Universidade de Aveiro Departamento de Biologia
Ano 2016

Micael Ferreira Mota Gonçalves

Development of novel short and long term studies in *Enchytraeus crypticus*

Desenvolvimento de novos estudos de curto e longo prazo em *Enchytraeus crypticus*

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Biologia Molecular e Celular, realizada sob a orientação científica da Doutora Mónica Amorim, Investigadora Auxiliar do CESAM, Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Susana Gomes, Investigadora em Pós-doutoramento do CESAM, Departamento de Biologia da Universidade de Aveiro.

Aos meus pais, irmão e pedaços de mim. *Obrigado*.

o júri

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Palavras-chave Teste de embriotoxicidade; Teste de longevidade; Regeneração; *Enchytraeus crypticus*.

Resumo

A maioria dos estudos ecotoxicológicos têm investigado os efeitos de curto prazo dos contaminantes. Comparativamente, tem sido desenvolvido pouco trabalho na avaliação dos efeitos a longo prazo dos químicos existindo portanto uma necessidade de preencher esta lacuna. De entre as espécies de solo usadas em ecotoxicologia estão os Enquitraídeos (Oligochaeta), membros importantes da mesofauna terrestre com diretrizes padrão para testar os efeitos ao nível da sobrevivência, reprodução e bioacumulação (ISO, 2004; OECD, 2010, 2004). Para a espécie *Enchytraeus crypticus*, existe também disponível o cDNA microarray com mais de 40 000 transcritos (Castro-Ferreira et al., 2014) sendo uma vantagem competitiva em relação a outras espécies padrão. O principal objetivo desta pesquisa foi desenvolver novos testes de curto e longo prazo, abrangendo novos/diferentes endpoints para *E. crypticus*.

A habilidade de regeneração do *E. crypticus* foi descoberta e o processo de regeneração foi descrito; este pode ser adicionalmente utilizado para avaliar os efeitos de tóxicos em testes de curto prazo.

O desenvolvimento embrionário de *E. crypticus* foi investigado e um teste de embriotoxicidade foi desenvolvido (pela primeira vez em um invertebrados do solo). O cádmio (Cd) foi usado como substância de teste para validar o teste embriotoxicidade dados os seus conhecidos efeitos embriotóxicos. Os resultados mostraram que o Cd causou uma diminuição no sucesso da eclosão devido a um atraso ou interrupção na formação de estruturas embrionárias.

Um teste de longevidade (com avaliação da sobrevivência e reprodução ao longo do tempo) foi desenvolvido para *E. crypticus*. Este ensaio de exposição de longo prazo foi utilizado para avaliar os efeitos de nanopartículas de óxido de cobre (CuO-NPs) em comparação com $CuCl_2$ revelando que CuO-NPs causou efeitos superiores (diminuindo a longevidade e reduzindo a reprodução) do que $CuCl_2$, a uma concentração de efeito semelhante. Este ensaio traz um novo conceito em ecotoxicidade, a longevidade. Este é um especto particularmente importante quando o assunto é a toxicidade de nanomateriais (NMs), onde se espera que o tempo de exposição a longo prazo revele efeitos imprevisíveis através dos testes correntes de curto/longo prazo.

O uso dos novos ensaios desenvolvidos podem melhorar a avaliação dos perigos dos produtos químicos.

Keywords Embryotoxicity test; lifespan test; regeneration; *Enchytraeus crypticus*

Abstract

Most of the ecotoxicity studies have investigated the short-term effects of chemicals. Comparatively, little work has been done in the assessment of the long-term effects of chemicals and there is a need to fill this gap. Among soil species used in ecotoxicology are Enchytraeids (Oligochaeta), important members of the terrestrial mesofauna with standard guidelines for testing effects at survival, reproduction and bioaccumulation level (ISO, 2004; OECD, 2010, 2004). For the species *Enchytraeus crypticus*, there is also available the cDNA microarray with more than 40 000 transcripts (Castro-Ferreira et al., 2014) being a competitive advantage in comparison to other standard species. The main goal of this research was to develop novel short and long-term tests, covering new/different endpoints, for *E. crypticus*.

Regeneration ability of *E. crypticus* was discovered and the regeneration process was described; this can be further used as endpoint to assess the effects of toxicants in short-term studies.

The embryonic development of *E. crypticus* was investigated and an embryotoxicity test was developed (for the first time in a soil invertebrate). Cadmium (Cd) was used as a test substance to validate the embryotoxicity test given its known embryotoxic effects. Results showed that Cd caused a decrease in the hatching success due to a delay or disruption in formation of embryonic structures.

A lifespan test (with assessment of survival and reproduction over time) was developed for *E. crypticus*. This long-term exposure assay was used to assess the effects of copper oxide nanoparticles (CuO-NPs) in comparison with $CuCl_2$ revealing that CuO-NPs caused higher effects (shortening lifespan and reducing reproduction) than $CuCl_2$, at similar effect concentration. This lifespan assay brings a novel concept in ecotoxicity, the longevity. This is a particularly important aspect when the subject is nanomaterials (NMs) toxicity, where longer term exposure time is expected to reveal unpredicted effects via the current short/long-term tests.

The use of the new assays developed can improve the hazard assessment of chemicals.

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Chapter 1

General Introduction

General Introduction

1. Ecotoxicology and Ecotoxicogenomics

Ecotoxicology is a multidisciplinary science which integrates toxicology and ecology (Hermens et al., 2004). Initially dedicated to the study of anthropogenic toxicants, the term was firstly used by René Truhaut in 1969, as "the branch of toxicology concerned with the study of toxic effects, caused by natural or synthetic pollutants, to the constituents of ecosystems, animal (including human), vegetable and microbial, in an integral context" (Truhaut, 1977). Now, ecotoxicology can be defined as the study of the adverse effects of chemicals on ecosystems structure, functions, and biodiversity in different levels of organization (individuals, populations, communities) (Hermens et al., 2004). The main goals of this discipline are identify, predict, control, and minimize the negative environmental consequences of the recent human industrial development. In this way, mandatory testing programs like those posed by REACH (Registration, Evaluation, Authorization and Restriction of Chemicals) in Europe and the U.S. Environmental Protection Agency (EPA) are essential to increase the information available for the chemicals where toxicity data are insufficient, like emerging contaminants (Ankley et al., 2006; Sanderson and Solomon, 2009).

Emerging contaminants result from: natural toxins, veterinary and human medicines, hormones, nanomaterials, human personal care products and paints (OECD, 2010a). Some of them, like transformation products of synthetic chemicals may be formed in the environment by biochemical processes in organisms (Boxall, 2012). Nowadays, they are a complex and pressing concern in environmental health, and new approaches, such as genomics, have significant implications in risk assessment for humans and ecosystems (Miracle and Ankley, 2005; Poynton and Vulpe, 2009).

The term genomics was first used in 1920, by Winkler, to describe the complete set of chromosomes and their associated genes (Snape et al., 2004). Nowadays, genomics is a broadly used term that encompasses numerous scientific disciplines and technologies. These disciplines include genome sequencing, assigning function to identified genes, determining genome architectures, studying gene expression at the transcriptome level, studying protein expression at the proteome level, and investigating metabolite flux (Snape

et al., 2004). Bioinformatics aim to link the massive and complex data generated from the "-omics" with population genetics, histopathology, and ecology to generate a landscape of events occurring within a given organism, or collection of organisms, from source of stressors through exposure and ultimately, to outcomes (Miracle and Ankley, 2005; Snape et al., 2004).

In 1999, Nuwaysir described the use of microarray technologies in toxicology, presenting the possibilities of a new field called toxicogenomics (Poynton and Vulpe, 2009). Toxicogenomics combines the fields of genomics and mammalian toxicology (Iguchi et al., 2007), studying the genes and their products in adaptive responses to chemical-derived exposures (Snape et al., 2004). It has three major goals: the elucidation of the relationship between molecular mechanisms underlying toxics responses to environmental contaminants; understanding the relationship between chemical exposure and adverse effects; and identification of useful biomarkers of exposure to toxic substances (Watanabe et al., 2008).

Ecotoxicogenomics describes the integration of toxicogenomics (transcriptomics, proteomics and metabolomics) into ecotoxicology (Iguchi et al., 2007; Watanabe et al., 2008), using organisms that are representative of ecosystems to study genes and protein expression in non-target organisms in response to environmental toxicant exposures. Standardized methods rely on measuring whole-organism responses (e.g. mortality, growth, reproduction) of generally sensitive indicator species at maintained concentrations, and deriving "endpoints" (e.g. median lethal concentrations, no observed effect concentrations, etc.), not providing understanding of the mechanism of chemical toxicity. Without this understanding, it is difficult to predict how toxic responses across the very broad diversity of the organisms present in aquatic and terrestrial ecosystems; to estimate how changes at one ecological level or organization will affect other levels (e.g. predicting population-level effects); and to predict the influence of time-varying exposure on toxicant responses (Snape et al., 2004). Ecotoxicogenomic tools may provide us with a better mechanistic understanding of this key challenges having a remarkable potential in ecological risk assessment. Figure 1 depicts the interactions between genomics and ecotoxicology.

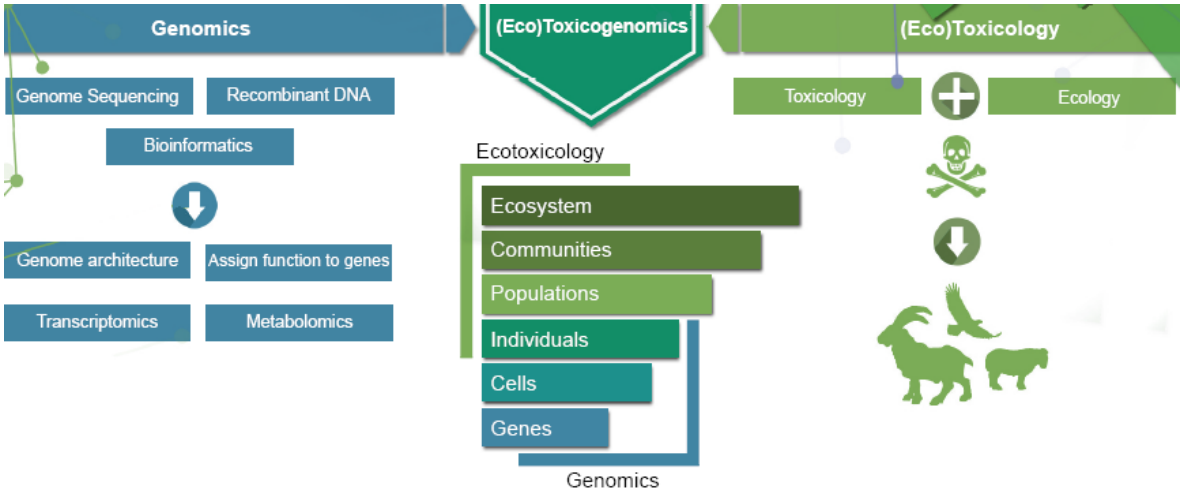

Figure 1: Diagram illustrating the interactions between genomics (blue) and ecotoxicology (green).

2. Short- and long-term tests

In ecotoxicology, the main objective of testing procedures is to assess hypotheses about the potential for chemicals that can cause adverse effects in organisms. Ecotoxicological tests are important for guiding decisions of pollutants relating to risk assessment and regulatory purposes allowing major improvements in environmental quality.

Standardized tests are one of the first steps in risk assessments of contaminants. Normally, they are sufficient for determining the ecological risk level of chemicals and the limit concentrations for humans and biota (Alves and Cardoso, 2016).

Ecotoxicological tests can be distinguished in two approaches (Van Gestel, 2012). The first approach includes analyses to determine the possible toxic effects of the substances. This approach is mainly used to test new substances for which the safe exposure in environment are unclear and can be used to regulate their use or prevent the entry in the market. The second approach involves analyses to determine the actual ecological risk or current damage using samples of contaminated media (Alves and Cardoso, 2016). These two approaches include many tests that can be classified according to exposure time (acute or chronic toxicity), observed effect (mortality, growth, reproduction, bioaccumulation and behavioural changes) or effective response (lethal or sublethal) (Kapanen and Itavaara,

2001). In these tests, representative species of the fauna/flora are exposed to contaminants and the effects are measured in one (single species) or several species (multispecies) to test dose-response relations (Jänsch et al., 2005).

