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Soil nanotoxicology: effect assessment using biomarkers and gene analysis

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palavras-chave

Nanopartículas de cobre, sais de cobre, biomarcadores de stress, perfil genético, microarrays, *Enchytraeus albidus*

resumo

Actualmente, os nanomaterias são utilizados extensivamente. As suas aplicações incluem a electrónica, consumíveis (p.e. cosméticos), medicina, remediação ambiental e catalisadores. Apesar do aumento na utilização de nanomaterias e consequente despejo no ambiente, os seus efeitos e comportamento são ainda potencialmente desconhecidos. A informação actualmente disponível diz respeito principalmente aos seus efeitos para a saúde humana. Comparativamente, tem sido desenvolvido pouco trabalho acerca do efeito nos sistemas ecológicos, especialmente na avaliação de risco em ecossistemas terrestres, existindo portanto uma necessidade de preencher esta lacuna. De entre as espécies de solo usadas em ecotoxicologia estão os Enquitraídeos (Oligochaeta), membros importantes da fauna do solo, que contribuem para a melhoria da sua estrutura porosa, e indirectamente na degradação da matéria orgânica. Além do facto de existir um protocolo padrão para testar efeitos ao nível da reprodução e sobrevivência (ISO, 2005), recentemente a utilização de ferramentas que estudam efeitos ao nível sub-celular (Howcroft et al., 2008) e dos genes (Amorim et al., 2009) foi optimizada e está disponível, sendo uma vantagem competitiva em relação a outras espécies padrão. O principal objectivo deste estudo foi comparar os efeitos de nanopartículas de cobre com um sal de cobre ($CuCl_2$), em *Enchytaeus albidus*, utilizando a análise da expressão genética, e de biomarcadores de stress e neuronais como parâmetro. Os resultados mostraram que a exposição, tanto a nanopartículas de cobre como aos sais de cobre, causou alterações significativas nos biomarcadores e no perfil de expressão genética. A análise dos biomarcadores mostrou a ocorrência de danos oxidativos, causados por ambas as formas de cobre (aos 8 dias de exposição), e resposta anti-oxidante (GPx, GSH e GSSG) aos 4 dias. Também foi observado um efeito dependente do tempo e da concentração testada entre os tratamentos. No entanto, não houve um biomarcador que permitisse discriminar entre tratamentos. Da análise dos efeitos ao nível dos genes foi possível observar expressão diferencial dos genes devido ao cobre (nano e sal). As principais funções afectadas verificaram-se nos genes relacionados com a transcrição e tradução, metabolismo energético e proteico, e resposta imunológica e de stress. Devido ao número reduzido de homologias com funções conhecidas, é difícil fornecer explicações mais detalhadas.

Dos resultados de ambas as ferramentas utilizadas não existe uma evidência forte de que uma das duas formas de cobre seja mais tóxica que a outra, no entanto, parecem indicar que os dois compostos têm modos de acção diferentes.

keywords

Copper nanoparticles, copper-salt, stress biomarkers, gene expression profile, microarrays, *Enchytraeus albidus*

abstract

Nanomaterials are widely used nowadays. Current applications include electronics, consumer products (e.g. cosmetics), medicine, environmental remediation and catalysts. Despite the increased use of nanomaterials, and consequent disposal in the environment, their behavior and effects are largely unknown. The current available information is mainly focused on the effetc on human health. Comparatively, little work has been done on ecological systems, especially in the assesment of the environmental risk to terrestrial ecossystems and there is a need to fill this gap. Among soil species used in ecotoxicology are the Enchytraeids (Oligochaeta), important members of the soil fauna, contributing to the improvement of the pore structure of the soil and, indirectly, to the degradation of the organic matter. Besides the fact that there is a standard guideline for testing effects at the survival and reproduction level (ISO, 2005), recently, the use of tools that study the effect at the subcelular (Howcroft et al, 2008) and gene level (Amorim et al, 2009) have been optimized and became available, being a competitive advantage in comparison to other standard species. The main goal of this research was to compare the effects of nanosized copper with Cu-salt ($CuCl_2$), in *Enchytareus albidus*, using gene expression, and oxidative stress and neuronal biomarkers as endpoints. The results showed that exposure to nano-Cu and Cu-salt caused significative changes on the biomarkers and the gene expression profile. Biomarkers analysis indicate oxidative damage caused by both forms of copper (at 8 days of exposure), and an anti-oxidative response (GPx, GSH and GSSG) at 4 days. There was also a time and concentration dependent effect between the treatments. Nevertheless, there was not one biomarker that provided the best discrimination. The analysis of effects at gene level showed differently expressed genes due to copper (nano or salt). Main functions affected were in transcription and translation, energy and protein metabolism and immune and stress response related genes. Due to the reduced number of homologies to known functions further explanations are difficult.

From results of both tools used there was no strong evidence of that one of the forms is significantly more toxic than the other, nevertheless these compounds seem to have different modes of action.

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Chapter 1

General Introduction

General Introduction

1. Introduction – Ecotoxicology and the use of molecular sciences

The term ecotoxicology was firstly used by Truhaunt, in 1969, as “the study of adverse effects of chemicals with the aim of protecting natural species and populations” (Twardowska 2004). This young branch of science has started to play a crucial role in providing the key knowledge of sustainability (Twardowska 2004). Since the beginning, ecotoxicology was recognized as an interdisciplinary science, integrating concepts of ecology, chemistry, biology, pharmacology, biochemistry and other disciplines. Nowadays, the main goal is not only the study of the effects, but also the understanding of how chemical stressors might affect organisms at individual, population and ecosystem levels (Hermens et al. 2004). This task becomes complicated by the fact that chemicals do not act in isolation but interact with non-chemical stressors (e.g. UV radiation, changes in temperature and CO_2 concentrations, pathogens, etc.) (Eggen et al. 2004; Hermens et al. 2004). Some decades of environmental management have reduced the concentrations of many well-known pollutants. The most recent challenges in ecotoxicology are related to: low concentrations of pollutants and long exposure periods (chronic effects), multiple effects induced by single compounds, complex mixtures of pollutants, multiple stressors (interaction between chemical and non-chemical stressors), and ecosystem complexity (Eggen et al. 2004). To deal with these challenges, a key element is a focus on a mechanistic understanding of toxic effects as well as interactions among stressors at multiple biological levels of organization (Eggen et al. 2004; Hermens et al. 2004). In this field, molecular approaches can provide a better understanding of underlying processes of ecotoxicology, necessary for this mechanism-based assessment of the risk and effects of stressors (Eggen et al. 2004).

During the past two decades, considerable efforts have been dedicated to develop and applying biomarkers for use in ecotoxicology and ecological risk assessment (Forbes et al. 2006). These efforts have resulted in part for the need of early warning indicators that respond before measurable effects on individuals and

populations occur (effects studied in traditional endpoints such as the impairment on growth, survival and reproduction) and in part as an aid to identifying the causes of effects observed at population and community level (Forbes et al. 2006). According to De Coen and co-authors (2000), biomarkers can be considered as measures of the initial changes (effects) caused by the interactions between the stressor and the (biological) receptor site. This interaction induces cascades of events starting at the sub-cellular level (e.g. disturbance of gene transcription, interference with metabolic pathways) and ultimately leads to adverse effects at higher level of biological organization. On the other hand, and despite biomarkers have higher sensitivity and specificity at molecular and cytological levels, physiological impairment are potentially the most effective markers for assessing pollution as they can serve as precursors to effects in populations or communities, and so, ecologically relevant (Nicholson and Lam 2005). As the targets of protection in ecological risk assessment most often are populations, communities and ecosystems, suites of biomarkers at different functional complexity (e.g. molecular, cytological) coupled with measures of higher levels of biological organization are recommended in monitoring programmes, and the biomarker response must be consistently linked to responses at these higher levels. Because, even if biomarkers were not able to provide reliable predictions of effects at higher levels of biological organization, they may reveal the mechanisms underlying these effects (Forbes et al. 2006). Thus, to provide both an early warning of pollution impacts and environmental relevance, measures of bioindicators (growth or reproductive output or energy utilization) should be associated with the molecular biomarker response (Depledge and Fossi 1994)

2. Test organism

For an adequate risk assessment, the appropriate selection of the test species is crucial. To be considered as a potential indicator organism for the assessment of soil quality, organisms should fulfil several criteria such as: play a key role in the functioning of the soil ecosystem, so that their response is relevant for conclusions on the system level; be present in a wide range of ecosystems, to allow

comparisons between systems; occur abundantly, so that they are widely available and their response is readily recordable; be easy to use both in field and laboratory conditions: they should be easily collectable and maintained in culture; come into contact with a variety of stress factors, via the soil solution, the solid phase, and the gaseous phase in soil; and be sufficiently sensitive to a wide range of environmental stresses but not so sensitive that they easily become extinct (Didden and Rombke 2001). Enchytraeids readily fulfill the first five of these criteria, thus they are useful as ecotoxicological test organisms (Didden and Rombke 2001; Rombke 2003).

Enchytraeids (Enchytraeidae) have been used in ecotoxicological laboratory tests for more than 30 years (Rombke and Moser 2002). The enchytraeids belong to the saprophagous mesofauna of the litter layer and the upper mineral soil. Through their feeding activity the soil assumes a fine-grained “crumb” structure with an often higher stability than that of the bulk soil. Their digging activity (despite more limited compared to most earthworms) may improve the small-scale water and air management of soil, especially when population levels are high (Jänsch et al., 2005). The family Enchytraeidae belongs to the order Oligochaeta (class Clitellata, phylum Annelida). There is about 200-300 species in Central Europe and 900 in worldwide (Jansch et al. 2005). In general, enchytraeids are small worms (1-40 mm), whitish, that reproduce sexually (but asexual fragmentation is also possible). They feed on decomposed plant residues and microorganisms. The pH of soil, the content of organic matter and the soil moisture are factors that influence their occurrence as well as their reaction to stress factors (Rombke and Moser 2002). Enchytraeus albidus is one of the biggest and well-known specie of the genus and. It is acidophobe (optimum pH between 6.8 to 7.0), likes a range of temperature between 15 to 20ºC, prefers 55 to 65% of dry mass (but is able to reproduce in soils with moisture content as high as 90%), and the reproduction is inhibit when the organic matter content is below 3% (Jansch et al. 2005). World-wide it occurs at places with a large amount of organic material, but can also be found at many soil types. Individuals of E. albidus reproduce quickly, can be kept in various substrates and feed with different foods (Rombke and Moser 2002), that make them so easy to keep in culture.

3. Test chemicals: copper (salt and nanosized form)

Copper (Cu) is a trace metal, classified as an essential element for most living organism, since trace amounts of copper serve essential biological functions, including respiration and oxidative stress protection by cytochrome oxidase and Cu, Zn-superoxide dismutase, respectively (Yasokawa et al. 2008). Nevertheless, in high concentrations it can be a very toxic pollutant, including for Enchytraieds (Amorim et al. 2008; Amorim et al. 2005; Maraldo et al. 2006) and earthworms (Scott-Fordsmand et al. 2000). Copper is sometimes detected in soil and water as a heavy metal pollutant close of mines and industrial areas, for e.g., at Hygum site in Denmark, soil copper concentration can reach up to 3,400 mg/Kg due to timber preservation with $CuSO_4$ (Maraldo et al. 2006).

Through the Haber-Weiss reaction, copper ions can catalyze the conversion of hydrogen peroxide (H_2O_2) to the powerful oxidant hydroxyl radical ($OH\cdot$), causing oxidative damage to lipids, DNA and proteins (Bremner 1998).

The recent investigation performed in –omics area allowed the unravel of molecular mechanisms underling copper toxicity (Bundy et al. 2008). Sublethal concentrations of copper caused mitochondrial dysfunction. As a result of this, the amount of energy available from oxidative phosphorylation is reduced resulting in a switch to metabolism of stored carbohydrates. Also, mitochondrial dysfunction leads to an increase in generation of reactive oxygen species (ROS). In higher levels o biological organization, the reduction in body weight was observed (Bundy et al. 2008). Also, genes coding for proteins involved in general response to metals, as metallothioneins and heat shock proteins were also found up-regulated in response to copper exposure (Bundy et al. 2008; Muller et al. 2007).

All these studies were performed with copper in its soluble form. Until the moment, very little is known about copper nanoparticles and their effects in the environment. An overview of the work performed in –nano field is given in the next chapter, including the work done with nano-copper.

4. Objectives

The main aim of this work was to compare the effects of copper nanoparticles with copper salt ($CuCl_2$) on *Enchytraeus albidus* using two different approaches: response of stress and neuro-muscular biomarkers, and gene expression analysis. Therefore, the present thesis is organized in two main chapters (papers) and general conclusion:

- Chapter 1: Introduction to ecotoxicology and use of molecular techniques in this science, test species, test chemicals and objectives of the thesis.
- Chapter 2: Review about the state of the art of the ecotoxicology of nanomaterials.
- Chapter 3: "Cu-Nanoparticles versus Cu-salt: analysis of stress and neuro-muscular biomarkers response in *Enchytraeus albidus* (Oligochaeta)" (Gomes, S.I.L., Novais, S., Gravato, C., Amorim, M.J.B., Guilhermino, L., Soares, A.M.V.M. & Scott-Fordsmand, J.J., in prep.)
- Chapter 4: "Effects of nanoparticles in *Enchytraeus albidus* (Oligochaeta): differential gene expression through microarray analysis" (Gomes, S.I.L, Novais, S., Amorim, M.J.B., Scott-Fordsmand, J.J., De Coen, W. & Soares, A.M.V.M., in prep.)
- Chapter 5: General Conclusions

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Chapter 2

State of the art: ecotoxicology of nanomaterials

State of the art: ecotoxicology of nanomaterials

1. Nanotechnology

Despite their increasing utilization, few formal definitions of nanoparticles have been published. The most often used is the definition of nanotechnology. For example, The Royal Society & The Royal Academy of Engineering (The Royal Society & The Royal Academy of Engineering 2004) defines nanotechnology as "the design characterization, production and application of structures, devices and systems by controlling shape and size at the nanometer scale". The Interagency Subcommittee on Nanoscale Science, Engineering and Technology (NEST) has proposed a similar definition: "Nanotecnhology is the creation and the use of materials, devices and systems through the control of matter on the nanometer-length scale at the level of atoms, molecules and supramolecular structures" (Report of the National Nanotechnology Initiative Workshop 2003). Currently, nanomaterials (NMs) are defined as materials with a size range between 1 and 100 nm, in at least one dimension (Fig 1). More recently, a good working definition of nanomaterials mentions not only their size range but also their exhibition of novel properties (Borm et al. 2006; Royal Commission on Environmental Pollution 2008).

