410, 83–95.

Li, R.; Guiney, L.M.; Chang, C.H.; Mansukhani, N.D.; Ji, Z.; Wang, X.; Liao, Y.P.; Jiang, W.; Sun, B.; Hersam, M.C.; et al. “Surface Oxidation of Graphene Oxide Determines

Membrane Damage, Lipid Peroxidation, and Cytotoxicity in Macrophages in a Pulmonary Toxicity Model". ACS Nano 2018, 12, 1390–1402.

Liu, S.; Zeng, T.H.; Hofmann, M.; Burcombe, E.; Wei, J.; Jiang, R.; Kong, J.; Chen, Y. "Antibacterial activity of graphite, graphite oxide, graphene oxide, and reduced graphene oxide: Membrane and oxidative stress". ACS Nano 2011, 5, 6971–6980.

Mendonça, M.C.P.; Soares, E.S.; De Jesus, M.B.; Ceragioli, H.J.; Batista, Â.G.; Nyúl-Tóth, Á.; Molnár, J.; Wilhelm, I.; Maróstica, M.R.; Krizbai, I.; et al. "PEGylation of reduced graphene oxide induces toxicity in cells of the blood-brain barrier: An *In Vitro* and *In Vivo* study". Mol. Pharm. 2016, 13, 3913–3924.

Nurunnabi, M.; Parvez, K.; Nafiujjaman, M.; Revuri, V.; Khan, H.A.; Feng, X.; Lee, Y.K. "Bioapplication of graphene oxide derivatives: Drug/gene delivery, imaging, polymeric modification, toxicology, therapeutics and challenges". RSC Adv. 2015, 5, 42141–42161.

OECD. Guidelines for the Testing of Chemicals. No. 220—Enchytraeid Reproduction Test; OECD: Paris, France, 2015.

Organisation for Economic Co-operation and Development. Series on the Safety of Manufactured Nanomaterials, No. 36: Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials; OECD: Paris, France, 2012.

Qu, Y.; He, F.; Yu, C.; Liang, X.; Liang, D.; Ma, L.; Zhang, Q.; Lv, J.; Wu, J. "Advances on graphene-based nanomaterials for biomedical applications". Mater. Sci. Eng. C 2018, 90, 764–780.

Ray, S.C. "Application and uses of graphene oxide and reduced graphene oxide". In Applications of Graphene and Graphene-Oxide Based Nanomaterials; Elsevier: Amsterdam, The Netherlands, 2015; pp. 39–55, ISBN 9780323375214.

Ren, X.; Zeng, G.; Tang, L.; Wang, J.; Wan, J.; Feng, H.; Song, B.; Huang, C.; Tang, X. "Effect of exogenous carbonaceous materials on the bioavailability of organic pollutants and their ecological risks". Soil Biol. Biochem. 2018, 116, 70–81.

ReportLinker Global and China Graphene Industry Report, 2018–2023. Available online: https://www.reportlinker.com/p04539093/Global-and-China-Graphene-Industry-Report.html (accessed on Day 10 Month March Year2019).

Santos, F.C.F.; Gomes, S.I.L.; Scott-Fordsmand, J.J.; Amorim, M.J.B. "Hazard assessment of nickel nanoparticles in soil - The use of a full life cycle test with *Enchytraeus crypticus"*. Environ. Toxicol. Chem. 2017, 36, 2934–2941.

Statista Market Volume of Graphene-Enhanced Composites Worlwide in 2017 and 2023, by Composite Type (in Tons). Available online: https://www.statista.com/statistics/960946/global-graphene-enhanced-composites-market-volume-by-type/ (accessed on Day 10 Marchonth 2019Year).

Seifati, S.M.; Nasirizadeh, N.; Azimzadeh, M. "Nano-biosensor based on reduced graphene oxide and gold nanoparticles, for detection of phenylketonuria-associated DNA mutation". IET Nanobiotechnol. 2018, 12, 417–422.

Zare-Zardini, H.; Taheri-Kafrani, A.; Ordooei, M.; Amiri, A.; Karimi-Zarchi, M. "Evaluation of toxicity of functionalized graphene oxide with ginsenoside Rh2, lysine and arginine on blood cancer cells (K562), red blood cells, blood coagulation and cardiovascular tissue: *In Vitro* and *In Vivo* studies". J. Taiwan Inst. Chem. Eng. 2018, 93, 70–78.

Supplementary Materials:

Figure IV-S1. Results of two-way analysis of variance of the effects of GO and rGO on life cycle parameters.

Parameter	Effect	P	F
Hatching	Treatment	0.0600	3.86
	Concentration	p < 0.0001	55.07
	Interaction	p < 0.0001	16.17
Size	Treatment	0.0932	2.83
	Concentration	0.0896	2.18
	Interaction	0.3037	1.21
Survival	Treatment	0.4793	0.516
	Concentration	p < 0.0001	1.06
	Interaction	0,1111	2.23
Reproduction	Treatment	0.0041	10.06
	Concentration	p < 0.0001	11.28
	Interaction	0.0026	6.318

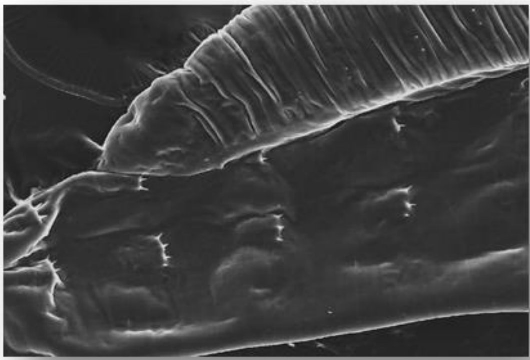


Photo by Natália Rodrigues

Chapter V

General Discussion and Final Remarks

In this work, we aimed for an integrated testing approach, for which (some of the) parameters/endpoints have been update; at the same time, we have always followed the standard tests, to allow comparisons between studies and to facilitate reproducibility. These updates will be explained, case by case.

Silver

Hatching test: Although hatching is not an endpoint standardized for *E. crypticus*, it has been optimized, by Bicho et al., 2015, in which it is recommended to be assessed at 11 days, when the juveniles are hatched in control conditions. Also, the same endpoint has been widely reported as an effective parameter for detecting effects of nanomaterials in early life stages (Bicho et al., 2016, 2017; Gomes et al., 2019; Santos et al., 2017). In Chapter II, the performance of the test in ISO water media, allowed the daily observation and photographic record of the cocoon (embryo) development (Figure II-S1). Based on this day-to-day assessment, we were able to realize that, in fact, the cocoons exposed to Ag NM300K had not only a hatching delayed, but also a real inability to hatch. This evaluation was possible by extending the test for an extra week, from 11 to 17 days, when it was noticed that, there would be no more possibility of hatching.

Still on water tests, there is a vast literature reporting that early life stages are time efficient and also very sensitive (e.g. juveniles can be more sensitive than adults) (Druart et al., 2010; Geffard et al., 2002; Truong and Tanguay, 2017). Results for *E. crypticus* exposed to NMs have evidenced to be in line with these findings, although still scarce (Bicho et al., 2017). In this context, we also studied the survival of juveniles of synchronized age exposed to Ag (NMs) in comparison with adults. As this test was performed in water, we could not rule out the hypothesis that enchytraeids are more sensitive there than in soil media. However, having found no effect for adults using this same via, our results corroborates the greater sensitivity of the juveniles when exposed to Ag(NMs).

The great advantage of this kind of test is to allow a visual monitoring of the organisms. It was through this monitoring that we could visualize the aggregation/agglomeration of AgNPs around the clitellum region of the adults in the survival test (see Figure II-S2 of the supplementary material in Chapter II). This data provide strong justifications for the decreased reproduction in ERT, as: 1) some dysfunction in the reproductive system of the parental generation (F0) affecting cocoon laying; 2) F0 were able to lay cocoons, however, these cocoons a) hatched later, b) did not hatch; c) hatched, but producing less juveniles, d) hatched with expected juvenile numbers, but they die during the test. However, combining the results of adults 'survival water test and hatching water test, we showed that the data support the hypotheses 2) a, b and c, depending on Ag(NMs) and concentrations tested.

The avoidance tests were also improved, adding three more sampling times (24 – 72 – 96h), in addition to the 48h exposure (see Table V-1), which is the standard test duration for avoidance tests in earthworms and collembolans (ISO - 17512-1, 2007; ISO - 17515-2, 2008). These extra sampling points were coupled with the evaluation of gene expression, which added a mechanistic explanation to the avoidance results.

Table V-1. Summary of the information on avoidance behavior regarding the highest tested concentrations for PVP – AgNMs, AgNMs, Ag NM300K and $AgNO_3$. Yes, means the organisms avoided the contaminated soil. No, means no avoidance behaviour. *, ** means gamma-aminobutyric acid receptor-associated protein (GABAAR) gene down- and up- regulation, respectively.

materials/conc (mg/kg) vs time		Did enchytraeids avoid?			
		24h	48h	72h	96h
PVP – AgNMs	640	no	no	no	no
	1000	no	no	no	no
AgNMs	640	yes*	yes	no	no**
	1000	yes	no	no	no
Ag NM300K	256	yes	no	no	no
	320	yes	no	no	no
$AgNO_3$	20	yes*	yes	no	no**
	64	yes	yes	yes	yes

Looking partially to these results, probably would lead us to its misinterpretation. For example, for the 24h, except for PVP-AgNM, all the other compounds caused organisms avoidance behavior. However, with the additional sampling times, we noticed that organisms tend to avoid less the contaminated soil along the test. Based only on these results, we could raise the following hypotheses: 1) the organisms increased tolerance to the contaminated soil during the test and so they tended do not avoid; 2) interaction of silver(NMs) with soil, decreased their toxicity, causing a decrease in avoidance behavior and/or a non-avoidance/attraction behaviour; 3) with increasing time, organisms decreased their ability to avoid and to return to the control soil. The results of gene expression analysis provided an answer to the third hypothesis and allowed a detailed comprehension for the mechanistic response of *E. crypticus* regarding the $AgNO_3$ and AgNMs. The answer is that *E. crypticus* were not able to avoid the contaminated soil at 96h, due to the inability of movements (paralysis), caused by GABA binding increase (as measured by the up regulation of the GABAAR gene).

However, while we were able to correlate the up-regulation of the GABA gene with the apparent attraction behavior of the organisms exposed to $AgNO_3$ and AgNMs at 20 and 640mg/kg, respectively, at the same time, we also could detect a different avoidance pattern between both contaminants, leading to not rule out some specificity of the nanoparticles. Also, since the non-avoidance response was similar for Ag NM300K and AgNMs after 72 hours, we would expect to have a similar response in terms of gene expression. However, for the PVP-coated material, more investigation would be required to clarify if the non-avoidance response is also linked to the GABA receptor up-regulation, as reported for the AgNMs without capping, or if there are other (coated related) mechanisms involved.

In general, for AgNPs, the longest exposure period (96h) induced a lower avoidance response than the shortest 24h, when the organisms were still able to avoid. This reduction of avoidance response can have implications in field scenarios, in which if the organisms would not be able to avoid contaminated soils their populations can be seriously affected. For $AgNO_3$, the opposite was observed. This fact highlights the need to extend test durations (or to perform longer tests) to better understand the toxicity profile of AgNPs.

Knowing that the toxicology of the 21st century advocates the use of fewer animals, less time-consuming, less expensive, modelling, among others (Council, 2007) in Chapter III, the first and fundamental objective of the study presented was to investigate whether the organism *E. crypticus* would be suitable for nanomaterials screening, before going deeper with vertebrate testing (in this case, rats). In this way, we reasoned from two points: 1) the toxicity results obtained in Chapter II and 2) the study performed by Mendonça et al., 2019, who showed that the combination of AgNPs with thiol antioxidants compounds could reverse the cytotoxicity of hepatocarcinoma cells induced by silver using *Rattus norvegicus* (vertebrate model Wistar rat). Thus, we wanted to assess the effect in an environmental species, guiding us by the following hypotheses: a) the combination of thiol groups with silver (NMs) would also reduce AgNMs toxicity to the *E. crypticus*, assessed via the standard ERT? After confirming the previous hypothesis, we aimed 2) to understand if the organisms would be able to choose between the control media and the soil treated with Ag(NM)+thiol in avoidance tests. We were able to realize that although silver binds with thiol, reducing its toxicity, the organisms still prefer the control side. Following this result, another question raised, 3) would the organisms choose the side containing soil treated with Ag(NM)+thiol groups, when the opposite side have been treated only with Ag(NM)? The results showed, as expected, the preference for soil spiked with Ag(NM)+thiol groups (AgNM avoidance), however, some questions to understand the possible mechanisms implicated in the organisms choice (e.g., research allocation) remained to be answered.

Graphene

To assess the toxicity of reduced graphene oxide (rGO) in comparison to the non-reduced nanoform (GO), as presented in Chapter IV, our approach, was once again, integrative, that is, combining several endpoints, as well as updating some of the standard procedures. Bicho et al., 2015, developed the FLCt. Here, we used a reduced version of this test, which includes, among other parameters, the hatching and the size of juveniles hatched. Size was assessed measuring juveniles' area for the first time. Other publications have used length to evaluate growth and/or size (Bicho et al., 2015, 2016, 2017; Gomes et al., 2019; Santos et al., 2017) however, through a series of measurements and optimizations for this parameter, we came to the conclusion that the area could be more representative of a potential effect than length. For instance, in organisms that choose to allocate their energy to growth in size but reducing mass (longer and skinnier organisms), when analyzing only its length, the results would show a false negative. The proposed measurement of area (significantly more affected by GO than rGO) might also be more sensitive than length in *E. crypticus*.

Conclusion

In this thesis, we opted for an integrated approach, combining various tests and methodologies, in parallel to the performance of standard procedures. Some of the standardized parameters were updated, meanwhile others were kept, with the intuition of allowing comparisons across studies and to facilitate reproducibility. Through this approach, we get closer to the toxicology of the 21st century, since we produce knowledge using 1) less organism; 2) more predictive technology, such as genomics; 3) intelligent testing strategies (ITS); 4) similar to the standard approaches for easier international harmonization of the current designs.

In the Chapter II and IV, we used a battery of tests, being the results not depending, but agreeing to each other. In Chapter III, we used the weight of evidence approach, i.e., the different/sequential tests were performed by taking decisions based in evidences. Both performances provided solid information for risk assessment of NMs.

To conclude, the combination of tests is more powerful than the reductionism provided by a single test and the integrated overview allows several advantageous procedures using *Enchytraeus crypticus*:

1) successful application of short-term exposure via water media to predict long term effects of Ag (nano)materials;

2) extending the exposure from hatching endpoint to reproduction allows to distinguish delay from impairment;

3) survival of juveniles was more sensitive than adults;

4) Ag interfered with the GABA mechanism, causing organisms ‘paralysis with gene up-regulation;

5) successful application of thiol groups to reduce/revert the toxic effects of silver materials on *Enchytraeus crypticus*, and good indications that *E. crypticus* can be a model for NMs toxicology screening, prior tests with vertebrate models*;*

6) GO caused a decrease in hatching and rFLC showed to be a good way to predict long-term effects;

References

Bicho, R., Ribeiro, T., Rodrigues, N., 2016. Effects of Ag nanomaterials (NM300K) and Ag salt ($AgNO_3$) can be discriminated in a full life cycle long term test with *Enchytraeus crypticus*. Journal of Hazard Materials, 318 608-614.

Bicho, R.C., Santos, F.C.F., Gonçalves, M.F.M., Soares, A.M.V.M., Amorim, M.J.B., 2015. Enchytraeid Reproduction TestPLUS: hatching, growth and full life cycle test—an optional multi-endpoint test with *Enchytraeus crypticus*. Ecotoxicology 24, 1053–1063. https://doi.org/10.1007/s10646-015-1445-5

Bicho, R.C., Santos, F.C.F., Scott-Fordsmand, J.J., Amorim, M.J.B., 2017. Effects of copper oxide nanomaterials (CuONMs) are life stage dependent – full life cycle in *Enchytraeus crypticus*. Environmental Pollution 224, 117–124. https://doi.org/10.1016/j.envpol.2017.01.067

Druart, C., Scheifler, R., de Vaufleury, A., 2010. Towards the development of an embryotoxicity bioassay with terrestrial snails: Screening approach for cadmium and pesticides. Journal of Hazard Matererials 184, 26–33. https://doi.org/10.1016/j.jhazmat.2010.07.099

Geffard, O., Budzinski, H., His, E., 2002. The effects of elutriates from PAH and heavy metal polluted sediments on *Crassostrea gigas* (Thunberg) embryogenesis, larval growth and bio-accumulation by the larvae of pollutants from sedimentary origin. Ecotoxicology 11, 403–416. https://doi.org/10.1023/A:1021024415695

Gomes, S., Scott-Fordsmand, J., 2015. Cellular energy allocation to assess the impact of nanomaterials on soil invertebrates (Enchytraeids): the effect of Cu and Ag. International Journal of Environmental Research Public Health

Gomes, S.I.L., Scott-Fordsmand, J.J., Campos, E.V.R., Grillo, R., Fraceto, L.F., Amorim, M.J.B., 2019. On the safety of nanoformulations to non-target soil invertebrates-an atrazine case study. Environmental Science: Nano 6, 1950–1958. https://doi.org/10.1039/c9en00242a

ISO - 17512-1, 2007. Soil quality—Avoidance test for testing the quality of soils and effects of chemicals on behaviour — Part 1: Test with earthworms (*Eisenia fetida* and *Eisenia andrei*), Geneva.

ISO - 17515-2, 2008. Soil quality — Avoidance test for testing the quality of soils and effects of chemicals — Part 2: Test with collembolans (*Folsomia candida*). ISO (International Organization for Standardization), Geneva, Switzerland.

Kittler, S., Greulich, C., Diendorf, J., Köller, M., Epple, M., 2010. Toxicity of silver nanoparticles increases during storage because of slow dissolution under release of silver ions. Chemical Materials. 22, 4548–4554. https://doi.org/10.1021/cm100023p

Mariyadas, J., Amorim, M.J.B., Jensen, J., Scott-Fordsmand, J.J., 2018. Earthworm avoidance of silver nanomaterials over time. Environ. Pollut. 239, 751–756. https://doi.org/10.1016/j.envpol.2018.04.059

Mendonça, M.C.P., Soares, E.S., de Jesus, M.B., Ceragioli, H.J., Ferreira, M.S., Catharino, R.R., da Cruz-Höfling, M.A., 2015. Reduced graphene oxide induces transient blood-brain barrier opening: An in vivo study. J. Nanobiotechnology 13. https://doi.org/10.1186/s12951-015-0143-z

Mendonça, M.C.P., Ferreira, L.B., Rizoli, C., Batista, Â.G., Maróstica Júnior, M.R., da Silva, E. do N., Cadore, S., Durán, N., Cruz-Höfling, M.A. da, de Jesus, M.B., 2019. N-Acetylcysteine reverses silver nanoparticle intoxication in rats. Nanotoxicology 13, 326–338. https://doi.org/10.1080/17435390.2018.1544302

Nacional Research Council., 2007. Toxicity Testing in the 21st Century: A Vision and a Strategy.