Concerning soil invertebrates, the main standardized tests by the norms of the Organization for Economic Cooperation and Development (OECD) and International Organization for Standardization (ISO) consist of exposing species to contaminated/spiked media. These protocols describe methods used to determine acute and chronic effects on earthworms (*Eisenia andrei* and *Eisenia fetida*), collembolans (*Folsomia candida* and *Folsomia fimetaria*), enchytraeids (*Enchytraeus albidus* and *Enchytraeus crypticus*), mites (*Hypoaspis aculeifer*, *Platynothrus peltifer*, and *Oppia nitens*), isopods (*Porcellio scabere* and *Porcellionides pruinosismolluscs*), molluscs (*Helix aspersa*), and insects (*Pterostichus oblongpunctatus*, *Poecilus cupreus* and *Oxythyrea funesta*) (Van Gestel, 2012).

Most of the existent data are based on acute toxicity tests (short-term tests) covering few hours up to a few weeks of duration (about 28-30 days) (Diez-Ortiz et al., 2015; Peters and Granek, 2016). They are useful for short-term identification of toxic contaminants and also used as initial evaluations to determine the concentration ranges to be used in definitive acute toxicity tests and/or the sublethal concentrations for chronic toxicity tests (Alves and Cardoso, 2016). Beyond that, when chemicals are released into the environment and depending of their persistence, can greatly exceed the duration of these toxicity test procedures and many authors have suggested more long-term studies with concentrations that reflect those detected in the environment (Baun et al., 2008; Kumar et al., 2014). Like this, Diez-Ortiz et al. (2015) and Oberdorster (2007) reported some concerns about the chemical persistence in the environment including the propagation of toxicity over-time, especially with nanomaterials when chemicals have high toxicokinetics which may continue accumulated in the tissues.

Chronic toxicity tests can be medium/long-term tests that measure the sublethal effects of toxic substances on organisms, such as changes in reproduction and growth. They are useful for assessing effects at the population level (Hoffman et al., 2003; Van Gestel, 2012). The first standard methods for chronic toxicity tests have been established in ISO (2012, 1999) and OECD (2009, 2004) guidelines. The objective of these standardized tests is similar for different groups of invertebrates. The only difference is in test duration because organisms have different reproductive characteristics (Alves and Cardoso, 2016).

In fact, the standardized guidelines for soil ecotoxicological tests are relatively new compared with the guidelines for aquatic environment and there is an increase in the number of tests based on these guidelines. This tests have been used to investigate the potential toxicity of several substances like fungicides, herbicides, insecticides, metals, nanomaterials, agro-industrial residues, and other substances in soil (Van Gestel, 2012).

To assess the hazards involved in the action of chemicals on soil organisms, several methods have been developed for many species. Also, the majority of these studies have already well-defined endpoints, such as, survival, reproduction, bioaccumulation and growth (Leppanen and Kukkonen, 1998). To our knowledge, in soil ecotoxicology with invertebrates there are no standardized tests specifically concerned with the effects of contaminants on embryonic development available (Druart et al., 2010). Embryotoxicity tests are included in short-term tests and they are important tools for risk assessment (Schirling et al., 2006) due to the level of detailed information that is provided. Further, their short duration and prediction power for effects on the individual at later stages is highly relevant and within the 3R (Replacement, Reduction and Refinement), and are also cost-effective. The 3Rs approach as formulated by Russel and Birch in 1959 and defined three strategies for reducing the number and the suffering of experimental animals used in research (Part et al., 2009). In an ecotoxicology perspective, few studies were performed using the terrestrial slugs *Deroceras reticulatum* (Iglesias et al., 2002) and snails *Monacha obstructa* and *H. aspersa* (Druart et al., 2010; Shoaib et al., 2009). For vertebrates, the test with *Danio rerio* is already standardized (OECD, 2013).

Another endpoint less used to assess the effects of toxicants as short-term study is the regeneration abilities of organisms (e.g. effects of tributyltin in planarian *Schmidtea mediterranea* regeneration (Ofoegbu et al., 2016)). Regeneration is the replacement of lost body parts, although the term has been applied to a broad range of processes (Bely and Nyberg, 2010). Regeneration can occur at multiple levels of biological organization (Fig. 2). Can occur at different parts of the life cycle triggered by a variety of stimuli and the structures regenerated can be relatively different to the original (Bely and Nyberg, 2010).
The capacity to regenerate lost body parts of animals is an aspect of biology poorly understood and highly variable (Bely and Nyberg, 2010; Tanaka and Reddien, 2011).

Many phyla have been investigated in relation to the regeneration abilities (Fig. 3). Bely and Nyberg (2010) reported that the broad variation in regeneration capabilities across animals is not easily explained. For example, many groups of animals such as birds, mammals, leeches and some nematodes are incapable of regenerate any lost structure (Goss, 1969) while other can, e.g. cnidarians, annelids, molluscs, platyhelminthes (Tanaka and Reddien, 2011). Moreover, the mechanisms of regeneration ability varies between species with respect to which parts of the body can be regenerated (Bely and Nyberg, 2010), e.g. planarians can replace a missing head or the entire body from small fragments (Tanaka and Reddien, 2011), many annelids can regenerate the whole body or only the anterior or the posterior segments (Bely, 2006), lizards are capable of replacing a missing tail but not a limb (Alibardi and Toni, 2005).

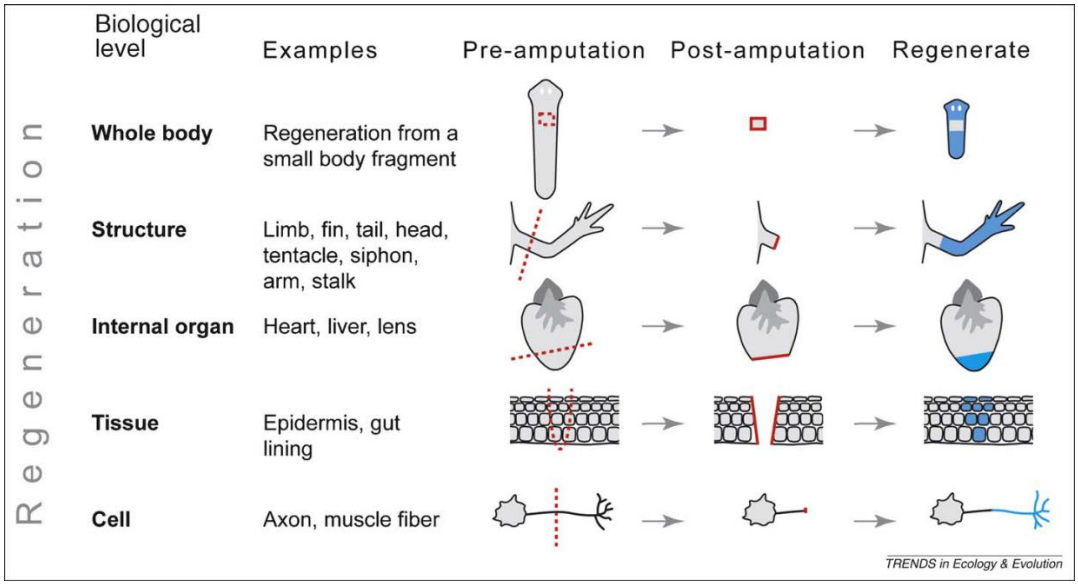

Figure 2: Regeneration at different levels of biological organization. A particular species might regenerate at all, none, or just a subset of these levels. Dashed red lines indicate amputation planes; solid red lines indicate wound surfaces; and blue fill indicates regenerated body parts (Bely and Nyberg, 2010).

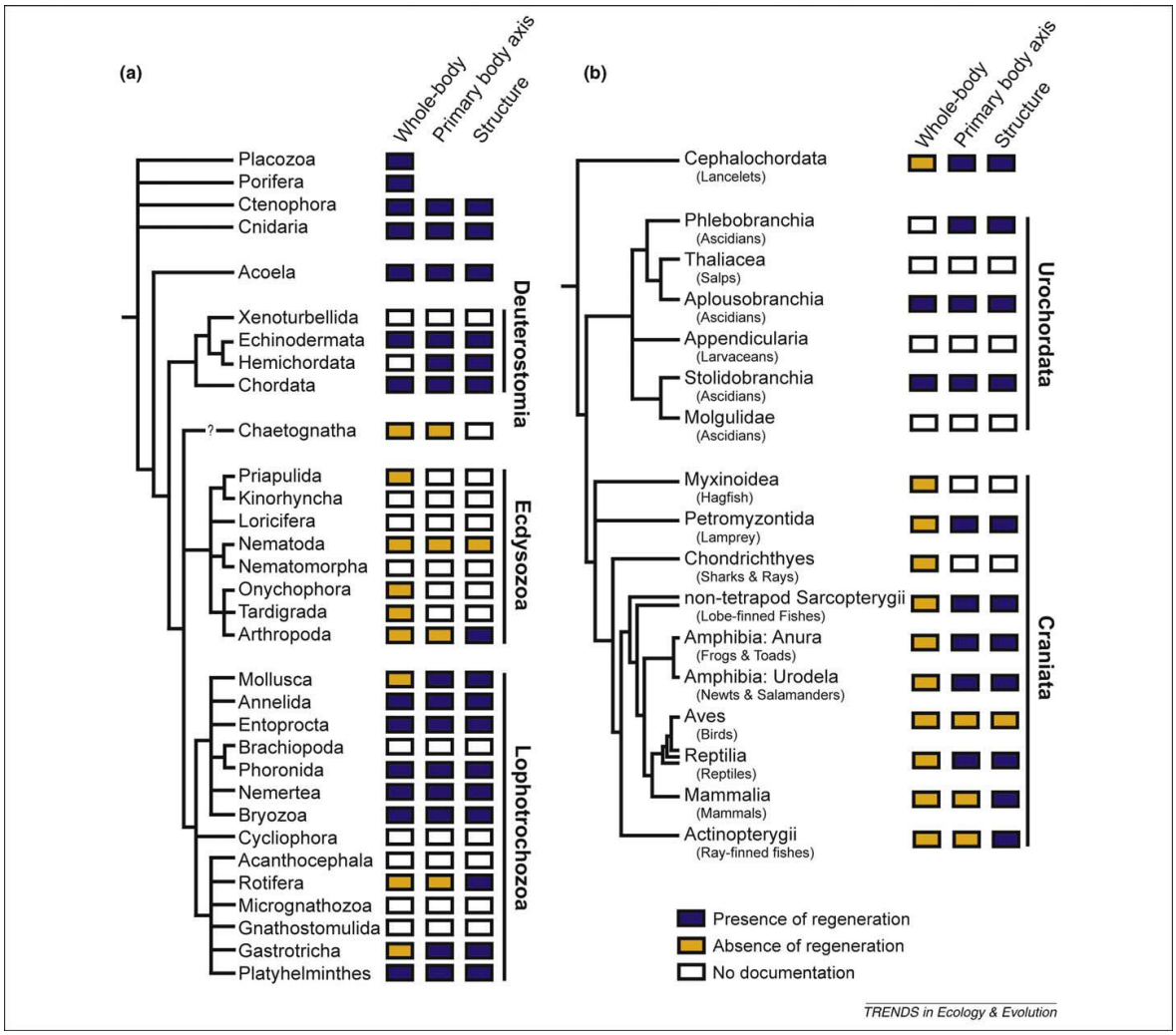

Figure 3: Phylogenetic distribution of regeneration across (a) the Metazoa and (b) the Chordata. Presence of regeneration indicates that at least one report exists for regeneration in that taxon (not imply that all species in that taxon can regenerate). Absence of regeneration indicates that there is at least one report for the lack of regeneration in that taxon (and none indicating the presence of regeneration) (Bely and Nyberg, 2010).