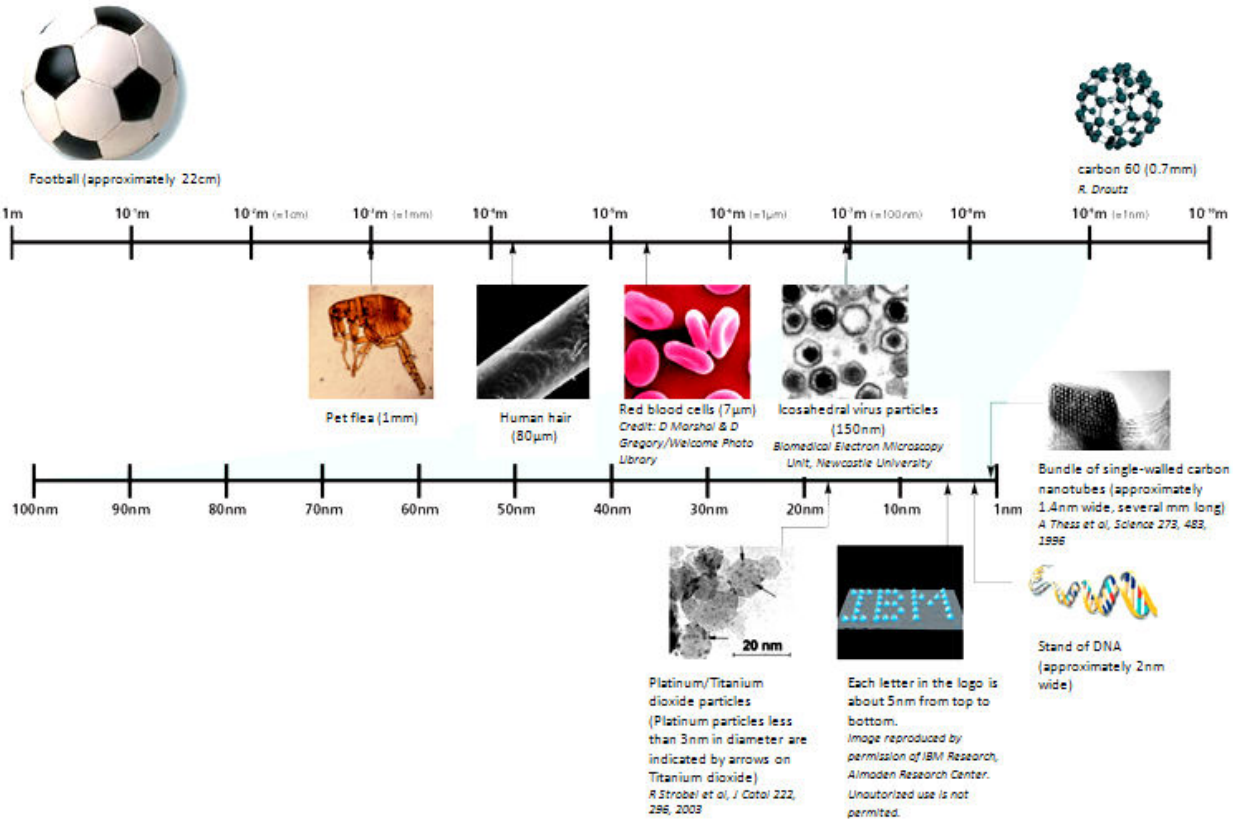

Figure 1: Length scale showing the nanometer in context (The Royal Society & The Royal Academy of Engineering 2004).

Nanomaterials can have one, two or three dimensions in the nanoscale. Materials having only one dimension in the nanoscale are layers such as surface coatings and thin films. Those which are nanoscaled in two dimensions include nanowires and nanotubes. And those which are nanoscaled in three dimensions are usually known as nanoparticles (NPs) and include colloids, quantum dots and nanocrystalline materials (Fig. 2) (The Royal Society & The Royal Academy of Engineering,(The Royal Society & The Royal Academy of Engineering 2004); Royal Commission on Environmental Pollution, (Royal Commission on Environmental Pollution 2008).

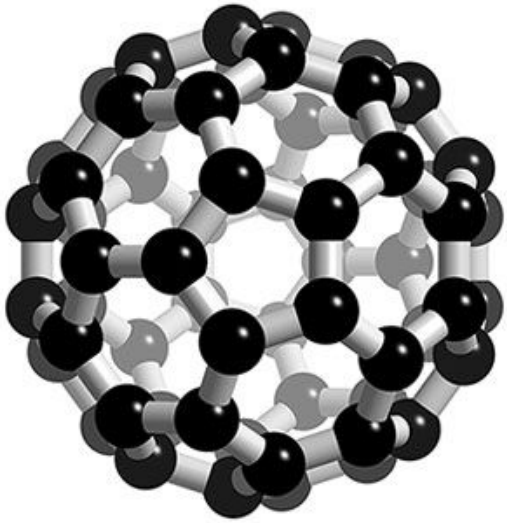

Figure 2: Tridimensional representation of C_{60} Buckminsterfullerene (also known as fullerene). It is a spherical molecule, with 1 nm of diameter, which comprises 60 carbon atoms arranged as the corners of 20 hexagons and 12 pentagons. It is perhaps the best known NP.
http://www.3dchem.com/moremolecules.asp?ID=217&othername=Buckminsterfullerene

2. Applications of nanomaterials

Nanomaterials are widely used in skin care products (titanium dioxide, iron oxide and other metal oxides); in structural applications such as coatings (titanium dioxide), catalysts and ceramics (silicon nitride and silicon carbide); in electronics (single walled nanotubes and metal oxides); in biotechnology on targeted drug delivery and biosensors; and in environmental remediation (Royal Comission on Environmental Pollution 2008; The Royal Society & The Royal Academy of Engineering 2004). At present, more than 800 nanotechnology-based consumer products are available in the market (Fig. 3) and the most common NMs used in consumer products are silver, carbon (which include fullerens), zinc (including zinc oxide), silica, titanium (including titanium dioxide) and gold (The Project on Emerging Technologies, 2009).

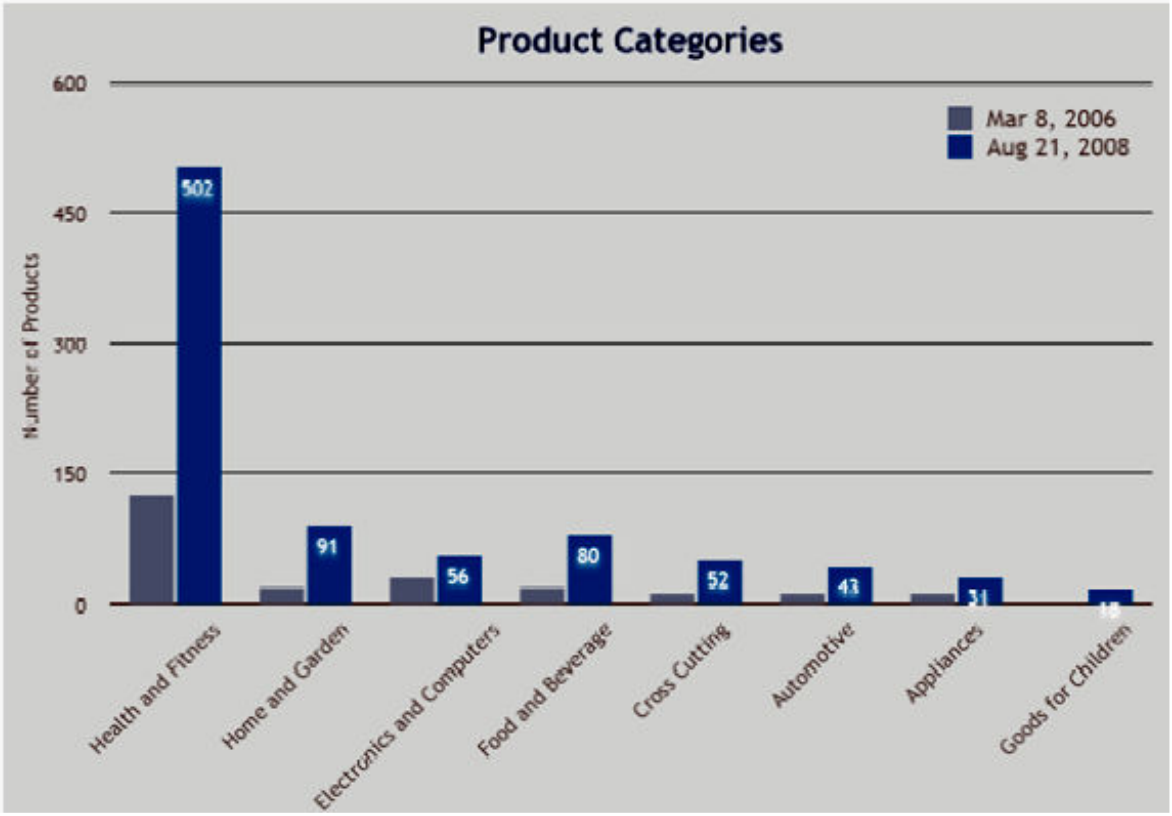

Figure 3: Number of consumer products, available in March 2008, containing nanomaterials. The products are grouped in categories (Health and Fitness, Home and Garden, Electronics and Computers, Food and Beverage, Cross Cutting, Automotive, Appliances and Goods for Children).
http://www.nanotechproject.org/inventories/consumer/analysis_draft/

Despite copper nanoparticles (Cu NPs) not being included in the most commonly used NMs (Fig. 4), they are being given considerable attention due to their interesting properties (e.g. high electrical and thermal conductivities) and potential applications in many areas of industry. Nowadays, metallic Cu NPs are used in modern electronic circuits (e.g. chip-package interconnections (Tummala et al. 2006) and conductive inks for printing electronic components (Lee et al. 2008b)), metallurgy, catalysis (Dhas et al. 1998; Samim et al. 2007) and sensors (Athanassiou et al. 2006).

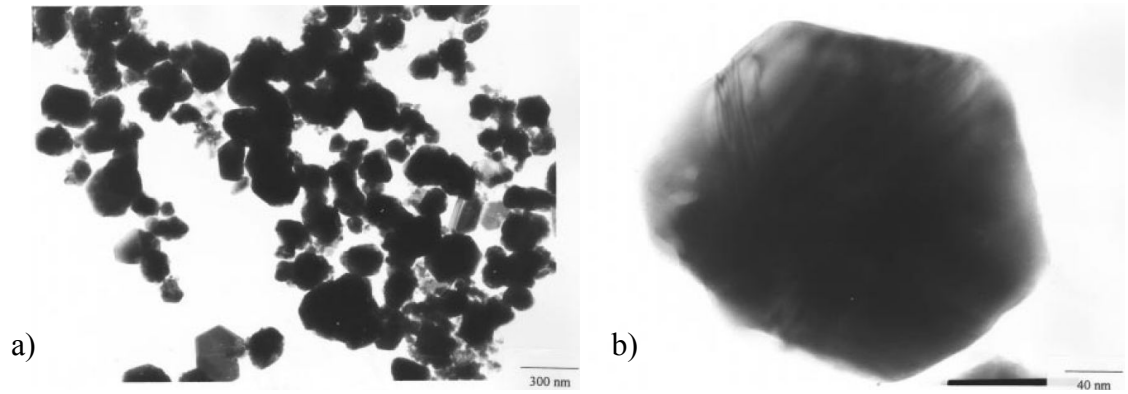

Figure 4: TEM micrograph of copper nanoparticles at two different magnifications: (a) bar = 300 nm, (b) bar = 40 nm (Dhas et al., 2008).

It is believed that NMs utilization will dramatically increase in the next few years (The Royal Society & The Royal Academy of Engineering 2004). Such increase in utilization will probably result in the entry of nonmaterial's wastes in the environment, which is scientist's main concern, because their effects and environmental behavior are largely unknown. In this context it appears the definition of Nanotoxicology as "the science of engineered nanodevices and nanostructures that deals with their effects in living organisms" (Oberdorster et al. 2005).

3. Novel properties and entry in the environment

Their small size and consequent larger surface area confer specific properties to nanomaterials. Increasing of surface reactivity predicts that NMs will exhibit greater biological activity per given mass comparatively to the respective bulk materials and that the interaction of nanoparticles with cells and their structures are likely to be very different from those of larger-sized particles (Oberdorster et al. 2005). With a reduction in size, several properties can be dramatically changed, for e.g. electrical conductivity, magnetic characteristics, hardness and, as mentioned above, active surface area, chemical reactivity and biological activity (Nel et al. 2006). The surface charge is an other characteristic that can change at the nanoscale. It is important because it determines the solubility and aggregation of nanomaterials which are key factors in determination of their fate and behavior in the environment (Royal Commission on Environmental Pollution 2008). These

properties have been explored for the different uses of nanomaterials, but can also become unwanted because of the possibility of causing toxic effects

Manufactured nanomaterials can reach the environment trough intentional releases (e.g. use of nanoparticles to remediate contaminated soil) as well as unintentional releases such as production facilities effluents and atmospheric emissions (Klaine et al. 2008; Oberdorster et al. 2005). Humans' uptake of nanoparticles can follow three main routes: inhalation (respiratory tract), ingestion (digestive tract) and dermal absorption (skin) (Oberdorster et al. 2005). Their uptake by organisms in the environment depends on a variety of processes that modify nanomaterials properties (e.g. aggregation, morphology and degradation) (Klaine et al. 2008; Royal Commission on Environmental Pollution 2008). Also, the different environmental matrices, such as air, soil/sediments, freshwater and seawater are very complex, leading to an increased difficulty in predicting NMs behaviour and toxicity (Handy et al. 2008). In figure 5 the routes of exposure, uptake, distribution and degradation of NMs in the environment are summarized.

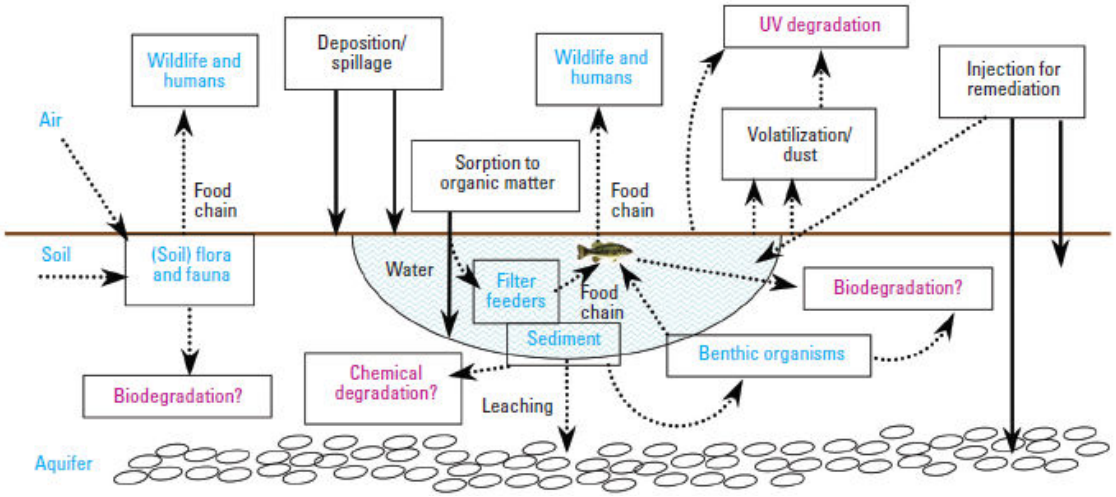

Figure 5: Routes of exposure, uptake, distribution, and degradation of NPs in the environment. Solid lines indicate routes that have been demonstrated in the laboratory or field or that are currently in use (remediation). Magenta lettering indicates possible degradation routes, and blue lettering indicates possible sinks and sources of NPs (Oberdorster et al. 2005).

4. Ecotoxicology of nanomaterials: state of the art

So far, most of the nanotoxicology studies are focused on human health (e.g. (Handy and Shaw 2007; Kreyling et al. 2006; Lewinski et al. 2008), especially on the effects of nanoparticles to respiratory system (e.g. (Borm and Kreyling 2004; Donaldson). Among the few published ecotoxicological studies with NMs or NPs, nearly all of these have been performed in organisms from the aquatic compartment, such as daphnids, zebrafish and algae. These studies refer to acute and chronic effects of several nanoparticles e.g. fullerenes (Lovern and Klaper 2006; Lovern et al. 2007; Oberdorster 2004; Zhu et al. 2006), TiO_2 and other metal oxides (Heinlaan et al. 2008; Hund-Rinke and Simon 2006; Lovern and Klaper 2006; Lovern et al. 2007; Velzeboer et al. 2008). In table 1 we present a brief review on ecotoxicology studies made with nanoparticles, after which a brief explanation is given with more details.

Table 1: Summary of literature review of toxicity studies with nanoparticles, indicating the nanoparticles, type of treatment, organism, dose or dose range and the measured parameters.