Nagy, A., Harrison, A., Sabbani, S., Munson, R.S., Dutta, P.K., Waldman, W.J., 2011. Silver nanoparticles embedded in zeolite membranes: release of silver ions and mechanism of antibacterial action. Int. J. Nanomedicine 6, 1833–1852. https://doi.org/10.2147/IJN.S24019

Ribeiro, M., Maria, V., 2015. Oxidative stress mechanisms caused by Ag nanoparticles (NM300K) are different from those of $AgNO_3$: Effects in the soil invertebrate *Enchytraeus* *crypticus*. International Journal of Environmental Research of Public Health 12, 9589-9602. https://doi:10.3390/ijerph120809589

Santos, F.C.F., Gomes, S.I.L., Scott-Fordsmand, J.J., Amorim, M.J.B., 2017. Hazard assessment of nickel nanoparticles in soil-The use of a full life cycle test with *Enchytraeus crypticus*. Environmental Toxicololy and Chemistry. 9999, 1–8. https://doi.org/10.1002/etc.3853

Truong, L., Tanguay, R.L., 2017. Evaluation of embryotoxicity using the zebrafish model, in: Methods in Molecular Biology. Humana Press Inc., pp. 325–333. https://doi.org/10.1007/978-1-4939-7172-5_18

Chapter VI

Supplementary Research

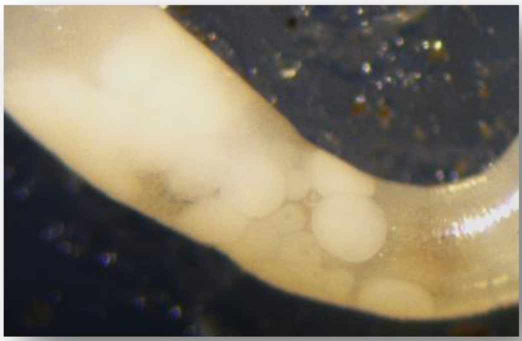


Photo by Natália Rodrigues

Annex I

Effects of Ag nanomaterials (NM300K) and Ag salt ($AgNO_3$) can be discriminated in a full life cycle long term test with Enchytraeus crypticus

Journal of Hazardous Materials 318 (2016) 608–614

Contents lists available at ScienceDirect

Journal of Hazardous Materials

journal homepage: www.elsevier.com/locate/jhazmat

Effects of Ag nanomaterials (NM300K) and Ag salt ($AgNO_3$) can be discriminated in a full life cycle long term test with *Enchytraeus crypticus*

Rita C. Bicho [a,*], Tânia Ribeiro [a], Natália P. Rodrigues [a], Janeck J. Scott-Fordsmand [b], Mónica J.B. Amorim [a]

[a] *Departamento de Biologia & CESAM, Universidade de Aveiro, 3810-193 Aveiro, Portugal*
[b] *Department of Bioscience, Aarhus University, Vejlsovej 25, PO BOX 314, DK-8600 Silkeborg, Denmark*

HIGHLIGHTS

- First full life cycle test with nanomaterials (NMs) with a soil invertebrate.
- Effects could be discriminated between AgNMs and Ag salt.
- AgNMs caused decrease in hatching whereas $AgNO_3$ caused a delay.
- Non-monotonic concentration-response to AgNMs, high effect to low concentration.

ARTICLE INFO

Article history:
Received 15 March 2016
Received in revised form 10 July 2016
Accepted 18 July 2016
Available online 19 July 2016

Keywords:
Hazard assessment
Oligochaete
Terrestrial compartment
Life stages
Long term

ABSTRACT

Information on effects of silver nanoparticles on soil invertebrates, especially using long-term exposures, is scarce. In this study we investigated the effects of the reference Ag (NM300K) (compared to $AgNO_3$) using the full life cycle test (FLCt) of the soil invertebrate *Enchytraeus crypticus*. Results showed that effects were higher compared to the standard reproduction test, which is shorter and does not cover the FLC. Both Ag forms caused a reduction on hatching success, juvenile and adult survival and reproduction with similar ECx. Differences between $AgNO_3$ and Ag NM300K could be discriminated using the FLCt: $AgNO_3$ decreased hatching success was shown to be a delay in the process, whereas Ag NM300K caused irreversible effects during the same time frame. These effects may have occurred during the embryo development, hatching (inhibition) or survival of hatched juveniles. Ag NM300K caused non-monotonic concentration-response effect as observed by the high effect of the lowest concentration (20 mg kg-1). It is known that dispersion is higher at lower concentrations – this could explain the increased effect at low concentration. Non monotonic responses are well described in the literature, where effects of high cannot predict for low concentrations, hence special attention should be given for NMs low concentration effects.

1. Introduction

Worldwide there has been a fast increase in production of nanomaterials (NMs) due to their exceptional physico-chemical properties. Silver nanomaterials (AgNMs) are among the most widely used in consumer products applications [1,2], such has electronics, medical devices, clothing, personal care products, cosmetics, detergents, among others [3,4]. With the increase use of these products there is a particular concern for environmental exposure. The terrestrial environment is one of the primary receivers of NMs [5–7] through the application of sewage sludge as fertilizer [7–10]. For example, during clothing laundry AgNMs can outflow to wastewater and end up in soils via sewage sludge [4,5,9]. Additional sources include landfills and waste incineration [5,11]. In Europe predictions indicate an annual increase for AgNMs of 1 μg/kg dry soil [12] and calculations showed predicted environmental concentrations (PECs) of 1.5–1.6 μg/kg for soil receiving sewage sludge [10,12]; for the U.S. predictions reach up to 13 μg/kg in the soil via sewage sludge [13]. A recent study [7] shows maximum PECs for AgNMs in different soils areas, 0.5 μg/kg for sludge

* Corresponding author.
E-mail address: ritabicho@ua.pt (R.C. Bicho).

http://dx.doi.org/10.1016/j.jhazmat.2016.07.040
0304-3894/

treated soil; 0.08 μg/kg for urban soils; 0.06 μg/kg for natural soils and 0.02 μg/kg for agricultural soils.

Despite the increasing number of studies currently available to assess the biological effects of AgNMs, these are still unclear and particularly scarce in the terrestrial environment. From available studies, indications are that current ISO and OECD test guidelines may underestimate effects of NMs and that e.g. longer-term exposures are required to assess the hazard of NMs [14–19]. For instance, standard reproduction tests (28 days) with earthworms (*Eisenia fetida*) and springtails (*Folsomia candida*) indicated that silver nitrate ($AgNO_3$) was more toxic than AgNMs. However, long-term exposures (52 and 310 days) with earthworms (*Lumbricus rubellus* and *Eisenia fetida*) showed that AgNMs toxicity increased with exposure time, whereas $AgNO_3$ toxicity decreased [14,17]. The need for more long-term relevant endpoints and more mechanistic studies has been highlighted [20–22]. In line with this, Bicho et al. [23] developed the *Enchytraeus crypticus* full life cycle test (FLCt) which has additional endpoints, e.g. hatching success and growth compared to the common 1–2 endpoint standard tests (survival, reproduction) hence being more comprehensive and allowing to discriminate effects between different life stages [24] while also providing life history information to model population dynamics [25,26]. Full life cycle studies with soil invertebrates are scarce, this being inexistent for nanoparticles. There is an example where a FLC study was performed with *Caenorhabditis elegans* exposed to AgNMs although exposure was performed in "Simulated Soil Pore Water" media rather than soil [27].

In the present study we investigated the effects of AgNMs on the various stages of the FLCt of *E. crypticus* (46 days). Further, $AgNO_3$ is tested for comparison purpose. Moreover, results are compared to the standard Enchytraeid Reproduction Test (ERT) (21 days) [28,29].

2. Material and methods

2.1. Test organisms

The test species *Enchytraeus crypticus* (Oligochaeta: Enchytraeidae) was used. Cultures are kept in agar plates prepared with a salt solution of $CaCl_2$, $MgSO_4$, KCl and $NaHCO_3$, fed ad libitum with oatmeal and maintained in laboratory under controlled conditions at 18 °C and a photoperiod of 16:8 (light:dark). Synchronized cultures were prepared as described in [23]. In short, adults with well-developed clitellum are transferred into fresh agar plates to lay cocoons. Synchronized 1–2 days old cocoons are used for test.

2.2. Test soil

The standard LUFA 2.2 natural soil (Speyer, Germany) was used. The main characteristics can be described as follows: pH (0.01 M $CaCl_2$) = 5.5, organic matter = 1.77 %, CEC (cation exchange capacity) = 10.1 meq/100 g, WHC (water holding capacity) = 41.8 %, grain size distribution of 7.3 % clay, 13.8 % silt, and 78.9 % sand.

2.3. Test materials and spiking

Silver nitrate ($AgNO_3$ > 99 % purity, Sigma-Aldrich) and the reference silver nanomaterial (NM300K) were used. The reference material Ag NM300K from the European Commission Joint Research Centre (JRC) is fully characterized [30]. In short Ag NM300K are spherical and consist of a colloidal dispersion with a nominal silver content of 10.2 w/w %, dispersed in 4 % w/w of polyoxyethylene glycerol trioleate and polyoxyethylene (20) sorbitan mono-laurat (Tween 20), having > 99 % number of particles with a nominal size of about 15 nm, with no coating. Transmisson Electron Microscopy (TEM) indicated a size of 17 ± 8 nm. Smaller nanoparticles of *ca.* 5 nm are also present. The dispersant was also tested alone.

The tested concentrations in the ERT were 0–36–48–60–72 mg Ag kg-1 soil (DW) for $AgNO_3$, and 0–dispersant–200–300–400–600–800 mg Ag kg-1 soil (DW) for Ag NM300K. For the FLCt concentrations were 0–24–48–72–96 mg Ag kg-1 soil (DW) for $AgNO_3$, and 0–dispersant–20–60–115–170 mg Ag kg-1 soil (DW) for Ag NM300K. Test chemical was spiked onto the pre-moistened soil as aqueous solution. Stock aqueous solution was prepared and serially diluted. For $AgNO_3$ soil batches per concentration were homogeneously mixed and split onto replicates, for Ag NM300K spiking was done per individual replicate. The control dispersant was made adding the same volume as used with the highest concentration of Ag NM300K. Soil was allowed to equilibrate for 3 days prior test start. Soil moisture was adjusted to 50 % of the WHCmax.

2.4. Test procedures

2.4.1. Enchytraeid Reproduction Test (ERT)

The ERT followed the procedures as described in the standard guideline [28,29]. In short, 10 adult organisms collected from cultures were selected and introduced in each test vessel containing 20 g of moist soil and food supply. Test runs at 20 °C and 16:8 h photoperiod. Food and water is replenished weekly. Four replicates per treatment were used.

To extract organisms from soil and counting, replicates were fixated with 96 % ethanol and Bengal red (1 % solution in ethanol). After 2 h, soil samples were sieved through 3 meshes (1.6, 0.5, 0.2 mm) to separate individuals from most of the soil and facilitate counting using stereo microscope.

2.4.2. Full life cycle test (FLCt)

A full life cycle test (FLCt) was performed following the procedures described in [23]. In short, exposure starts with synchronized (1–2 days old) cocoons (n = 10 per replicate) selected and introduced in each test vessel containing 10 g of moist soil. Test runs at 20 °C and 16:8 h photoperiod. Food is added at day 11 and is replenished weekly as well as water loss. Four replicates per treatment were used. Sampling days included the following: 11, 14, 22, 25 and 46 days, where organisms were counted and measured (length). The presence of clitellum (maturity status) was also recorded at days 22 and 25. Extraction of organisms was performed as described above.

2.5. Data analysis

One-way analysis of variance (ANOVA) followed by Dunnettís comparison post-hoc test ($p \leq 0.05$) was used to assess differences between controls and treatments with SigmaPlot 11.0 ed. Effect Concentrations (ECx) calculations were performed for the various endpoints modelling data to logistic or threshold sigmoid 2 parameters regression models, as indicated in Table 1, using the Toxicity Relationship Analysis Program (TRAP v1.22) software.

Further, as described [20], Cocoon production (CoP) and Population growth rate were calculated. For CoP:

$$CoP = Jt * (Jh/Js))/(Jh/Coi)$$

where, Jt is the number of juveniles at test end (day 46), Jh is the number of juveniles hatched (day 11), Js is the number of juveniles hatched that survived (day 25), and Coi is the initial number of cocoons. Population growth rate was calculated as the instantaneous population rate (r_i):

$$r_i = \ln(Nf/No)/t$$

where, Nf and No is respectively the final and initial numbers (of cocoons or juveniles), and t is the time (total number of days of the test).

The effects of Ag on population growth were also presented for each of the life stages in the FLCt, being calculated from the initial number of cocoons, the hatched juveniles, the juvenile survival and the final number (surviving adults and number of juveniles from reproduction).

3. Results

3.1. Biological characterization

Overall, no significant changes occurred in soil pH within concentrations and during the test. The validity criteria from the standard test were fulfilled, i.e. for juveniles coefficient of variation was < 20% and the number of juveniles was ≥ 25, for adults mortality was ≤ 20%.

3.1.1. Enchytraeid Reproduction Test (ERT)

Results can be observed in Fig. 1.

Effects occurred for both survival and reproduction in a concentration-response manner within the tested range for both Ag forms. $AgNO_3$ significantly reduced the number of adults at concentrations ≥ 60 mg Ag kg-1 (d.f. = 4 and 20; F = 4.4; p = 0.014). The number of juveniles was significantly lower at concentrations ≥ 48 mg Ag kg-1 (d.f. = 4 and 20; F = 29.6; p < 0.001). Results for Ag NM300K showed a significantly reduction for the number of adults at concentrations ≥ 600 mg Ag kg-1 (d.f. = 5 and 26; F = 15.1; p < 0.001). The number of juveniles was significantly lower at concentrations ≥ 200 mg Ag kg-1 (d.f. = 5 and 26; F = 22.9; p < 0.001). There was no effect of the tested control solvent. ECx values can be observed in Table 1.

3.1.2. Full life cycle test (FLCt)

Results can be observed in Fig. 2.

No significant differences were observed between control and control-dispersant, hence only control dispersant was used for calculations. Further, for Ag NM300K the lowest tested concentration (20 mg Ag kg-1) was not used for the ECx calculation given the deviation from the concentration-response models. This is discussed in the next section.

Results showed a decrease in a concentration-response manner in the number of hatched, survived juveniles and reproduction within the tested range to both Ag forms.

$AgNO_3$ significantly reduced the number of hatched juveniles at concentrations ≥ 72 mg Ag kg-1 (d.f. = 4 and 19; F = 20.2; p < 0.001). The number of survived juveniles at 14 days was significantly

Table 1
Summary of the effect concentrations, for *Enchytraeus crypticus* when exposed to $AgNO_3$ and Ag NM300K in LUFA 2.2 soil. Results show ECx (Effect Concentration) estimates and the 95 % confidence intervals (in brackets). Model and parameters include the values for slope (S) and intercept (Y0). EC values (mg kg-1) are given per endpoint and test material. n.d.: not determined; n.e.: no effect. "par" = parameter; NOEC: No Observed Effect Concentration; LOEC: Lowest Observed Effect Concentration.

Test	Endpoint	Time (days)	EC_{10}	EC_{20}	EC_{50}	EC_{80}	NOEC/LOEC	Model and parameters
	$AgNO_3$							
F	Hatching	11	**42**	**48**	**58**	**65**	24/<48	Threshold 2 par
L			(30–54)	(38–57)	(51–65)	(54–76)		(S: 0.04; Y0: 29.8)
C	Growth		n.e.	n.e.	n.e.	n.e.	–	–
	Maturity status	25	**69**	**79**	**98**	**117**	24/<48	Log 2 par
			(25–112)	(53–106)	(78–118)	(71–163)		(S: 0.02; Y0: 76)
	Survival	14	**41**	**47**	**57**	**65**	24/<48	Threshold 2 par
			(28–55)	(37–57)	(50–65)	(53–77)		(S: 0.03; Y0: 26.5)
		22	**21**	**33**	**54**	**75**	<24/<24	Log 2 par
				(16–51)	(43–65)	(57–93)		(S: 0.02; Y0: 27.8)
		25	**29**	**40**	**62**	**78**	<24/<24	Threshold 2 par
			(6–51)	(24–56)	(52–73)	(62–95)		(S: 0.02; Y0: 25.5)
		46	**85**	**109**	**149**	**189**	<24/<24	Log 2 par
			(20–151)					(S: 0.01; Y0: 29)
	Reprod	46	**61**	**68**	**83**	**93**	24/<48	Thresh 2 par
			(47–75)	(58–78)	(76–89)	(82–103)		(S: 0.02; Y0: 802.5)
E	Survival	21	**52**	**61**	**75**	**90**	36/<48	Log 2 par
R			(38–67)	(52–69)	(66–84)	(70–110)		(S: 0.02; Y0: 9.2)
T	Reprod	21	**38**	**47**	**62**	**78**	<36/<36	Log 2 par
			(24–51)	(37–56)	(57–68)	(67–88)		(S: 0.02; Y0: 389.8)
	Ag NM300K							
F	Hatching	11	**28**	**40**	**61**	**82**	20/<60	Log 2 par
L				(22–59)	(55–67)	(62–101)		(S: 0.02; Y0: 34.5)
C	Growth		n.e.	n.e.	n.e.	n.e.	–	–
	Maturity status	22	**85**	**119**	**178**	**237**	<20/<20	Log 2 par
			(10–160)	(73–165)	(131–225)	(136–338)		(S: 0.006; Y0: 58.4)
		25	**89**	**105**	**131**	**157**	20/<60	Log 2 par
			(57–121)	(84–126)	(112–149)	(120–194)		(S: 0.02; Y0: 76)
	Survival	14	**28**	**48**	**83**	**118**	20/<60	Log 2 par
				(22–74)	(66–100)	(92–145)		(S: 0.01; Y0: 28.2)
		22	**16**	**40**	**81**	**122**	<20/<20	Log 2 par
				(17–62)	(67–95)	(99–144)		(S: 0.008; Y0: 33)
		25	**43**	**56**	**82**	**100**	<20/20	Log 2 par
			(20–65)	(39–72)	(70–94)	(82–119)		(S: 0.01; Y0: 37.5)
		46	**51**	**69**	**99**	**130**	<20/<20	Log 2 par
			(12–90)	(40–97)	(82–117)	(105–155)		(S: 0.01; Y0: 31.2)
	Reprod	46	**56**	**73**	**103**	**133**	20/<60	Log 2 par
			(21–91)	(48–99)	(88–118)	(111–154)		(S: 0.01; Y0: 1417)
E	Survival	21	**356**	**467**	**657**	**846**	<200/<200	Log 2 par
R			(195–517)	(353–580)	(578–735)	(709–984)		(S: 0.018; Y0: 8.2)
T	Reprod	21	n.d.	**52**	**161**	**270**	<200/<200	Log 2 par
				(35–139)	(102–220)	(192–348)		(S: 0.003; Y0: 274.5)

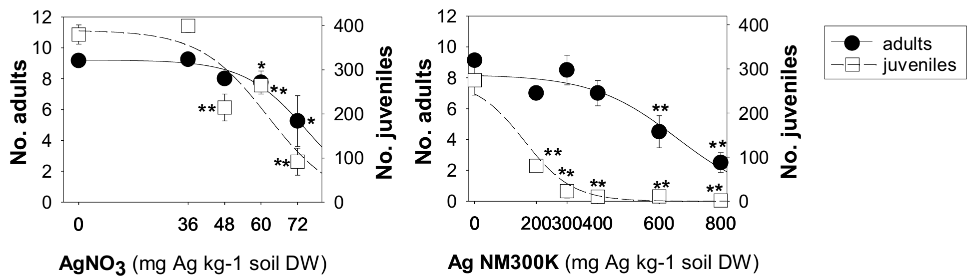


Fig. 1. Results of the Enchytraeid Reproduction Test (ERT) standard test in terms of survival and reproduction of *Enchytraeus crypticus* when exposed to $AgNO_3$ and Ag NM300K (mg Ag kg-1 DW soil) in LUFA 2.2 soil. All values are expressed as average ± standard error (Av ± SE). The lines represent the model fit to data. * p < 0.05 and **p < 0.001, Dunnets'.