To our knowledge there are no standardized tests for regeneration as endpoint available for soil or aquatic invertebrates. Among the many species that are recognized as models for regeneration research, developmental biology and used for ecotoxicological studies, the freshwater planarian *Schmidtea mediterranea* is the most used (Ofoegbu et al., 2016). Although there are not standardized protocols for freshwater planarians they have been

used in several studies to measure regeneration and locomotor behaviour (Knakievicz, 2014; Pagan et al., 2009). Regeneration in planarians involves proliferation of the totipotent stem cells, neoblasts, to form new tissues at wound sites (blastema formation) and remodelling of old tissues (Reddien and Sanchez-Alvarado, 2004).
Since many chemicals are known to have cytotoxic and teratogenic effects (Hagger et al., 2002; Velma et al., 2009), they may alter the regeneration process. In this way, the regeneration capacity can also be used (as short-term test) to evaluate the effects of chemicals.

On the other hand and as mentioned before, long-term studies are also very important for risk assessment. They represent a continuous exposure to toxicants during a long period or the whole life, similar to what happens in the natural environment, creating a potential realistic scenario of adverse ecological effects caused by toxicants (Coutellec and Barata, 2013; Van Gestel, 2012). However the cost and time involved in these tests are frequently high.
Lifespan tests are included in the category of long-term exposures. Despite the recognized importance of understand the long-term effects of contaminants, relatively few studies have focused on the lifespan of organisms (Harada et al., 2007). Some model organisms are frequently used to study lifespan effects, such as, *Drosophila melanogaster*, *Mus musculus*, *Saccharomyces cerevisiae* and the most commonly used the "soil" organism *Caenorhabditis elegans* (Buffenstein et al., 2008). However, none of the organisms described are used in a real ecotoxicological context. Most of these studies (using *M. musculus*, *D. melanogaster*, S. cerevisiae and *C. elegans*) were performed to discover genetic, environmental and pharmacologic modulators of aging for the lifespan extension purpose, providing new insights for human therapy (Hamilton and Miller, 2016; Lucanic et al., 2013). Studies that assess the effects of contaminants in lifespan are still limited, the few examples use *C. elegans* to investigate lifespan effects of metals and detergents (Harada et al., 2007; Wang et al, 2010).

3. Test organism

Enchytraeids have been used in ecotoxicological laboratory tests for more than 30 years (Rombke and Moser, 2002). Enchytraeids belongs to the family Enchytraeidae, order Oligochaeta, class Clitellata and phylum Annelida. They are whitish small oligochaetes (1-40 mm), typical of the saprophagous mesofauna, feeding on decomposed plant residues and microorganisms (Jänsch et al., 2005) and found in a large variety of soils (Didden, 1993). They are ecologically important, because they contribute to the decomposition of organic matter and nutrient cycling and improve soil pore structure (Didden, 1993). Despite of that, this organisms had been practically ignored as test organisms during many years until to find the sensitivity to stress in field studies caused by human activity (Römbke, 2003) and one of the main invertebrates used in standardized terrestrial ecotoxicological tests are enchytraeids (Rombke and Moser, 2002).

Worldwide, about 950 species of enchytraeids have been described (Didden et al., 1997) and in Central Europe there is appreciably between 200 to 300 species (Jänsch et al., 2005). In temperate regions enchytraieds' population density may range between a few thousand up to 100 000 individuals/m^2, and outside of these regions, the density can vary even more (Jänsch et al., 2005). The influence of abiotic factors on enchytraeids' population density is well known. Temperature, moisture, pH, and organic matter can influence the spatial and temporal distribution of these organisms (Didden, 1993; Graefe and Schmelz, 1999; Jänsch and Römbke, 2003) as well as their reaction to stress factors (Rombke and Moser, 2002).

In general, enchytraeids reproduce sexually by cross-fertilization (the most common mode of copulation in oligochaetes) and/or self-fertilization, but asexual fragmentation with subsequent regeneration is also possible (Jänsch et al., 2005).

E. crypticus (Fig. 4) is a good model in ecotoxicology due to its short life cycle (25 days from cocoon release until the production of new offspring), and a rapid embryogenesis (9–11 days) (Bicho et al., 2015; Westheide and Müller, 1996). This species is also easy to manipulate, maintain, monitor and the cocoons are transparent which facilitates the visualization and study of the embryonic development. *E. crypticus* has standardized ecotoxicity tests to evaluate survival, reproduction and bioaccumulation (ISO, 2004; OECD, 2010b, 2004), moreover the hatching success, growth and maturity status in the

form of a full life cycle test (Bicho et al, 2015). *E. crypticus* has an average size of about 7 mm (Westheide and Müller, 1996), reproduces relatively fast, and are very tolerant in terms of soil properties (for instance pH from 4.4 to 8.2, clay content from 1 to 29% and organic matter from 1.2 to 42% (Kuperman et al., 2006)), preferring temperatures around 25 to 30 ºC (Jänsch et al., 2005).

Figure 4: *Enchytraeus crypticus* (adult and juvenile).

4. Test chemicals

4.1. Cadmium

Cadmium (Cd) is a relatively abundant transition metal classified as a nonessential element (Fernández et al., 2003; Wang et al., 2004) and is included in the list of priority pollutants of EPA (Blechinger et al., 2002). The distribution of Cd in the environment has dramatically increased over the past decades due to its extensive use in industries and anthropogenic activities, like in agriculture, electroplating and galvanizing, batteries, colour pigment in paints and metal coatings and plastics (Gonzalez et al., 2006; Trinchella et al., 2010; Wang et al., 2004). It is also introduced in the atmosphere, water and soil as a result of the burning of fossil fuels, waste incineration and as a by-product of zinc and lead

mining and smelting (Gonzalez et al., 2006; Hassoun and Stohs, 1996). Thus Cd is a toxicant of considerable environmental and occupational concern.

Currently, most of the knowledge on Cd toxicity covers the "adults' stages" and in vitro testing (cell cultures), for which the mechanisms of toxicity of Cd are better known. For instance, Cd is known to cause oxidative stress, increasing the production of reactive oxygen species (ROS), metallothioneins (MTs) and heat shock proteins (HSPs), protein denaturation and lipid peroxidation (e.g. (Bertin and Averbeck, 2006; Dabas et al., 2013; Faverney et al., 2001; Jia et al., 2011; Muangphra and Gooneratne, 2011)). Also, Cd inhibit the enzymes involved in DNA synthesis and repair, and can cause the overexpression of the proto-oncogenes, some translation factors and apoptosis mechanisms (Bertin and Averbeck, 2006; Cambier et al., 2010; Gao et al., 2013; Gonzalez et al., 2006; Lag et al., 2002). Castro-Ferreira et al. (2012) showed the effects of Cd on survival and reproduction in *E. crypticus* (LC_{50} > 320 mg/kg and EC_{50} = 35 mg/kg).

On the other hand, the molecular mechanisms behind Cd embryotoxicity remains poorly known (Blechinger et al., 2002; Cheng et al., 2000; Yamamoto et al., 2012). For example, for embryos of the vertebrates *Danio rerio* (zebrafish) and *Podarcis sicula*, Cd promotes oxidative stress and affects pathways associated with membrane traffic, cytoskeletal organization, apoptosis and cell-cycle regulation include cell proliferation, differentiation and cellular metabolism (Chan and Cheng, 2003; Hsu et al., 2013; Pereira et al., 2013; Simoniello et al., 2011; Trinchella et al., 2010). For invertebrates, the knowledge is more limited, nevertheless, induction of oxidative stress and apoptosis were also identified for *Paracentrotus lividus* embryos (Agnello et al., 2007, 2006; Roccheri et al., 2004; Russo et al., 2003).

Cd was used as a test substance in the development of embryotoxicity test (Chapter 3) given its known embryotoxicity (Brasfield et al., 2004; Coeurdassier et al., 2003; Druart et al., 2010; Gomot, 1998).

4.2. Copper salt

Copper (Cu) is a trace metal classified as essential element for most living organisms (Mortimer et al., 2010). It is crucial for biological functions and processes, involved in

growth, development, respiration and oxidative stress protection, functioning as a co-factor in many enzymes systems and proteins, like cytochrome oxidase and superoxide dismutase (Yasokawa et al., 2008).

However this metal can be toxic above certain concentrations and/or if organisms are exposed chronically in the environment (Gaetke and Chow, 2003). For example, free Cu ions, participate in the formation of ROS that can cause mitochondrial dysfunction and inactivation of some proteins (Gomes et al., 2014; Maria and Bebianno, 2011). Also it was found that Cu caused up-regulation of MT's and HSPs and reduction of body weight in *Lumbricus rubellus* (Bundy et al., 2008). Cu toxicity to enchytraeids is relatively well studied (Amorim and Scott-Fordsmand, 2012; Amorim et al., 2008, 2005; Gomes et al., 2015a, 2015b; Maraldo et al., 2006; Menezes-Oliveira et al., 2011).

Cu-salt ($CuCl_2$) was used as test substance in the long-term exposure (lifespan) test (Chapter 4) to be compared with nanosized CuO.

4.3. Copper oxide nanoparticles

NPs are particles with a size range between 1 and 100 nm, in at least one dimension. After more than two decades of basic and applied research, nanotechnologies are growing increasingly and used in many commercial products, like in cosmetics industry, antimicrobial paints and coatings, textile products, electronic devices, disinfectants, medicines, gene therapy, food and packaging, bioremediation, fuel catalysts and water purification (Vance et al., 2015).

NPs are characterized by size, structure, toxicity and specific physical and chemical properties (e.g. chemical composition, solubility, agglomeration, mobility, density, concentration and charge) (Frenk et al., 2013; Gomes et al., 2014). These specific properties make the application of NPs useful in several different products as mentioned above (Adam et al., 2015). However, there are many NPs that are of growing concern due to their increased use and release into the environment. After such releases, it is known that NPs can suffer reactions and transformations that will change their form, surface properties and characteristics (Pan and Xing, 2012).

Metal oxide NPs belongs to the family of nanomaterials, which include CuO, TiO2, ZnO among others that are extensively used in a variety of applications (Chang et al., 2012; Frenk et al., 2013). These NPs affect the environmental differently when compared with their equivalents (bulk size and dissolved ion), because of their intrinsic characteristics, like an amplified surface area and the charge and reactivity are more stronger, which may lead to increased bioavailability and toxicity, which makes them more hazardous to the organisms (Peralta-Videa et al., 2011; Qafoku, 2010). However is need take into account other variables that are involved in toxicity of these NPs, such as, oxidative state, exposure time, particle concentration, the target organism and the environmental matrices (habitat) (Klaine et al., 2008). For example, in soil compartment features such as the soil type, soil water content, soil organic matter and mineral composition need to be considered (Ben-Moshe et al., 2013). Over the last decade, there are many reports about the potential toxicity of nanometal oxides and some of them can be found in the OECD guidance manual for the testing of manufactured nanomaterials (OECD, 2010a).

CuO-NPs is not included in the OECD list (Pradhan et al., 2012) but it is one of the metal nanoparticles (NPs) that are commonly used with wide range of industrial and commercial applications, such as, gas sensor (Chowdhuri et al., 2004); metallic and plastic coatings (Hernández Battez et al., 2010); circuits, batteries and semiconductor devices (Zhang et al., 2005) and solar energy conversion (Yin et al., 2005). Due to their antimicrobial and antifungal properties these NPs are also used in textiles industries, paints, plastics, food preservation, additives in lubricants, skin products and medical science (Dastjerdi and Montazer, 2010; Delgado et al., 2001; Gabbay et al., 2006; Jin and Ye, 2007). The increased production of CuO-NPs increase the risk of their introduction into the environment and human health (Buffet et al., 2013; Siddiqui et al., 2013), where their small size and specific properties can cause adverse effects (Adam et al., 2015). Therefore, its potential toxicity combined with its relatively low dissolution rate should not be ignored and it is important investigate the toxicity of these materials (Blinova et al., 2010; Buffet et al., 2011; Saison et al., 2010; Stone et al., 2010).

In the literature, majority of the ecotoxicological studies regarding ecotoxicity of CuO-NPs is relative to aquatic compartment (Chang et al., 2012), and is mostly based on “short-term”/acute effects (Mortimer et al., 2010; Nations et al., 2011; Pradhan et al., 2012; Zhao

et al., 2011). Moreover these studies focus on comparisons between CuO-NPs of different sizes, Cu in ionic/dissolved form and/or with CuO in bulk form (Ramskov et al., 2014).