Reference	Nanoparticle	Treatment	Target Organism	Dose	Parametre
(Oberdorster 2004)	fullerenes (C_{60})	THF method	juvenile largemouth bass	0.5 and 1.0 ppm	↑ LPO in the brain at 0.5 ppm; no changes in protein oxidation or GSH levels in any tissue (brain, liver and gill)
(Fortner et al. 2005)	C_{60}	THF method	Bacteria (Gram + and -) in water	0,04 mg/L and ≥0,4mg/L	The first concentration did no affect significantly the grow of bacteria; the second one inhibit completely bacteria grow
(Hund-Rinke and Simon 2006)	Titanium dioxide(TiO_2) (in product 1 and 2)	photocatalytic activity induced by pre-illumination	algae	3,1; 6,25; 12,5; 25 and 50 mg/L of product1 (25nm) and product2 (100nm)	↓ of growth; EC_{50}= 40mg/L (for the first product)
			Daphnia magna	1; 1,5; 2; 2,5 and 3 mg/L of product1 (25nm) and product2 (100nm)	↓movements

Reference	Nanoparticle	Treatment	Target Organism	Dose	Parametre
(Kashiwada 2006)	Fluorescent polystyrene microspheres (latex) (39,4; 474; 932; 18600 and 42000 nm)		Medaka (*Oryzias latipes)*	1, 10 and 30 mg/L (depends on the test performed)	Fluorescence in all eggs (chorion and oil droplets). The fluorescence was the highest for the 474 nm nanoparticles. Larvae exhibit fluorescence in the yolk and gallbladder. The nanoparticles concentration ↓ with ↑ in salinity, which ↑ eggs mortality. In adults, fluorescence observed in gills, kidney, liver, intestine, gonads, brain and blood.
(Lovern and Klaper 2006)	C_{60}	filtered in THF	*D. magna*	40, 180, 260, 350, 440, 510, 700 and 880 ppb	LC_{50}= 460ppb; LOEC= 260ppb; NOEC= 180ppb; LC_{100}= 880ppb
	C_{60}	sonicated		0,2; 0,45; 0,9; 2,25; 4,5; 5,4; 7,2 and 9 ppm	LC_{50}= 7,9ppm; LOEC= 0,45ppm; NOEC= 0,18ppm
	TiO_2	filtered in THF		0,2; 1; 2; 5; 6; 8 and 10 ppm	LC_{50}= 5,5 ppm; LOEC= 2,0ppm; NOEC= 1,0ppm; LC_{100}= 10ppm
	TiO_2	sonicated		50, 200, 250, 300, 400 and 500 ppm	survival was not significantly affected
(Lyon et al. 2006)	C_{60}	4 methods of water suspension: THF/C60; son/C60; aq/C60 and PVC/C60	*Bacillus subtilis* (bacteria)		All FWS (fullerene water suspensions) displayed antibacterial activity, being THF/C60 the most potent. For all FSW except PVC/C60 (no different sizes separated), the smaller fractions exhibit more antibacterial activity than the larger ones.
(Oberdorster et al. 2006)	C_{60}	water-stirred method	*D. magna*	2,5; 5 and 35 ppm	2,5 and 5 ppm of nC60 (during 21 days) decrease the number of offspring; 35ppm (in fresh water) increase the mortality, but never more than 50%
			Hyalella	7 ppm	no toxic effects were observed
			Fathead Minnows	0,5 ppm	PMP70 (protein) was down regulated
			Medaka	0,5 ppm	no toxic effects were observed

Reference	Nanoparticle	Treatment	Target Organism	Dose	Parametre
(Templeton et al. 2006)	Single-walled carbon nanotubes (SWCNTs), and small fluorescent nanocarbon byproducts	Produced by arc-discharge method and purified	*Amphiascus tenuiremes* (estuarine meiobentic copepod)	0,58; 0,97;1,6 and 10 mg/L of	10 mg/L of SWCNTs caused ↑ life cycle mortality, ↓ fertilization rate and ↓number of viable offspring. Purified SWCNTs showed no effects on parameters tested. Small fluorescent nanocarbon byproducts exposure caused ↑ life cycle mortality, ↓ fertilization rate and ↓number of viable offspring for all the concentrations tested.
(Zhu et al. 2006)	C_{60}	THF method	*D. magna*	5, 10, 25, 100, 500, 1000 and 2000 ppb	LC_{50}≈ 0,8ppm (800ppb)
		water-stirred method		0,5; 1; 2,5; 5; 35ppm	LC_{50}> 35ppm
		THF method	FHM (fatead minnow)	0,5 ppm	all die before 18 hours of exposition
		water-stirred method		0,5 ppm	induction of CYP2 hepatic isozymes; ↑ LPO(lipid peroxidation)
(Cheng et al. 2007)	carbon black nanotubes (CNTs)	water-stirred method	zebrafish embryos	120 and 240 mg/L	No effects on hatching, embryonic development or survival.
	SWCNTs				↓ hatching. No effects on embryonic development or survival.
	double-walled carbon nanotubes (DWCNTs)				↓ hatching (shorter in comparison with caused by SWCNTs). No effects on embryonic development or survival.
(Federici et al. 2007)	TiO_2	sonication in water	rainbow trout	0.1, 0.5 and 1.0 mg/L	↑ incidence of odema and other gill injuries; ↓Na^+K^+-ATPase activity in the intestine at the lowest concentration; ↑ TBARS in the gill; ↑ TG in the gill and ↓ in the liver. No mortality or accumulation on internal organs was observed.

Reference	Nanoparticle	Treatment	Target Organism	Dose	Parametre
(Griffitt et al. 2007)	copper		zebrafish	0,25 and 1,5 mg/L	LC_{50}= 1,56mg/L; inhibition of Na^+/K^+ ATPase activity in gills; induction of HIF-1, HSP70 and CTR genes
(Lin and Xing 2007)	multi-walled carbon nanotubes (MWCNTs)	dispersed by ultrasonic vibration on water	*Brassica napus, Raphanus sativus, Lolium perenne, Lactuca sativa, Zea mays e Cucumis sativus*	2000 mg/L	no effects on seed germination or root growth
	Al_2O_3				↓ root growth only for Zea mays
	Al				↑ root growth of Brassica napus and Raphanus sativus; ↓root growth for Lolium perenne and Lactuca sativa
	ZnO				↓root growth of Zea mays. For the other treated plants no root growth was observed.
	Zn				↓root growth of Lolium perenne. For the other treated plants no root growth was observed.
(Lovern et al. 2007)	TiO2	THF method	*D. magna*	2,0ppm	hopping frequency, feeding appendage, post abdominal curling movement and heart rate did not suffer significant alterations
	C_{60}			260ppb	significant ↑ of hopping frequency, feeding appendage and heart rate, and no significant of post abdominal curling movement
	$C_{60}H_xC_{70}H_x$			260ppb	significant increase of hopping frequency and feeding appendage and no significant of post abdominal curling movement; no significant decrease of heart rate

Reference	Nanoparticle	Treatment	Target Organism	Dose	Parametre
(Roberts et al. 2007)	Lipid-coated carbon nanotubes	SWCNTs dissolved in water using lysophophadity lcholine (LPC) coating	*D. magna*	O; 2,5; 10 and 20 mg/L of SWCNTs	Mortality of 20 and 100% at 10 and 20 mg/L, respectively. Black precipitates in test vessels and adhering to the external surface of daphnids. *D. magna* decreases the solubility of LPC-SWCNTs due to its ingestion, using LPC as food and excreting SWCNTs.
(Tong et al. 2007)	C_{60}	THF method	soil bacteria (Gram+ and -)	1µg/g	there was no significant alterations in respiration levels
		granular form		1000µg/g	there was no significant alterations in respiration levels
(Zhang et al. 2007)	TiO_2	In cadmium (Cd) contaminated water	*Cyprius carpio* (carp)	10 mg/L of TiO2 (in 10-100 µg/L of Cd)	Nano TiO2 has a stronger adsorption capability for Cd than natural sediment particles (SP). Adsorption caused ↓ in Cd concentration. Carp exposed to Cd+TiO2 accumulated more Cd than when exposed at Cd or Cd+SP, in viscera, gills, skin and scales and muscle.
(Asharani et al. 2008)	Ag	capped with BSA and with starch	zebrafish embryos	5, 10, 25, 50 and 100 µg/ml	↑mortality rate (LC_{50} =25-50 µg/ml), ↓hearth rate and ↓hatching; severe phenotypic changes (bent and twisted nothocord, accumulation of blood near the tail, pericardial edema and degradation of body parts)

Reference	Nanoparticle	Treatment	Target Organism	Dose	Parametre
(Baun et al. 2008)	C_{60}	Water stirred and mixed with: atrazine, methyl parathion, pentachlophenol and phenanthrene	*Pseudokirchneriella subcapitata* (algae) and *D. magna* (crustacean)		Phenanthrene showed significant sorption to C_{60} aggregates whereas the other model compounds stayed in the aqueous phase. C_{60} adsorb to daphnids and algae. When phenanthrene was mixed with C_{60}, there was ↑ toxicity in algae test (↓ of EC_{50} value from 720 to 430 µg/L without and with C_{60}, respectively); and ↓ toxicity in daphnids test (↑ of EC_{50} value of 500 to 680 µg/L without and with C_{60}, respectively). A non-significant ↑ in antrazine toxicity in test with algae was observed, with addition of C_{60}. ↓ in toxicity of pentachlorophenol in the presence of C_{60}. No changes in toxicity of methyl parathion.
(Canesi et al. 2008)	carbon black (NCB)	sonication in water	blue mussels hemocytes	1.5 and 10 µg/ml	NCB aggregates accumulate into hemocytes, stimulate the release of lysossomal hydrolotic enzymes and induce phosphorylation of different stress-activated MAPK members.
(Heinlaan et al. 2008)	bulk TiO_2 and nano-TiO_2	sonication in water	*Vibrio fischeri* (bacteria) and *Thamnocephalus platyurus* (crustacean)	0-20000 mg/L	TiO_2 EC_{50}>20000mg/L and nano-TiO_2 EC_{50}>20000mg/ for both specie tested
	bulk ZnO, nano-ZnO and ionic Zn ($ZnSO_4.7H_2O$)		*V. fischeri* (bacteria), *T. platyurus* and *D. magna* (crustaceans)	0-100000 mg/L	ZnO, nano-ZnO and $ZnSO_4.7H_2O$ EC_{50} are, to *V. fischeri*:1.8, 1.9 and 1.1 mg/L, respectively. To *T. platyurus* 0.24, 0.18 and 0.98 mg/L, respectively. And to *D. magna* 8.8, 3.2 and 6.1, respectively.
	bulk CuO, nano-CuO and ionic Cu ($CuSO_4$)		*V. fischeri* (bacteria), *T. platyurus* and *D. magna* (crustaceans)	0-100000 mg/L	CuO, nano-CuO and $CuSO_4$ EC_{50} are, to *V. fischeri*: 3811, 79 and 1.6 mg/L, respectively. To *T. platyurus* 94.5, 2.1 and 0.11 mg/L, respectively. And to *D. magna* 164.8, 3.2 and 0.17, respectively.
(Johansen et al. 2008)	C_{60} (50 nm to µm-size)	water-stirred method applied to soil	bacteria and protozoans	0, 5, 25, and 50 mg/Kg	↓number of bacteria only immediately after incorporation of C_{60}, after 23 days of incubation the differences were decreased; protozoans were not affected

Reference	Nanoparticle	Treatment	Target Organism	Dose	Parametre
(Lee et al. 2008a)	copper	exposure in agar culture media	*Phaseolus radiatus* and *Triticum aestivum*	0, 200, 400, 600, 800 and 1000 mg/L during 2 days	↓seedling growth for both plants (*P. radiatus* was more sensitive); bioaccumulation was observed
(Velzeboer et al. 2008)	TiO_2, ZrO_2, Al_2O_3, CeO_2, C_{60} , SWCNTs, PMMA (0.06; 0.41 and 1.08 µm)	Microtox test	Bioluminescent bacteria *V. fischeri*	1, 10 and 100 mg/L of TiO_2, ZrO_2, Al_2O_3 and CeO_2; 1 and 100 mg/L of C_{60} and PMMA; unknown concentratation of SWCNTs	EC_{50} (15 min.) >100 mg/L for TiO_2, ZrO_2, $Al2O_3$, CeO_2 and for different sizes of PMMA; >1 mg/L for C_{60}; and no effect observed for SWCNTs
	TiO_2, ZrO_2, Al_2O_3, CeO_2, C_{60} , SWCNTs, PMMA (0.06; 0.41 and 1.08 µm)	PAM test	*Pseudokirchneriella subcapitata* (green algae)	100 mg/L for all the nanoparticles	EC_{50} (4.5h) > 100 mg/L for all the nanoparticles tested
	TiO_2, Al_2O_3, CeO_2, PMMA (0.06; 0.41 and 1.08 µm)	Chydotox test	*Chydorus sphaericus*(bentic cladoceran)	100 mg/L for all the nanoparticles	LC_{50} (48h) > 100 mg/L for all the nanoparticles tested
	TiO_2	Biolog test	mix of soil bacteria	100 mg/L	EC_{50} (7days)> 100 mg/L
(Zhu et al. 2008)	C_{60} aggregates(average diamaeter of 349 and/or 1349 nm)	water-stirred method	*Carassius auratus* (juvenile carp)	0.04, 0.20 and 1.0 mg/L	Attending on growth inhibition: 0.20 mg/L caused ↓lenght; 1.0 mg/L caused ↓lenght and ↓body weight. Attending on oxidative stress response: ↑SOD in the liver for all the concentrations; ↑ CAT on gill and liver for all the concentrations; ↓GSH on brain, gill and liver for all the concentrations; ↓LPO on brain, gill and liver (except on 1.0 mg/L were increases in the liver)

The generation of reactive oxygen species (ROS) and cell internalization are reported as causes of citotoxicity of NPs (Lewinski et al. 2008). Some ecotoxicological studies reported the induction of oxidative stress after exposure to fullerenes (Oberdorster 2004; Zhu et al. 2006; Zhu et al. 2008), TiO_2 (Federici et al. 2007) and carbon black NPs in fish species (Canesi et al. 2008). In the study performed by Zhu and co-authors (2008) the levels of both SOD and CAT activities (the primary defense against ROS) were significantly induced in the liver

of juvenile carp for all the concentrations of C_{60} tested (0.04, 0.2 and 1 mg/L). In gill and brain tissues, the other organs tested, only CAT was induced, suggesting that the liver is the target or the most susceptible organ to the C_{60} exposure. However, studies performed with different nanoparticles and different test species (TiO_2 in rainbow trout and Cu in zebra fish) reported the gill as the primary organ affected by NPs exposure (Federici et al. 2007; Griffitt et al. 2007). In a study with blue mussels' hemocytes (Canesi et al. 2008) it was showed that carbon black NPs (NCB) entered into hemocytes in a dose-dependent way, and caused oxidative stress to the cells (stimulating the release of lysossomal hydrolytic enzymes, oxidative burst and nitric oxide production) and inflammatory response (mediated by activation of stress-activated MAPK members). This is the only ecotoxicological study that simultaneously reports cell internalization and oxidative damage after NMs exposure. However, investigation is needed to allow the correlation between the modulation of antioxidant enzymes and physiological responses (e.g. survival, growth and reproduction).

Some other studies reported the assimilation and/or bioaccumulation of NMs by exposed organisms. Kashiwada (2006) results showed that fluorescent polystyrene microspheres were visible in exposed eggs of medaka, and persist in the larvae spawned from exposed eggs. The accumulation was size dependent and higher for particles with 474 nm (when testing sizes of 39.4, 474, 932, 18600 and 42000 nm). An other study performed by Asharani and co-authors (2008) showed that silver (Ag) NPs (capped with BSA or starch), with a size ranging between 5-20 nm, entered in the chorion of zebrafish embryos and were toxic in a dose-dependent way. Ag NPs exposure also caused a decrease in heart rate and hatching as well as severe malformations. Cheng and co-authors (2007) have studied the effects of carbon nanotubes in zebrafish embryos. According to their results no effects on mortality or embryonic development occurred, whereas a hatching delay was observed. They also showed that chorion forms a barrier protecting zebrafish embryos from the aggregates of single-walled carbon nanotubes (in the range of 10 μm^2) which were to large to enter the chorion.