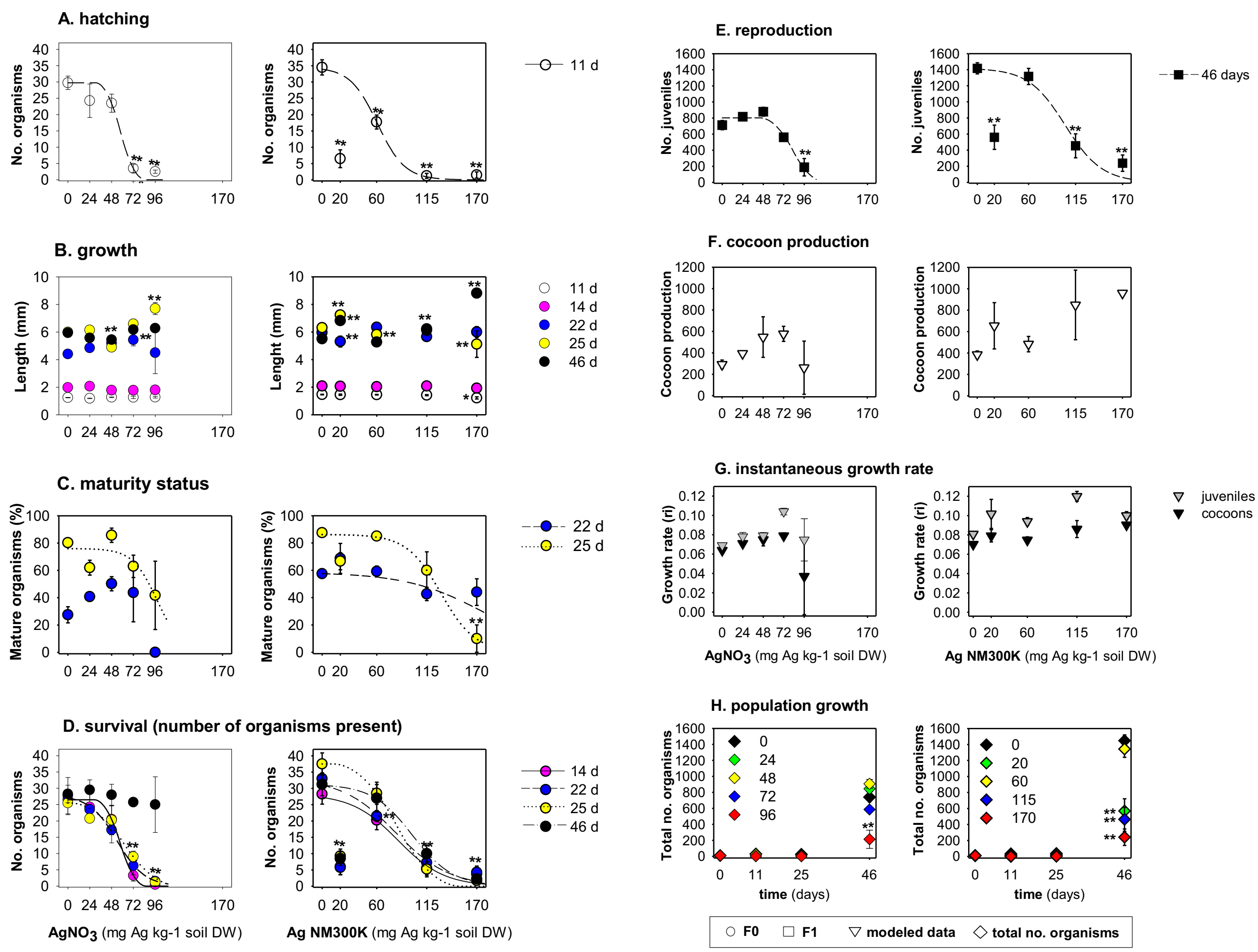


Fig. 2. Results of the full life cycle test (FLCt) for *Enchytraeus crypticus* when exposed to $AgNO_3$ and Ag NM300K (mg Ag kg-1 DW soil) in LUFA 2.2 soil including the various endpoints: **A**: hatching; **B:** growth; **C:** maturity status; **D:** survival (number of organisms present) **E**: reproduction; **F**: cocoon production; **G:** instantaneous population growth rate (r_i) and **H**: population growth. All values are expressed as average ± standard error (Av ± SE). The solid lines represent the model fit to data. * p < 0.05, and **p < 0.001, Dunnets'. d = days.

lower at concentrations ≥ 72 mg Ag kg-1 (d.f. = 4 and 19; F = 16.3; p < 0.001). Whereas reproduction was significantly decreased at concentrations ≥ 96 mg Ag kg-1 (d.f. = 4 and 19; F = 18.7; p < 0.001). Results for Ag NM300K showed a significant reduction for the number of hatched juveniles at concentrations ≥ 20 mg Ag kg-1 (d.f. = 4 and 19; F = 52.6; p < 0.001). The number of survived juveniles at 14 days was significantly lower at concentrations ≥ 72 mg Ag kg-1 (d.f. = 4 and 19; F = 20.3; p < 0.001). Whereas reproduction was significantly decreased at concentrations ≥ 96 mg Ag kg-1 (d.f. = 4 and 19; F = 20.3; p < 0.001).

In terms of growth no clear pattern was identified, but e.g. at 46 days for Ag NM300K organisms exposed to 170 mg Ag kg-1 were significantly longer than control organisms (d.f. = 4 and 239; F = 76.8; p < 0.001).

Concerning the maturity, organism exposed to $AgNO_3$ showed a tendency to decrease the percentage of clitellate organisms only at 96 mg Ag kg-1 (p > 0.05). For Ag NM300K effects are more pronounced at day 25 and show a clear decrease in a dose-related manner. Population growth decreased significantly: for $AgNO_3$ from ≥ 96 mg Ag kg-1 (d.f. = 4 and 19; F = 17.7; p < 0.001), and for Ag NM300K from 115 mg Ag kg-1 (and also for 20 mg Ag kg-1) (d.f. = 4 and 19; F = 20.7; p < 0.001). In terms of instantaneous growth rate (r_i) and cocoon production no significant effects occurred.

Results from the concentration-response modelling are summarised in Table 1, including details on the models for EC (Effect Concentration) estimations and associated confidence intervals.

4. Discussion

Comparison between the ERT and FLCt results show the higher sensitivity and specificity of the FLCt. This is not surprising considering the main differences in terms of life stages and exposure time: organisms are exposed from the cocoon stage (instead of adults) and during 46 days (instead of 21). On the other hand, the level of effect measured at the hatching success (11 days) during the FLCt showed to be a good predictor of effects in terms of reproduction (46 days). Further studies are needed testing a wider range of compounds, but the hatching success could be a good alternative or screening test for chemicals.

Major differences between FLCt and ERT were observed when testing the Ag NM300K, e.g. in terms of survival ECx (i.e. LCx) for Ag NM300K, ERT-$LC_{10/50}$ = 350/650 mg Ag kg-1 and FLC-$LC_{10/50}$ = 50/100 mg Ag kg-1 soil. When testing $AgNO_3$ the differences between the ERT and FLCt were relatively smaller. Hence, results show that the current standard test under-estimates effects in particular for NMs. This has been reported before by e.g. van der Ploeg et al. [14], where $AgNO_3$ was more toxic than Ag NM300K for earthworms (*Lumbricus rubellus*) during a short term exposure, whereas at longer term Ag NM300K was the most toxic: four weeks exposure to $AgNO_3$ (15.4 mg Ag kg-1) and Ag NM300K (15.4 mg Ag kg-1) caused a 40 % and 8 % reduction in reproduction respectively; predictions for 10 months exposure estimated population growth rate significant decreased to 93.1 % and 91.8 % when exposed to $AgNO_3$ and Ag NM300k respectively.

Curiously, for $AgNO_3$ the FLCt showed lower effect. One hypothesis could be that organisms exposed to $AgNO_3$ from cocoon stage develop some sort of resistance and hence survive higher levels of Ag, although, this was not the case in Ag NM300K exposure. Therefore, maybe this illustrates differences in mechanisms of action between the two Ag forms, i.e. organisms exposed to $AgNO_3$ in cocoon stage could have activated antioxidant defence mechanisms, allowing them to detoxify, hatch and survive high exposure levels. The maintenance of this high level of antioxidant protection system could be transferred to their offspring. With Ag NM300K this protection system may not have been activated, hence organisms never hatched. Differences in terms of oxidative stress response mechanisms between $AgNO_3$ and Ag NM300K have been shown by Ribeiro et al. [31] in a study with *E. crypticus* exposed to the $EC_{20,50,80}$. Overall, there was a delayed increase in the antioxidant enzymes responses for Ag NM300K exposure compared to $AgNO_3$ and some specific activation for Ag NM300K, e.g. metallothionein. Dissimilar oxidative stress mechanisms have also been observed for another soil invertebrate, *Folsomia candida*, when also exposed to the $EC_{20,50,80}$ of Ag NM300K and $AgNO_3$ indicating a combined effect of released Ag ions (from Ag NM300K) and Ag NM300K specific effects [32].

Since the level of detail of the FLCt is considerably higher, this allows increased interpretation of results. The differences observed in terms of adults survival between Ag forms seems to indicate that Ag NM300K caused a) embryotoxic effect or inability to hatch, hence decreased hatching success, or b) the juveniles hatch later (25 < days < 46) and their survival is affected, hence the decreased number of adults at test end. On the other hand, the effects observed for $AgNO_3$ in terms of reduced hatching (11–25 days) reflected a delay in hatching since longer exposure (46 days) showed that adults were present and at similar numbers to controls. Again this indicates that there are differences in the mechanisms of action between $AgNO_3$ and Ag NM300K and highlights the importance of the additional test endpoints and longer term exposures when testing NMs such as Ag, as also referred by other authors [14,15,17,18]. As documented [14,17,30] AgNMs tend to oxidise and Ag ions are released, with AgNMs possibly providing a continuous source of ions [33]. Nevertheless, the released Ag ions in soil media are immediately bound to its constituents, such as organic matter (OM) and clays [33–35]. Coutris et al. [33] compared the "bioaccessible" fraction of AgNMs and $AgNO_3$ along 70 days in two soils: an organic soil with 14 % OM and, a mineral soil with 1.5 % OM [LUFA 2.2 used in our experiment has 2 % OM and 7 % Clay]. Ag ions bind slower to soil components in mineral soil. For AgNMs it was observed that "bioaccessible" fraction increases with time, but this process is slower for the mineral soi [33]. This could partly justify the observed differences between Ag forms, i.e., longer time required for AgNMs effects as caused by Ag ions. The results by Gomes et al. [36] with *E. crypticus* also suggest slower oxidation rate effect of AgNMs compared to $AgNO_3$. Li et al. [37] also studied the uptake of AgNM, over 96 h in artificial soil solution; they observed that in artificial soil solution the hydrodynamic size of AgNMs increased probably due to higher strength, and they further found evidence that AgNMs toxicity cannot be completely attributed only to the dissolution of the AgNMs and release of Ag ions.

Nanoparticulate specific effects should not be excluded, possibly because of the following: Ag NM300K may have a) damaged the membrane of cocoons with consequent embryo mortality, or b) crossed the membrane of cocoons and damaged the embryonic tissues by either a slow release of Ag ions, or a boom release of Ag ions, i.e. *trojan horse* effect [38]. There are examples of studies where specific NM effect is confirmed, e.g. Ong et al. [39] showed with zebrafish embryos that the inability to hatch was due to NMs specific effects, since the "free metals" solutions used as controls did not affect embryos morphology and movement or the hatching enzyme activity. In another study with zebrafish embryos [40] it was shown that AgNMs (5–46 nm) reached the embryonic structures by crossing the chorion through pore canals. In the same study it is suggested that the increase of AgNMs in embryos could affect gene expression by modifying the charge or interactions of biomolecules, like nucleic acids and transcription factors. In another study with zebrafish embryos [41] it was shown that AgNMs (13 nm) toxicity depended on the stage of embryonic development: the earlier the exposure the higher the effect and the hatched embryos had increased resistance to AgNMs with high success rate. In our study cocoons are exposed with 1–2 days old after cocoon laying and at this stage embryos are at first cells division [23] so the worst case scenario should be expected. Further studies, e.g. at the embryotoxicity level via histology [42] should help clarifying some of these aspects.

At the population level, i.e. combining the effects at various life stages, modelling showed that both Ag forms cause a decrease in population growth with increasing concentrations [despite no effect in r_i and cocoon production], this being more pronounced for Ag NM300K, i.e., larger differences between the control and treatments.

One particular aspect that requires attention is the high effect caused by the lowest tested concentration of Ag NM300K (20 mg Ag kg-1). In line with these results, and for the same material, van der Ploeg at al. [14] observed that for 15 mg Ag kg-1 the worms had higher tissue concentration than at the highest concentration (154 mg Ag kg-1), and this could not be directly linked to pore-water measurements. It is described that aggregation and agglomeration can increase with higher concentrations in different media, including in soil [5,43,44]. These processes interfere with NMs dissolution, e.g. lower agglomeration/aggregation, higher dissolution of NMs [5,43]. Additionally, lower agglomeration/aggregation, higher amount of single NMs [5]. In our study it is possible that at low dose (20 mg Ag kg-1), Ag dissolution and the amount of single NMs was higher. This could explain that the increased effect measured was a combined increased effect of Ag ions and nanoparticulate specific effects. Such results could be compared to the low concentration effect or non-monotonic concentration-response curves as observed in the context of endocrine disrupting compounds. Non monotonic responses are well described in the literature where the slope of the curve changes sign within the range of tested concentrations, hence effects of high doses cannot predict for low doses [45–47].

In summary, the FLCt offered considerable advantages compared to the ERT, including more endpoints, being more sensitive, and showing longer term effects. Further, the FLCt with *E. crypticus* provides information that is comparable to vertebrate models like *Danio rerio*, being potentially useful to read across species, while complying with the 3 R – refinement, reduction and replacement of animal testing.

5. Conclusions

The novel FLCt allowed assessing the effects of nanomaterials (Ag NM300K) to a much higher extent (extra endpoints, higher sensitivity, longer-term effects) than using the standard ERT, presenting a good improvement and alternative for NM hazard assessment.

Effects of Ag NM300K occur either during the embryo development/hatchability or at the survival of juvenile stage and at a slower rate than for $AgNO_3$. Adults' survival was less affected by $AgNO_3$ than Ag NM300K and the effects on reproduction indicate different underlying mechanisms. Special attention should be given for NMs low concentration effects as non-monotonic concentration-response effect was observed for Ag NM300K – high effect at low concentration (20 mg kg-1).

Acknowledgements

This study was supported by EU FP7–MARINA (G.A. no. 263215) funds and SUN (G.A. no. 604305) funds, and thanks are due to CESAM (UID/AMB/50017), to FCT/MEC through national funds, and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020 and by FCT by a PhD grant to Rita Bicho (SFRH/BD/102702/2014).