Studies on chronic effects of CuO-NPs showed that this NPs inhibited *Daphnia magna* growth and reproduction (21 days test) (Adam et al., 2015; Rossetto et al., 2014); and induced mortality and decreased growth in *Xenopus laevis* above 0.3 mg Cu/L in a 47 days test, however lower concentrations (below 0.15 mg Cu/L) were beneficial to the organisms (higher growth and no mortality) (Nations et al., 2015). Regarding soil compartment, most of the literature available is relative to plants (Da Costa and Sharma, 2015; Peng et al., 2015; Shi et al., 2014) with virtually no information on CuO-NPs toxicity to soil dwelling invertebrates (in opposition to some studies on Cu-NPs that become oxidized (Gomes et al., 2015a; Heckmann et al., 2011; Unrine et al., 2010)).

5. Objectives

The overall objective of the present thesis was to develop new assays, covering novel endpoints, for the soil species *Enchytraeus crypticus*. The specific aims (addressed in the different chapters of the thesis) were: 1) to investigate the regeneration ability of *E. crypticus*; 2) understand the embryonic development in of *E. crypticus* for the implementation of an embryotoxicty test; 3) understand the parameters survival and reproduction over the entire lifespan of *E. crypticus* for the implementation of a long-term (lifespan) assay covering those parameters.
The present thesis is organized as follows:

- Chapter 1: Introduction to ecotoxicology and ecotoxicogenomics; short and long term tests; test species; test chemicals; aspects of ecotoxicology of nanomaterials; and the objectives of the thesis.

- Chapter 2: "*Enchytraeus crypticus* (Oligochaeta) is able to regenerate – considerations for a standard ecotoxicological species" (Gonçalves, M.F.M., Gomes, S.I.L., Soares, A.M.V.M., Amorim, M.J.B., submitted).

- Chapter 3: "Development of an embryotoxicity test for *Enchytraeus crypticus* – The effect of Cd" (Gonçalves, M.F.M., Bicho, R.C., Rêma, A., Soares, A.M.V.M., Faustino, A.M.R., Amorim, M.J.B., 2015. Chemosphere 139, 386–392. doi:10.1016/j.chemosphere.2015.07.021).

- Chapter 4: "Lifespan (all life) term test exposure using *Enchytraeus crypticus* – the effect of CuO NMs – longevity as novel endpoint" (Gonçalves, M.F.M., Gomes, S.I.L., Janeck J. Scott-Fordsmand, Amorim, M.J.B., submitted).

- Chapter 5: General discussion and final considerations.

References

Adam, N., Vakurov, A., Knapen, D., Blust, R., 2015. The chronic toxicity of CuO nanoparticles and copper salt to *Daphnia magna*. J. Hazard. Mater. 283, 416–422.

Agnello, M., Filosto, S., Scudiero, R., Rinaldi, A.M., Roccheri, M.C., 2006. Cadmium accumulation induces apoptosis in *P* . *Lividus* embryos. Caryologia 59, 403–408.

Agnello, M., Filosto, S., Scudiero, R., Rinaldi, A.M., Roccheri, M.C., 2007. Cadmium induces an apoptotic response in sea urchin embryos. Cell Stress Chaperones 12, 44–50.

Alibardi, L., Toni, M., 2005. Wound keratins in the regenerating epidermis of lizard suggests that the wound reaction is similar in the tail and limb. J. Exp. Zool. 303A, 845–860.

Alves, P.R.L., Cardoso, E.J.B.N., 2016. Overview of the Standard Methods for Soil Ecotoxicology Testing, in: Larramendy, M.L., Soloneski, S. (Eds.), Invertebrates - Experimental Models in Toxicity Screening. InTech.

Amorim, M.J.B., Novais, S., Rombke, J., Soares, A.M.V.M., 2008. *Enchytraeus albidus* (Enchytraeidae): A test organism in a standardised avoidance test? Effects of different chemical substances. Environ. Int. 34, 363–371.

Amorim, M.J.B., Römbke, J., Schallnass, H.J., Soares, A.M.V.M., 2005. Effect of soil properties and aging on the toxicity of copper for *Enchytraeus albidus*, *Enchytraeus luxuriosus*, and *Folsomia candida*. Environ. Toxicol. Chem. 24, 1875–1885.

Amorim, M.J.B., Scott-Fordsmand, J.J., 2012. Toxicity of copper nanoparticles and $CuCl_2$ salt to *Enchytraeus albidus* worms: survival, reproduction and avoidance responses. Environ. Pollut. 164, 164–168.

Ankley, G.T., Daston, G.P., Degitz, S.J., Denslow, N.D., Hoke, R.A., Kennedy, S.W., Miracle, A.L., Perkins, E.J., Snape, J., Tillitt, D.E., Tyler, C.R., Versteeg, D., 2006. Toxicogenomics in Regulatory Ecotoxicology. Env. Sci Technol 40, 4055–4065.

Baun, A., Hartmann, N.B., Grieger, K., Kusk, K.O., 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. Ecotoxicology 17, 387–395.

Bely, A.E., 2006. Distribution of segment regeneration ability in the Annelida. Integr. Comp. Biol. 46, 508–518.

Bely, A.E., Nyberg, K.G., 2010. Evolution of animal regeneration: re-emergence of a field. Trends Ecol. Evol. 25, 161–170.

Ben-Moshe, T., Frenk, S., Dror, I., Minz, D., Berkowitz, B., 2013. Effects of metal oxide nanoparticles on soil properties. Chemosphere 90, 640– 646.

Bertin, G., Averbeck, D., 2006. Cadmium: cellular effects, modifications of biomolecules, modulation of DNA repair and genotoxic consequences (a review). Biochimie 88, 1549–59.

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

Blechinger, S.R., Warren, J.T., Kuwada, J.Y., Krone, P.H., 2002. Developmental Toxicology of Cadmium in Living Embryos of a Stable Transgenic Zebrafish Line. Environ. Health Perspect. 110, 1041–1046.

Blinova, I., Ivask, A., Heinlaan, M., Mortimer, M., Kahru, A., 2010. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. Environ. Pollut. 158, 41–47.

Boxall, A.B.A., 2012. New and Emerging Water Pollutants arising from Agriculture.

Brasfield, S.M., Bradham, K., Wells, J.B., Talent, L.G., Lanno, R.P., Janz, D.M., 2004. Development of a terrestrial vertebrate model for assessing bioavailability of cadmium in the fence lizard (*Sceloporus undulatus*) and in ovo effects on hatchling size and thyroid function. Chemosphere 54, 1643–1651.

Brodin, T., Piovano, S., Fick, J., Klaminder, J., Heynen, M., Jonsson, M., 2014. Ecological effects of pharmaceuticals in aquatic systems—impacts through behavioural alterations. Philos. Trans. R. Soc. B-Biological Sci. 369, 20130580.

Buffenstein, R., Edrey, Y.H., Larsen, P.L., 2008. Animal Models in Aging Research - A Critical Examination, in: Sourcebook of Models for Biomedical Research. pp. 499–506.

Buffet, P.-E., Richard, M., Caupos, F., Vergnoux, A., Perrein-Ettajani, H., Luna-Acosta, A., Akcha, F., Amiard, J.-C., Amiard-Triquet, C., Guibbolini, M., Risso-De Faverney, C., Thomas-Guyon, H., Reip, P., Dybowska, A., Berhanu, D., Valsami-Jones, E., Mouneyrac, C., 2013. A mesocosm study of fate and effects of CuO nanoparticles on endobenthic species (*Scrobicularia plana*, *Hediste diversicolor*). Environ. Sci. Technol. 47, 1620–1228.

Buffet, P.-E., Tankoua, O.F., Pan, J.-F., Berhanu, D., Herrenknecht, C., Poirier, L., Amiard-Triquet, C., Amiard, J.-C., Bérard, J.-B., Risso, C., Guibbolini, M., Roméo, M., Reip, P., Valsami-Jones, E., Mouneyr, C., 2011. Behavioural and biochemical responses of two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* to copper oxide nanoparticles. Chemosphere 84, 166–174.

Bundy, J.G., Sidhu, J.K., Rana, F., Spurgeon, D.J., Svendsen, C., Wren, J.F., Stürzenbaum, S.R., Morgan, A.J., Kille, P., 2008. "Systems toxicology" approach identifies coordinated metabolic responses to copper in a terrestrial non-model invertebrate, the earthworm *Lumbricus rubellus*. BMC Biol. 6.

Burkal, J.Z., Gregersen, N.F., Reventlow, J.T., 2015. Long-term Effects of CuO Nanoparticles on *Hyalella azteca*. Roskilde University.

Cambier, S., Gonzalez, P., Durrieu, G., Bourdineaud, J.-P., 2010. Cadmium-induced genotoxicity in zebrafish at environmentally relevant doses. Ecotoxicol. Environ. Saf. 73, 312–9.

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

Cava, R.J., 1990. Structural chemistry and the local charge picture of copper oxide superconductors. Science (80-.). 247, 656–662.

Chan, P.K., Cheng, S.H., 2003. Cadmium-induced ectopic apoptosis in zebrafish embryos. Arch. Toxicol. 77, 69–79.

Chang, H., Jwo, C., Lo, C., Tsung, T., Kao, M., Lin, H., 2005. Rheology of CuO nanoparticle suspension prepared by ASNSS. Rev. Adv. Mater. Sci. 10, 128–132.

Chang, Y.-N., Zhang, M., Xia, L., Zhang, J., Xing, G., 2012. The Toxic Effects and Mechanisms of CuO and ZnO Nanoparticles. Materials (Basel). 5, 2850–2871.

Cheng, S.H., Wai, A.W.K., So, C.H., Wu, R.S.S., 2000. Cellular and Molecular basis of Cadmium-induced deformities in zebrafish embryos. Environ. Toxicol. Chem. 19, 3024–3031.

Chowdhuri, A., Gupta, V., Sreenivas, K., Kumar, R., Mozumdar, S., Patanjali, P.., 2004. Response speed of SnO_2-based H_2S gas sensors with CuO nanoparticles. Appl. Phys. Lett. 84, 1180–1182.

Coeurdassier, M., De Vaufleury, a, Badot, P.M., 2003. Bioconcentration of cadmium and toxic effects on life-history traits of pond snails (*Lymnaea palustris* and *Lymnaea stagnalis*) in laboratory bioassays. Arch. Environ. Contam. Toxicol. 45, 102–9.

Coutellec, M.A., Barata, C., 2013. Special issue on long-term ecotoxicological effects: an introduction. Ecotoxicology 22, 763–766.

Da Costa, M.V.J., Sharma, P.K., 2015. Effect of copper oxide nanoparticles on growth, morphology, photosynthesis, and antioxidant response in *Oryza sativa*. Photosynthetica 54, 110–119.

Dabas, A., Nagpure, N.S., Mishra, R.M., Kushwaha, B., Kumar, R., Kumar, P., 2013. Investigation of Cadmium-Induced Genotoxicity and Oxidative Stress Response in Indian Major Carp, *Labeo rohita*. Hum. Ecol. Risk Assess. An Int. J. 20, 510–526.

Dastjerdi, R., Montazer, M., 2010. A review on the application of inorganic nanostructured materials in the modification of textiles: focus on antimicrobial properties. Colloids Surf B Biointerfaces 79, 5–18.

Delgado, K., Quijada, R., Palma, R., Palza, H., 2001. Polypropylene with embedded copper metal or copper oxide nanoparticles as a novel plastic antimicrobial agent. Lett Appl Microbiol 53, 50–54.

Didden, W.A.M., 1993. Ecology of terrestrial Enchytraeidae. Pedobiologia (Jena). 37, 2–29.

Didden, W.A.M., Frond, H.-C., Graefe, U., 1997. Enchytraeids, in: Benckiser, G. (Ed.), Fauna in Soil Ecosystems: Recycling Processes, Nutrient Fluxes, and Agricultural Production. Marcel Dekker, New York, pp. 135–172.

Diez-Ortiz, M., Lahive, E., George, S., Ter Schure, A., Van Gestel, C.A.M., Jurkschat, K., Svendsen, C., Spurgeon, D.J., 2015. Short-term soil bioassays may not reveal the full toxicity potential for nanomaterials; bioavailability and toxicity of silver ions ($AgNO_3$) and silver nanoparticles to earthworm *Eisenia fetida* in long-term aged soils. Environ. Pollut. 203, 191–198.