Also in aquatic ecotoxicology, the toxicity of NMs has been related to their action as vectors for the transport of other chemicals. Zhang and co-authors (2007)

results showed that TiO_2 NPs adsorb to cadmium (Cd) increasing its accumulation in carp. Nano-TiO_2 facilitated the transport of Cd ions in a positive correlation between Cd and TiO_2 concentrations. As showed by Baun and co-authors (2008) C_{60} easily adsorb to phenanthrene (85%) while only 10% of atrazine, methyl-parathion and pentachlorophenol adsorb to C_{60}. The toxicity of phenanthrene to algae was increased following sorption to C_{60} aggregates, but decreased in daphnids. The toxicity of pentachlorophenol decreases in both organisms tested with the addition of C_{60}. This study shows that the interaction between NMs/NPs and other chemicals, which will eventually occur in natural environments, should be taken into account in the nano-ecotoxicology research.

The effects of C_{60} at lethal and sublethal levels are relatively well studied in bacteria. Thong and co-authors (2007) results showed that the introduction of C_{60} has no significant impact in soil microbial communities. No significant differences could be observed between controls and treated samples in terms of soil respiration, microbial biomass and soil enzymatic activities. Johansen and co-authors (2008) results showed that 0.5, 25 or 50 mg/Kg of soil of C_{60} only inhibit the growth of fast-growing bacteria, which have recovered until the end of exposure (23 days). Studies on microbial effects have also been performed in water with C_{60} (Fortner et al. 2005). Gram-positive and Gram-negative bacteria were exposed to 0,04mg/L and 4mg/L of C_{60}: no effect on bacterial growth was observed at the smaller concentration of C_{60} and there was a complete inhibition of growth in the highest concentration. These studies indicate that different exposure media (water or soil) have an important role in ecotoxicity of NPs. Lyon and co-authors (2006) have studied the effects of C_{60} water suspensions, on *Bacillus subtilis*, prepared by four different methods. Their results showed that despite all the suspensions possessed antibacterial activity, some of them were more potent than others, which is in accordance with other studies showing that different methods of NMs/NPs preparation could lead to differences in toxicity (Lovern and Klaper 2006; Zhu et al. 2006).

Heinlaan and co-authors (2008) performed a study comparing the effects of several sizes (bulk, nano and ionic) of different particles (CuO, ZnO and TiO_2) to the bacteria *Vibrio fischeri* and the crustaceans *Thamnocephalus platyurus* and *D.*

magna. Their results showed that there were no pronounced differences between the tree forms of Zn and TiO2 was not toxic. In the Cu group, different forms of the material caused different effects (to e.g. *V. fischeri* EC_{50} of 1.6 mg/L to $CuSO_4$, 76 mg/L to nano CuO and 3811 mg/L to CuO).

Some studies were focused on nanosized copper. Results from Griffit and co-authors (2007) showed that the gill was the primary target and no evidence for damage to other organs was observed. Exposure to nanocopper caused inhibition of Na^+/K^+ ATPase activity and a slight increase of plasma urea nitrogen (BUN) levels. Acute toxicity evaluation results showed that copper sulfate (soluble copper) was more toxic than nanocopper with a 48h EC_{50} of 0,25 mg Cu/L. Microarray results showed that 48h exposure to sub lethal concentrations of nanocopper resulted in 82 genes significantly differently expressed and that the transcriptional response induced by nanocopper was divergent from the one that was generated by the soluble copper, which means that the effects caused by nanocopper are not only due to solubility. The only knew study about the toxicity of copper NPs to terrestrial organisms was performed in plants (*Phaseolus radiatus* and *Triticum aestivum*), but in agar media instead of soil, by Lee and co-authors (2008a). Their results showed that exposure to Cu NPs caused a reduction in seedling growth of both test species, although *P. radiatus* was more sensitive with an EC_{50} value of 335 mg/L compared to 570 mg/L of *T. aestivum*. Bioaccumulation of nano Cu was observed in both plants (in the cytoplasm and cell wall of the root cells) and it was concentration dependent. To ensure that the toxic effect caused was the result of Cu NPs and not due to their solubility, the amount of cupric ions released during the preparation of Cu NPs was measured. The results showed that 1 mg/L of Cu nanoparticles released 0.3 mg/L of cupric ions, which was subsequently proved as non toxic to the test plants.

Despite the fact of not being mentioned in the table above, because they are about toxicology (to humans) and not ecotoxicology, there are some interesting studies reporting the effects of nano-Cu. Chen and co-authors (2006) and Meng and co-authors (2007) performed studies with mice. The results of the first study indicated that nanocopper (such as ion copper particles) is moderately toxic with 48h EC_{50} of 414 mg/kg body weight and micro-copper is nearly non-toxic. Pathological

examinations revealed that kidney, liver and spleen are target organs for Cu NPs, suffering serious injuries also demonstrated by measurements of the blood biochemical indexes (BUN, Cr, TBA and ALP). Both studies explain the toxicity of nanocopper as a result of massive formation of copper ions, consumption of H^+ and formation of HCO^{3-}. Nanocopper reacts with hydrogen ions of gastric juice resulting in formation of Cu ions and HCO^{3-} (leading to the alkanescence of the medium). The overload of copper ions causes damage on the hepatic cells and the alkanescence of the medium reduces the solubility of copper salts that is deposited in the kidney. An other study (Karlsson et al. 2008) compares the toxicity of several metal oxide nanoparticles (including CuO) and carbon nanotubes to human lung epithelial cells. Their results showed that between CuO, TiO_2, ZnO, $CuZnFe_2O_4$, Fe_2O_3, Fe_3O_4, carbon and nanotubes nanoparticles, the nano CuO (which was not the smaller NP) was the one that caused more damage in all the parameters evaluated, which includes percentage of non-viable cells, oxidative DNA lesions, DNA damage and intracellular ROS. It was also compared the effect of CuO NPs and Cu ions ($CuCl_2$) in the same cell line and the results showed that nano CuO was much more toxic than $CuCl_2$. The most important finding in this study was the high toxicity and ability of nano CuO to cause DNA damage and oxidative stress, because Cu NPs are widely used in several industries and their toxicity has not been sufficiently investigated.

5. Knowledge gaps and future perspectives

In the current literature available, the reported effect concentrations are often very different, not only between laboratories using similar tests and organisms, but also depending on the method used to prepare the NMs for testing. On the other hand, based on the current understanding, the traditional methods used to assess the chemical toxicity are a good starting point for NMs/NPs testing. However new test strategies need to be delineated to lead with the novel toxicity mechanisms that can emerge from NMs/NPs (Nel et al. 2006). To adequately manage NMs in the environment, quantitative measures of both exposure and effects are needed. To enable the progression of the study about the NMs/NPs behavior, fate and effects

in the environment, Klaine and co-authors (Klaine et al. 2008) recommended the harmonization of several issues:

. availability of standardized and characterized materials to researchers;

. development of standardized methods to the creation of test media (both soil/sediment or water) for conducting fate and effects testing;

. standardization of characterization requirements for particles and particle suspensions.

That will give scientists the minimum information requirements to interpret their own and others results. Also needed is the development of high-sensitive techniques capable of the measurement of NMs in the environment (against a high background of naturally-occurring materials) to allow the testing of realistic exposure scenarios, including relevant test species (Royal Comission on Environmental Pollution 2008). Figure 6 schematizes the key challenges and knowledge gaps in the ecotoxicology of NMs that must be fulfilled to perform a correct environmental risk assessment and monitoring.

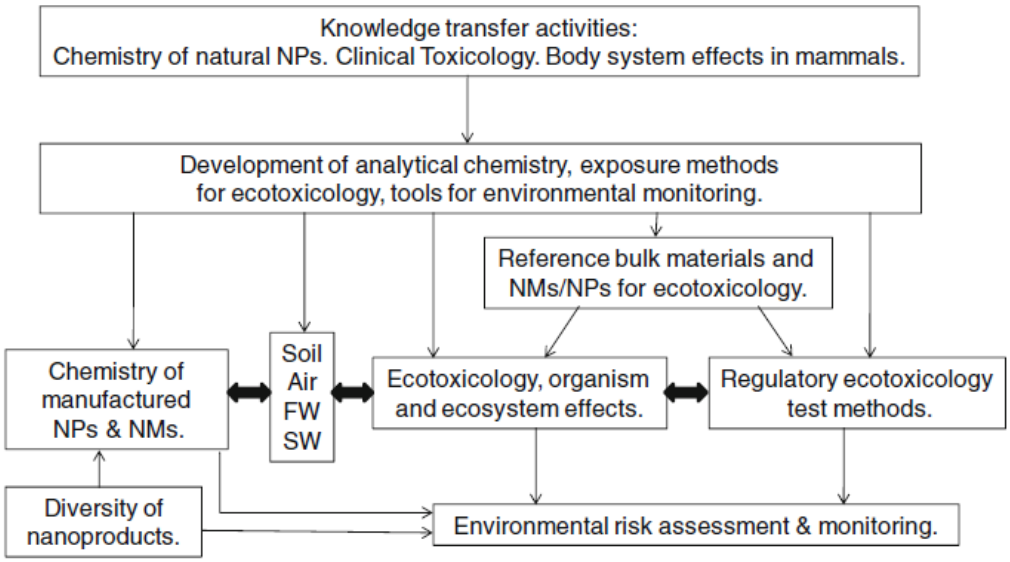

Figure 6: Key challenges and knowledge gaps in the ecotoxicology of NPs and NMs. At this early stage, some uncertainty in data interpretation seems likely given the need for method development in both the ecotoxicology and chemistry. Investigation is needed about the behaviour of NMs in natural matrices (soil, air, freshwater (FW) and seawater (SW)). Also, the large variety of nanoproducts, and lack of knowledge on adverse effects on a wide variety of wildlife, adds uncertainty about what products to prioritise for risk

assess first; and whether or not the concept of protecting ‘‘most of the organisms most of the time’’ is achievable in the immediate future (Handy et al. 2008).

Despite all the recent investigation performed in order to demonstrate the possible effects of NMs, the truth is that the largest fraction refers to the human concern. Although most people are concerned with effects on large wildlife (particularly the human being), the basis of many food chains depends on the benthic and soil flora and fauna, which could be dramatically affected by NMs release in the environment (Oberdorster et al. 2005). It is generally accepted that research is needed in water and soil ecosystems, but since the ultimate sink of NMs may be sediment and soil it is of outmost importance to study the effects on terrestrial environment and organisms (Klaine et al. 2008).

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Chapter 3

Effect of Cu-Nanoparticles versus Cu-salt: analysis of stress and neuro-muscular biomarkers response in *Enchytraeus albidus* (Oligochaeta)

Effect of Cu-Nanoparticles versus Cu-salt: analysis of stress and neuro-muscular biomarkers response in *Enchytraeus albidus* (Oligochaeta)

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Abstract

Nanotechnology is an emergent discipline with applications in science, technology and medicine. Despite the increased use of nanomaterials, their behaviour and effects in the environment are largely unknown. The information currently available is mostly focused on effects on human health and comparatively very little is known in ecological systems, terrestrial in particular. Enchytraeids (Oligochaeta) are important members of the soil fauna that indirectly contribute to the regulation and degradation of organic matter. These organisms are used in standard testing (ISO 2003) and the measurement of several biomarkers has currently been optimized for this species (Howcroft et al. 2009). In the present study, the main goal was to compare the effects of ionic copper versus copper nanoparticles in *Enchytraeus albidus* assessing the effect at the biomarker level, testing different concentrations and exposure time. Measured parameters were lipid peroxidation (LPO), total, reduced and oxidized glutathione content (TG, GSH and GSSG), the enzymatic activity of superoxide dismutase (SOD), catalase (CAT), glutathione reductase (GR), glutathione peroxidase (GPx), glutathione-S-transferase (GST) and cholinesterases (ChEs). Results showed that both salt- and nano-copper caused oxidative stress and damage to *E. albidus*, and effects could be discriminated between the copper forms. Nevertheless and despite the visible discrimination between nano and the salt form (time and exposure dependent), there was not one or a set of biomarkers that provided the best discrimination.

Keywords: oxidative stress, enzymatic and non-enzymatic antioxidants, copper nanoparticles, copper, Enchytraeids

1. Introduction

Copper nanoparticles (Cu NPs) are being given considerable attention due to their properties e.g. high electrical and thermic conductivities. Cu NPs are used in modern electronic circuits e.g. chip-package interconnections (Tummala et al. 2006) and conductive inks for printing electronic components (Lee et al. 2008b), metallurgy, catalysis (Dhas et al. 1998; Samim et al. 2007) and sensors (Athanassiou et al. 2006)). Increased use of nanomaterials will lead to their possible distribution in the environment, including the potential to contaminate soil, migrate into surface and groundwater, and interact with biota (Klaine et al. 2008). Despite the increased interest and recent investigation in this area, very little is known about the environmental effects and behaviour of nanoparticles especially for terrestrial ecosystems (Klaine et al. 2008).

The available information about the ecotoxicology of nanoparticles includes mainly studies in organisms of the aquatic compartment (Fortner et al. 2005; Griffitt et al. 2007; Heinlaan et al. 2008; Hund-Rinke and Simon 2006; Lovern and Klaper 2006; Lovern et al. 2007; Oberdorster 2004; Velzeboer et al. 2008; Zhu et al. 2006; Zhu et al. 2008), with very few studies on soil organisms (Johansen et al. 2008; Scott-Fordsmand et al. 2008a; Scott-Fordsmand et al. 2008b; Tong et al. 2007; Lee et al. 2008a)).

Despite the fact that copper is an essential element for most living organisms it is also well documented to cause toxicity in various organisms, including the enchytraeid *E. albidus* (Amorim et al. 2008; Amorim et al. 2005; Howcroft et al. 2009). Copper is known to cause oxidative damage, catalysing the formation of hydroxyl radicals via the Haber-Weiss reaction (Bremner 1998): $O2\cdot - + Cu2+ \rightarrow O2 + Cu+$; $Cu+ + H2O2 \rightarrow Cu2+ + OH- + OH\cdot$. To protect organisms from oxidative stress there are antioxidant defences, present in all aerobic organisms, which include antioxidant enzymes (superoxide dismutase, catalase, glutathione peroxidases and glutathione reductase) and free radical scavengers (vitamins C and E, carotenoids, glutathione) whose function is to remove reactive oxygen species (ROS) (Doyotte et al. 1997). Enzymatic and non-enzymatic antioxidants play a crucial role in maintaining cell homeostasis. Thus the balance between

prooxidant endogenous and exogenous factors and antioxidant defences in biological systems can be used to assess toxic effects under stressful environmental conditions (Howcroft et al. 2009).

Comparatively, very little is known about the effects of copper nanoparticles. Studies reporting acute toxicity results comparing ionic copper with copper nanoparticles show that the first was more toxic to mice (LD50 of 110 and 413 mg/Kg respectively) (Chen et al. 2007; Chen et al. 2006; Meng et al. 2007) and zebrafish (EC50 of 0.25 mg/L and 1.6 mg/L respectively)(Griffitt et al. 2007). Microarray experiments (Griffitt et al. 2007) showed that 48h exposure to sub lethal concentrations of nanocopper resulted in 82 genes significantly differently expressed and that the transcriptional response induced by nanocopper was divergent from the one that was generated by the soluble copper, which suggests that the effects caused by nanocopper are not only due to solubility.