References

[1] Y. Ju-Nam, J.R. Lead, Manufactured nanoparticles: an overview of their chemistry, interactions and potential environmental implications, Sci. Total Environ. 400 (2008) 396–414.
[2] Q.H. Tran, V.Q. Nguyen, A.T. Le, Silver nanoparticles: synthesis, properties, toxicology, applications and perspectives, Adv. Nat. Sci.: Nanosci. Nanotechnol. 4 (2013) 033001.
[3] M. Ahamed, M.S. AlSalhi, M.K.J. Siddiqui, Silver nanoparticle applications and human health, Clin. Chim. Acta 411 (2010) 1841–1848.
[4] R. Kessler, Engineered nanoparticles in consumer products: understanding a new ingredient, Environ. Health Perspect. 119 (3) (2011) A120–A125.
[5] P.S. Tourinho, C.A.M. van Gestel, S. Lofts, C. Svendsen, A.M.V.M. Soares, S. Loureiro, Metal-based nanoparticles in soil: fate, behavior, and effects on soil invertebrates, Environ. Toxicol. Chem. 31 (2012) 1679–1692.
[6] S. Frenk, T. Ben-Moshe, I. Dror, B. Berkowitz, D. Minz, Effect of metal oxide nanoparticles on microbial community structure and function in two different soil types, PLoS One 8 (12) (2013) e84441.
[7] F. Gottschalk, C. Lassen, J. Kjoelholt, F. Christensen, B. Nowack, Modeling flows and concentrations of nine engineered nanomaterials in the danish environment, Int. J. Environ. Res. Public Health 12 (2015) 5581.
[8] A.E. Pradas del Real, H. Castillo-Michel, R. Kaegi, B. Sinnet, V. Magnin, N. Findling, J. Villanova, M. Carrière, C. Santaella, A. Fernández-Martıínez, C. Levard, G. Sarret, Fate of Ag-NPs in sewage sludge after application on agricultural soils, Environ. Sci. Technol. 50 (4) (2016) 1759–1768.
[9] B. Kim, C.-S. Park, M. Murayama, M.F. Hochella, Discovery and characterization of silver sulfide nanoparticles in final sewage sludge products, Environ. Sci. Technol. 44 (19) (2010) 7509–7514.
[10] N.C. Mueller, B. Nowack, Exposure modeling of engineered nanoparticles in the environment, Environ. Sci. Technol. 42 (2008) 4447–4453.
[11] D.E. Meyer, M.A. Curran, M.A. Gonzalez, An examination of existing data for the industrial manufacture and use of nanocomponents and their role in the life cycle impact of nanoproducts, Environ. Sci. Technol. 43 (5) (2009) 1256–1263.
[12] K. Schlich, T. Klawonn, K. Terytze, K. Hund-Rinke, Hazard assessment of a silver nanoparticle in soil applied via sewage sludge, Environ. Sci. Eur. 25 (2013) 17.
[13] F. Gottschalk, T. Sonderer, R.W. Scholz, B. Nowack, Modeled environmental concentrations of engineered nanomaterials (TiO_2, ZnO, Ag, CNT, fullerenes) for different regions, Environ. Sci. Technol. 43 (2009) 9216–9222.
[14] M.J. van der Ploeg, R.D. Handy, P.L. Waalewijn-Kool, J.H. van den Berg, Z.E. Herrera Rivera, J. Bovenschen, B. Molleman, J.M. Baveco, P. Tromp, R.J. Peters, G.F. Koopmans, I.M. Rietjens, N.W. van den Brink, Effects of silver nanoparticles (NM-300K) on *Lumbricus rubellus* earthworms and particle characterization in relevant test matrices including soil, Environ. Toxicol. Chem. 33 (2014) 743–752.
[15] S.I.L. Gomes, A.M.V.M. Soares, J.J. Scott-Fordsmand, M.J.B. Amorim, Mechanisms of response to silver nanoparticles on *Enchytraeus albidus* (Oligochaeta): survival, reproduction and gene expression profile, J. Hazard. Mater. 254–255 (2013) 336–344.
[16] M. Mrakovcic, M. Absenger, R. Riedl, C. Smole, E. Roblegg, L.F. Frohlich, E. Frohlich, Assessment of long-term effects of nanoparticles in a microcarrier cell culture system, PLoS One 8 (2013) e56791.
[17] M. Diez-Ortiz, E. Lahive, S. George, A. Ter Schure, C.A.M. Van Gestel, K. Jurkschat, C. Svendsen, D.J. Spurgeon, Short-term soil bioassays may not reveal the full toxicity potential for nanomaterials; bioavailability and toxicity of silver ions ($AgNO_3$) and silver nanoparticles to earthworm *Eisenia fetida* in long-term aged soils, Environ. Pollut. 203 (2015) 191–198.
[18] S.I. Gomes, D. Hansen, J.J. Scott-Fordsmand, M.J.B. Amorim, Effects of silver nanoparticles to soil invertebrates: oxidative stress biomarkers in *Eisenia fetida*, Environ. Pollut. 199 (2015) 49–55.
[19] M.J.B. Amorim, The daunting challenge of ensuring sustainable development of nanomaterials, Int. J. Environ. Res. Public Health 13 (2) (2016) 245.
[20] J.J. Scott-Fordsmand, S. Pozzi-Mucelli, L. Tran, K. Aschberger, S. Sabella, U. Vogel, C. Poland, D. Balharry, T. Fernandes, S. Gottardo, S. Hankin, M.G.J. Hartl, N.B. Hartmann, D. Hristozov, K. Hund-Rinke, H. Johnston, A. Marcomini, O. Panzer, D. Roncato, A.T. Saber, H. Wallin, V. Stone, A unified framework for nanosafety is needed, Nano Today 9 (2014) 546–549.
[21] C. Schultz, K. Powell, A. Crossley, K. Jurkschat, P. Kille, A.J. Morgan, D. Read, W. Tyne, E. Lahive, C. Svendsen, D. Spurgeon, Analytical approaches to support current understanding of exposure, uptake and distributions of engineered nanoparticles by aquatic and terrestrial organisms, Ecotoxicology 24 (2015) 239–261.
[22] M.P. Castro-Ferreira, T.E. de Boer, J.K. Colbourne, R. Vooijs, C.A.M. van Gestel, N.M. van Straalen, A.M.V.M. Soares, M.J.B. Amorim, D. Roelofs, Transcriptome assembly and microarray construction for *Enchytraeus crypticus*, a model oligochaete to assess stress response mechanisms derived from soil conditions, BMC Genomics 15 (2014) 302.
[23] R.C. Bicho, F.C.F. Santos, M.F.M. Gonçalves, A.M.V.M. Soares, M.J.B. Amorim, Enchytraeid Reproduction Test^{PLUS}: hatching, growth and full life cycle test—an optional multi-endpoint test with *Enchytraeus crypticus*, Ecotoxicology 24 (2015) 1053–1063.
[24] C.G. Ingersoll, T.H. Hutchinson, M. Crane, S. Dodson, T. DeWitt, A. Geis, M.C. Huet, C.L. McKenney, E. Oberdö rster, D. Pascoe, D.J. Versteeg, O. Warwick, Laboratory tests for evaluating potential effects of endocrine disrupting compounds, in: P.L. deFur, M. Crane, C.G. Ingersoll, L.J. Tattersfield (Eds.), Endocrine Disruption in Invertebrates, SETAC Technical Publication, Brussels, Belgium, 1999, pp. 107–109.
[25] W.K. Walthall, J.D. Stark, Comparison of two population-level ecotoxicological endpoints: the intrinsic (r_m) and instantaneous (r_i) rates of increase, Environ. Toxicol. Chem. 16 (1997) 1068–1073.
[26] M. Brinke, P. Heininger, W. Traunspurger, Effects of a bioassay-derived ivermectin lowest observed effect concentration on life-cycle traits of the nematode *Caenorhabditis elegans*, Ecotoxicology 22 (2013) 148–155.
[27] W. Tyne, S. Little, D.J. Spurgeon, C. Svendsen, Hormesis depends upon the life-stage and duration of exposure: examples for a pesticide and a nanomaterial, Ecotoxicol. Environ. Saf. 120 (2015) 117–123.
[28] ISO, ISO: 16387 Soil Quality—Effects of Pollutants on Enchytraeidae (*Enchytraeus* sp.)—Determination of Effects on Reproduction and Survival, ISO (International Organization for Standardization), Geneva, Switzerland, 2004.
[29] OECD, Test No. 220: Guidelines for Testing of Chemicals—Enchytraeid Reproduction Test, OECD (Organization for Economic Cooperation and Development), OECD Publishing, Paris, France, 2004.
[30] C.L. Klein, S. Comero, B. Stahlmecke, J. Romazanov, T.A. Kuhlbusch, E. v. Doren, P.-J. d. Temmerman, J. Mast, P. Wick, H. Krug, G. Locoro, K. Hund-Rinke, W. Kördel, S. Friedrichs, G. Maier, J. Werner, T. Linsinger, B.M. Gawlik, NM-series of Representative Manufactured Nanomaterials. NM-300 Silver. Characterisation, Stability, Homogeneity, Publications Office of the European Union, Luxembourg, 2011.
[31] M.J. Ribeiro, V.L. Maria, J.J. Scott-Fordsmand, M.J.B. Amorim, Oxidative stress mechanisms caused by Ag nanoparticles (NM300K) are different from those

of $AgNO_3$: effects in the soil invertebrate *Enchytraeus crypticus*, Int. J. Environ. Res. Public Health 12 (2015) 9589.
[32] L.A. Mendes, V.L. Maria, J.J. Scott-Fordsmand, M.J.B. Amorim, Ag nanoparticles (Ag NM300K) in the terrestrial environment: effects at population and cellular level in *Folsomia candida* (Collembola), Int. J. Environ. Res. Public Health 12 (2015) 12530.
[33] C. Coutris, E.J. Joner, D.H. Oughton, Aging and soil organic matter content affect the fate of silver nanoparticles in soil, Sci. Total Environ. 420 (2012) 327–333.
[34] W.A. Shoults-Wilson, B.C. Reinsch, O.V. Tsyusko, P.M. Bertsch, G.V. Lowry, J.M. Unrine, Role of particle size and soil type in toxicity of silver nanoparticles to earthworms, Soil Sci. Soc. Am. J. 75 (2011) 365–377.
[35] E. Smolders, K. Oorts, P. Van Sprang, I. Schoeters, C.R. Janssen, S.P. McGrath, M.J. McLaughlin, Toxicity of trace metals in soil as affected by soil type and aging after contamination: using calibrated bioavailability models to set ecological soil standards, Environ. Toxicol. Chem. 28 (2009) 1633–1642.
[36] S.I. Gomes, J.J. Scott-Fordsmand, M.J.B. Amorim, Cellular energy allocation to assess the impact of nanomaterials on soil invertebrates (Enchytraeids): the effect of Cu and Ag, Int. J. Environ. Res. Public Health 12 (2015) 6858.
[37] L. Li, H. Wu, W.J.G.M. Peijnenburg, C.A.M. van Gestel, Both released silver ions and particulate Ag contribute to the toxicity of AgNPs to earthworm *Eisenia fetida*, Nanotoxicology 9 (6) (2015) 792–801.
[38] E.-J. Park, J. Yi, Y. Kim, K. Choi, K. Park, Silver nanoparticles induce cytotoxicity by a Trojan-horse type mechanism, Toxicol. In Vitro 24 (2010) 872–878.
[39] K.J. Ong, X. Zhao, M.E. Thistle, T.J. MacCormack, R.J. Clark, G. Ma, Y. Martinez-Rubi, B. Simard, J.S.C. Loo, J.G.C. Veinot, G.G. Goss, Mechanistic insights into the effect of nanoparticles on zebrafish hatch, Nanotoxicology 8 (2014) 295–304.
[40] K.J. Lee, P.D. Nallathamby, L.M. Browning, C.J. Osgood, X.H. Xu, In vivo imaging of transport and biocompatibility of single silver nanoparticles in early development of zebrafish embryos, ACS Nano 1 (2007) 133–143.
[41] K.J. Lee, L.M. Browning, P.D. Nallathamby, C.J. Osgood, X.-H.N. Xu, Silver nanoparticles induce developmental stage-specific embryonic phenotypes in zebrafish, Nanoscale 5 (2013) 11625–11636.
[42] M.F.M. Gonçalves, R.C. Bicho, A. Rêma, A.M.V.M. Soares, A.M.R. Faustino, M.J.B. Amorim, Development of an embryotoxicity test for *Enchytraeus crypticus*—the effect of Cd, Chemosphere 139 (2015) 386–392.
[43] L. Stebounova, E. Guio, V. Grassian, Silver nanoparticles in simulated biological media: a study of aggregation, sedimentation, and dissolution, J. Nanopart. Res. 13 (2011) 233–244.
[44] N.G. Bastús, E. Casals, S. Vázquez-Campos, V. Puntes, Reactivity of engineered inorganic nanoparticles and carbon nanostructures in biological media, Nanotoxicol 2 (2008) 99–112.
[45] L.N. Vandenberg, T. Colborn, T.B. Hayes, J.J. Heindel, D.R. Jacobs, D.-H. Lee, T. Shioda, A.M. Soto, F.S. vom Saal, W.V. Welshons, R.T. Zoeller, J.P. Myers, Hormones and endocrine-disrupting chemicals: low-dose effects and nonmonotonic dose responses, Endocr. Rev. 33 (2012) 378–455.
[46] D. Fagin, Toxicology: the learning curve, Nature 490 (2012) 462–465.
[47] L.N. Vandenberg, Non-monotonic dose responses in studies of endocrine disrupting chemicals: bisphenol A as a case study, Dose Response 12 (2014) 259–276.

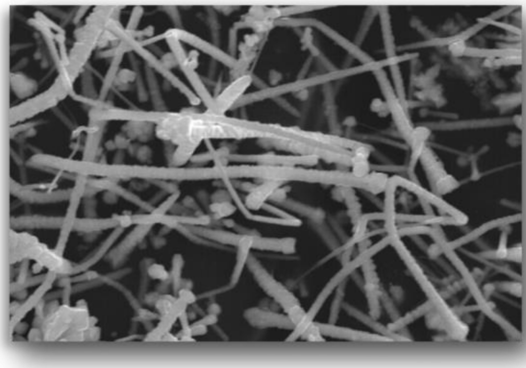


Photo by Natália Rodrigues

Annex II

High-throughput tool to discriminate effects of NMs (Cu-NPs, Cu-nanowires, $CuNO_3$, and Cu salt aged): transcriptomics in Enchytraeus crypticus

Nanotoxicology

ISSN: 1743-5390 (Print) 1743-5404 (Online) Journal homepage: http://www.tandfonline.com/loi/inan20

High-throughput tool to discriminate effects of NMs (Cu-NPs, Cu-nanowires, $CuNO_3$, and Cu salt aged): transcriptomics in Enchytraeus crypticus

Susana I. L. Gomes, Carlos P. Roca, Natália Pegoraro, Tito Trindade, Janeck J. Scott-Fordsmand & Mónica J. B. Amorim

To cite this article: Susana I. L. Gomes, Carlos P. Roca, Natália Pegoraro, Tito Trindade, Janeck J. Scott-Fordsmand & Mónica J. B. Amorim (2018): High-throughput tool to discriminate effects of NMs (Cu-NPs, Cu-nanowires, $CuNO_3$, and Cu salt aged): transcriptomics in Enchytraeus crypticus, Nanotoxicology, DOI: 10.1080/17435390.2018.1446559

To link to this article: https://doi.org/10.1080/17435390.2018.1446559

NANOTOXICOLOGY, 2018
https://doi.org/10.1080/17435390.2018.1446559

Taylor & Francis
Taylor & Francis Group

ARTICLE

Check for updates

High-throughput tool to discriminate effects of NMs (Cu-NPs, Cu-nanowires, $CuNO_3$, and Cu salt aged): transcriptomics in *Enchytraeus crypticus*

Susana I. L. Gomes[a], Carlos P. Roca[b,c], Natália Pegoraro[a], Tito Trindade[d], Janeck J. Scott-Fordsmand[c] and Mónica J. B. Amorim[a]

[a]Department of Biology & CESAM, University of Aveiro, Aveiro, Portugal; [b]Department of Chemical Engineering, Universitat Rovira i Virgili, Tarragona, Spain; [c]Department of Bioscience, Aarhus University, Silkeborg, Denmark; [d]Department of Chemistry & CICECO, Aveiro Institute of Materials, University of Aveiro, Aveiro, Portugal

ABSTRACT

The current testing of nanomaterials (NMs) via standard toxicity tests does not cover many of the NMs specificities. One of the recommendations lays on understanding the mechanisms of action, as these can help predicting long-term effects and safe-by-design production. In the present study, we used the high-throughput gene expression tool, developed for *Enchytraeus crypticus* (4 × 44k Agilent microarray), to study the effects of exposure to several copper (Cu) forms. The Cu treatments included two NMs (spherical and wires) and two copper-salt treatments ($CuNO_3$ spiked and Cu salt field historical contamination). To relate gene expression with higher effect level, testing was done with reproduction effect concentrations (EC_{20}, EC_{50}), using 3 and 7 days as exposure periods. Results showed that time plays a major role in the transcriptomic response, most of it occurring after 3 days. Analysis of gene expression profiles showed that Cu-salt-aged and Cu-nanowires (Nwires) differed from $CuNO_3$ and Cu-nanoparticles (NPs). Functional analysis revealed specific mechanisms: Cu-NPs uniquely affected senescence and cuticle pattern formation, which can result from the contact of the NPs with the worms' tegument. Cu-Nwires affected reproduction via male gamete generation and hermaphrodite genitalia development. $CuNO_3$ affected neurotransmission and locomotory behavior, both of which can be related with avoidance response. Cu salt-aged uniquely affected phagocytosis and reproductive system development (via different mechanisms than Cu-Nwires). For the first time for Cu (nano)materials, the adverse outcome pathways (AOPs) drafted here provide an overview for common and unique effects per material and linkage with apical effects.

ARTICLE HISTORY
Received 13 December 2016
Revised 16 January 2018
Accepted 29 January 2018

KEYWORDS
Nanomaterials; high-throughput; toxicogenomics; mechanisms of response; adverse outcome pathway

Introduction

The testing of nanomaterials (NMs) under the current framework (Regulation (EC) No 1907/2006 2006; Regulation (EC) No 440/2008 2008; European Commission 2013), as within the standard toxicity tests (e.g. OECD220/ISO16387 Enchytraeid Reproduction Test), only provide mortality/reproduction. No information on the mechanisms of action is provided and hence it is not possible to correlate the organism phenotype to mechanistics of particle properties. On the other hand, much of the uncertainty is due to the lack of knowledge on the interactions between NMs and the organisms/cells, the nano-bio interactions. The understanding of mechanisms is one of the alternatives, which can provide information that allows prediction of effects and safer-by-design production. In this regard, high-throughput -omics tools help to understand the mechanisms of toxic-mediated responses, i.e. by knowing the biological target it is possible, in an intelligent manner, to modify the NM to avoid the toxicity (i.e. based on knowledge), also considering whether the original desired function remains. Further, establishing the link between alterations in critical macromolecules (e.g. genes, proteins, metabolites) and their possible biological implications represents one of the major milestones of 'predictive toxicology' (Singh et al. 2010).

Copper NMs can be synthesized in different shapes, ranging from spherical (NPs) (Wu and Chen 2004) to wires (Chang, Lye, and Zeng 2005), and the shape of NMs is known to influence toxicity

CONTACT Susana I. L. Gomes susana.gomes@ua.pt; Mónica J. B. Amorim mjamorim@ua.pt Department of Biology & CESAM, University of Aveiro, 3810-193 Aveiro, Portugal

Supplemental data for this article can be accessed here.