Druart, C., Scheifler, R., de Vaufleury, A., 2010. Towards the development of an embryotoxicity bioassay with terrestrial snails: screening approach for cadmium and pesticides. J. Hazard. Mater. 184, 26–33.

Faverney, C.R., Devaux, A., Lafaurie, M., Girard, J.P., 2001. Cadmium induces apoptosis and genotoxicity in rainbow trout hepatocytes through generation of reactive oxygene species. Aquat Toxicol. 53, 65–76.

Fernández, E.L., Gustafson, A.-L., Andersson, M., Hellman, B., Dencker, L., 2003. Cadmium-Induced Changes in Apoptotic Gene Expression Levels and DNA Damage in Mouse Embryos Are Blocked by Zinc. Toxicol. Sci. 76, 162–170.

Frenk, S., Ben-Moshe, T., Dror, I., Berkowitz, B., Minz, D., 2013. Effect of metal oxide nanoparticles on microbial community structure and function in two different soil types. PLoS One 8, e84441.

Gabbay, J., Borkow, G., Mishal, J., Magen, E., Zatcoff, R., Shemer-Avni, Y., 2006. Copper oxide impregnated textiles with potent biocidal activities. J Ind Text 35, 323–335.

Gaetke, L.M., Chow, C.K., 2003. Copper toxicity, oxidative stress, and antioxidant nutrients. Toxicology 189, 147–163.

Gao, D., Xu, Z., Qiao, P., Liu, S., Zhang, L., He, P., Zhang, X., Wang, Y., Min, W., 2013. Cadmium induces liver cell apoptosis through caspase-3A activation in purse red common carp (*Cyprinus carpio*). PLoS One 8, e83423.

Gomes, S.I.L., Murphy, M., Nielsen, M.T., Kristiansen, S.M., Amorim, M.J.B., Scott-Fordsmand, J.J., 2015a. Cu-nanoparticles ecotoxicity-explored and explained? Chemosphere 139, 240–245.

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

Gomes, T., Chora, S., Pereira, C.G., Cardoso, C., Bebianno, M.J., 2014. Proteomic response of mussels *Mytilus galloprovincialis* exposed to CuO NPs and Cu^{2+}: an exploratory biomarker discovery. Aquat. Toxicol. 155, 327–36.

Gomot, A., 1998. Toxic effects of cadmium on reproduction, development, and hatching in the freshwater snail *Lymnaea stagnalis* for water quality monitoring. Ecotoxicol. Environ. Saf. 41, 288–97.

Gonzalez, P., Baudrimont, M., Boudou, A., Bourdineaud, J.-P., 2006. Comparative effects of direct cadmium contamination on gene expression in gills, liver, skeletal muscles and brain of the zebrafish (*Danio rerio*). Biometals 19, 225–235.

Goss, R.J., 1969. Principles of Regeneration. Academic Press.

Graefe, U., Schmelz, R.M., 1999. Indicator values, strategy types and life forms of terrestrial Enchytraeidae and other microannelids. Newsl. Enchytraeidae 6, 59–67.

Griffitt, R.J., Hyndman, K., Denslow, N.D., Barber, D.S., 2009. Comparison of molecularand histological changes in zebrafish gills exposed to metallic nanoparticles. Toxicol. Sci. 107, 404–415.

Griffitt, R.J., Weil, R., Hyndman, K.A., Denslow, N.D., Powers, K., Taylor, D., Barber, S.D., 2007. Exposure to copper nanoparticles causes gill injury and acute lethality in zebrafish (*Danio rerio*). Environ. Sci. Technol. 41, 8178–8186.

Guogang, R., Hu, D., Cheng, E.W.C., Vargas-Reus, M.A., Reip, P., Allaker, R.P., 2009. Characterisation of copper oxide nanoparticles for antimicrobial applications. Int. J. Antimicrob. Agents 33, 587–590.

Hagger, J.A., Fisher, A.S., Hill, S.J., Depledge, M.H., Jha, A.N., 2002. Genotoxic, cytotoxic and ontogenetic effects of tri-n-butyltin on the marine worm Platynereis dumerilii (*Polychaeta Nereidae*). Aquat. Toxicol. 57, 243–255.

Hamilton, K.L., Miller, B.F., 2016. What is the evidence for stress resistance and slowed aging? Exp. Gerontol. 82, 67–72.

Harada, H., Kurauchi, M., Hayashi, R., Eki, T., 2007. Shortened lifespan of nematode *Caenorhabditis elegans* after prolonged exposure to heavy metals and detergents. Ecotoxicol. Environ. Saf. 66, 378–383.

Hassoun, E.A., Stohs, S.J., 1996. Cd-induced production of superoxide anion and nitric oxide, DNA single strand breaks and lactate dehydrogenase leakage in J77A.1 cell cultures. Toxicology 112, 219– 226.

Heckmann, L.-H., Hovgaard, M.B., Sutherland, D.S., Autrup, H., Besenbacher, F., Scott-Fordsmand, J.J., 2011. Limit-test toxicity screening of selected inorganic nanoparticles to the earthworm *Eisenia fetida*. Ecotoxicology 20(1), 226–233.

Hermens, J.L.M., Ankley, G.T., Sumpter, J.P., 2004. Ecotoxicology - A multidisciplinary, problem-driven science. Environ. Sci. Technol. 38, 446a– 447a.

Hernández Battez, A., Viesca, J.., González, R., Blanco, D., Asedegbega, E., Osorio, A., 2010. Friction reduction properties of a CuO nanolubricant used as lubricant for a NiCrBSi coating. Wear 268, 325–328.

Hoffman, D.J., Rattner, B.A., Burton, G.A., Cairns, J., 2003. Handbook of Ecotoxicology, Dordrecht. ed. London: Blackwell.

Hsu, T., Huang, K.-M., Tsai, H.-T., Sung, S.-T., Ho, T.-N., 2013. Cadmium(Cd)-induced oxidative stress down-regulates the gene expression of DNA mismatch recognition proteins MutS homolog 2 (MSH2) and MSH6 in zebrafish (*Danio rerio*) embryos. Aquat. Toxicol. 126, 9–16.

Iglesias, J., Castillejo, J., Ester, A., Castro, R., Lombardia, M.J., 2002. Susceptibility of the eggs of the field slug *Deroceras reticulatum* to contact with pesticides and substances of biological origin on artificial soil. Ann. Appl. Biol. 140, 53–59.

Iguchi, T., Watanabe, H., Katsu, Y., 2007. Toxicogenomics and ecotoxicogenomics for studying endocrine disruption and basic biology. Gen. Comp. Endocrinol. 157, 25–29.

ISO, 1999. ISO: 11267 Soil Quality - Inhibition of Reproduction of Collembola (*Folsomia candida*) by Soil Pollutants. ISO (International Organ. Stand. Geneva, Switzerland).

ISO, 2004. ISO: 16387 Soil Quality - Effects of Pollutants on Enchytraeidae (*Enchytraeus* sp.) - Determination of Effects on Reproduction and Survival. ISO (International Organ. Stand. Geneva, Switz).

ISO, 2012. ISO: 11268-2 Soil Quality - Effects of pollutants on earthworms - Part 2: Determination of effects on reproduction of *Eisenia fetida*/*Eisenia andrei*. ISO (International Organ. Stand. Geneva, Switzerland).

Jänsch, S., Amorim, M.J.B., Römbke, J., 2005. Identification of the ecological requirements of important terrestrial ecotoxicological test species. Environ. Rev. 13, 51–83.

Jänsch, S., Römbke, J., 2003. Ecological characterization of selected enchytraeid species (Enchytraeidae, Oligochaeta). A literature research. Newsl. Enchytraeidae 8, 57–68.

Jia, X., Zhang, H., Liu, X., 2011. Low levels of cadmium exposure induce DNA damage and oxidative stress in the liver of Oujiang colored common carp *Cyprinus carpio* var. color. Fish Physiol. Biochem. 37, 97–103.

Jin, S., Ye, K., 2007. Nanoparticle-mediated drug delivery and gene therapy. Biotechnol. Prog. 23, 32–41.

Kapanen, A., Itavaara, M., 2001. Ecotoxicity tests for compost applications. Ecotoxicol. Environ. Saf. 49, 1–16.

Klaine, S.J., Alvarez, P.J.J., Batley, G.E., Fernandes, T.F., Handy, R.D., Lyon, D.Y., Mahendra, S., McLaughlin, M.J., Lead, J.R., 2008. Nanomaterials in the environment: behavior, fate, bioavailability, and effects. Env. Toxicol Chem 27, 1825–1851.

Knakievicz, T., 2014. Planarians as invertebrate bioindicators in freshwater environmental quality: the biomarker approach. Ecotoxicol. Environ. Contam. 9, 1–12.

Kuperman, R.G., Amorim, M.J.B., Rombke, J., Lanno, R., Checkai, R.T., Dodard, S.G., Sunahara, G.I., Scheffczyk, A., 2006. Adaptation of the enchytraeid toxicity test for use with natural soil types. Eur. J. Soil Biol. 42, S234–S243.

Kumar, A., Kumar, P., Anandan, A., Fernandes, T.., Ayoko, G.., Biskos, G., (2014), 2014. Engineered nanomaterials: knowledge gaps in fate, exposure, toxicity, and future directions. J. Nanomater. 2014, 16.

Lag, M., Westly, S., Lerstad, T., Björnsrud, C., Refsnes, M., Schwarze, P.E., 2002. Cadmium-induced apoptosis of primary epithelial lung cells : Involvement of Bax and p53 , but not of oxidative stress 29–42.

Leppanen, M.., Kukkonen, J.V.., 1998. Relationship between reproduction, sediment type, and feeding activity of *Lumbriculus variegatus* (Muller): implications for sediment toxicity testing. Env. Toxicol Chem 17, 2196–202.

Lucanic, M., Lithgow, G.J., Alavez, S., 2013. Pharmacological lifespan extension of invertebrates. Ageing Res. Rev. 12, 445–58.

Manusadžianas, L., Caillet, C., Fachetti, L., Gylytė, B., Grigutytė, R., Jurkonienė, S., Karitonas, R., Sadauskas, K., Thomas, F., Vitkus, R., 2012. Toxicity of copper oxide nanoparticle suspensions to aquatic biota. Env. Toxicol Chem 31, 108–114.

Maraldo, K., Christensen, B., Strandberg, B., Holmstrup, M., 2006. Effects of copper on enchytraeids in the field under differing soil moisture regimes. Environ. Toxicol. Chem. 25, 604–612.

Maria, V.L., Bebianno, M.J., 2011. Antioxidant and lipid peroxidation responses in *Mytilus galloprovincialis* exposed to mixtures of benzo(a)pyrene and copper. Comp. Biochem. Physiol. Part C Toxicol. Pharmacol. 154, 56–63.

Menezes-Oliveira, V.B., Scott-Fordsmand, J.J., Rocco, A., Soares, A.M.V.M., Amorim, M.J.B., 2011. Interaction between density and Cu toxicity for *Enchytraeus crypticus* and *Eisenia fetida* reflecting field scenarios. Sci. Total Environ. 409, 3370–3374.

Miracle, A.L., Ankley, G.T., 2005. Ecotoxicogenomics: linkages between exposure and effects in assessing risks of aquatic contaminants to fish. Reprod. Toxicol. 19, 321–326.

Mortimer, M., Kasemets, K., Kahru, A., 2010. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. Toxicology 269, 182–189.

Muangphra, P., Gooneratne, R., 2011. Comparative Genotoxicity of Cadmium and Lead in Earthworm Coelomocytes. Appl. Environ. Soil Sci. 2011, 1–7.

Nations, S., Long, M., Wages, M., Maul, J.D., Theodorakis, C.W., Cobb, G.P., 2015. Subchronic and chronic developmental effects of copper oxide (CuO) nanoparticles on *Xenopus laevis*. Chemosphere 135, 166–174.

Nations, S., Wages, M., Cañas, J.E., Maul, J., Theodorakis, C., Cobb, G.P., 2011. Acute effects of Fe_2O_2, TiO_2, ZnO and CuO nanomaterials on *Xenopus laevis*. Chemosphere 83, 1053–1061.

Oberdorster, G., 2007. Biokinetics and Effects of Nanoparticles, in: Nanotechnology – Toxicological Issues and Environmental Safety and Environmental Safety. pp. 15–51.