In the present study the effect of two copper forms in *Enchytaeus albidus*, copper-salt and nanosized copper, were assessed and compared. Enchytraeids (Oligochaeta) are important members of the soil fauna that have been used in ecotoxicological laboratory tests for more than 30 years (Rombke and Moser 2002) and have established standard guideline (ISO 2003). They are ecologically relevant, being abundant in many soils contributing to the improvement of the pore structure of the soil and, indirectly, to the degradation of the organic matter. Effects were assessed in terms of oxidative stress (lipid peroxidation, superoxide dismutase, catalase, glutathione_total, oxidized and reduced, glutathione reductase, glutathione peroxidase and glutathione-S-transferase) and neuro-muscular (cholinesterases) biomarkers. Additionally, three different exposure times (2, 4 and 8 days) and two exposure concentrations of copper and nano copper were analysed.

2. Material and Methods

2.1. Test organism

The test organism used belongs to the species *Enchytraeus albidus*, Henle 1837. The individuals were maintained in laboratory cultures under controlled conditions, e.g. photoperiod 16:8h light:dark and a temperature of 18ºC. Details of culturing are given in (Rombke and Moser 2002).

2.2. Test soil

The test soil used was from the natural site in Hygum, Denmark. Soil was homogenized and sterilized. The general physico-chemical characteristics of soil from the Hygum-site are as follows: 20-32% coarse sand (>200Am), 20-25% fine sand (63- 200Am), 11-20% coarse silt (20-63Am), 12-20% silt (20-20Am), 12-16% clay (<2Am) and 3.6-5.5% humus. The soil was sampled to a depth of 20 cm. To exclude soil animals already present, the soil was dried at 80°C for 24 h in an oven (Memmert, Type UL40, Braunschweig, Germany) and then sieved through a 2-mm mesh to remove larger particles.

2.3. Chemical substances

The nanoparticles were made of Cu with a mean diameter of 80 nm, (PW-XRD <74 nm, DLS = 419±1 nm, Zeta = 15.3±0.3) and a purity of 99.5%. The nanoparticles were added to the soil following transfer to deionised water and ultrasonic-steering for 15 min. The Cu-salt was obtained from $CuCl_2$ solubilised in water. The concentrations used were 450 and 750 mg/Kg of copper and nanosized copper.

2.4. Test procedure

After homogeneous mixing, sub-samples of the batch of soil were introduced into the test vessels. Fifteen adult worms with well developed clitellum were introduced in each test vessel, each containing 25 g moist soil (40 to 60% of the maximum WHC). The vessel was covered with a lid (containing small holes) and the worms

were exposed for two, four and eight days (in the absence of food) at 20ºC and a 16:8h photoperiod. Seven replicates per treatment were used. At test end, animals of each replicate were carefully removed, rinsed in deionised water, weighed and frozen in liquid Nitrogen. Samples were stored at -80ºC till further analysis.

2.5. Biochemical analysis

Each sample containing 15 organisms was homogenized in 1500µL K-Phosphate 0,1M buffer, pH 7,4. Part of the tissue homogenate (300µL) was separated into a microtube with 5µL BHT (2,6-dieter-butyl-4-metylphenol) 4% in methanol for lipid peroxidation (LPO) determination. The remaining tissue homogenate (1200µL) of each sample was centrifuged at 10000g for 20 min at 4ºC, to isolate the Post-Mithocondrial Supernatant (PMS). The PMS was divided into eight microtubes, stored at -80ºC, for posterior analysis of biomarkers and protein quantification.

2.5.1. Determination of lipid peroxidation

The extent of LPO was measured as thiobarbituric acid-reactive substances (TBARS) at 535nm (Bird and Draper 1984; Ohkawa et al. 1979).

2.5.2. Quantification of enzymatic and nonenzymatic antioxidants

Superoxide Dismutase (SOD) activity was determined based on the reduction of cytochrome c by superoxide radicals generated by the xanthine-xanthine oxidase system. Measurements were recorded at 550nm (McCord and Fridovich 1969) adapted to microplate. Catalase (CAT) activity was measured following the decrease in absorbance at 240nm due to H_2O_2 (substrate) decomposition (Clairborne 1985). Glutathione Peroxidase (GPx) activity was determined following the oxidation of NADPH, at 340nm, when GSSG is reduced back to GSH by glutathione reductase, using H_2O_2 as substrate (Mohandas et al. 1984). Glutathione Reductase (GR) activity was measured assessing the decrease of NADPH level, at 340nm (Cribb et al. 1989). Glutathione-S-Transferase (GST) activity was measured at 340nm, following the conjugation of GSH with CDNB (the

substrate with the broadest range of GST isozymes detectability) (Habig et al. 1974). For more details see (Howcroft et al. 2009). Total glutathione (TG, GSH + GSSG) and oxidized glutathione (GSSG) were measured at 412 nm, using the recycling reaction of reduced glutathione (GSH) with DTNB in the presence of GR excess (Baker et al. 1990; Tietze 1969). 2-Vinyl-pyridine was used to conjugate GSH for the GSSG determination (Griffith 1980). The GSH content was calculated by subtraction of GSSG from the total glutathione.

2.5.3. Quantification of Cholinesterases activity

Cholinesterases (ChEs) activity was measured using Ellman method (Ellman et al. 1961) adapted to microplate (Guilhermino et al. 1996). ChE activity was determined in the PMS, using 50 µL of sample and 250 µL of reaction buffer (30 mL K-phosphate buffer 0.1 M pH 7.2, 1 mL DTNB 10 mM in K-phosphate buffer with 7.5 mg $NaHCO_3$ and 0.2 mL acetylthiocoline 0.075 M solution as the substrate). The absorbance was measured at 414 nm.

2.5.4. Protein quantification

Protein concentration was assayed using the Bradford method (Bradford 1976), adapted from BioRad's Bradford micro-assay set up in a 96 well flat bottom plate, using bovine γ-globuline as a standard.

2.6. Statistical analysis

The main aim of this experiment was to analyse differences in response induction between the salt form ($CuCl_2$) compared to the nano form (NP-Cu). Multivariate analysis (Principal Component Analysis, PCA) was performed using SAS 9.1.3 (2002-2004). Data was standardized (mean between 0 and 1). Univariate statistical analysis was also performed using the software SigmaPlot11 (SPSS, 1997): one-way ANOVA (Dunnett's Method) was used to compare differences between control and treatments, for each individual exposure time. A t-test was used to compare differences between Cu versus Np-Cu within the same

concentration and different concentration of the same compound. One-way ANOVA (Holm-Sidak) was used to compare differences due to different times of exposure.

3. Results

3.1. Multivariate analysis – trends in data

Multivariate analysis (Principal Component Analysis, PCA) was performed with the aim of finding trends. Trends in PCA are based on the individual samples and not on mean values as performed in the uni-variate analysis (see below). When pooling all data the first three PCA axes explained 60-70% of the variation, with a clear indication of that data (graph not shown) were grouped according to exposure durations and exposure concentration. Analysis of the control organisms showed a clear time related enzymatic response. Given this, the data were subsequently treated in accordance with the test design i.e. analysing difference between Cu-NP and Cu-salt within all combinations (termed “treatment”) of days and concentrations. For each of these treatments there was a clear discrimination between the three groups: the control, Cu-salt and Cu-NP exposed organisms (graph not shown), but in the subsequent description only the possible difference between the Cu-salt and Cu-NP is described as this is the main aim of the study. Performing the PCA for each treatment showed that 70-80% of the variation could be explained by the first three axis of the PCA.

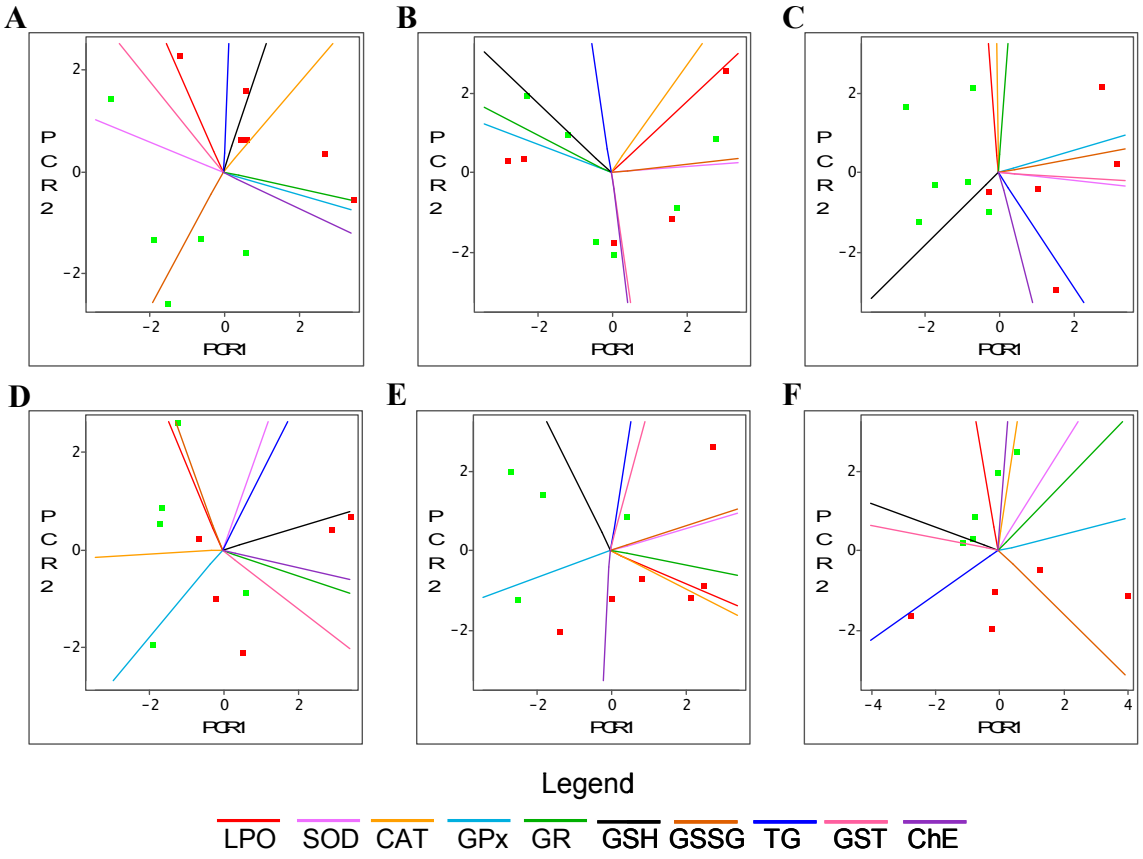


Figure 1: PCA graphs for the combinations of exposure time and concentration. Red dots represent copper nanoparticles and green dots copper-salt. Top row figures show the concentration of 450 mg/Kg at 2 days (A), 4 days (B) and 8 days (C);lower row figures show the concentration of 750 mg/Kg at 2 days (D), 4 days (E) and 8 days (F). The biomarkers represented are: lipid peroxidation (LPO), superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), Glutathione reductase (GR), reduced glutathione (GSH), oxidized glutathione (GSSG), total glutathione (TG), glutathione-S-transferase (GST) and cholinesterases (ChE).

As seen from the graphs, clear separation between Cu-salt (green dots) and NP-Cu (red dots) exposed organisms (each dot representing one organism sample) was possible for all except for day 4 of the 450mg Cu/kg exposed and for the day 2 of the 750 mg Cu/kg exposed (see Fig 1). The separation pattern and which enzymes were correlated best with a discrimination, was both dependent on the exposure concentration and the exposure time, that is, no single enzymatic response could be used to identify either the Cu-salt or the NP-Cu at all concentrations and times. For the 450 mg Cu/kg exposure, day 2 showed that the

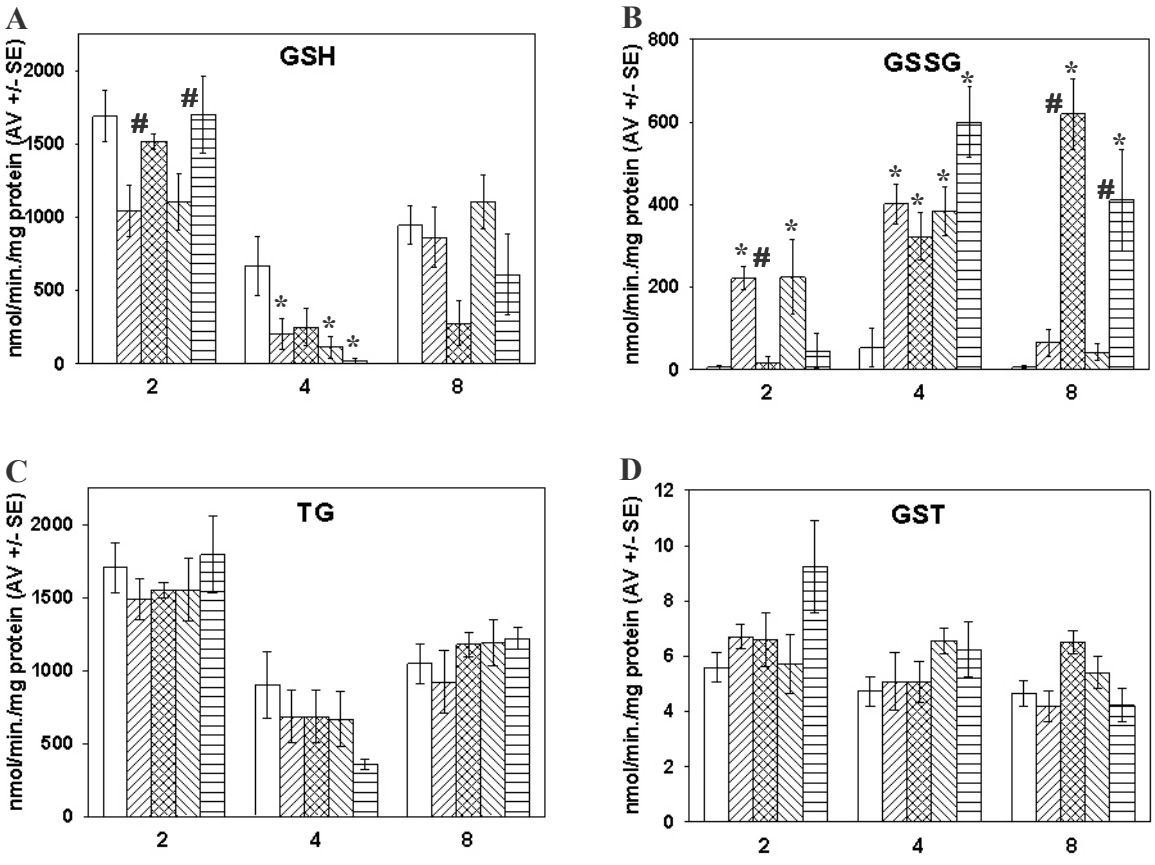
discrimination was best determined by the difference between GSSG, CAT GSH and TG. GSSG had a strong positive response (increase compared to Cu-NP) for the Cu-salt, whereas CAT, GSH and TG had a strong positive response in the Cu-NP (increased compared to Cu-salt). LPO, SOD, GST, GR, GPx and ChE were of little importance in discriminating between the groups. For the latter group the LPO, SOD and GST displayed an inverse response to GR, GPx and ChE, that is, when the former group were high the latter was generally low. For day 4 of the 450mg Cu/kg exposure, TG showed an opposite response to ChE and GST, and GSH, GR and GPx an opposite response to CAT, LPO, GSSG and SOD. No clear separation of the group was possible. A clear separation was possible for the two groups in the day 8 exposure of the 450 mg Cu/kg. In general there was a strong positive response of GPx, LPO, GST and SOD for NP-Cu exposed organisms and a strong (although less strong) positive response of GSH for Cu-salt exposed organisms, compared of course to the other group. Although, not highly important for discriminating between the two groups GR, CAT and GSSG showed an inverse response to TG and ChE.