(Pal, Tak, and Song 2007; Ispas et al. 2009). Data on 'spherical' Cu-NPs showed that NPs were comparatively five times less toxic than Cu-salt to *Enchytraeus crypticus* (Gomes, Murphy, et al. 2015) and to *Eisenia fetida* (Heckmann et al. 2011), but three times more toxic to *Enchytraeus albidus* (Amorim and Scott-Fordsmand 2012). A study in *E. albidus* has shown that Cu-NPs affect antioxidant enzymatic response while Cu-salt also caused lipid peroxidation (Gomes, Novais, Gravato, et al. 2012). Unrine et al. (2010) found that exposure to Cu-NPs induced metallothionein expression, in *E. fetida*, but the expression of other oxidative stress related genes (i.e. superoxide dismutase, catalase, and heat shock proteins) was not affected. Also at transcriptomic level, gene expression profiles (energy metabolism involved) were different for Cu-NPs and $CuCl_2$ exposed organisms (Gomes, Novais, Scott-Fordsmand, et al. 2012). This is in line with observations by Griffitt et al. (2009) who showed that for zebrafish exposed to Cu-NPs gene expression profile was different from Cu-salt.

In the present study, we aimed to assess the effects of Cu materials varying in type, shape and history, anchoring gene expression, and population effect (reproduction). The Cu based materials included Cu-NPs (spherical), Cu-nanowires (wires), $Cu(NO_3)_2$ (salt), and Cu(II) salt from a historically contaminated field (Cu salt-aged). The selected test species, the potworm *E. crypticus*, is a soil ecotoxicology model (OECD 2004) and is the one with the most gene sequences [ca. 44 000 Expressed Sequence Tags (ESTs)] in the present high density microarray tool (Castro-Ferreira et al. 2014), compared to other soil oligochaetes [*Lumbricus rubellus* has 17000 ESTs (Bundy et al. 2008), *E. fetida* has 4032 ESTs (Pirooznia et al. 2007) and *E. albidus* with 2100 ESTs (Novais, Howcroft, et al. 2012)]. Ecotoxicity tests were performed using the standard test for survival and reproduction. For the gene expression, exposures were performed during 3 and 7 days to concentrations corresponding to the EC_{20} and EC_{50} (20 and 50% effect concentrations on reproduction) (Gomes, Murphy, et al. 2015). To note that the historically Cu salt contaminated field has a gradient up to ca. 3000 mg Cu/kg soil and the tested ECx were well-below this, hence within environmental relevance.

Materials and methods

Test organism

The test species *Enchytraeus crypticus* (Westheide and Graefe 1992) was used. Individuals were cultured in Petri dishes containing agar medium, consisting of a sterilized mixture of four different salt solutions ($CaCl_2 \cdot 2H_2O$; $MgSO_4$; KCl; $NaHCO_3$) and a Bacti-Agar medium (Oxoid, Agar No. 1). The cultures were kept under controlled conditions, at 19 °C and photoperiod 16:8 h light:dark. Organisms were feed on ground and autoclaved oats twice a week.

Test materials

Copper nitrate ($Cu(NO_3)_2.3H_2O$, Sigma Aldrich, 99%) was used.

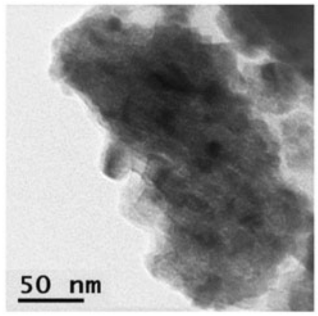
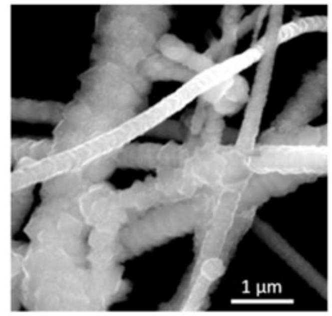
Two morphological distinct copper nanostructures were tested: Cu-nanoparticles (Cu-NPs) and Cu-nanowires (Cu-Nwires). Cu-NPs (American elements, purity 99.8%, Cu-M-028 M-NP.025 N) had a size between 20 and 30 nm. XANES studies showed that, before addition to soil, the oxidation state distribution of the Cu-NPs was approximately 86% Cu(0), 1% Cu(I), and 13% Cu(II) (Gomes, Murphy, et al. 2015). Cu-Nwires were synthesized following the procedure described by Chang, Lye, and Zeng (2005) by reduction of copper (II) nitrate with hydrazine in alkaline medium. Further details on the synthesis and characterization procedures are presented in Supplementary Material. The characteristics of the tested NMs are presented on Table 1.

The amount of Cu was measured in the test soil by Graphite Furnace Atomic Absorption Spectroscopy (AAS-GF) and in soil solution by $9#AAS-GF and free active form by ion-selective electrode (Cu-ISE). For method details, see (Gomes, Murphy, et al. 2015).

Test soil and spiking procedure

The test soil consisted of a natural soil collected at the Hygum site, Jutland, Denmark. In this site, the soil has been historically exposed to contamination with $CuSO_4$ (due to activities of timber preservation, ceased more than 80 years ago), originating a well-known Cu gradient along the field, ranging from the natural background levels of 30 up to 2900 mg Cu/kg dry soil (Scott-Fordsmand, Weeks, and Hopkin

Table 1. Characteristics of the tested Cu NMs: Cu-NPs and Cu-Nwires including manufacturer, size (nominal and Transmission/Scanning Electron Microscopy –TEM/SEM-based), shape, purity, and solubility/dispersability.

	Cu-NPs	Cu-Nwires
TEM/SEM pictures		
Manufacturer	American elements	Synthesized*
Nominal size (nm)	20–30	
TEM/SEM (nm)	17 ± 5 (AV ± SE, n = 60)	365 ± 100 (AV ± SE, n = 74) diameter, >10 000 length
Shape	Spheres	Wires
Purity (%)	99.8	89:Cu (9.4:C; 1.4:O)
Solubility/dispersability	Not dispersible in water	Not dispersible in water

*Chang, Lye, and Zeng (2005).

Table 2. Exposure concentrations (values in mg/kg) used for population level ERT (enchytraeid reproduction test) and gene level microarray studies.

	Population level (ERT)	Gene level (microarray) Control	EC_{20}	EC_{50}	Data source
$CuNO_3$	0–100–200–400–600–800–1000–1500	0	290	360	(Gomes, Murphy, et al. 2015)
Cu salt-aged	0–252–1155–2880–3920	0	500	1400	(Gomes, Murphy, et al. 2015)
Cu-NPs (25 nm)	0–980–1760	0	980	1760	Present data
Cu-Nwires	0–100–400–600–800–1000–1500	0	850	1610	Present data

EC_{20} and EC5$_0$: reproduction effect concentration for 20 and 50%.

2000). Soil was sampled in the field to a depth of 20 cm, dried at 80 °C for 24 h in an oven (Memmert, Type UL40, Braunschweig, Germany) to exclude soil fauna, and then sieved through a 2 mm mesh to remove larger particles. The general physico-chemical characteristics of the soil are as follows: 20–32% coarse sand (>200 µm), 20–25% fine sand (63–200 µm), 11–20% coarse silt (20–63 µm), 12–20% silt (20–20 µm), 12–16% clay (<2 µm), 3.6–5.5% organic matter, Cation Exchange Capacity 6.8–10 (cmolc/kg dw), pH = 5, N 0.25–0.31% and P 0.10–0.12%. The clay mineralogy analyzed by X-ray diffraction was dominated by illite, kaolinite, chlorite, and vermiculite.

For the treatments $CuNO_3$, Cu-NPs, and Cu-Nwires, spiking was done in soil from control area [with natural background levels of 30 mg Cu/kg (Scott-Fordsmand, Weeks, and Hopkin 2000)]. All the Cu materials were added to soil as dry powders, following the recommendations for nanomaterials (OECD 2012). Briefly, of 2 g of dry soil per replicate were mixed with the quantity of the test materials, as dry powders, to obtain the corresponding concentration range. The spiked soil was added to the remaining pre-moistened soil (18 g dry soil +2 ml demineralized water) and mixed manually. After that, demineralized water was added until 50% of soil water holding capacity. The procedure was repeated for each replicate individually to ensure the total nominal amounts per replicate (Gomes, Novais, Gravato, et al. 2012).

For the treatment Cu salt-aged, the soil was collected along the Cu gradient in Hygum site, at the concentrations presented in Table 2 (as determined by AAS-GF).

The concentrations tested for population level and gene level studies are shown on Table 2, mostly from previous experiments (Gomes, Murphy, et al. 2015). To test the range for effects of the 25 nm Cu-NPs, a limit test was conducted for 980 and 1760 mg/kg.

For the gene level studies, the exposure concentrations besides the control soil included the reproduction effect concentrations EC_{20} and EC_{50}

(within 95% confidence intervals). With this approach, we aim to study comparable apical effects (i.e. reproduction EC20/50) while also ensuring that concentrations are sub-lethal, an essential aspect to study mechanisms, and that these are relevant at the phenotype level.

Experimental procedure

Population level: survival and reproduction – Cu-Nwires

The test followed the standard procedures in the Enchytraeid Reproduction Test (ERT) guideline (OECD 2004). In short, 10 adult organisms with well-developed clitellum and similar size were selected and introduced in each test vessel (8 × 4 ⊘ cm) containing 20 g of moist soil and fed with 25 mg of finely ground and autoclaved rolled oats. The vessels were covered with a lid containing small holes (for aeration) and the test ran for 3 weeks, at 20 ± 1 °C and 16:8 h light:dark photoperiod. Four replicates per treatment were used. Weekly, 12.5 mg of food was supplied and soil moisture was adjusted by replenishing weight loss. At the test end, the organisms were fixated with ethanol and colored with Bengal rose (1% in ethanol). After 24 h, soil samples were sieved through meshes with decreasing pore size (1.6, 0.5, and 0.3 mm) to separate the enchytraeids from most of the soil and facilitate counting. Adult and juvenile organisms were counted using a stereo microscope to assess survival (number of adults, from the 10 starting, found at the end of exposure) and reproduction (number of juveniles produced).

Gene level – transcriptomics: differentially expressed genes (Microarray). For the gene expression assay, exposure followed the same procedures as the standard ERT (OECD 2004) with adaptations as follows: 20 adults with well-developed clitellum were place in each test vessel, containing 20 g of moist soil (control or contaminated/spiked). The organisms were exposed for 3 and 7 days under controlled conditions of photoperiod (16:8 h light:dark) and temperature (20 ± 1 °C) without food. Four replicates per treatment and exposure period were used. After the exposure period, the organisms were carefully removed from the soil, rinsed in distilled water, and frozen in liquid nitrogen. The samples were stored at −80 °C, until analysis.

RNA extraction, labeling, and hybridizations. RNA was extracted from each replicate containing a pool of 20 animals. Three biological replicates per test treatment (including controls) were used. Total RNA was extracted using SV Total RNA Isolation System (Promega, Madison, WI). The quantity and purity of the isolated RNA were measured spectrophotometrically with a nanodrop (NanoDrop ND-1000 Spectrophotometer, Wilmington, DE) and its quality was checked on a denaturing formaldehyde agarose gel electrophoresis. A single-color design was used. In brief, 500 ng of total RNA was amplified and labeled with Agilent Low Input Quick Amp Labelling Kit (Agilent Technologies, Palo Alto, CA). Positive controls were added with the Agilent one-color RNA Spike-In Kit (Agilent Technologies, Palo Alto, CA). Purification of the amplified and labeled cRNA was performed with the RNeasy columns (Qiagen, Valencia, CA).

The cRNA samples were hybridized on the Custom Gene Expression Agilent Microarray (4 × 44k format) developed for this species (Castro-Ferreira et al. 2014). Hybridizations were performed using the Agilent Gene Expression Hybridization Kit (Agilent Technologies, Palo Alto, CA) and each biological replicate was individually hybridized on one array. The arrays were hybridized at 65 °C with a rotation of 10 rpm, during 17 h. After that, the microarrays were washed using Agilent Gene Expression Wash Buffer Kit (Agilent Technologies, Palo Alto, CA) and scanned with the Agilent DNA microarray scanner G2505B (Agilent Technologies).

Acquisition and microarray data analysis. Fluorescence intensity data were obtained with Agilent Feature Extraction Software v. 10.7.3.1 (Agilent Technologies). Quality control was done by inspecting the reports on the Agilent Spike-in control probes. Background correction was provided by Agilent Feature Extraction software v. 10.7.3.1, using recommended protocol GE1 107 Sep09. To ensure an optimal comparison between the different normalization methods, only gene probes with good signal quality (flag IsPosAndSignif = True) in all samples were included in the analyzes. Analyses were performed with R (R-Project 2015) v. 3.3.1 and

Bioconductor (Huber et al. 2015) v. 3.3 package limma (Ritchie et al. 2015) v. 3.28.20. Data was normalized with SVCD normalization Roca et al. (2017). Differential expression between control and treated samples was assessed with limma methodology. The Benjamini–Hochberg's (BH) method (Benjamini and Hochberg, 1995) was used for multiple testing correction between genes, controlling the false discovery rate below 5% (adjusted p value <0.05, independently for each comparison of treatment versus control). The Minimum Information About a Microarray Experiment (MIAME) compliant data from this experiment was submitted to the Gene Expression Omnibus (GEO) at the National Center for Biotechnology Information (NCBI) website (platform: GPL20310; series: GSE69792).

Cluster analysis on differentially expressed genes was performed using MultiExperiment Viewer (MeV, TIGR). The differentially expressed genes for each treatment were analyzed separately for GO (Gene Ontology) term enrichment analysis (Alexa, Rahnenfuhrer, and Lengauer 2006) using the Blast2GO software.

Quantitative real-time PCR confirmations. Total RNA (500 ng) of the same samples used for the microarray hybridizations was converted into cDNA through a reverse transcription reaction using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen, Thermo Fisher Scientific corporation, Waltham, MA). Quantitative real-time Polymerase Chain Reaction (qPCR) was carried out on 7500 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific corporation, Waltham, MA), using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Primer sets were designed for nine target genes and one housekeeping gene (Supplementary Table S1) with the software Oligo Explorer (v. 1.1.0). The target genes were selected based on: (i) being annotated, (ii) involved in relevant biological processes, and (iii) relatively higher variations in expression (fold change >2). Determination of PCR efficiency and specificity was done by observing the obtained standard and melting curves, respectively, for all primer sets. The cDNA was diluted four times and 2 μl were used in 20 μl PCR reaction volumes containing 2 μl of forward and 2 μl of reverse primers (2 μM), 10 μl of Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen) and 4 μl of DEPC water. qPCR was performed in triplicate for each sample, on a 96-well optical plate (GeneAmp, Applied Biosystems). Reaction conditions consisted of one initial cycle at 50 °C for 2 min, followed by one denaturation step at 95 °C for 2 min and 40 cycles at 95 °C for 10 s and 60 °C for 1 min; a melting curve was done for each primer pair. A mean normalized expression value was calculated from the obtained Ct values with Relative Expression Software Tool (REST-MSC), using 'zgc:174506 protein' as a reference gene for normalization of input cDNA. Spearman's rho correlation was performed on log2 ratios using SigmaPlot software (v. 11) to compare microarray and qPCR data.

Survival and reproduction data analysis

Data were checked for normality and homogeneity of variances. One-way analysis of variance (ANOVA) with Post Hoc Dunnett's test was used to assess differences between control and treatments and to determine the No Observed Effect Concentration (NOEC) and the Lowest Observed Effect Concentration (LOEC), respectively, as the highest tested concentration with no statistically significant effect and the lowest tested concentration with a statistically significant effect (SigmaPlot 11.0). The reproduction effect concentrations (EC_x) were calculated using the Toxicity Relationship Analysis Program (TRAP 1.21) fitting the 2-parameters Logistic model ($Y = \frac{Y0}{1+e^{4S(X-X50)}}$, $Y0$: top point; S: slope).

Results

In situ *characterization*

The background Cu concentration in soil (i.e. in control soil) was 30 mg Cu/kg. For the Cu salt-aged treatments the Cu concentrations measured (AAS-FG) were 502 and 1398 mg Cu/kg. For $CuNO_3$ and Cu-NPs treatments, the total Cu concentration was within 5–7% of the nominal. The active Cu^{2+} fraction (Cu-ISE) in soil-solution extracts was below the detection limit & quantification (3×10^{-9} M Cu^{2+}). Although maybe possible, Single Particle Inductively Coupled Plasma Mass Spectrometry was not pursued due to the small size of the particles and the complex matrix (Navratilova et al. 2015).

Population level: survival and reproduction – Cu-Nwires and Cu-NPs

There were no differences, in terms of survival and reproduction, between the Cu-Nwires, the particles tested here (25 nm Cu-NPs) (Figure 1), or with respect to the particles (80 nm Cu-NPs) studied earlier (Gomes, Murphy, et al. 2015).

The test fulfilled the validity criteria of the OECD guideline (OECD 2004) [controls' mortality lower than 20%, number of juveniles produced per replicate higher than 50, and coefficient of variation below 50%].

Cu-Nwires and Cu-NPs did not affect adults' survival within the tested concentrations. Reproduction was significantly decreased at 1000 and 1500 mg Cu/kg of Cu-Nwires. The estimated ECx values are summarized in Table 3.

Gene level – transcriptomics: differentially expressed genes (microarray)

A total of 13 165 transcripts (out of the 29 383 probes that passed the quality criteria) were significantly differentially expressed (adjusted $p < 0.05$) after 3 or 7 days of exposure to the different Cu treatments, in at least one condition. The number of differentially expressed genes (DEGs) affected by each test condition is shown in Figure 2 and the list of DEGs is provided in Supplementary Table S3.

Results show that exposure for 3 days consistently caused a higher number of DEGs compared to 7 days (Figure 2), in fact, only Cu salt-aged showed a response after 7 days of exposure. Comparing the different copper forms, $CuNO_3$ was the treatment affecting fewer transcripts, followed by Cu-NPs, with Cu-Nwires and Cu salt-aged affecting the highest number of genes.

Clustering analysis (Pearson's uncentered with average linkage) was performed on genes and samples (dendrogram on genes not shown, for the complete heat map Supplementary Figure S1), using all the DEGs (Figure 3). Because of the clear separation by the factor time of exposure, the analysis was also done for each time individually based on the 12 903 and 1918 DEGs affected at 3 and 7 days, respectively, which did not change the result (data not shown).