OECD, 2004. Test No. 220: Guidelines for Testing of Chemicals - Enchytraeid Reproduction Test. OECD (Organization Econ. Coop. Dev. Publ. Paris, Fr).

OECD, 2009. Test No. 232: Guidelines for Testing of Chemicals - Collembolan Reproduction Test in Soil. OECD (Organization Econ. Coop. Dev. Publ. Paris, Fr).

OECD, 2010a. Test No. 25: Guidance Manual for the Testing of Manufactured Nanomaterials: OECD Sponsorship Programme: First Revision. OECD (Organization Econ. Coop. Dev. Publ. Paris, Fr).

OECD, 2010b. Test No. 317: Guidelines for Testing of Chemicals - Bioaccumulation in Terrestrial Oligochaetes. OECD (Organization Econ. Coop. Dev. Publ. Paris, Fr).

OECD, 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test. OECD Guidel. Test. Chem. Sect. 2, OECD Publ.

Ofoegbu, P.U., Simão, F.C.P., Cruz, A., Mendo, S., Soares, A.M.V.M., Pestana, J.L.T., 2016. Toxicity of tributyltin (TBT) to the freshwater planarian *Schmidtea mediterranea*. Chemosphere 148, 61–67.

Pagan, O.R., Coudron, T., Kaneria, T., 2009. The flatworm planarian as a toxicology and behavioural pharmacology animal model in undergraduate research experiences. J. Undergrad. Neurosci. Educ. 7, A48–A52.

Pan, B., Xing, B.S., 2012. Applications and implications of manufactured nanoparticles in soils: a review. Eur. J. Soil Sci. 63, 437–456.

Part, P., Castano, A., Bengtsson, B.-E., 2009. Has the 3R's concept a future in ecotoxicology? Comp. Biochem. Physiol. Part A Mol. Integr. Physiol. 153, S86.

Peng, C., Duan, D., Xu, C., Chen, Yongsheng, Sun, L., Zhang, H., Yuan, X., Zheng, L., Yang, Y., Yang, J., Zhen, X., Chen, Yingxu, Shi, J., 2015. Translocation and biotransformation of CuO nanoparticles in rice (*Oryza sativa L.*) plants. Environ. Pollut. 197, 99–107.

Peralta-Videa, J.., Zhao, L., Lopez-Moreno, M.., de la Rosa, G., Hong, J., Gardea-Torresdey, J.L., 2011. Nanomaterials and the environment: a review for the biennium 2008-2010. J Hazard Mater 186, 1–15.

Pereira, S., Cavalie, I., Camilleri, V., Gilbin, R., Adam-Guillermin, C., 2013. Comparative genotoxicity of aluminium and cadmium in embryonic zebrafish cells. Mutat. Res. 750, 19–26.

Peters, J.R., Granek, E.F., 2016. Long-term exposure to fluoxetine reduces growth and reproductive potential in the dominant rocky intertidal mussel, *Mytilus californianus*. Sci. Total Environ. 545-546, 621–628.

Poynton, H.C., Vulpe, C.D., 2009. Ecotoxicogenomics: Emerging Technologies for Emerging Contaminants. J. Am. Water Resour. Assoc. 45, 83–96.

Pradhan, A., Seena, S., Pascoal, C., Cássio, F., 2012. Copper oxide nanoparticles can induce toxicity to the freshwater shredder *Allogamus ligonifer*. Chemosphere 89, 1142–1150.

Qafoku, N.., 2010. Terrestrial nanoparticles and their controls on soil-/geo-processes and reactions. Adv Agron 107, 33–91.

Ramskov, T., Selck, H., Banta, G., Misra, S.K., Berhanu, D., Valsami-Jones, E., Forbes, V.E., 2014. Bioaccumulation and effects of different-shaped copper oxide nanoparticles in the deposit-feeding snail *Potamopyrgus antipodarum*. Environ. Toxicol. Chem. 33, 1976–1987.

Reddien, P.W., Sanchez-Alvarado, A., 2004. Fundamentals of planarian regeneration. Annu. Rev. Cell Dev. Biol. 20, 725–757.

Roccheri, M.C., Agnello, M., Bonaventura, R., Matranga, V., 2004. Cadmium induces the expression of specific stress proteins in sea urchin embryos. Biochem. Biophys. Res. Commun. 321, 80–7.

Römbke, J., 2003. Ecotoxicological laboratory tests with enchytraeids: A review. Pedobiologia (Jena). 47, 607–616.

Rombke, J., Moser, T., 2002. Validating the enchytraeid reproduction test: organisation and results of an international ringtest. Chemosphere 46, 1117–1140.

Rossetto, A.., Melegari, S.., Ouriques, L.., Matias, W.., 2014. Comparative evaluation of acute and chronic toxicities of CuO nanoparticles and bulk using *Daphnia magna* and *Vibrio fischeri*. Sci Total Environ. 490, 807–814.

Russo, R., Bonaventura, R., Zito, F., Schro, H., Mu, I., Matranga, V., 2003. Stress to cadmium monitored by metallothionein gene induction in *Paracentrotus lividus* embryos 8, 232–241.

Saison, C., Perreault, F., Daigle, J.C., Fortin, C., Claverie, J., Morin, M., Popovic, R., 2010. Effect of core-shell copper oxide nanoparticles on cell culture morphology and photosynthesis (photosystem II energy distribution) in the green alga, *Chlamydomonas reinhardtii*. Aquat. Toxicol 96, 109–114.

Sanderson, H., Solomon, K., 2009. Contaminants of emerging concern challenge ecotoxicology. Environ. Toxicol. Chem. 28, 1359–1360.

Schirling, M., Bohlen, A., Triebskorn, R., Köhler, H.-R., 2006. An invertebrate embryo test with the apple snail *Marisa cornuarietis* to assess effects of potential developmental and endocrine disruptors. Chemosphere 64, 1730–8.

Scott-Fordsmand, J.J., Weeks, J.M., Hopkin, S.P., 2000. Importance of contamination history for understanding toxicity of copper to earthworm *Eisenia fetica* (Oligochaeta: Annelida), using neutral-red retention assay. Environ. Toxicol. Chem. 19, 1774–1780.

Shi, J., Peng, C., Yang, Y., Yang, J., Zhang, H., Yuan, X., Chen, Y., Hu, T., 2014. Phytotoxicity and accumulation of copper oxide nanoparticles to the Cu-tolerant plant *Elsholtzia splendens*. Nanotoxicology 8, 179–188.

Shoaib, M. a., Mahmoud, M.F., Loutfy, N., Tawfic, M. a., Barta, M., 2009. Effect of botanical insecticide Nimbecidine® on food consumption and egg hatchability of the terrestrial snail *Monacha obstructa*. J. Pest Sci. (2004). 83, 27–32.

Siddiqui, M.A., Alhadlaq, H.A., Ahmad, J., Al-Khedhairy, A.A., Musarrat, J., Ahamed, M., 2013. Copper oxide nanoparticles induced mitochondria mediated apoptosis in human hepatocarcinoma cells. PLoS One 8, e69534.

Simoniello, P., Motta, C.M., Scudiero, R., Trinchella, F., Filosa, S., 2011. Cadmium-induced teratogenicity in lizard embryos: correlation with metallothionein gene expression. Comp. Biochem. Physiol. C. Toxicol. Pharmacol. 153, 119–27.

Snape, J.R., Maund, S.J., Pickford, D.B., Hutchinson, T.H., 2004. Ecotoxicogenomics: the challenge of integrating genomics into aquatic and terrestrial ecotoxicology. Aquat. Toxicol. 67, 143–154.

Stone, V., Nowack, B., Baun, A., van den Brink, N., Kammer, F., Dusinska, M., Handy, R., Hankin, S., Hassellöv, M., Joner, E., Fernandes, T.., 2010. Nanomaterials for environmental studies: classification, reference material issues, and strategies for physico-chemical characterisation. Sci Total Env. 408, 1745–1754.

Tanaka, E.M., Reddien, P.W., 2011. The cellular basis for animal regeneration. Dev. Cell 21, 172–185.

Tranquada, J.M., Sternlieb, B.J., Axe, J.D., Nakamura, Y., Uchida, S., 1995. Evidence for stripe correlations of spins and holes in copper oxide superconductors. Nature 375, 561–563.

Trinchella, F., Cannetiello, M., Simoniello, P., Filosa, S., Scudiero, R., 2010. Differential gene expression profiles in embryos of the lizard *Podarcis sicula* under in ovo exposure to cadmium. Comp. Biochem. Physiol. Part C 151, 33–39.

Truhaut, R., 1977. Ecotoxicology: Objectives, principles and perspectives. Ecotoxicol. Environ. Saf. 1, 151–173.

Unrine, J.M., Tsyusko, O. V, Hunyadi, S.E., Judy, J.D., Bertsch, P.M., 2010. Effects of Particle Size on Chemical Speciation and Bioavailability of Copper to Earthworms (*Eisenia fetida*) Exposed to Copper Nanoparticles. J. Environ. Qual. 39, 1942–1953.

Vance, M.E., Kuiken, T., Vejerano, E.P., McGinnis, S.P., Hochella, M.F., Rejeski, D., Hull, M.S., 2015. Nanotechnology in the real world: Redeveloping the nanomaterial consumer products inventory. Beilstein J. Nanotechnol. 6, 1769–1780.

Van Gestel, C.A.M., 2012. Soil ecotoxicology: State of the art and future directions. Zookeys 176, 275–296.

Velma, V., Vutukuru, S.S., Tchounwou, P.B., 2009. Ecotoxicology of hexavalent chromium in freshwater fish: a critical review. Rev. Environ. Heal. 24, 129–145.

Wang, Y., Fang, J., Leonard, S.S., Rao, K.M.K., 2004. Cadmium inhibits the electron transfer chain and induces reactive oxygen species. Free Radic. Biol. Med. 36, 1434–1443.

Watanabe, H., Kobayashi, K., Kato, Y., Oda, S., Abe, R., Tatarazako, N., Iguchi, T., 2008. Transcriptome profiling in crustaceans as a tool for ecotoxicogenomics. Cell Biol. Toxicol. 24, 641–647.

Westheide, W., Müller, M.C., 1996. Cinematographic documentation of enchytraeid morphology and reproductive biology. Hydrobiologia. 263–267.

Yamamoto, F.Y., Filipak Neto, F., Freitas, P.F., Oliveira Ribeiro, C. a, Ortolani-Machado, C.F., 2012. Cadmium effects on early development of chick embryos. Environ. Toxicol. Pharmacol. 34, 548–55.

Yasokawa, D., Murata, S., Kitagawa, E., Iwahashi, Y., Nakagawa, R., Hashido, T., Iwahashi, H., 2008. Mechanisms of copper toxicity in *Saccharomyces cerevisiae* determined by microarray analysis. Environ. Toxicol. 23, 599–606.

Yin, M., Wu, C.-K., Lou, Y., Burda, C., Koberstein, J.T., Zhu, Y., O'Brien, S., 2005. Copper oxide nanocrystals. J. Am. Chem. Soc. 127, 9506–9511.

Zhang, X., Zhang, D., Ni, X., Song, J., Zheng, H., 2008. Synthesis and electrochemical properties of different sizes of the CuO particles. J. Nanopart. Res. 10, 839–844.

Zhang, D.-W., Yi, T.-H., Chen, C.-H., 2005. Cu nanoparticles derived from CuO electrodes in lithium cells. Nanotechnology 16, 2338–2341.

Zhao, H., Lu, G., Xia, J., Jin, S., 2012. Toxicity of Nanoscale CuO and ZnO to *Daphnia magna*. Chem. Res. Chin. Univ. 28, 209–213.

Zhao, J., Wang, Z., Liu, X., Xie, X., Zhang, K., Xing, B., 2011. Distribution of CuO nanoparticles in juvenile carp (*Cyprinus carpio*) and their potential toxicity. J. Hazard. Mater. 197, 304–310.