For the 750 mg Cu/kg exposure, it was not possible to make a clear separation of the two groups for 2 days exposed organisms. For these organisms SOD and TG showed an inverse response compared to GPx, CAT and inverse compared to GSH, and although not so strong GST, GR and ChE had an inverse response to LPO and GSSG. For the day 4 exposure of the 750 mg Cu /kg CAT, LPO and ChE showed a strong relationship with Cu-NP exposure with GSH showing an inverse relationship, GSH had a strong positive response in Cu-salts. Less clearly discriminating and less expressed in the Cu-salt organisms was GST and TG. Not important for discrimination between the two groups GR, SOD and GSSG had an inverse response compared to GPx. Finally for the day 8 of the 750 mg Cu/kg, the NP exposed organisms had a strong expression of GSSG, whereas for the Cu-salt GSH and GST, and to a lesser extend LPO and ChE was stronger expressed. Little important for discrimination GR, SOD and CAT was inversely expressed compared to TG.

3.2. Univariate analysis – detailed approach to data

3.2.1. Effect of the test chemicals (Cu and Np-Cu) and different concentrations (450/750 mg/kg) on the enzymatic activity

Results (Fig. 2) show significant differences in the majority of biomarkers, due to exposure to both Cu-salt and Cu-NP. Changes with higher level of significance were observed in terms of increase in LPO levels (Fig. 2J) after 8 days of exposure, except for Cu-NP 750. Also at 8 days exposure, GSSG (Fig. 2B) was stimulated by Cu-NP treatments while GPx activity decreases (Fig. 2E), except for Cu-NP 450. At 4 days exposure, GPx activity increases with concentration increase and is higher for all NP versions. Similarly GSSG activity increases in both concentrations of both chemicals, while GSH activity decreases (Fig. 2A). At 2 days of exposure GSSG activity was stimulated by ionic Cu-salt treatments while GR decreases (Fig. 2F).



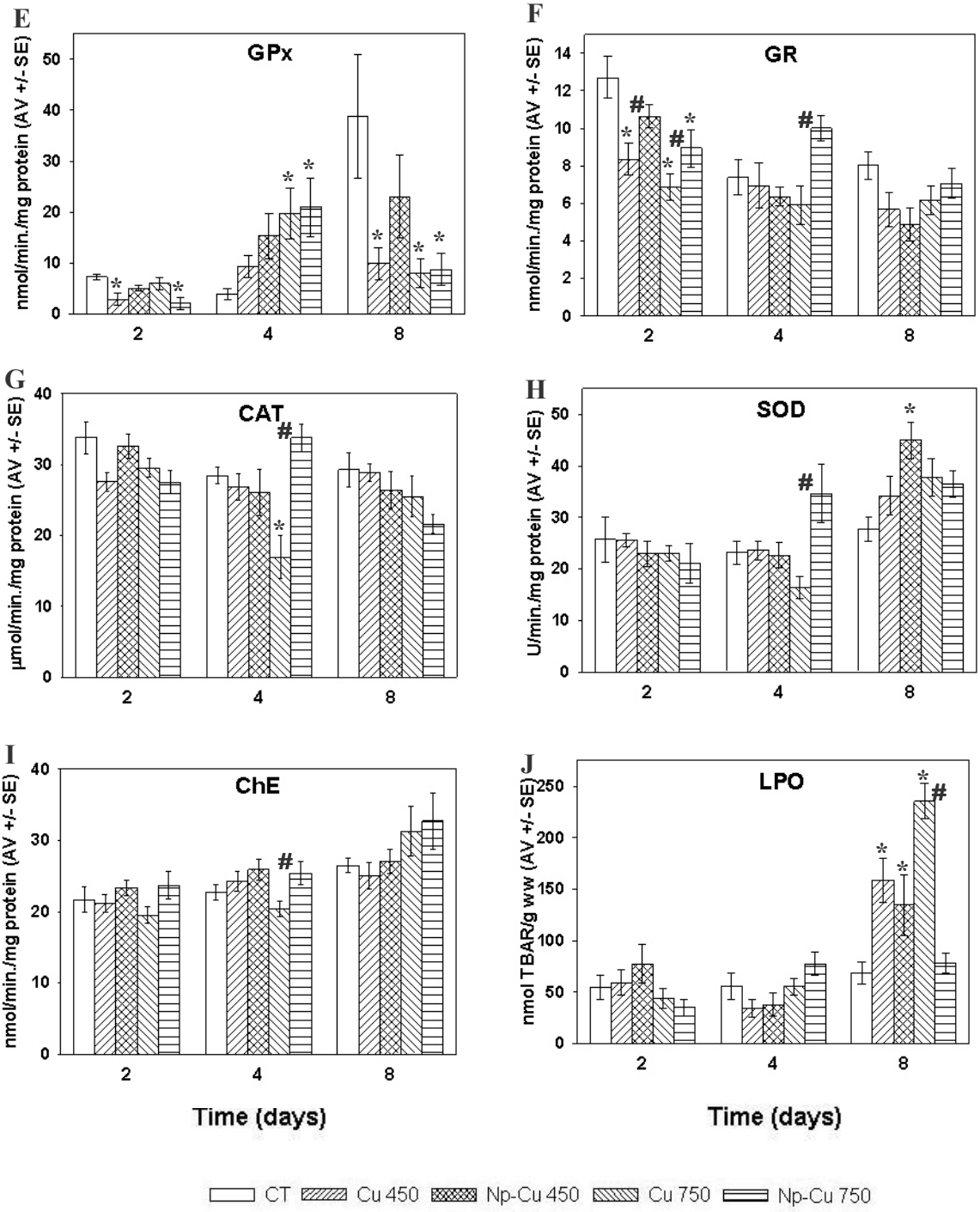


Figure 2: Results of reduced glutathione (GSH), oxidized glutathione (GSSG), total glutathione (TG), glutathione-S-transferase (GST), glutathione peroxidase (GPx), glutathione reductase (GR), catalase (CAT), superoxide dismutase (SOD), colinesterases (ChE) and lipid peroxidation (LPO) expressed as mean values ± standard error (Av±SE) for *E. albidus* exposed at 450 and 750mg/Kg of cooper-salt and nanosized copper, during

2, 4 and 8 days. (* indicates statistical significant differences compared to control, Holm-Sydak $p<0.05$; and # indicates statistical significant differences between chemicals within the same concentration $p<0.05$).

3.2.2. Effects of copper-salt versus nanosized copper

Some of the biomarkers assessed showed different responses depending on the chemical size applied (Cu or Cu-NP) (Fig. 2). At 2 days exposure, GR and GSH activities (Fig. 2F, 2A) were more inhibited by Cu than by Cu-NP. The activity of CAT at 4 days exposure (Fig. 2G) was inhibited by Cu 750, which was significantly different form Np-Cu 750. The same response, at 4 days exposure, was observed for the biomarkers GR, SOD and ChE, being the activities significantly smaller when exposed at Cu 750 when compared to Cu-NP 750.

3.2.3. Effects of concentration (450 versus 750 mg/Kg)

The different toxic concentration used caused different effects on some of the biomarkers. SOD and ChE activities at 4 days exposure, were significantly reduced in Cu 750 in comparison to Cu 450. CAT levels, at 4 days of exposure were reduced for the highest concentration of Cu and LPO levels, at 8 days of exposure were significantly higher for Cu 750 when compared to Cu 450. For the NPs, lower concentration has more effect than the highest on GSSG activity and LPO levels.

3.2.4. Effects of exposure time on biomarkers response

As mentioned before in the text, PCA analysis showed an exposure time related enzymatic response. Univariate analysis performed confirmed also that biomarkers responses varied with the time of exposure: GPx activity on controls was significantly different between the three exposure times. GR, GSH and TG activity decreased on controls at 4 and 8 days of exposure. Cu 450 caused a different response in SOD between 4 and 8 days, GSH and TG between 2 and 4 days, GSSG different between the three times of exposure and LPO is different at 8 days in comparison to 2 and 4 days. When exposed to Cu-NP 450, GSH, TG and GSSG activities were different between all exposure times, SOD activity was

different at 8 days, GR activity differed only at 2 days, CAT response differ between 2 and 4 days and LPO levels between 4 and 8 days. Cu 750 caused an increase in SOD, LPO and ChE levels at 8 days; GPX activity was increased at 4 days; CAT activity was different between all the times of exposure; GSH and TG responses were different between 2 and 4 days and GSSG response differ between 4 and 8 days. Cu-NP 750 caused a GSH and TG responses that were different between all the times of exposure; GSSG and LPO levels differ at 2 days, comparing to 4 and 8 days; GPx activity was different between 2 and 4 days and GST between 2 and 8 days.

4. Discussion

The subsequent discussion is based on the assumption that the effects present in the Cu-NP exposed organisms is indeed caused by nano-copper fully or to some extend and not by dissolved Cu: it must however be realised that this may not be the case but, at present, there is no method to verify and quantify this. Nevertheless, the results do indicate which responses should be further studied to discriminate between Cu-salt and Cu-NP. It was observed (although not shown) that the response in all the exposed organisms were different from that of the controls.

Results show that both copper-salt and nano-copper caused changes in all parameters evaluated and in the different times of exposure, revealing that organisms are facing an oxidative stress status but this is changing over time. In fact, the biomarkers response showing the best discrimination of the nano and the salt form was dependent on the exposure duration and concentration *i.e.* there was not one biomarker that provided the best discrimination.

The current results show that SOD activity increased, after 8 days of exposure at Cu-NP 450. SOD is an antioxidant enzyme that protects tissues against superoxide anion radical catalysing its conversion in H_2O_2, which is detoxified by CAT and GPx. Increased SOD activity as a response to metals, including copper, has already been observed in several organism (Drazkiewicz et al. 2007; Jing et al. 2006; Sampaio et al. 2008).

A previous study performed with *E. albidus* exposed to Cu (320 mg/Kg) (Howcroft et al. 2009) showed no differences in SOD activity, at 2 days and 3 weeks exposure. The differences found between the present results and literature could be explained by the different soil type used (Gomes et al. 2009). Hygum soil affects reproduction, being itself a possible cause of stress. This fact can be a possible explanation to the fact that in our results the controls of GPx, GR, GSH and TG varied between times of exposure. The increase in GPx activity (a common response at hydroperoxides accumulation as will be discussed bellow) plus the inhibition of GR and GSH are signals of stress. The increased levels of LPO at 8 days of exposure, indicated that the organisms are suffering oxidative damage and that the effects caused by copper-salt (Cu) were more pronounced than those caused by nano-copper (Cu-NP), especially at the highest concentration tested. The results show that the higher concentration of Cu caused significant increase in LPO levels while the same did not occur for Cu-NP. Results in LUFA 2.2 soil (Howcroft et al. 2009) show that the antioxidant defence processes in *E. albidus* were able to prevent oxidative damage (LPO) on short exposures to copper (2 days), but not after 3 weeks. Our data suggests that 8 days of exposure at a sub-lethal concentration of copper (450 mg/Kg) are already causing oxidative damage.

CAT activity was inhibited at 4 days of exposure when exposed to Cu 750. CAT and GPx are antioxidant enzymes responsible for the detoxification of hydroperoxides (H_2O_2). It is suggested by Atli and co-authors (2006) that CAT inhibition may be related to the inactivation of the enzyme due to the direct binding of metal ions to –SH groups on the enzyme molecule. It is also suggested that no changes in CAT activity may be related to the increase in other antioxidant enzymes and/or substrates, such as GPx and GSH.

At 4 days exposure, GPx increased as GSH decreased: attending to the fact that GPx activity comes as an attempt to detoxification of H_2O_2 (and other organic hydroperoxides) using GSH as substrate (and producing GSSG which is then regenerated into GSH by GR) (Thomas et al. 1990), the decrease in GSH activity observed at 4 days of exposure can result from its use as substrate by GPx in its antioxidant activity. This can be supported by the fact that, at this time of exposure

no significant changes were observed in GR, TG and GST. The results also show that the depletion of GSH is not being compensated by GR, resulting in the increase of GSSG observed.

No changes occurred in ChEs, at any treatment or time of exposure. ChEs are a group of enzymes related with neuronal functions and in recent decades some studies have been published regarding the effect of metals in AChE activity (the main cholinesterase present in six earthworms' whole body (Rault et al. 2007)), but results are contradictory: Frascos' results (Frasco et al. 2005) show that metals (including copper) caused AChE inhibition *in vitro*.

Regarding the different concentrations tested, Cu showed a dose effect, with the highest concentration causing more effects than the lower. For Cu-NP, LPO levels were higher for 450mg/kg (Cu-NP 750 did not cause lipid peroxidation). This could be indicative of a bell-shape response which was already observed for other stress biomarkers (Dagnino et al. 2007; Dazy et al. 2009), nevertheless it is hard to confirm such conclusion without testing more concentrations.

In general, the results seem to indicate that, at 2 days of exposure, the organisms start to respond to the oxidative stress imposed by copper-salt and copper nanoparticles. At 4 days of exposure GPx was the main defence against oxidative damage, for which *E. albidus* could not compensate for at 8 days of exposure (resulting in LPO increase).

At 4 days of exposure to 750 mg/kg differences between Cu and Cu-NP are very pronounced, while those observed between the concentrations of 450mg/kg at 2 days of exposure do not persist after that. Cu-salt exposure caused lower values of CAT compared to Cu-NP; attending to the fact that copper can bind directly to CAT that could mean that copper binding is higher for Cu-salt than for Cu-NP. Reduced activity of CAT results in the accumulation of hydroperoxides, which can be removed by GPx until a saturation point leading ultimately to the higher LPO levels (observed at 8 days). On the other hand, higher values of CAT and GR at Cu-NP in comparison to Cu-salt, associated with the activity of GPx, seem to have avoided the ultimate cellular damage, LPO.

5. Conclusions

Both salt- and nano-copper caused oxidative stress and damage to *E. albidus*, and effects could be discriminated between the copper forms. Nevertheless and despite the visible discrimination between nano and the salt form (time and exposure dependent), there was not one or a set of biomarkers that provided the best discrimination. In general, effects of Cu-salt were more pronounced especially at LPO level. GPx seemed to be the main antioxidant acting against the stress imposed, and associated with GSH and GSSG were the biomarkers with more robust response. The selected time intervals were adequate to follow the evolution of the stress response until oxidative damage. To better characterize these responses a broader range of concentrations should be tested. Further research is needed to link these effects with higher levels of biological organization.

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Chapter 4

Differential gene expression analysis in *Enchytraeus albidus* exposed to natural and chemical stressors: effect of different exposure periods

Effect of Cu-Nanoparticles versus Cu-salt in *Enchytraeus albidus* (Oligochaeta): differential gene expression through microarray analysis

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Abstract

Nanotechnology is an emergent technology with applications in science, technology and medicine. Despite the increased utilization of nanoparticles, the behavior and effects in the environment is largely unknown and few resources are available for health and environmental effects studies. Enchytraeids are extensively used in studies of soil toxicology (ISO No. 16387, 2005) and recently, a cDNA microarray for *E. albidus*, was developed and toxicogenomic studies are also possible. These organisms are small worms that indirectly contribute to the regulation and degradation of organic matter, being ecologically relevant. In this study we compared the gene expression profiles of *Enchytraeus albidus* when exposed to copper-salt ($CuCl_2$) and copper nanoparticles spiked soil. The worms were exposed for 48 hours to a range of concentrations in soil. Microarray hybridizations revealed a different response patterns between copper-salt and copper nanoparticles, with a higher number of differentially expressed genes due to the copper salt exposure, however the discrimination between copper nanoparticles and copper-salt was not possible, neither the unravelling of the underlying mechanisms of toxicity.