There was no clear separation by effect concentration (ECx) (Figure 3). For 3 days of exposure – two main groups can be discriminated: (1) Cu-Nwires and Cu salt-aged treatments (clustered very closely, at a correlation level of around 0.9), and (2) $CuNO_3$ and Cu-NPs treatments. For 7 days of exposure – Cu-NPs EC_{20} and $CuNO_3$ EC_{20} were clearly separated from the other treatments (correlation of -0.26); within the other treatments, Cu-Nwires EC_{20} and EC_{50} formed a sub-group of samples.

An overview in terms of number of genes affected by each Cu form (independently of the EC) and shared among the treatments per time of exposure is depicted in the Venn diagrams of Figure 4.

Results show that, for 3 days of exposure, there are more genes commonly affected by some of the Cu forms (among Cu salt-aged, Cu-NPs, and Cu-

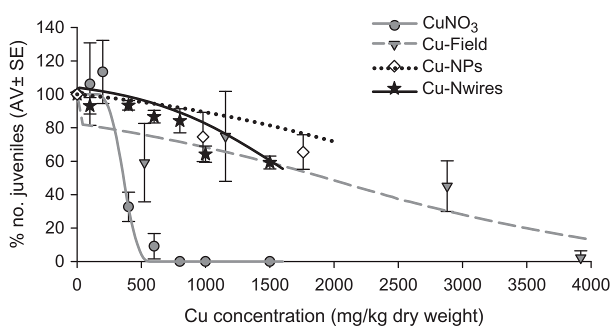


Figure 1. Results in terms of reproduction of *Enchytraeus crypticus* exposed to copper-nanowires (Cu-Nwires), copper-nanoparticles 25 nm (Cu-NPs) expressed as % of control. Results from copper nitrate ($CuNO_3$) and copper salt aged in the field (Cu salt-aged) are from (Gomes, Murphy, et al. 2015). Data are presented as average ± standard error ($n = 4$). $^*p < 0.05$ (Dunnett's method).

Table 3. Survival and reproduction effect concentrations (ECx) of Cu-Nwires and Cu-NPs for *Enchytraeus crypticus*, with the 95% confidence interval (in brackets) and the model applied with the respective parameters (S: slope, Y0: top point).

	Survival		Reproduction				
	LC_{50}	NOEC/LOEC	EC_{10}	EC_{20}	EC_{50}	NOEC/LOEC	Model and parameters
Cu-Nwires	>1500	>1500	398 (120–676)	846 (680–1013)	1613 (1372–1855)	800/1000	Logistic 2 Param (S:4.5 × $10^{-4,}$ Y0: 414)
Cu-NPs	>1760	>1760	106 nd	1081 nd	2748 nd	>1760	Logistic 2 Param (S:2.1 × $10^{-4,}$ Y0: 401)

LC: lethal concentration; EC: reproduction effect concentration; NOEC: no observed effect concentration; LOEC: lowest observed effect concentration; nd: not determined.

Nwires) than uniquely affected by each of these. Cu salt-aged and Cu-Nwires treatments share around half of all the DEGs affected at 3 days of exposure, which corroborates the close clustering observed between these two Cu forms. Seven days of exposure only caused response in the case of the Cu salt-aged (EC_{50}), as the other Cu forms affected very few genes.

Enrichment analysis on GO terms was performed to identify biological processes significantly affected by each copper treatment. For that, 13 gene lists with the annotated transcripts affected by each copper form, ECx, and time of exposure were analyzed versus the entire gene library (annotated transcripts present in the microarray). The GO terms (of the category Biological Processes) significantly affected in each gene list are listed in Supplementary Table S4. $CuNO_3$ was the Cu-form affecting fewer processes and Cu-Nwires and Cu salt-aged were the treatments affecting the most processes.

qPCR confirmations

The microarray results were validated by comparing the *M* values (log2 ratio) of nine transcripts in several treatments, with a total of 24 confirmations (Supplementary Table S2). The results from microarray and qPCR had a significant correlation (Spearman's rho) $\rho = 0.882$, $p = 2^{-7}$, $n = 24$.

Discussion

Population level

There were no differences, in terms of survival and reproduction effect between the Cu-Nwires, the 25 nm Cu-NPs and the 80 nm Cu-NPs (Gomes, Murphy, et al. 2015), which indicates that for these Cu-NMs size ranges and shape have little influence on the measured toxicity at organism level. Several studies have shown size dependent toxicity (e.g. mobility,

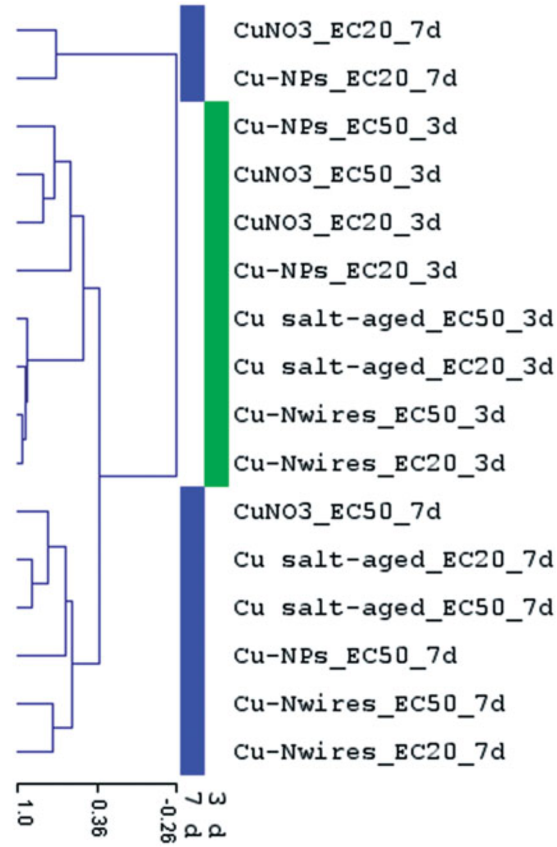


Figure 3. Dendrogram of samples hierarchically clustered using Pearson' Uncentered and average linkage, based on the 13165 differentially expressed genes (adjusted $p < 0.05$) after exposure of *Enchytraeus crypticus* for 3 and 7 days to copper: copper nitrate ($CuNO_3$), copper salt aged in the field (Cu salt-aged), copper nanoparticles (Cu-NPs), and copper nanowires (Cu-Nwires). EC20, 50: reproduction effect concentration of 20 and 50%; d: days.

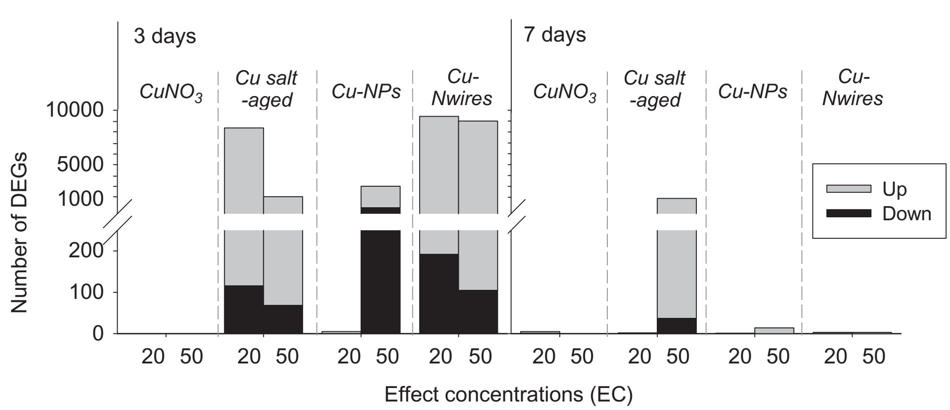


Figure 2. Results in terms of number of differentially expressed genes (DEGs) in *Enchytraeus crypticus* affected by exposure to the EC_{20} and EC_{50} (reproduction effect concentrations) of copper nitrate ($CuNO_3$), copper salt aged in the field (Cu salt-aged), copper nanoparticles (Cu-NPs), and copper nanowires (Cu-Nwires), after 3 and 7 days. Y scale shows the break between 250 and 500 to improve visualization.

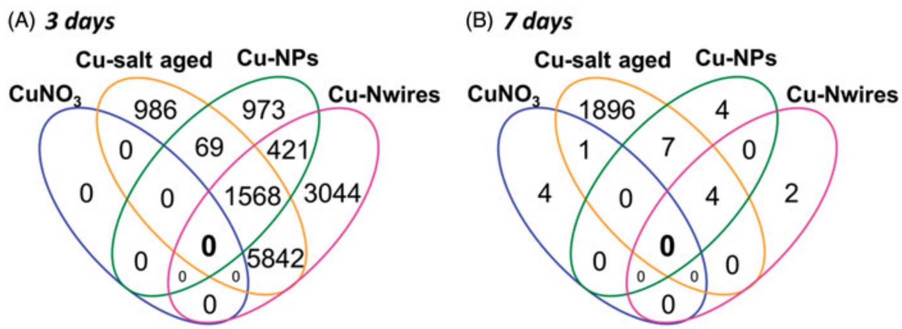


Figure 4. Venn diagram representation of the differentially expressed genes (adjusted $p < 0.05$) after exposure to the several copper treatments ($CuNO_3$: copper nitrate, Cu salt-aged: copper salt aged in the field, Cu-NPs: copper nanoparticles, and Cu-Nwires: copper nanowires) at 3 days (A) and 7 days of exposure (B).

growth) of NPs by testing several sizes of NPs, for example, Ag-NPs to crustaceans, algae, yeast, and a cell line (Ivask et al. 2014) and Au-NPs and TiO_2-NPs to several cell lines (Pan et al. 2007; Cai et al. 2011); however, none of these studies were performed in soil. Shoults-Wilson et al. (2011) investigated the effects of 10 and 30–50 nm Ag-NPs to *Eisenia fetida* in soils and did not find differences in toxicity (reproduction, bioaccumulation, and sub-chronic lethality) between the NPs, even though size may be a factor in the bioaccumulation of Ag or in the dissolution of Ag nanoparticles. Our results show that the ERT did not allow the discrimination between Cu-NPs of different sizes and shape. This is likely due to the limitations of the test design itself, and it does not imply that there are no differences. As observed by Bicho et al. (2016), a full life cycle test, compared to the ERT, allowed discrimination of effects between $AgNO_3$ and Ag NM300K (dispersed Ag-NPs) and also increased sensitivity, which shows the possibility for improvement of the test design at the organism level. Recent results on a full life cycle using Cu materials (Bicho et al. 2017) showed that CuO NMs mainly affected growth or juveniles' development, whereas $CuCl_2$ mainly affected embryo development and/or hatching success and adults survival. This is currently being further studied in terms of embryotoxicity.

Gene level

The high number of DEGs shown after 3 days of exposure indicates the capture of a peak in gene regulation, whereas at day 7 there is a more stable stage. For instance, results for the same species in response to zinc chloride showed a variation between 250–2500–500 DEGs for 2–3–4 days exposure, respectively (unpublished). Similarly, this time-dependency of gene regulation has been reported in previous studies (Nota et al. 2008; Novais, Arrais, et al. 2012; Gomes et al. 2017). Hence, these results were also influencing the clustering by time of exposure.

The lower number of DEGs in response to $CuNO_3$ in comparison to all other treatments could be related with differences in uptake rates. For example, the uptake of Cu from $CuNO_3$ will likely be faster than for the other Cu forms, and the peak of gene regulation is likely to have occurred earlier than later, e.g. after 1 or 2 days of exposure.

Regarding the differences between the four Cu forms, there is a main separation between two sets: Cu salt-aged and Cu-Nwires versus $CuNO_3$ and Cu-NPs. In the precursor study (Gomes, Murphy, et al. 2015), it was found that in the Cu salt-aged contaminated soil, 40% of the Cu was bound to carbonates, the more easily extractable fraction, and hence also relatively more bioavailable, compared to ca. 70–80% for Cu-NPs and $CuNO_3$. The clustering could be partly related to this aspect, i.e. the higher bioavailability of Cu from $CuNO_3$ and Cu-NPs in comparison to Cu salt-aged.

In the following, we discuss the functions/biological processes associated with the genes affected by the Cu forms, resulting from Enrichment Analysis of Gene Ontology (GO) terms, and independently of the exposure period.

The differences in the absolute number of genes affected by each test condition is reflected in the number of functions affected, with more functions being affected by Cu-Nwires and Cu salt-aged, followed by Cu-NPs (in particular at the EC_{50}) and very few functions associated with $CuNO_3$ (only at EC_{20}, 7 days) due to the very low number of (or null) DEGs.

GO terms affected by $CuNO_3$ include locomotory behavior, which can be associated with the function neurotransmitter transport, also unique to $CuNO_3$. This may be linked with the known avoidance behavior observed in oligochaetes, as reported for *Enchytraeus albidus* (Amorim and Scott-Fordsmand 2012), in an attempt to avoid the contaminated soil. Uniquely affected by $CuNO_3$ are the functions: glutathione metabolic process and glutathione conjugation reaction, associated with detoxification mechanisms, for instance, to remove reactive oxygen species (ROS) that cause oxidative damage to enchytraeids (Gomes, Novais, Gravato, et al. 2012).

Affected across all Cu forms, except $CuNO_3$, are numerous processes associated with DNA damage response: cellular response to DNA damage stimulus, DNA double strand breaking process, DNA repair, double strand break repair, nucleotide excision repair, and DNA gap filling, by the up-regulation of several transcripts including ubiquitin-conjugating enzyme e2n and f-box wd repeat-containing protein 7 isoform 1 (confirmed by qPCR). The induction of DNA damage by Cu-NPs has already been reported, *in vitro*, for NPs of about 100 nm (Midander et al. 2009) and 4–5 nm (Jose et al. 2011) as well as for Cu-salts (Oikawa and Kawanishi 1998; Lloyd and Phillips 1999); our results indicate that *in vivo* exposed enchytraeids are facing induction of DNA damage. Induction of DNA repair mechanisms was also reported for *E. crypticus* exposed to several Ag materials (salt and nano forms) exposed to similar EC20/EC50 (Gomes et al. 2017), suggesting this could be a common or general response to sub-lethal concentrations. On the other hand, DNA repair was impaired in *E. albidus* exposed to lethal concentrations of $AgNO_3$ (Gomes et al. 2013).

Mitotic cell cycle is being strongly regulated in response to all Cu forms (except $CuNO_3$), through the biological processes: cell cycle, cell cycle process, cell cycle G2/M transition, regulation of G1/S transition of mitotic cell cycle, regulation of G2/M transition of mitotic cell cycle, by the up-regulation of several transcripts including cyclin A and B, and a DNA helicase recq4 (qPCR confirmed). Other NPs such as Ag (Eom and Choi 2010) and TiO_2 (Wu, Sun, and Xue 2010), induced cell cycle arrest which was considered one of the toxicity mechanisms for those NPs. In our results, indications are that cell cycle arrest is not a mechanism of Cu toxicity; on the other hand, it is not clear if the regulation of cell cycle observed here at gene level would cause negative effects to the organisms or if it is a regulatory process without further direct consequences. Interestingly, a study on gene expression showed that Ni-salt ($NiCl_2$) induced cell cycle arrest in *E. albidus*, but not $CuCl_2$ (Gomes, Scott-Fordsmand, and Amorim 2014), which strengthens the hypothesis that Cu does not cause toxicity via cell cycle arrest mechanisms.

Notch signaling pathway was also affected by all Cu forms (except $CuNO_3$). Notch signaling is involved in neurogenesis and plays a major role in the regulation of embryonic development. The fact that it was affected only at the EC_{50} indicates a potential correlation with negative effects on reproduction. Also affected across Cu forms (except $CuNO_3$) was the negative regulation of neuron death/negative regulation of neuron apoptotic process. Apoptosis (or programed cell death) is a mechanism to eliminate damaged cells (Davies 2001) and its negative regulation caused by Cu exposure can lead to the accumulation of aberrant neurons with further neuronal defects.

Copper, independently of the form, affected several protein post-transcriptional modifications, i.e. methylation, alkylation (methylation is the most common type of alkylation) and ubiquitination, via the up-regulation of several transcripts including methyltransferases (e.g. protein arginine methyltransferase 1 and 7) and ubiquitin conjugating enzymes (for protein ubiquitination), as the ubiquitin-conjugating enzyme e2n (qPCR confirmed). Both protein methylation and ubiquitination play important roles in the regulation of intracellular signaling events (Paik, Paik, and Kim 2007; Komander 2009), in addition methylation is involved in chromatin remodeling, which has been linked with epigenetics (Molina-Serrano, Schiza, and Kirmizis 2013). It is not clear how these post-transcriptional modifications can further impact organisms, however, based on this, longer-term and transgenerational effects should be investigated.

The similar expression patterns of Cu-Nwires and Cu salt-aged was also reflected in the high number of commonly enriched biological processes. Among those processes is cell redox homeostasis, with the up-regulation of several transcripts (e.g. glutaredoxin 3, selenoprotein t, dihydrolipoamide dehydrogenase, thioredoxin peroxidase, etc.). Cu, including Cu-NPs, are known to induce oxidative stress to enchytraeids (Gomes, Novais, Gravato, et al. 2012) and the current results do indicate that potworms are responding to oxidative stress when exposed to Cu-Nwires and Cu salt-aged. Also affected by Cu-Nwires and Cu salt-aged is the endoplasmic reticulum dependent peroxisome organization, with involvement of the transcript coding for peroxisomal biogenesis factor 16 (qPCR confirmed). Among other functions, peroxisomes are involved in ROS regulation in the cells (Bonekamp et al. 2009), thus this process may be linked with cell redox homeostasis. The observed indication of oxidative stress for Cu-Nwires and Cu salt-aged, and not for Cu-NPs and $CuNO_3$, is probably related with temporal variations in gene expression response and not a difference in the mechanism.

Endocytosis was affected by exposure to Cu-Nwires and Cu salt-aged (via the up-regulation of actin, amphiphysin, syntaxin-binding protein 1, adp-ribosylation factor 1, etc.). NPs can enter the cells by endocytosis, which is in fact a property exploited in nanomedicine [i.e. using NPs/NMs to deliver medicines; Sahay, Alakhova, and Kabanov (2010)]. On the other hand, it is known that excess of copper induces the endocytosis of Cu transporters (CTR family) and its degradation, to prevent the entry of Cu into the cells (Petris et al. 2003). Knowing that the process of endocytosis is also being affected by Cu salt-aged, the last option is the most likely scenario.