Chapter 2

Enchytraeus crypticus (Oligochaeta) is able to regenerate – considerations for a standard ecotoxicological species

Enchytraeus crypticus (Oligochaeta) is able to regenerate – considerations for a standard ecotoxicological species

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Abstract

Certain invertebrates (and vertebrates) are able to regenerate lost body parts. In the present study we investigated if *Enchytraeus crypticus* (Oligocheata), a species used for standard ecotoxicological testing, shows regeneration ability. Artificial amputation was induced. The results showed that regeneration occurs for this species, this being promoted by the presence of a mass of undifferentiated cells (blastema) in the wound site. This only occurred in the anterior fragment resulting in the posterior regeneration (formation of the tail), i.e. the posterior fragment does not regenerate the head and degenerates. The regenerated organisms were tested for fertility, which was confirmed. This is an important knowledge, that *E. crypticus* has an additional survival strategy to deal, for instance with mechanical stress/injuries. Further, given this is an ecotoxicological model species and the possible implications thereof – *E. crypticus* can fragment and use regeneration as a survival strategy.

Keywords: Posterior regeneration; Amputation; Oligochaeta; Enchytraeids

1. Introduction

The capacity to regenerate lost body parts of animals is an aspect of biology poorly understood and highly variable (Bely and Nyberg, 2010; Tanaka and Reddien, 2011). Many groups of animals such as birds and mammals are incapable of regenerating any lost structure (Goss, 1969) while other can, e.g. cnidarians, annelids, molluscs, platyhelminthes (Tanaka and Reddien, 2011). Moreover, the mechanisms of regeneration ability varies

between species with respect to which parts of the body can be regenerated (Bely and Nyberg, 2010), e.g. planarians can replace a missing head or the entire body from small fragments (Tanaka and Reddien, 2011), many annelids can regenerate the whole body or only the anterior or the posterior segments (Bely, 2006), lizards are capable of replacing a missing tail but not a limb (Alibardi and Toni, 2005).

Worldwide, about 950 species of enchytraeids (Family: Enchytraeidae) have been described (Jänsch et al., 2005) and only five species of the genus *Enchytraeus* have been reported to reproduce asexually by fragmentation and subsequent regeneration: *E. fragmentosus* (Bell, 1959); *E. bigeminus* (Nielsen and Christensen, 1963); *E. variatus* (Bouguenec and Giani, 1989) *E. japonensis* (Nakamura, 1993); and *E. dudichi* (Dózsa-Farkas, 1995). Only one study reports the regeneration of an enchytraeid (*E. buchholzi*) that reproduces exclusively sexually (Myohara, 2012). Enchytraeids are small oligochaetes typical of the saprophagous mesofauna (Jänsch et al., 2005) found in a large variety of soils (Didden, 1993). They are ecologically important because they contribute to the decomposition of organic matter and improve soil pore structure (Didden, 1993).

E. crypticus is a standard species in soil ecotoxicology with standardized protocols to evaluate survival, reproduction and bioaccumulation (ISO, 2005; OECD, 2010, 2004). Additionally, other endpoints such as avoidance behaviour (Bicho et al., 2015a), embrytoxicity (Gonçalves et al., 2015), hatching, growth and maturity status in the form of a full life cycle test (Bicho et al, 2015b) were studied in this species. It is a small enchytraied, with around 7 mm long (Westheide and Graefe, 1992), with a large tolerance range in terms of soil properties (for instance pH from 4.4 to 8.2, clay content from 1 to 29% and organic matter from 1.2 to 42% (Kuperman et al., 2006)) and temperature preferences ranging from 25 to 30 ºC (Jänsch et al, 2005). *E. crypticus* has a relatively short life cycle (25 days from cocoon release until the production of new offspring) (Bicho et al., 2015b); it reproduces sexually by self-fertilization and, although not confirmed, possibly also by cross-fertilization (the most common for oligochaetes) (Schmelz and Collado, 2012). Interestingly, we have sporadically observed in agar plate's cultures and soil test vessels fragments of enchytraeids with the same body thickness as a grown adult. This suggested that these organisms were able to self-amputate. Same observations were reported for *E. buchholzi* by Nakamura and Shiraishi (1999) currently explained as a mechanism of detoxification. In the present study we investigated the regeneration of *E.*

crypticus after artificial fragmentation by amputation and further assessed their viability. The implications at ecological and ecotoxicological levels were discussed.

2. Materials and Methods

2.1. Test organism

Cultures (*Enchytraeus crypticus*, Westheide and Graefe, 1992) were kept in agar plates fed *ad libitum* with grinded and autoclaved oats and maintained under controlled conditions of 16:8 hours (light: dark) and 20 ± 1ºC. Organisms for test were of synchronized age (20 days old) following the procedures as described in Bicho et al. (2015b).

2.2. Test procedures

To assess regeneration capacity organisms were artificially cut and monitored daily during 42 days. Organisms were randomly selected from cultures and cut at approximately the 7th segment (ca. 1 to 2 mm from the head) using a scalpel blade. This procedure was done in a Petri dish containing water placed on ice to reduce their mobility. The two parts (anterior and posterior) were transferred to individual Petri dishes with agar media. Food (grinded and autoclaved oats) and water were added. The test was maintained at a photoperiod of 16:8 h light:dark and at 20 ± 1 ºC. A total of 50 organisms were amputated. After 1, 2, 4 and 6 days, ten posterior and anterior parts were collected and photographed for macroscopic monitoring and whole-mount staining. Photographs were taken using a Dino-Eye camera and Dino-Lite software (Corp, 2010) under a stereo microscope (Zeiss Stemi 2000-C). Ten replicates were maintained up to 42 days to assess reproduction. At day 28, after the first cocoon laying, adults were removed from the Petri dish and their length was recorded; all produced cocoons were left. At day 42, hatched juveniles were fixated with ethanol and Bengal rose for about 24 h and counted using a stereo microscope (Zeiss Stemi 2000-C).

Whole-mount staining followed the procedures described in Myohara et al. (1999). This technique allows a three-dimensional visualization of the morphology and distribution of the cells due to the almost complete transparency of the enchytraeids. The sampled enchytraeids' fragments were fixated in a mixture of acetic acid, glycerol and ethanol (4:1:2) for 15–20 min, stained in 4% orcein in acetic acid and glycerol (4:1) for 20 min,

washed with the acetic acid:glycerol:ethanol solution, mounted in 70% glycerol and photographed using a microscope (Olympus BX51) with an attached camera (Olympus).

3. Results

None of the fragmented enchytraeids died during the 6 days. Representative pictures along time are shown in figure 1, including the macroscopic visualization and whole-mount staining with orcein.

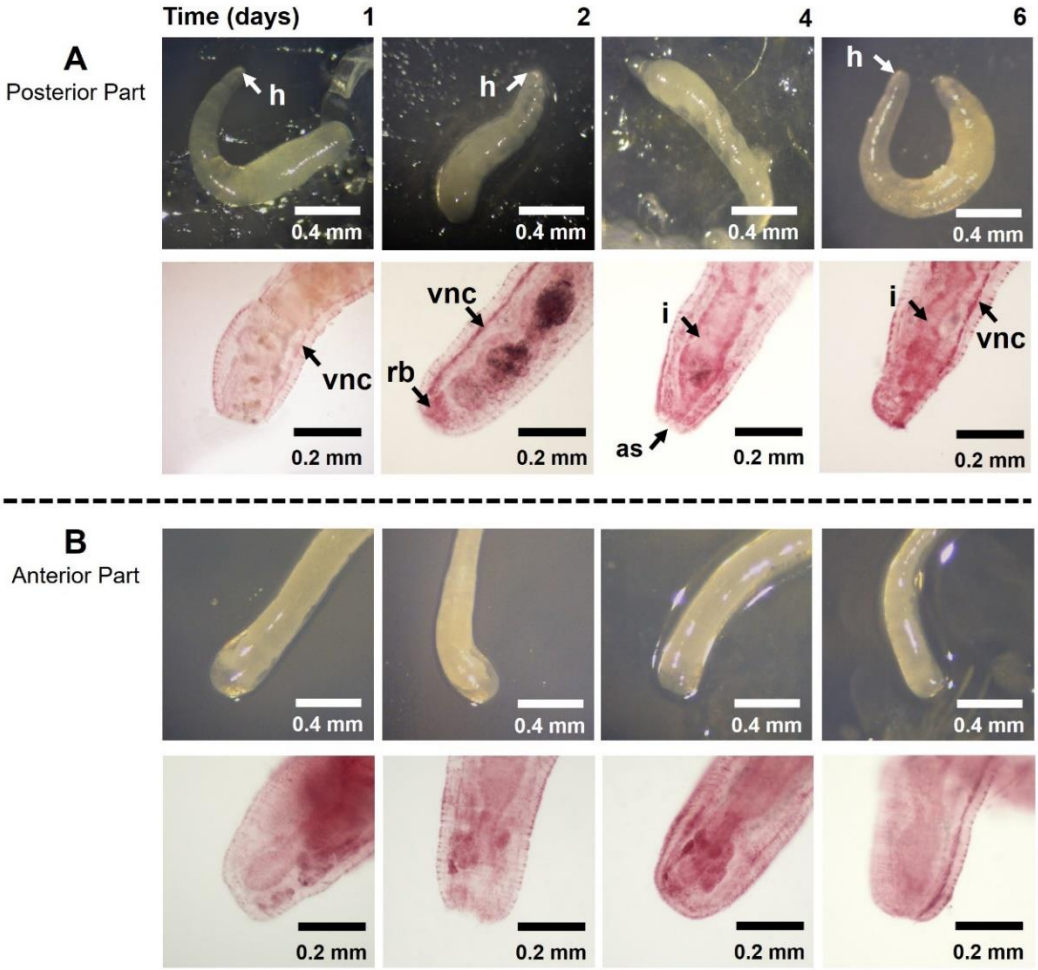

Figure 1: Visualization of the regeneration process of cut *Enchytraeus crypticus* along time (1, 2, 4, 6 days) of the A) posterior part and B) anterior part. Macroscopic visualization is showed on top row, whole-mount staining with orcein is on bottom row. as: anal segment; h: head; i: intestine; rb: regeneration blastema; vnc: ventral nerve cord.

According to our observations, the regeneration of the posterior part was completed in 6 days (Fig. 1A). At day 1 the surrounding epidermal cells are covered and wound is healed; day 2 regeneration blastema is observed; day 4 the anal segment is formed; day 6 beginning of elongation/formation of new segments. During the days after (around 16 days) the organisms grew normally (reaching "normal" adults' size) and reached maturity. The anterior part (Fig. 1B) did not regenerate. Observations showed that the wound was healed, given the establishment of the connection between the body wall and intestinal wall, and the tissues were alive between one and three weeks before degeneration.

At day 28, 100% of the regenerated organisms survived, and were 5.34 ± 0.26 mm long (average ± standard error, n = 10). At day 42 the reproductive output was 3.1 ± 0.9 juveniles/adult (average ± standard error, n = 10).

4. Discussion

Results showed regenerative ability in the anterior part (head segments), after artificial amputation, whereas the posterior part was not able to regenerate. The presence of blastema is usually known to be necessary in annelids for regeneration (Müller, 2004). Similarly to what has been described for *E. buchholzi* (Myohara, 2012), amputation in *E. crypticus* immediately after the head segments resulted in the regeneration of a tail. However, head regeneration was not observed. Same observations were made for *E. variatus* by Bouguenec and Giani (1989). It has also been described for earthworms' species that only anterior fragments are viable and capable of regenerating the missing segments (Cameron, 1932).

Regarding the time required for regeneration, our results showed that the regeneration of basic body components is completed within 6 days, which was identical to what was observed for other enchytraeids (Müller, 2004; Yoshida-Noro and Tochinai, 2010). The fast healing of the wounds caused by amputation could reduce fluid loss and necrosis in adjacent segments to the autotomy site. Interestingly, the time required for regeneration plus the time required to grow and reach maturity (6 + 16 days), is equivalent to the time required from hatching with normal reproduction as described by Bicho et al. (2015b) (see Fig. 2), i.e. 22 days. Moreover, the time that the amputee organisms took to grow is similar to the 2 weeks observed in *E. japonensis* (Yoshida-Noro and Tochinai, 2010). After the full regeneration and production of new clitellum, organisms reached more than 5 mm in

length which is about the same length as observed by Bicho et al. (2015b) in 25 days old adults. Regarding the reproductive output, the number of juveniles produced (around 3 per adult) is lower than expected (unpublished data). One hypothesis is that amputated *E. crypticus* may need more time (after regeneration) to recover the fitness of the non-amputated organisms or the resources that they need for reproduction are being used for regeneration, but further tests are needed to understand this.