***Keywords**:* toxicogenomics, gene expression profile, copper, copper nanoparticles, Oligochaeta

1. Introduction

Nanotechnology is an emergent discipline with several applications (electronics, consumer products, medicine, environmental remediation, and catalysts), with rapidly increasing investments from businesses and from governments in many parts of the world (The Royal Society & The Royal Academy of Engineering 2004). The use of nanomaterials is dramatically increasing and will continue over the next years, producing “nanowastes” that will probably enter in the environment from manufacturing effluent, from washing off consumer products or through landfills and other methods of disposal. Despite the fact that most people are concerned with effects on large wildlife (particularly the human being), the basis of many food chains depends on the benthic and soil fauna and flora, which could be dramatically affected by the release of nanomaterials in the environment, whose behavior and effects are largely unknown (Oberdorster et al. 2005).

Copper nanoparticles (Cu NPs) have been given considerable attention due to their properties e.g. high electrical and thermic conductivities which make them very useful in modern electronics (Lee et al. 2008b; Tummala et al. 2006), metallurgy (Dhas et al. 1998; Samim et al. 2007) and catalysis. Copper is known to cause oxidative damage in organisms via the Haber-Weiss reaction, catalyzing the formation of the powerful oxidant hydroxyl radical (OH·) (Bremner 1998) and recent investigation has shown that sublethal concentrations of copper caused mitochondrial dysfunction resulting in the reduction of the amount of energy available and a switch to metabolism of stored carbohydrates (Bundy et al. 2008). The environmental effects of copper nanoparticles are still poorly known. When compared to soluble copper, copper nanoparticles were less toxic to mice (LD50 of 110 and 413 mg/Kg respectively) (Chen et al. 2007; Chen et al. 2006; Meng et al. 2007) and zebrafish (EC50 of 0.25 mg/L and 1.6 mg/L respectively). Results obtained by Griffitt and co-authors (Griffitt et al. 2007) suggests that the transcriptional response induced by copper nanoparticles was divergent from the induced by the soluble copper, indicating that the effects are caused by the copper nanoparticles and not (only) due to their solubilization.

At the moment, very little information is available about the effects of nanomaterials in terrestrial ecosystems (Johansen et al. 2008; Lee et al. 2008a; Scott-Fordsmand et al. 2008a; Scott-Fordsmand et al. 2008b; Tong et al. 2007).
Among soil species used in ecotoxicology are the Enchytraeids (Oligochaeta), important members of the soil fauna (Rombke and Moser 2002). They are abundant in many soils contributing to the improvement of the pore structure of the soil and, indirectly, to the degradation of the organic matter (Amorim et al. 2005). Effect assessment is traditionally made at the individual level (ISO 2005), evaluating e.g. survival and reproduction. Recently, a cDNA microarray for *E. albidus*, was developed and toxicogenomic studies are also possible (Amorim et al. 2009). DNA microarrays provide a powerful tool to analyze global responses of an organism to stressors present in the environment. Trough the quantification of the expression of thousands of genes in a single experiment (Lettieri 2006; Rockett and Dix 1999), we can identify cellular components that may be damaged and repaired during the stress exposure, as well as mechanisms involved in cellular responses. The measurement of these responses can serve as a signal/marker for potential effects at population level.
Therefore, in the present study the main objective was to compare the effect of copper salt versus nanosized copper, at the gene level in *E. albidus*. The test species was exposed to varying concentrations of copper and nanosized copper and its gene expression profile was analyzed through the cDNA microarray.

2. Material and Methods

2.1. Test organism

The test organism used belongs to the specie *Enchytraeus albidus*, Henle 1837. The individuals were maintained in laboratory cultures under controlled conditions, e.g. photoperiod 16:8h light:dark and a temperature of 18ºC, details of culturing in (Rombke and Moser 2002).
Laboratory cultures of the test species *Enchytraeus albidus* (Henle, 1837) were maintained in moist soil (50% OECD soil, 50% natural garden soil), at 18ºC with a

photoperiod of 16:8h (light:dark), and fed once a week with finely ground and autoclaved rolled oats (Cimarrom, Portugal). Details of the culturing process are given in Römbke and Möser (2002).

2.2. Test soil

The test system was based on homogenized and sterilized field collected soil from Hygum, Denmark. The general physico-chemical characteristics of soil from the Hygum-site are as follows: 20-32% coarse sand (>200µm), 20-25% fine sand (63-200µm), 11-20% coarse silt (20-63µm), 12-20% silt (20-20µm), 12-16% clay (<2µm) and 3.6-5.5% humus. The soil was sampled to a depth of 20 cm. To exclude soil animals already present, the soil was dried at 80°C for 24 h in an oven (Memmert, Type UL40, Braunschweig, Germany) and then sieved through a 2 mm mesh to remove larger particles.

2.3. Chemical substances

The nanoparticles were made of Cu with a mean diameter of 80 nm, (PW-XRD <74 nm, DLS = 419±1 nm, Zeta = 15.3±0.3) and a purity of 99.5%. The nanoparticles were added to the soil following transfer to deionised water and ultrasonic-steering for 15 min. The Cu-salt was obtained from $CuCl_2$ solubilised in water. The concentrations used 400, 600, 800 and 1000 mg/Kg of copper and nanosized copper.

2.4. Experimental procedure

After homogeneous mixing, sub-samples of the batch of soil were introduced into the test vessels. Fifteen adult worms with well developed *clitellum* were introduced in each test vessel, each containing 25 g moist soil (40 to 60% of the maximum WHC). The vessel is covered with a lid (containing small holes) and the worms were exposed for 48h at 20ºC and a 16:8h photoperiod. Four replicates per treatment were used. At test end, animals were carefully removed, rinsed in deionised water, stored in RNA later (Ambion) and frozen in liquid Nitrogen.

Samples were stored at -80ºC till further analysis. Hybridization experiments, control versus exposed, were always made in triplicate, using biological replicates.

2.5. RNA extraction

Isolation of total RNA from enchytraeids was performed through the Trizol extraction method (Invitrogen, Belgium). RNA extraction was followed by a DNAse treatment (Fermentas Germany). The quantity and purity of the isolated RNA were measured spectrophotometrically with Nanodrop and its quality was checked on a denaturing agarose gel.

2.6. cDNA synthesis and Amminoallyl labeling

Equal quantities of RNA were pooled for all control and exposed organisms samples. mRNA spike mix (lucidea controls) (GE Healthcare, Amersham) and random hexamer primers (Invitrogen) were added to the RNA. To convert RNA in cDNA, the amminoallyl-dNTPs mix (containing a 2:3 ratio of aminoallyl-dUTP (Invitrogen)) and 200U/µL Superscript II RT (Invitrogen) were incorporated. Unincorporated amminoallyl-dUTPs were removed using modified Qiagen PCR spin column protocol. Amminoallyl labeled cDNA was covalently bound to appropriate NSH-esters Cy Dyes (Amersham). Cy3 was coupled with control and Cy5 with treatments. Uncoupled dyes were removed using the QIAquick PCR purification kit (Qiagen). The efficiency of labeling reaction was measured spectrophotometrically with a Nanodrop. Dye incorporation per sample of approximately 150pmol and a frequency of dye incorporation (FOI) less than 50 are near optimal for hybridization.

2.7. Microarrays

Previously developed cDNA normalized library (Amorim et al. 2009) was spotted onto Generoma microarray slides (Asper Biotech, Estonia) and used in the present study.

2.8. Hybridization of microarrays

Before the hybridization, the arrays were incubated in pre-hybridization buffer, consisting of 50% formamide, 5×SSC, 0,1% SDS and 0,1 BSA, at 42ºC for 60 minutes. After that, the arrays were washed by immersion in ultra pure water followed by immersion in isopropanol, and immediately dried with compressed N2. The vacuum dried samples were resolved in hybridization solution consisting of 50% formamide, 5×SSC, 0,1% SDS and 0,1 mg of Salmon sperm (SIGMA), a nucleic-acid blocker. The probe solution (that results of the combination of Cy3 and Cy5 labeling probes), incubated at 95ºC for 5 minutes, was applied on the cover-slides in the array. The array was placed in Genetix hybridization chamber where the hybridization was performed at 42ºC overnight. After the hybridization, the arrays were immersed in four wash solutions containing decreasing concentrations of SSC and SDS, finally the arrays were rinsed in MilliQ water and isopropanol for 1 minute and immediately dried in compressed N2. Three replicates of each treatment were done, hybridizing each treatment versus the control sample.

2.9. Scanning and analysis of microarray data

Scanning was performed using an Agilent Microarray Scanner (Agilent Technologies) at 532 and 635 nm for Cy3 and Cy5 respectively. The images acquired were assessed using Quant Array (Packard Biochip Technologies) for spot identification and for quantification of fluorescent signal intensities. Subsequently data were analyzed with limmaGUI package (based on limma (Smyth 2005)) in the R (2.8.0) software environment (http://www.R-project.org). The fluorescent signal intensity for each DNA spot was calculated using background subtraction and saturated signal were excluded from further analysis. After that, a within and between array normalization was applied using global loess (Yang et al. 2002) and scale methods respectively. Quality control was done by making MA-plots and box plots of each array. Differential expression was assessed using linear models and Benjamini-Hochberg's (BH) method to correct for multiple testing (Benjamini and Hochberg 1995) (adjusted $p<0.05$ was considered significant). Assessment of differential expression of genes was done

for each treatment separately, and resulted in a mean log2 expression ratio (treated/untreated) and a p-value for each probe on the array. All cDNA fragments correspondent to differentially expressed genes (adjusted $p<0.05$) were identified based on their homology to sequences from the National Center for Biotechnology Information (NCBI) database as determined by the Basic Local Alignment Search Tool (BLAST). Both nucleotide–nucleotide and nucleotide–protein homology searches were done to identify the isolated clones (BLASTN and BLASTX).

2.10. Quantitative RT PCR

Total RNA (500ng) from the samples used for microarrays was converted into cDNA through a reverse transcription reaction using the SuperScript First-Strand Synthesis System for RT-PCR (Invitrogen). Real time PCR was carried out on 7500 Real-Time PCR System (Applied Biosystems), using Platinum SYBR Green qPCR SuperMix-UDG (Invitrogen). Primer sets were designed for 5 target genes and one endogenous control gene (table 1) with the software Oligo Explorer™ (version 1.1.0). Determination of PCR efficiency and specificity was done by the obtaining standard and melting curves, respectively for all primer sets. cDNA was diluted 1:4 and 2 µL was 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 50ºC for 2 min, followed by one denaturation step at 95ºC for 2 min, 40 cycles at 95 ºC for 10 sec, and a dissociation step consisting of 15 sec at 95 ºC, 1 min at 60 ºC, and 15 sec at 95 ºC.

A mean normalized expression value was calculated from the obtained Ct values with Relative Expression Software Tool (REST-MSC) using actin as a reference gene (housekeeping) for normalization of input cDNA.

Tabela 1: RT-PCR primer sequences

Gene homolog	5' – 3' Forward primer	5' – 3' Reverse primer
Superoxide dismutase (SOD)	GCGGAAGTGGAAATGGAAG	CGACATGGGGACTGAAGTG
Calcium dependent protein kinase	AAAGGGTCGGCAGAATTGG	AAGGCGCGGAACTCTATCC
Citocrome c	CGGAAGGTGATGCTGAGAAG	CGTCTCCTTCGTCCATTTG
Heat Shock Protein 8 (HSPA8)	TGTTCAGGTTCTTTAACC	GTTTTCTTTTATTGCACC
Transcription factor	AGCTTTCTCAAATCATCAAA	AGACTTCACCAACCTATTGCG
Actine	CAGGGAAAAGATGACCCAAA	AGGGCGTAACCCTCGTAGAT

3. Results and Discussion

Using linear models and empirical Bayes methods (Smyth 2005), a total of 127 in 480 gene fragments were identified as differently expressed (BH adjusted $p<0.05$). Cu salt treatment caused 68, 32, 10 and 4 significant transcripts and Cu nanoparticles 26, 2, 39 and 1, for the concentrations 400, 600, 800 and 1000 mg/Kg respectively. No additional fold-change cut-off was used to identify differently expressed genes because, as stated in Nota (Nota et al. 2008), slight changes in gene expression could also result in major physiological effects. Figure 1 shows an overview of differently expressed genes for the different treatments.

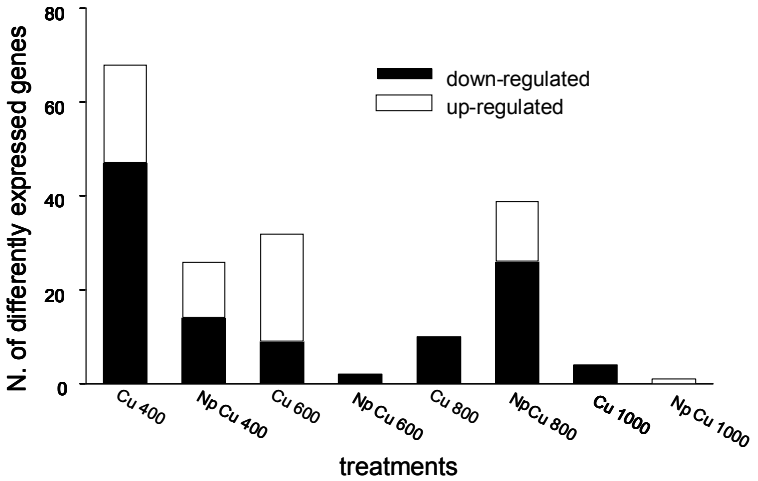

Figure 1. Number of differently expressed genes after exposure to different concentrations (400, 600, 800 and 1000 mg/Kg) of Cu-salt (Cu) and copper nanoparticles (Np-Cu).

In general, there are more down than up-regulated genes, as also been observed in several studies (Bakel et al. 2005; Lewis and Keller 2009; Nota et al. 2008). Cu salt caused a dose-response like decrease in the number of differently expressed genes. Nano-Cu does not show a pattern.

All the differently expresses transcripts had been previously sequenced (Amorim et al, 2009) and were subjected to recent BLAST homology search (BLASTX as well as BLASTN). Only 28 of 127 differentially expressed gene fragments had homology with sequences in public databases. From these 28 sequences, 2 had unknown function and the remaining 26 could be classified in functional categories (% of total no. of known genes): transcription and translation (28.6%), protein metabolism (21.4%), energy metabolism (17.9%), immune and stress response (10.7%), cell structure and cytoskeletal organization (3.6%), carbohydrate and fat metabolism (3.6%), signal transduction (3.6%) and development (3.6%) (Fig. 2). All the differently expressed genes with homology to sequences in public data are listed in Table 2.

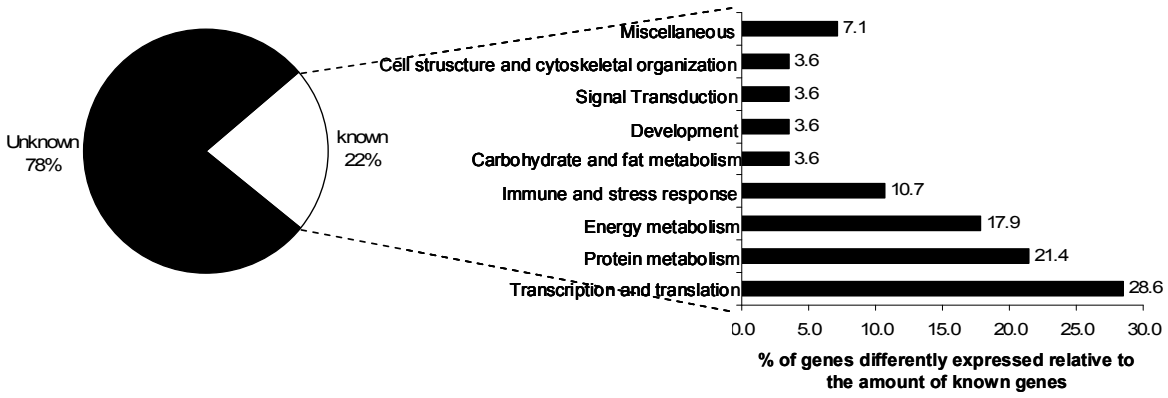

Figure 2: All unique and known differently expressed genes subdivided into functional classes according to their GO annotation.