In addition to the protein modifications affected for all Cu forms except $CuNO_3$ (as discussed above), Cu-Nwires and Cu salt-aged are involved in protein lipidation, geranylgeranylation, and neddylation. Geranylgeranylation is a type of lipidation (or prenylation) that adds hydrophobic groups to a protein, and plays an important role in protein–protein and protein–membrane interactions. In the current study, both protein lipidation and the most specific protein geranylgeranylation are being affected, since different genes were involved in the two biological processes. Lipidation processes, together with membrane budding and membrane docking (also affected by Cu-Nwires and Cu salt-aged) might be associated with endocytosis. The inhibition of both protein geranylgeranylation and protein neddylation has been associated with increase in apoptosis (Li et al. 2002; Wang et al. 2015). In our case, however, the genes involved in these processes (e.g. protein geranylgeranyltransferase type beta subunit and nedd8 activating enzyme e1 subunit 1) were up-regulated, indicating induction and not inhibition, for which posteriori effects are unknown.

Also affected by Cu-Nwires and Cu salt-aged are the histone modifications: histone ubiquitination and histone H3 acetylation. Histone ubiquitination is being induced by the up-regulation of several transcripts (e.g. ubiquitin-conjugating enzyme e2a, ubiquitin-conjugating enzyme e2n). The involvement of E2 ubiquitin conjugating enzyme family suggests the implication in DNA damage repair (Cao and Yan 2012), which was found triggered by Cu independently of its form (see discussion above). Histone H3 acetylation is a known epigenetic marker associated with gene regulation (Yan and Boyd 2006). Decrease in histone acetylation has been associated with nickel carcinogenesis (Arita and Costa 2009), however, our current results indicate increase in histone H3 acetylation in response to Cu (Nwires and salt-aged). Again, the investigation of longer-term effects of Cu, including multi-generational should be pursued.

The effects on blastocyst development and body morphogenesis by Cu-Nwires and Cu salt-aged indicate negative effects on embryo development, which can be linked with impairment in reproduction. Bicho et al. (2017) reported that Cu salt inhibited hatching in *E. crypticus,* followed by a decrease in reproduction, hence supporting embryotoxicity. Additionally, unique to Cu-Nwires were effects on meiotic cell cycle, male meiosis/male gamete generation, and hermaphrodite genitalia development. While Cu salt-aged uniquely affected: reproductive system development and male sex differentiation. These results indicate that Cu-Nwires and Cu salt-aged can affect reproductive output via effects at two different stages, i.e. directly on embryos and on adults' reproductive system. However, while the effects on embryos involved the same processes (and the same genes), the effects on adults are Cu-form specific, i.e. with involvement of different genes.

The number of functions found commonly affected by Cu-NPs and Cu salt-aged was much smaller than between Cu-Nwires and Cu salt-aged, reflecting the different mechanisms of toxicity of the two Cu-NMs, not distinguishable based on the

ERT results. Interestingly, those functions, lysosome organization and intracellular pH reduction, seem to be related. The mechanism of toxicity called 'lysosome-enhanced Trojan horse effect' as the internalization of NPs by endocytosis followed by degradation and ions release in the lysosomes has been attributed to several metal containing NPs (Sabella et al. 2014). On the other hand, it is also known that Cu(ions) might be sequestered into lysosomes (van den Berghe et al. 2007), thus it is not surprising to find these functions activated by Cu-NPs and Cu salt-aged.

Based on the affected functions for Cu-NPs and Nwires, there are indications that these cause: (i) similar mechanisms, and (ii) different mechanisms, hence shape has an important role in Cu-NMs toxicity, as shown by the very distinct expression pattern of Cu-Nwires and Cu-NPs (more similar to Cu salt-aged).

In the next paragraphs, the uniquely affected functions will be summarized.

Exposure to Cu salt-aged exclusively affected biological processes related with energy metabolism, i.e. ATP synthesis coupled electron transport (via the up-regulation of several cytochrome c oxidase transcripts and NADH dehydrogenase) and carbohydrate metabolism. This is in line with an increase in energy production for, for instance detoxification processes, which was previously described at subcellular level (Gomes, Scott-Fordsmand, and Amorim 2015), and at transcriptomic level for Cu (Bundy et al. 2008) and other metals such as cadmium (Novais, De Coen, and Amorim 2012). In fact, we found the process cell redox homeostasis affected not only by Cu salt-aged but also by Cu-Nwires. A high energy demanding process that was exclusively affected by Cu salt-aged was phagocytosis. Trace elements, such as Cu, inhibited phagocytosis in *in vitro* exposed coelomocytes of terrestrial and aquatic annelids (Sauvé et al. 2002). On the other hand, Pipe et al. (1999) showed that, to the mussel *Mytilus edulis*, phagocytic activity was stimulated at 0.2 ppm of Cu, but not at 0.5 ppm. In our study, phagocytosis was being stimulated at transcriptomic level indicating stimulation of immune system in response to Cu salt-aged EC_{50}.

Also exclusively affected by Cu salt-aged was the brain segmentation/central nervous system segmentation. It is not clear how this process was being affected (due to the involvement of only one transcript, i.e. up-regulation of ribosomal protein S1). Cu exposure has been associated with poorer motor performance and altered structure of the basal ganglia in children (Pujol et al. 2016), suggesting that Cu salt-aged might be impairing nervous system of the organisms (in addition to the negative regulation of neuron death, as discussed above).

The cuticle pattern formation was uniquely affected by Cu-NPs, with the down-regulation of myosin heavy chain, disheveled associated activator of isoform d, and protein transport protein sec23a. Given that the enchytraeids' cuticle/tegument is directly exposed to NPs in soil, this might be a direct effect of the contact with the NPs.

Another biological process affected by Cu-NPs is senescence. A study with *Caenorhabditis elegans* showed that organisms fed with silica-NPs exhibited a reproductive senescence, even though lifespan remained unaffected (Pluskota et al. 2009). In our study, it is not possible to know if the affected senescence (at transcriptomic level) will cause age or reproductive senescence at the apical or organism level. However, in another study with *E. crypticus*, exposed to CuO-NPs the lifespan and reproduction over time was decreased (Gonçalves et al. 2017) confirming the apical effect.

Among the functions exclusively affected by Cu-Nwires were copper ion transport (with the up-regulation of several transcripts including copper chaperone for superoxide dismutase and heavy metal translocating p-type ATPase) and response to hypoxia/response to decreased oxygen levels (with the involvement of zinc-binding dehydrogenase family protein, confirmed by qPCR). Interestingly both processes have been previously described for Cu(salt) exposure (Prohaska 2008; Song, Li, and Freedman 2009) suggesting similar mechanisms between the Cu forms.

As discussed above, Cu-Nwires seem to affect enchytraeids reproduction via effects on its reproductive system due to the affected functions: hermaphrodite genitalia development, meiotic cell cycle, and male meiosis/male gamete generation.

Effect level integration and adverse outcome pathway (AOP)

For overview, data on gene and population level was integrated, and an AOP was drafted (Figure 5).

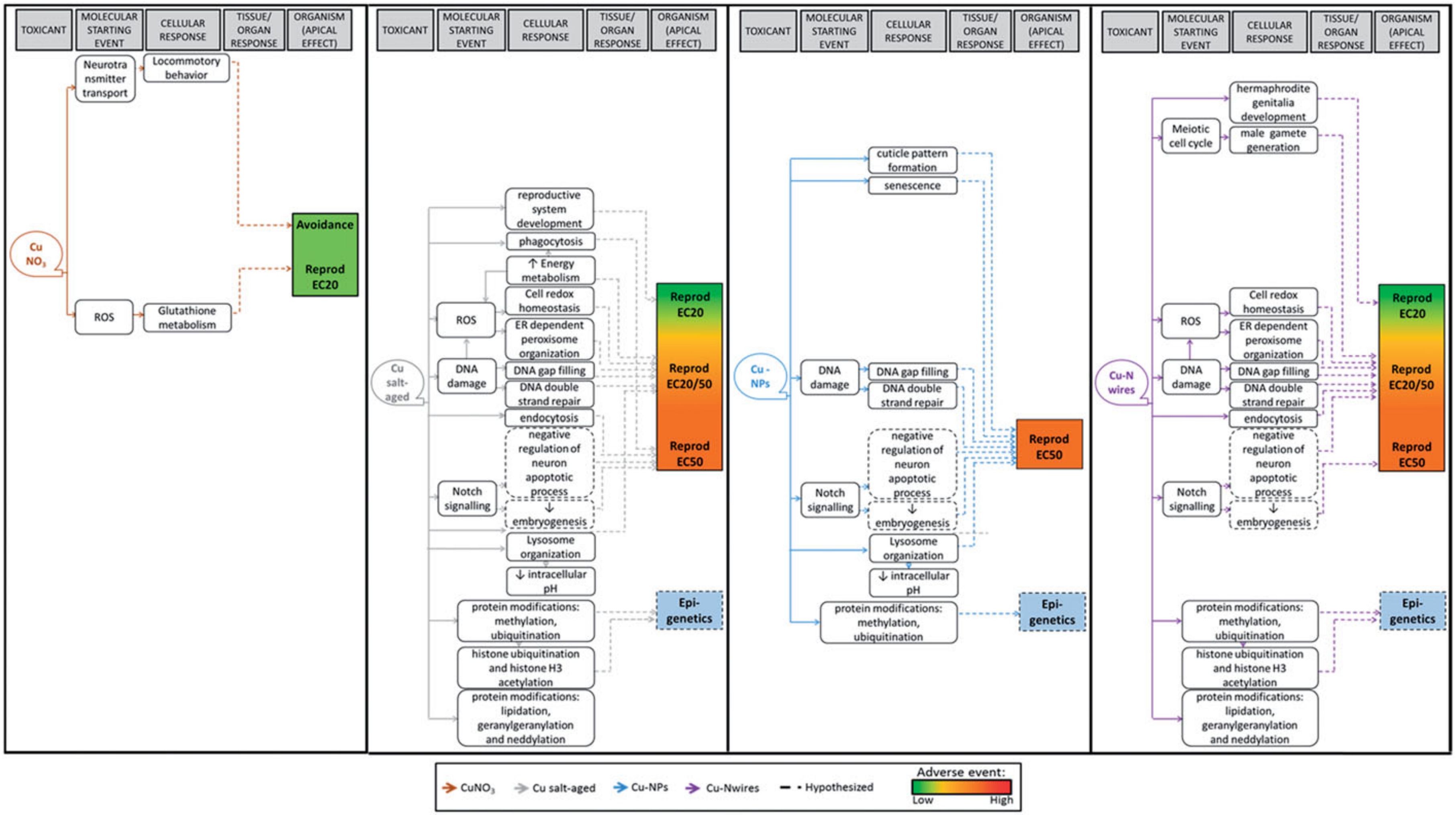


Figure 5. Adverse outcome pathway (AOP) for *Enchytraeus crypticus* when exposed to copper nitrate ($CuNO_3$), copper salt aged in the field (Cu salt-aged), copper nanoparticles (Cu-NPs), and copper nanowires (Cu-Nwires) in LUFA 2.2 soil based on transcript-expression data. Square boxes represent final states for the organism and rounded boxes represent intermediate states. Dashed line represents relationships hypothesized.

This is the first time such an AOP format is provided for the effects of Cu (nano)materials, providing mechanistic hypothesis for the apical effects. As can be depicted, there are common starting events across all Cu forms (except for $CuNO_3$ for the reasons discussed above) (e.g. DNA damage and Notch signaling). Interestingly, ROS was associated with both forms of Cu salt (freshly spiked and aged) and Cu-Nwires, but not to Cu-NPs. Differentiation between materials occurred at predicted cellular processes, which are more difficult to link to starting events, for instance cuticle pattern formation and senescence for Cu-NPs or reproductive system development for Cu salt-aged.

Conclusions

The current study shows the benefits of relating organism and gene effect levels allowing to further understand the mechanisms of toxicity. Additionally, it provides information to integrate in an AOP framework, as drafted here for the first time for Cu (nano)-materials. Such AOP, besides giving the overview on the unique effects per material, serves the purpose of guiding future research based on mechanistic hypothesis for the apical effects. Some of the discriminating effects, unique to each material, include e.g. senescence and cuticle pattern formation for Cu-NPs, which can be caused by the contact of the NPs with the worms' tegument. Cu-Nwires affected reproduction, which may be via male gamete generation and hermaphrodite genitalia development. $CuNO_3$ was associated with neurotransmission and locomotory behavior, both of which can be related with avoidance response. And, finally, Cu salt-aged may be linked with affected phagocytosis and reproductive system development (via different mechanisms than Cu-Nwires). Common across Cu forms (except $CuNO_3$) were the response to ROS and DNA damage, and protein modifications (i.e. methylation and ubiquitination) which indicate longer-term/epigenetic effects.

Exposure time was a key factor in the detection of effects at gene level (higher activity at 3 days compared to 7 days), which was possibly related with different Cu uptake rates.

Finally, effects were discriminated at the gene level (3 days), whereas at organism level this was not possible (21 days): the selected endpoints (survival, reproduction) do not vary. The use of transcriptomic HTP tools offers significant power for the hazard assessment of NMs, especially if combined with the standard ecotoxicity test endpoints.

Disclosure statement

The authors report no conflict of interest. The authors alone are responsible for the content and writing of the paper.

Funding

This study was supported by the European Commission EU-FP7 SUN (G.A. No. 604305), MARINA (G.A. No. 263215) and MODERN (Ref. 309314- 2). By funding FEDER through COMPETE Programa Operacional Factores de Competitividade, and by National funding through FCT-Fundação para a Ciência e Tecnologia, within the research project NANOkA FCOMP-01-0124- FEDER-008944 (Ref. FCT PTDC/BIA-BEC/103716/2008), project POCI-01-0145-FEDER-007679 (FCT ref. UID/CTM/50011/2013), project NM_OREO (POCI-01-0145-FEDER-016771, PTDC/AAG-MAA/4084/2014), through the post-doc grant to Susana Gomes (SFRH/BPD/95775/2013) and CESAM (UID/AMB/50017/2013 - POCI-01-0145-FEDER-007638), to FCT/MCTES through national funds (PIDDAC), and the co-funding by the FEDER, within the PT2020 Partnership Agreement and Compete 2020.

ORCID

Susana I. L. Gomes http://orcid.org/0000-0001-7537-2341
Carlos P. Roca http://orcid.org/0000-0003-0230-1926
Natália Pegoraro http://orcid.org/0000-0002-0076-4188
Tito Trindade http://orcid.org/0000-0002-5456-7243
Janeck J. Scott-Fordsmand http://orcid.org/0000-0002-2260-1224
Mónica J. B. Amorim http://orcid.org/0000-0001-8137-3295

References

Alexa, A., J. Rahnenfuhrer, and T. Lengauer. 2006. "Improved Scoring of Functional Groups from Gene Expression Data by Decorrelating GO Graph Structure." *Bioinformatics (Oxford, England)* 22 (13): 1600–1607. doi:10.1093/bioinformatics/btl140.

Amorim, M. J. B., and J. J. Scott-Fordsmand. 2012. "Toxicity of Copper Nanoparticles and $CuCl_2$ Salt to *Enchytraeus albidus* Worms: Survival, Reproduction and Avoidance Responses." *Environmental Pollution* 164: 164–168. doi:10.1016/j.envpol.2012.01.015.

Arita, A., and M. Costa. 2009. "Epigenetics in Metal Carcinogenesis: Nickel, Arsenic, Chromium and Cadmium." *Metallomics* 1 (3): 222–228. doi:10.1039/B903049b.

Benjamini, Y., and Y. Hochberg. 1995. "Controlling the False Discovery Rate: A Practical and Powerful Approach to

Multiple Testing." *Journal of the Royal Statistical Society. Series B, Statistical Methodology* 57: 289–300.

Bicho, R. C., T. Ribeiro, N. P. Rodrigues, J. J. Scott-Fordsmand, and M. J. B. Amorim. 2016. "Effects of Ag Nanomaterials (NM300K) and Ag Salt (AgNO3) Can Be Discriminated in a Full Life Cycle Long Term Test with *Enchytraeus Crypticus*." *Journal of Hazardous Materials* 318: 608–614. doi:10.1016/j.jhazmat.2016.07.040.

Bicho, R. C., F. C. F. Santos, J. J. Scott-Fordsmand, and M. J. B. Amorim. 2017. "Effects of Copper Oxide Nanomaterials (CuONMs) Are Life Stage Dependent: full Life Cycle in *Enchytraeus Crypticus*." *Environmental Pollution* 224: 117–124. doi:10.1016/j.envpol.2017.01.067.

Bonekamp, N. A., A. Völkl, H. D. Fahimi, and M. Schrader. 2009. "Reactive Oxygen Species and Peroxisomes: Struggling for Balance." *Biofactors (Oxford, England)* 35 (4): 346–355. doi:10.1002/biof.48.

Bundy, J. G., J. K. Sidhu, F. Rana, D. J. Spurgeon, C. Svendsen, J. F. Wren, S. R. Stürzenbaum, A. J. Morgan, and P. Kille. 2008. "Systems Toxicology" Approach Identifies Coordinated Metabolic Responses to Copper in a Terrestrial Non-Model Invertebrate, the Earthworm *Lumbricus rubellus*." *BMC Biology* 6: 25. doi:10.1186/1741-7007-6-25.

Cai, K., Y. Hou, Y. Hu, L. Zhao, Z. Luo, Y. Shi, M. Lai, W. Yang, and P. Liu. 2011. "Correlation of the Cytotoxicity of TiO_2 Nanoparticles with Different Particle Sizes on a Sub-200-Nm Scale." *Small (Weinheim an Der Bergstrasse, Germany)* 7 (21): 3026–3031. doi:10.1002/smll.201101170.

Cao, J., and Q. Yan. 2012. "Histone Ubiquitination and Deubiquitination in Transcription, DNA Damage Response, and Cancer." *Frontiers in Oncology* 2: 26. doi:10.3389/fonc.2012.00026.

Castro-Ferreira, M. P., T. E. de Boer, J. K. Colbourne, R. Vooijs, C. A. van Gestel, N. M. van Straalen, A. M. Soares, M. J. Amorim, and D. Roelofs. 2014. "Transcriptome Assembly and Microarray Construction for *Enchytraeus crypticus*, a Model Oligochaete to Assess Stress Response Mechanisms Derived from Soil conditions." *BMC Genomics* 15: 302. doi:10.1186/1471-2164-15-302.

Chang, Y., M. L. Lye, and H. C. Zeng. 2005. "Large-Scale Synthesis of High-Quality Ultralong Copper Nanowires." *Langmuir: The ACS Journal of Surfaces and Colloids* 21 (9): 3746–3748. doi:10.1021/la050220w.

Davies, K. 2001. "Oxidative Stress, Antioxidant Defenses, and Damage Removal, Repair, and Replacement Systems." *IUBMB Life* 50 (4): 279–289. doi:10.1080/713803728.