Table 2. Differently expressed genes with homology to known sequences. Results presented include the gene homology, E value, species match and information about which gene was affected in each treatment (↓: down regulated; ↑: up regulated; ↔: not differently expressed).

			Different exposure treatments							
Gene per functional category	**E-value**	**Species match**	**Cu 400**	**Cu-NP 400**	**Cu 600**	**Cu-NP 600**	**Cu 800**	**Cu-NP 800**	**Cu 1000**	**Cu-NP 1000**
Transcription and translation										
replication protein a3	4.16E-17	*Cryptosporidium parvum*	↓	↔	↔	↔	↔	↔	↔	↔
transcription factor	2.94E-05	*Humulus lupulus*	↔	↔	↔	↔	↓	↓	↔	↔
mitochondrial ribosomal	3.66E-27	*Aedes aegypti*	↔	↑	↔	↔	↔	↔	↔	↔
ribosomal protein l7a	8.80E-10	*Ciona intestinalis*	↓	↓	↑	↔	↔	↔	↔	↔
16s ribosomal rna partial sequence	1.00E-04	-	↔	↓	↑	↔	↔	↔	↔	↔
small nuclear ribonucleoprotein d2	3.93E-19	*Salmo salar*	↔	↓	↔	↔	↓	↓	↔	↔
poly (adp-ribose) polymerase member 14	6.80E-18	*Branchiostoma floridae*	↔	↔	↓	↔	↔	↓	↔	↔
elongation factor-1 alpha	8.81E-75	*Antonospora locustae*	↓	↔	↔	↔	↔	↔	↔	↔
Immune and stress response										
mannose C type 1-like 1	4.27E-09	*Thrasops jacksoni*	↓	↔	↔	↔	↔	↔	↔	↔
superoxide dismutase	5.16E-31	*Chlamys farreri*	↓	↔	↔	↔	↔	↓	↔	↔
heat shock hspa8	2.84E-08	*Numida meleagris*	↑	↔	↔	↔	↔	↔	↔	↔
Energy metabolism										
short-chain dehydrogenase	1.03E-30	*Strongylocentrotus purpuratus*	↓	↔	↔	↔	↔	↔	↔	↔
nadh dehydrogenase fe-s protein 4	2.18E-44	*Xenopus laevis*	↔	↔	↔	↔	↔	↑	↔	↔
adipokinetic hormone akh2	3.48E-04	*bombyx mori*	↔	↔	↓	↔	↔	↔	↔	↔
cytochrome c	5.95E-39	Tribolium castaneum	↔	↔	↔	↔	↔	↑	↔	↔
atp synthase e chain	9.79E-09	*Amblyomma americanum*	↑	↔	↔	↔	↔	↓	↔	↔
Protein metabolism										
serine threonine kinase	1.29E-04	*Nicotiana tabacum*	↔	↔	↔	↔	↓	↔	↔	↔
calmodulin-domain protein kinase	1.00E-04	*Cryptosporidium parvum Iowa II*	↓	↔	↔	↔	↔	↔	↔	↔
peptidoglycan recognition protein sc2	5.92E-63	*Branchiostoma floridae*	↔	↑	↔	↔	↔	↔	↔	↔
calcium-dependent protein kinase	3.60E-09	*Trichomonas vaginalis*	↔	↔	↔	↔	↓	↓	↔	↔
arginine	1.69E-07	*Malus domestica*	↑	↔	↔	↔	↔	↔	↔	↔
chaperonin subunit 8	1.00E-39	*Xenopus tropicalis*	↑	↔	↔	↔	↔	↔	↔	↔
Carbohydrate and fat metabolism										
monosaccharide-transporting ATPase	4.15E-52	*Burkholderia phymatum STM815*	↔	↑	↔	↔	↔	↑	↔	↔
Development										
regeneration-upregulated protein	3.47E-04	*Enchytraeus japonensis*	↓	↔	↔	↔	↔	↔	↔	↔
Signal Transduction										
protein tyrosine phosphatase 4a2 of regenerating liver	4.55E-72	*Canis familiaris*	↔	↔	↑	↔	↔	↑	↔	↔

Cell struscture and cytoskeletal organization										
microtubule-associated protein tau	1.96E-20	*Branchiostoma floridae*	↓	↔	↔	↔	↔	↔	↔	↔
Unknown										
diuretic hormone 34	3.15E-06	*Bombyx mori*	↔	↑	↔	↔	↔	↔	↔	↔
leukocyte receptor cluster member 1	6.04E-15	*Branchiostoma floridae*	↓	↔	↑	↔	↔	↑	↔	↔

Several genes encoding translational proteins were down-regulated by both copper and nano-copper exposure, including replication protein A3 (RPA3), transcription factor, small nuclear ribonucleoprotein D2, poly (ADP-ribose) polymerase member 14, and elongation factor-1 α. RPA3 is the smallest subunit of Replication Protein A (RPA) and is believed to have a structural role in the assembly of RPA heterotrimer. RPA is a nuclear ssDNA binding protein which appears to be involved in all aspects of DNA metabolism including replication, recombination, and repair. A reduction in the amount of RPA could be indicative of DNA damage, since the recruitment of RPA to DNA damaged sites may contribute to its down-regulation (Wang et al. 1999). Poly ADP-ribose polymerase member 14 (PARP14) is a multifunctional protein implicated in many cellular functions. PARP proteins are important components in the cellular responses to various kinds of insults to genomic DNA, including oxidative DNA damage, telomere erosion, or improper segregation of chromosomes, thus its down-regulation could cause severe damage to *E. albidus*. Elongation factor-1alpha (EF-1α) is an actin binding protein involved in the shuttling of aa-tRNA. It was also found down-regulated in fathead minnow larvae when exposed to copper (Lewis and Keller 2009) and in recent investigations, its down-regulation has been related with cell death (Byun et al. 2009; Kobayashi and Yonehara 2009) A gene coding for a ribosomal protein (ribosomal protein L7A) was found down-regulated by the treatments Cu e Cu-NP 400 and up-regulated by Cu 600.

Energy metabolism was also affected by copper-salt and nano-copper. Short-chain dehydrogenase and adipokinetic hormone akh2 where found down-regulated while NADH dehydrogenase Fe-S protein 4 and cytochrome c were up-regulated (tab 2). ATP synthase e chain was found up-regulated and down-regulated by different treatments (tab 2). The disruption of genes associated with mitochondrial electron transport and consequent dysfunction due to copper

exposure has already been reported in the earthworm *Lumbricus rubellus* (Bundy et al. 2008). However, our data show the up-regulation of genes coding for NADH dehydrogenase Fe-S protein 4, ATP synthase e chain and cytochrome c (mitochondrial proteins). On the other hand, the up-regulation of monosaccharide-transporting ATPase (in the treatments Cu-NP 400 and 800) is in accordance with Bundy's results (Bundy et al. 2008), which suggests a switch to metabolism of stored carbohydrates after copper exposure.

In the group of transcripts involved in protein metabolism, there are down-regulated genes (serine-threonine kinase; calmodulin-domain protein kinase; and calcium-dependent protein kinase) and up-regulated genes (peptidoglycan recognition protein sc2, arginine and chaperonin subunit 8). Protein kinases play a role in a multitude of cellular processes, including division, proliferation, apoptosis and differentiation, which could be affected by the down-regulation of these three kinases. Peptidoglycan recognition proteins (PGRPs) are involved in innate immunity. They are pattern recognition receptors that bind, and in certain cases, hydrolyze peptidoglycans (PGNs) of bacterial cell walls. The immune response of *E. albidus* can be compromised in the treatment Cu 400 due to the down-regulation of the gene encoding for manose C type 1-like.This is a type I membrane receptor protein highly related to the mannose receptor C type 1 protein (MRC1) which mediates the endocytosis of glycoproteins by macrophages and acts as phagocytic receptor for bacteria, fungi and other pathogens.

Both chaperonins and heat shock proteins are involved in assisting the folding of proteins into their functional state, and genes coding for these proteins where found up-(tab 2). The gene HSPA8 encodes a heat shock cognate protein (that belongs to the heat shock protein 70 family) which binds to nascent polypeptides to facilitate correct folding. The up-regulation of transcripts encoding a heat shock protein (HSPA8), has already been observed in response to metals (Nota et al. 2008). As suggested to cadmium (Nota et al. 2008), copper ions probably disturb the normal folding of proteins or cause indirect damage to proteins, e.g. trough the generation of reactive oxygen species (ROS). Although copper is known to cause oxidative damage through the formation of hydroxyl radicals via the Haber-Weiss

reaction (Bremner 1998), the transcript encoding superoxide dismutase (SOD), an enzyme involved in antioxidant defence, was found down-regulated (tab. 2).

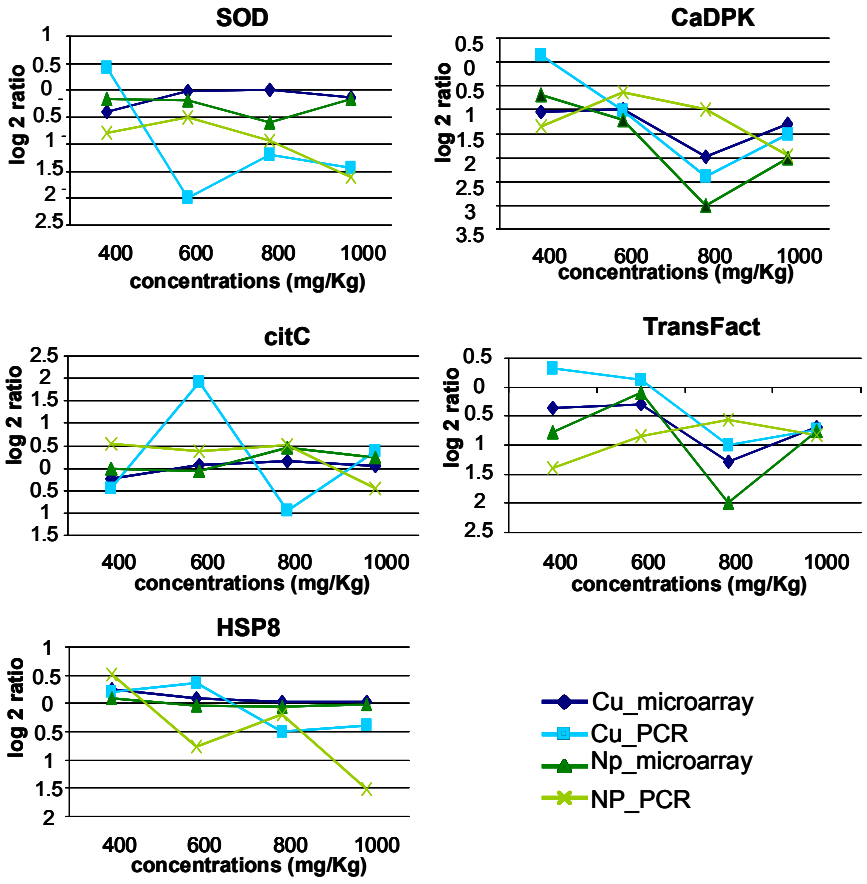

Figure 3: Gene expression changes observed in microarray and RT-PCR for superoxide dismutase (SOD), calcium dependent protein kinase (CaDPK), cytochrome c (citC), transcription factor (TransFact) and heat shock protein 8 (HSP8). Results are expressed by means of log2 ratio exposed/control.

In Fig. 3, results from the RT-PCR analysis are shown. Except for SOD, in the treatment Cu 400, which showed to be, up-regulated in the RT-PCR, the expression of all genes in the microarray (tab 2) was confirmed in the RT-PCR.

It was confirmed that there was no concentration dependent response in any of the tested genes, which was in agreement with the microarray data (tab 2). For Cu (copper-salt) treatments, in general genes show the highest expression value at 400mg/Kg and the lowest expression at 800 mg/Kg. In NP (nano-copper) treatments, the higher down-regulation (lowest expression value) was always

observed in 1000 mg/Kg, which suggest that the higher concentration of copper nanoparticles caused higher effects in gene expression of *E. albidus*.

Based on differently expressed genes (BH adjusted p<0,05), a cluster analysis (Pearson's Uncentered) was performed to both genes and treatments. The cluster analysis of genes did not cluster according to function (GO homology), suggesting that either form of copper affected the same functions. However this information is poorly supported at the moment due to the fact that it is not know in which processes the "unknown" genes are involved.

The cluster between treatments is shown in figure 4.

Figure 4: Hierarchical cluster of samples (Perarson's uncentered), based on differently expressed genes (BH adjusted p<0.05).

No separation between treatments (Cu-salt or nano-Cu) is observed based on the cluster analysis. However, there is a trend to cluster by concentration range: the two smaller (400 and 600 mg/Kg, with the exception of Cu 600) and the two higher (800 and 1000 mg/Kg) concentrations are clustered separately.

4. Conclusions

DNA microarray analysis was an important tool in analysing effects, showing that there was differential gene expression in response to the different treatments. Additionally, transcriptional response induced by copper nanoparticles was different from those induced by the copper-salt. At the moment, in part due to the fact that *E. albidus* is a non-model organism and that the function of the great part of the genes is unknown, the discrimination between copper nanoparticles and copper-salt was not possible, neither the unravelling of the underlying mechanisms of toxicity.

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Chapter 5

General Discussion and Conclusions

General discussion and conclusions

The results presented in this Masters thesis show a piece of research done regarding the use of sub-cellular and molecular biomarkers (oxidative stress, neuro-muscular and gene expression) in ecotoxicology. The purpose was to compare the responses in *Enchytraeus albidus* of the two copper forms: copper-salt ($CuCl_2$) and copper nanoparticles. Results show that both tools were adequate to assess effects, revealing oxidative stress and differently expressed genes although, not able to fully discriminate and interpret effects.

The battery of biomarkers analysed in this study was chosen to have a broad range of enzymes: being the first time nanomaterials are studied in *E. albidus* we wished to obtain the general response of the organism. A clear separation between the two treatments was observed, which could indicate that the effects caused by nano-copper are not only due to the solubility. A time and concentration dependent response was also inferred. However, it was not possible to choose one or a group of biomarkers which could characterize the response. Nevertheless, glutathione-peroxidase and non-enzymatic glutathiones (reduced glutathione, oxidised glutathione) seemed to act as the main anti-oxidant defence against stress induced by copper (salt and nano form) at 4 days of exposure, even if cellular damage was occurring after 8 days, observed by the increased levels of lipid peroxidation. To discriminate between copper-salt and copper nanoparticles, a broader range of concentrations should be tested.

At the gene expression level, in general, the biological functions affected were transcription and translation, protein metabolism, energy metabolism, immune and stress response, carbohydrate and fat metabolism, development, signal transduction and cell structure and cytoskeletal organization, all involving key functions that can lead to effects at higher levels of biological organization. In general, copper-salt caused a higher number of differently expressed transcripts. The proportion of genes in each category was different between the copper forms, however conclusions cannot be drawn based on these results, due to a large percentage of “unknown” gene functions. The fact that *E. albidus* is a non-model

organism (attending their use in molecular techniques since its genome has not been sequenced, and not according to the ecotoxicological point of view where it is, in fact, a model organism) and thus the analysis of the results is restricted by the homology of DNA fragments in other organisms, is a limiting factor in the interpretation of the results obtained.

Further research is needed to link these effects with higher levels of biological organization, to allow the integration of effects at population, sub-cellular and molecular levels. More metal-based nanomaterials could be studied, using these parameters, to determine if the response is related to copper nanoparticles and, if so, to establish a group of biomarkers suitable to characterize this response.

This study represents an important contribution on the field of ecotoxicology of nanomaterials to soil ecosystems/organisms.