Eom, H.-J., and J. Choi. 2010. "p38 MAPK Activation, DNA Damage, Cell Cycle Arrest and Apoptosis as Mechanisms of Toxicity of Silver Nanoparticles in Jurkat T Cells." *Environmental Science & Technology* 44 (21): 8337–8342. doi:10.1021/es1020668.

European Commission. 2013. General Report on REACH. Report from the Commission to the European Parliament, the Council, the European Economic and Social Committee and the Committee of the Regions in Accordance with Article 117(4) of REACH and Article 46(2) of CLP, and a review of certain elements of REACH in line with Articles 75(2), 138(2), 138(3) and 138(6) of REACH.

Gomes, S. I. L., S. C. Novais, C. Gravato, L. Guilhermino, J. J. Scott-Fordsmand, A. M. Soares, and M. J. Amorim. 2012. "Effect of Cu-Nanoparticles versus One Cu-Salt: Analysis of Stress and Neuromuscular Biomarkers Response in *Enchytraeus Albidus* (Oligochaeta)." *Nanotoxicology* 6 (2): 134–143. doi:10.3109/17435390.2011.562327.

Gomes, S. I. L., S. C. Novais, J. J. Scott-Fordsmand, W. De Coen, A. M. Soares, and M. J. Amorim. 2012. "Effect of Cu-Nanoparticles versus Cu-Salt in *Enchytraeus Albidus* (Oligochaeta): Differential Gene Expression through Microarray Analysis." *Comparative Biochemistry and Physiology Part C: Toxicology & Pharmacology* 155 (2): 219–227. doi:10.1016/j.cbpc.2011.08.008.

Gomes, S. I. L., A. M. V. M. Soares, J. J. Scott-Fordsmand, and M. J. B. Amorim. 2013. "Mechanisms of Response to Silver Nanoparticles on *Enchytraeus albidus* (Oligochaeta): Survival, Reproduction and Gene Expression Profile." *Journal of Hazardous Materials* 254–255: 336–344. doi:10.1016/j.jhazmat.2013.04.005.

Gomes, S. I. L., J. J. Scott-Fordsmand, and M. J. B. Amorim. 2014. "Profiling Transcriptomic Response of *Enchytraeus albidus* to Cu and Ni: Comparison with Cd and Zn." *Environmental Pollution* 186: 75–82. doi:10.1016/j.envpol.2013.11.031.

Gomes, S. I. L., M. Murphy, M. T. Nielsen, S. M. Kristiansen, M. J. Amorim, and J. J. Scott-Fordsmand. 2015. "Cu-Nanoparticles Ecotoxicity-Explored and Explained?" *Chemosphere* 139: 240–245. doi:10.1016/j.chemosphere.2015.06.045.

Gomes, S. I. L., J. J. Scott-Fordsmand, and M. J. B. Amorim. 2015. "Cellular Energy Allocation to Assess the Impact of Nanomaterials on Soil Invertebrates (Enchytraeids): The Effect of Cu and Ag." *International Journal of Environmental Research and Public Health* 12 (12): 6858–6878. doi:10.3390/ijerph120606858.

Gomes, S. I. L., C. P. Roca, J. J. Scott-Fordsmand, and M. J. B. Amorim. 2017. "High-Throughput Transcriptomics Reveals Uniquely Affected Pathways: AgNPs, PVP-Coated AgNPs and Ag NM300K Case Studies." *Environmental Science Nano* 9: e102108. doi:10.1039/C6EN00652C.

Gonçalves, M. F. M., S. I. L. Gomes, J. J. Scott-Fordsmand, and M. J. B. Amorim. 2017. "Shorter Lifetime of a Soil Invertebrate Species When Exposed to Copper Oxide Nanoparticles in a Full Lifespan Exposure Test." *Scientific Reports* 7 (1): 1355.

Griffitt, R. J., K. Hyndman, N. D. Denslow, and D. S. Barber. 2009. "Comparison of Molecular and Histological Changes in Zebrafish Gills Exposed to Metallic Nanoparticles." *Toxicological Sciences* 107 (2): 404–415. doi:10.1093/toxsci/kfn256.

Heckmann, L.-H., M. B. Hovgaard, D. S. Sutherland, H. Autrup, F. Besenbacher, and J. J. Scott-Fordsmand. 2011. "Limit-Test Toxicity Screening of Selected Inorganic Nanoparticles to the Earthworm *Eisenia fetida*."

Ecotoxicology (London, England) 20 (1): 226–233. doi:10.1007/s10646-010-0574-0.

Huber, W., V. J. Carey, R. Gentleman, S. Anders, M. Carlson, B. S. Carvalho, H. C. Bravo, et al. 2015. "Orchestrating High-Throughput Genomic Analysis with Bioconductor." *Nature Methods* 12 (2): 115–121. doi:10.1038/nmeth.3252.

Ispas, C., D. Andreescu, A. Patel, D. V. Goia, S. Andreescu, and K. N. Wallace. 2009. "Toxicity and Developmental Defects of Different Sizes and Shape Nickel Nanoparticles in Zebrafish." *Environmental Science & Technology* 43 (16): 6349–6356. doi:10.1021/es9010543.

Ivask, A., I. Kurvet, K. Kasemets, I. Blinova, V. Aruoja, S. Suppi, H. Vija, et al. 2014. "Size-Dependent Toxicity of Silver Nanoparticles to Bacteria, Yeast, Algae, Crustaceans and Mammalian Cells In Vitro." *PLoS One* 9 (7): e102108. doi:10.1371/journal.pone.0102108.

Jose, G., S. Santra, S. Mandal, and T. Sengupta. 2011. "Singlet Oxygen Mediated DNA Degradation by Copper Nanoparticles: Potential towards Cytotoxic Effect on Cancer cells." *Journal of Nanobiotechnology* 9: 9. doi:10.1186/1477-3155-9-9.

Komander, D. 2009. "The Emerging Complexity of Protein ubiquitination." *Biochemical Society Transactions* 37 (Pt 5): 937–953. doi:10.1042/BST0370937.

Li, X., L. Liu, J. C. Tupper, D. D. Bannerman, R. K. Winn, S. M. Sebti, A. D. Hamilton, and J. M. Harlan. 2002. "Inhibition of Protein Geranylgeranylation and RhoA/RhoA Kinase Pathway Induces Apoptosis in Human Endothelial Cells." *The Journal of Biological Chemistry* 277 (18): 15309–15316. doi:10.1074/jbc.M201253200.

Lloyd, D. R., and D. H. Phillips. 1999. "Oxidative DNA Damage Mediated by Copper(II), Iron(II) and Nickel(II) Fenton Reactions: Evidence for Site-Specific Mechanisms in the Formation of Double-Strand Breaks, 8-Hydroxydeoxyguanosine and Putative Intrastrand Cross-Links." *Mutation Research – Fundamental and Molecular Mechanism of Mutagenesis* 424 (1–2): 23–36. doi:10.1016/S0027-5107(99)00005-6.

Midander, K., P. Cronholm, H. L. Karlsson, K. Elihn, L. Möller, C. Leygraf, and I. O. Wallinder. 2009. "Surface Characteristics, Copper Release, and Toxicity of Nano- and Micrometer-Sized Copper and Copper(II) Oxide Particles: A Cross-Disciplinary Study." *Small (Weinheim an Der Bergstrasse, Germany)* 5 (3): 389–399. doi: 10.1002/smll.200801220.

Molina-Serrano, D., V. Schiza, and A. Kirmizis. 2013. "Cross-Talk among Epigenetic Modifications: Lessons from Histone Arginine Methylation." *Biochemical Society Transactions* 41 (3): 751–759. doi:10.1042/BST20130003.

Navratilova, J., A. Praetorius, A. Gondikas, W. Fabienke, F. von der Kammer, and T. Hofmann. 2015. "Detection of Engineered Copper Nanoparticles in Soil Using Single Particle ICP-MS." *International Journal of Environmental Research and Public Health* 12 (12): 15756–15768. doi:10.3390/ijerph121215020.

Nota, B., M. J. T. N. Timmermans, O. Franken, K. Montagne-Wajer, J. Mariën, M. E. De Boer, T. E. De Boer, et al. 2008. "Gene Expression Analysis of Collembola in Cadmium Containing Soil." *Environmental Science & Technology* 42 (21): 8152–8157. doi:10.1021/es801472r.

Novais, S. C., J. Arrais, P. Lopes, T. Vandenbrouck, D. W. Coen, D. Roelofs, A. M. Soares, and M. J. Amorim. 2012a. "*Enchytraeus albidus* Microarray: Enrichment, Design, Annotation and Database (EnchyBASE)." *PLoS One* 7 (4): e34266. doi:10.1371/journal.pone.0034266.

Novais, S. C., C. F. Howcroft, L. Carreto, P. M. Pereira, M. A. Santos, D. W. Coen, A. M. Soares, and M. J. Amorim. 2012b. "Differential Gene Expression Analysis in *Enchytraeus albidus* Exposed to Natural and Chemical Stressors: Effect of Different Exposure Periods." *Ecotoxicology* 21 (1): 213–224. doi:10.1007/s10646-011-0780-4.

Novais, S. C., W. De Coen, and M. J. B. Amorim. 2012. "Transcriptional Responses in *Enchytraeus albidus* (Oligochaeta): Comparison between Cadmium and Zinc Exposure and Linkage to Reproduction Effects." *Environmental Toxicology and Chemistry* 31 (10): 2289–2299. doi:10.1002/etc.1946.

Organization for Economic Cooperation and Development (OECD) 2004. "*Guidelines for the Testing of Chemicals No. 220. Enchytraeid Reproduction Test*." Paris, France: Organization for Economic Cooperation and Development.

Organization for Economic Cooperation and Development (OECD) 2012. "*OECD Environment, Health and Safety Publications Series on the Safety of Manufactured Nanomaterials No 36. Guidance on Sample Preparation and Dosimetry for the Safety Testing of Manufactured Nanomaterials*." Paris, France: Organization for Economic Cooperation and Development.

Oikawa, S., and S. Kawanishi. 1998. "Distinct Mechanisms of Site-Specific DNA Damage Induced by Endogenous Reductants in the Presence of Iron(III) and Copper(II)." *Biochimica et Biophysica Acta - Gene Structure and Expression* 1399 (1): 19–30. doi:10.1016/S0167-4781(98)00092-X.

Paik, W. K., D. C. Paik, and S. Kim. 2007. "Historical Review: The Field of Protein Methylation." *Trends in Biochemical Sciences* 32 (3): 146–152. doi:10.1016/j.tibs.2007.01.006.

Pal, S., Y. K. Tak, and J. M. Song. 2007. "Does the Antibacterial Activity of Silver Nanoparticles Depend on the Shape of the Nanoparticle? A Study of the Gram-Negative Bacterium *Escherichia coli*." *Applied and Environmental Microbiology* 73 (6): 1712–1720. doi:10.1128/Aem.02218-06.

Pan, Y., S. Neuss, A. Leifert, M. Fischler, F. Wen, U. Simon, G. Schmid, W. Brandau, and W. Jahnen-Dechent. 2007. "Size-Dependent Cytotoxicity of Gold Nanoparticles." *Small (Weinheim an Der Bergstrasse, Germany)* 3 (11): 1941–1949. doi:10.1002/smll.200700378.

Petris, M. J., K. Smith, J. Lee, and D. J. Thiele. 2003. "Copper-Stimulated Endocytosis and Degradation of the Human Copper Transporter, hCtr1." *The Journal of Biological Chemistry* 278 (11): 9639–9646. doi:10.1074/jbc.M209455200.

Pipe, R. K., J. A. Coles, F. M. M. Carissan, and K. Ramanathan. 1999. "Copper Induced Immunomodulation in the Marine Mussel, *Mytilus edulis*." *Aquatic Toxicology* 46 (1): 43–54. doi:10.1016/S0166-445X(98)00114-3.

Pirooznia, M., P. Gong, X. Guan, L. S. Inouye, K. Yang, E. J. Perkins, and Y. Deng. 2007. "Cloning, Analysis and Functional Annotation of Expressed Sequence Tags from the Earthworm *Eisenia fetida*." *BMC Bioinformatics* 8 (Suppl 7): S7. doi:10.1186/1471-2105-8-S7-S7.

Pluskota, A., E. Horzowski, O. Bossinger, and A. von Mikecz. 2009. "In *Caenorhabditis elegans* Nanoparticle-Bio-Interactions Become Transparent: Silica-Nanoparticles Induce Reproductive Senescence." *PLoS One* 4 (8): e6622. doi:10.1371/journal.pone.0006622.

Prohaska, J. R. 2008. "Role of Copper Transporters in Copper Homeostasis." *American Journal of Clinical Nutrition* 88 (3): 826S–829S.

Pujol, J., R. Fenoll, D. Macià, G. Martínez-Vilavella, M. Alvarez-Pedrerol, I. Rivas, J. Forns, et al. 2016. "Airborne Copper Exposure in School Environments Associated with Poorer Motor Performance and Altered Basal Ganglia." *Brain and Behavior* 6 (6): e00467. doi:10.1002/brb3.467

R-Project. 2015. "*R: A Language and Environment for Statistical Computing*." Vienna: R Foundation for Statistical Computing. http://www.R-project.org/.

Regulation (EC) No 1907/2006. 2006. Regulation (EC) No 1907/2006 of 18 December 2006 of the European Parliament and of the Council concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

Regulation (EC) No 440/2008. 2008. Regulation (EC) No 440/2008 of 30 May 2008 laying down test methods pursuant to Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH)

Ritchie, M. E., B. Phipson, D. Wu, Y. Hu, C. W. Law, W. Shi, and G. K. Smyth. 2015. "Limma Powers Differential Expression Analyses for RNA-Sequencing and Microarray Studies." *Nucleic Acids Research* 43 (7): e47. doi:10.1093/nar/gkv007.

Roca, C. P., S. I. L. Gomes, M. J. B. Amorim, and J. J. Scott-Fordsmand. 2017. "Variation-Preserving Normalization Unveils Blind Spots in Gene Expression Profiling." *Scientific Reports* 7: 42460. doi:10.1038/srep42460.

Sabella, S., R. P. Carney, V. Brunetti, M. A. Malvindi, N. Al-Juffali, G. Vecchio, S. M. Janes, et al. 2014. "A General Mechanism for Intracellular Toxicity of Metal-Containing Nanoparticles." *Nanoscale* 6 (12): 7052. doi:10.1039/c4nr01234h.

Sahay, G., D. Y. Alakhova, and A. V. Kabanov. 2010. "Endocytosis of nanomedicines." *Journal of Controlled Release* 145 (3): 182–195. doi:10.1016/j.jconrel.2010.01.036.

Sauvé, S., M. Hendawi, P. Brousseau, and M. Fournier. 2002. "Phagocytic Response of Terrestrial and Aquatic Invertebrates following in Vitro Exposure to Trace Elements." *Ecotoxicology and Environmental Safety* 52 (1): 21–29. doi:10.1006/eesa.2001.2125.

Scott-Fordsmand, J. J., J. M. Weeks, and S. P. Hopkin. 2000. "Importance of Contamination History for Understanding Toxicity of Copper to Earthworm *Eisenia fetida* (Oligochaeta: Annelida), Using Neutral-Red Retention Assay." *Environmental Toxicology and Chemistry* 19 (7): 1774–1780. doi:10.1002/etc.5620190710.

Shoults-Wilson, W. A., B. C. Reinsch, O. V. Tsyusko, P. M. Bertsch, G. V. Lowry, and J. M. Unrine. 2011. "Role of Particle Size and Soil Type in Toxicity of Silver Nanoparticles to Earthworms." *Soil Science Society of America Journal* 75 (2): 365. doi:10.2136/sssaj2010.0127nps.

Singh, S., N. K. Singhal, G. Srivastava, and M. P. Singh. 2010. "Omics in Mechanistic and Predictive Toxicology." *Toxicology Mechanisms and Methods* 20 (7): 355–362. doi:10.3109/15376510903559976.

Song, M. O., J. Y. Li, and J. H. Freedman. 2009. "Physiological and Toxicological Transcriptome Changes in HepG2 Cells Exposed to Copper." *Physiol Genomics* 38 (3): 386–401. doi:10.1152/physiolgenomics.00083.2009.

Unrine, J. M., O. V. Tsyusko, S. E. Hunyadi, J. D. Judy, and P. M. Bertsch. 2010. "Effects of Particle Size on Chemical Speciation and Bioavailability of Copper to Earthworms (*Eisenia fetida*) Exposed to Copper Nanoparticles." *Journal of Environmental Quality* 39 (6): 1942–1953. doi:10.2134/jeq2009.0387.

van den Berghe, P. V. E., D. E. Folmer, H. E. M. Malingré, E. van Beurden, A. E. Klomp, B. van de Sluis, M. Merkx, R. Berger, and L. W. Klomp. 2007. "Human Copper Transporter 2 Is Localized in Late Endosomes and Lysosomes and Facilitates Cellular Copper Uptake." *Biochemical Journal* 407 (1): 49–59. doi:10.1042/BJ20070705.

Westheide, W., and U. Graefe. 1992. "Two New Terrestrial Enchytraeus Species (Oligochaeta, Annelida)." *Journal of Natural History* 26 (3): 479–488.

Wang, Y., Z. Luo, Y. Pan, W. Wang, X. Zhou, L. S. Jeong, Y. Chu, J. Liu, and L. Jia. 2015. "Targeting Protein Neddylation with an NEDD8-Activating Enzyme Inhibitor MLN4924 Induced Apoptosis or Senescence in Human Lymphoma Cells." *Cancer Biology & Therapy* 16 (3): 420–429. doi:10.1080/15384047.2014.1003003.

Wu, J., J. Sun, and Y. Xue. 2010. "Involvement of JNK and P53 Activation in G2/M Cell Cycle Arrest and Apoptosis Induced by Titanium Dioxide Nanoparticles in Neuron Cells." *Toxicology Letters* 199 (3): 269–276. doi:10.1016/j.toxlet.2010.09.009.

Wu, S.-H., and D.-H. Chen. 2004. "Synthesis of High-Concentration Cu Nanoparticles in Aqueous CTAB Solutions." *Journal of Colloid and Interface Science* 273 (1): 165–169. doi:10.1016/j.jcis.2004.01.071.

Yan, C., and D. D. Boyd. 2006. "Histone H3 Acetylation and H3 K4 Methylation Define Distinct Chromatin Regions Permissive for Transgene Expression." *Molecular and Cellular Biology* 26 (17): 6357–6371. doi:10.1128/MCB.00311-06.