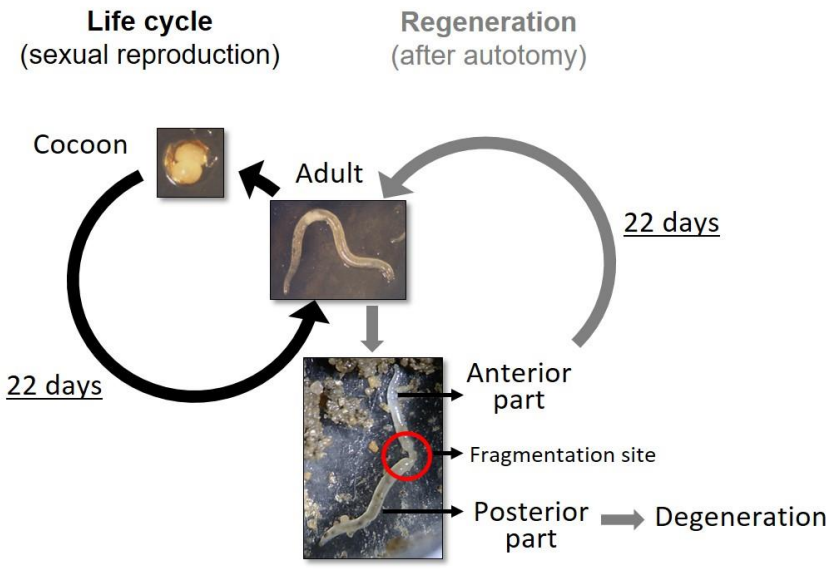

Figure 2: Diagram illustrating aspects of the reproduction and regeneration of *Enchytraeus crypticus*, indicating time to reach maturity.

The current results confirm the capacity of regeneration (posterior regeneration) in *E. crypticus*. Nevertheless, we believe that the spontaneous amputation and posterior regeneration is not the most common reproductive strategy, based on the fact that the observation of fragments in the cultures and test vessels is sporadic. One hypothesis is that autotomy can be used by this specie as a self-defence mechanism in response to natural/environmental stress or injuries from physical or chemical stimuli. This mechanism has been observed among a variety of phyla such as Cnidaria, Annelida, Mollusca, Arthropoda and Echinodermata (Fleming et al., 2007); the result is a quick separation and discard of a body part from the main body (Lesiuk and Drewes, 1999). In fact, several

studies report the benefits of self-amputation. In many oligochaetes this can serve as a detoxification process by the accumulation of the contaminants in the caudal segments followed by its disposal by autotomy, e.g. in *E. buchholzi* (Nakamura and Shiraishi, 1999), *Lumbriculus variegatus* (Lesiuk and Drewes, 1999), *Lutodrilus multivesiculatus* (McMahan, 1998), *Megascolides australis* (Jones et al., 1994), *Tubifex tubifex* (Bouché et al., 1999), and *Sparganophilus pearsei* (Vidal and Horne, 2003).

Another hypothesis is that fragmentation could be a rare reproductive alternative in this species (instead of autotomy) which possibly lost the capacity for full anterior regeneration, similarly to what is described for *Paranais litoralis* by Martínez and Levinton (1992) (further details in Bely and Sikes (2010)).

This is the first study reporting and describing regeneration in *E. crypticus*. *E. crypticus* can fragment and regenerate as a response to stress. This may have consequences for the interpretation of ecotoxicity results (e.g. in terms of survival). In natural populations, regeneration ability can be an advantage to deal with mechanical injuries, but the fitness of those organisms can be temporarily affected.

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References

Alibardi, L., Toni, M., 2005. Wound keratins in the regenerating epidermis of lizard suggests that the wound reaction is similar in the tail and limb. J. Exp. Zool. 303A, 845–860.

Bateman, P.W., Fleming, P.A., 2009. To cut a long tail short: a review of lizard caudal autotomy studies carried out over the last 20 years. J. Zool. 277, 1–14.

Bell, A.W., 1959. *Enchytraeus fragmentosus*: a new species of naturally fragmenting oligochaete worm. Science (80). 129, 1278.

Bely, A.E., 2006. Distribution of segment regeneration ability in the Annelida. Integr. Comp. Biol. 46, 508–518.

Bely, A.E., Nyberg, K.G., 2010. Evolution of animal regeneration: re-emergence of a field. Trends Ecol. Evol. 25, 161–170.

Bely, A.E., Sikes, J.M., 2010. Latent regeneration abilities persist following recent evolutionary loss in asexual annelids. Proc. Natl. Acad. Sci. U. S. A. 107, 1464–9.

Bicho, R.C., Gomes, S.I.L., Soares, A.M.V.M., Amorim, M.J.B., 2015a. Non-avoidance behaviour in enchytraeids to boric acid is related to the GABAergic mechanism. Environ. Sci. Pollut. Res. 22, 6898–6903.

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

Bouché, M.L., Risbourg, S.B., Arsac, F., Vernet, G., 1999. An original decontamination process developed by the aquatic oligochaete *Tubifex tubifex* exposed to copper and lead. Aquat Toxicol 45, 9–17.

Bouguenec, V., Giani, N., 1989. Biological studies upon *Enchytraeus variatus* Bouguenec & Giani 1987 in breeding cultures. Hydrobiologia 180, 151–165.

Cameron, G.R., 1932. Inflammation in earthworms. J. Pathol. Bacteriol. 35, 933–972.

Didden, W.A.M., 1993. Ecology of terrestrial Enchytraeidae. Pedobiologia (Jena). 37, 2–29.

Dózsa-Farkas, K., 1995. *Enchytraeus dudichi* sp. n., a new fragmenting Enchytraeus species from Iran (Enchytraeidae, Oligochaeta). Opusc. Zool. Budapest 27-28, 41–44.

Fleming, P.A., Muller, D., Bateman, P.W., 2007. Leave it all behind: a taxonomic perspective of autotomy in invertebrates. Biol. Rev. 82, 481–510.

Goss, R.J., 1969. Principles of Regeneration. Academic Press.

ISO, 2005. Soil Quality - Effects of pollutants on Enchytraeidae (*Enchytraeus* sp.). Determination of effects on reproduction and survival. Guideline No 16387. International Organization for Standardization. Geneva,Switzerland.

Jänsch, S., Amorim, M.J., Römbke, J., 2005. Identification of the ecological requirements of important terrestrial ecotoxicological test species. Environ. Rev. 13, 51–83.

Jones, D.R., Bushnell, P.G., Evans, B., Baldwin, K., 1994. Circulation in the gippsland giant earthworm *Megascolides australis*. Physiol Zoo 67, 1383–1401.

Kuperman, R.G., Amorim, M.J.B., Rombke, J., Lanno, R., Checkai, R.T., Dodard, S.G., Sunahara, G.I., Scheffczyk, A., 2006. Adaptation of the enchytraeid toxicity test for use with natural soil types. Eur. J. Soil Biol. 42, S234–S243.

Lesiuk, N.M., Drewes, C.D., 1999. Autotomy reflex in a freshwater oligochaete, *Lumbriculus variegatus* (Clitellata: Lumbriculidae). Hydrobiologia 406, 253–261.

Maginnis, T.L., 2006. The costs of autotomy and regeneration in animals: a review and framework for future research. Behav. Ecol. 17, 857–872.

Martínez, D.E., Levinton, J.S., 1992. Asexual metazoans undergo senescence. Proc. Natl. Acad. Sci. U. S. A. 89, 9920–9923.

McMahan, M.L., 1998. Ecology of the limicolous megadrile *Lutodrilus multivesiculatus* (Annelida: Oligochaeta). Megadrilogica 7, 40–44.

Müller, M.C.M., 2004. Nerve development, growth and differentiation during regeneration in *Enchytraeus fragmentosus* and *Stylaria lacustris* (Oligochaeta). Dev. Growth Differ. 46, 471–478.

Myohara, M., 2012. What Role Do Annelid Neoblasts Play? A Comparison of the Regeneration Patterns in a Neoblast-Bearing and a Neoblast-Lacking Enchytraeid Oligochaete. PLoS One 7, e37319.

Myohara, M., Yoshida-noro, C., Kobari, F., 1999. Fragmenting oligochaete *Enchytraeus japonensis*: A new material for regeneration study. Dev. Growth Differ. 41, 549–555.

Nakamura, Y., 1993. new fragmenting enchytraeid species, *Enchytraeus japonensis* from a cropped Kuroboku soil in Fukushima, Northern Japan (enchytraeids in Japan 5). Edaphologia 50, 37–39.

Nakamura, Y., Shiraishi, H., 1999. The nodule nickel in posterior segments of *Enchytraeus buchholzi* (Enchytraeidae: Oligochaeta). Edaphologia 62, 93–96.

Nielsen, C.O., Christensen, B., 1963. The Enchytraeidae. Critical revision and taxonomy of European species. Suppl. 2. Nat. Jutl. 10, 1–19.

OECD, 2010. Guidelines for the testing of chemicals No317. Bioaccumulation in Terrestrial Oligochaetes. Organization for Economic Cooperation and Development. Paris, France.

OECD, 2004. Guidelines for the testing of chemicals No. 220. Enchytraeid Reproduction Test. Organization for Economic Cooperation and Development. Paris, France.

Schmelz, R.M., Collado, R., 2010. A guide to European terrestrial and freshwater species of Enchytraeidae (Oligochaeta). Soil Org. 82, 1–176.

Schmelz, R.M., Collado, R., 2012. Guide to European Terrestrial and Freshwater Species of Enchytraeidae (Oligochaeta): First supplement, in: Schrader, S., Schmelz, R. M. (Eds.), Newsletter on Enchytraeidae No. 12. Proceedings of the Ninth International Symposium on Enchytraeidae, 14–16 July 2010, Braunschweig, Germany. Landbauforschung vTI Agriculture and Forestry Research, Special Issue 357.

Tanaka, E.M., Reddien, P.W., 2011. The cellular basis for animal regeneration. Dev. Cell 21, 172–185.

Vidal, D.E., Horne, a. J., 2003. Mercury toxicity in the aquatic oligochaete *Sparganophilus pearsei* II: Autotomy as a novel form of protection. Arch. Environ. Contam. Toxicol. 45, 462–467.

Westheide, W., Graefe, U., 1992. Two new terrestrial *Enchytraeus* species (Oligochaeta, Annelida). J. Nat. Hist. 26, 479–488.

Yoshida-Noro, C., Tochinai, S., 2010. Stem cell system in asexual and sexual reproduction of *Enchytraeus japonensis* (Oligochaeta, Annelida). Dev. Growth Differ. 52, 43–55.

Chapter 3

Development of an embryotoxicity test for *Enchytraeus crypticus* – the effect of Cd

Development of an embryotoxicity test for *Enchytraeus crypticus* – the effect of Cd

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Abstract

The existing standard enchytraeid reproduction test (ERT) concerns the assessment of effects on survival and reproduction. In the present study we optimized and propose an embryotoxicity test using *Enchytraeus crypticus.* Cadmium (Cd) was used as a test substance. Endpoints evaluated were embryo development, number of embryonic structures, Calcium (Ca) channels quantification and hatching success with macroscopic monitoring, histological and immunohistochemistry analysis. Results showed that Cd is embryotoxic for this species, causing a decrease in the hatching success (EC_{50}=3.1 mg/kg), a delay or disruption in formation of embryonic structures depending on concentrations (<5 mg Cd/kg or ≥16 mg Cd/kg). Results from immunohistochemistry suggest a competitive binding between Cd and Ca for Ca channels, resulting in changes in Ca homeostasis. The use of the *E. crypticus* embryotoxicity test with the combination of histological and immunohistological tools provided a good option towards mechanistic information enhancing the importance of these tests to evaluate the hazard of chemicals and possible use in risk assessment.

Keywords

Embryonic development, Cadmium, Embryotoxicity, Histology, Immunohistochemistry.

1. Introduction

In recent years, embryotoxicity tests have become increasingly interesting as tools for risk assessment (Schirling et al., 2006) given their short duration, prediction power for effects on the individual, and cost-effectiveness.

In soil ecotoxicology with invertebrates, embryonic development tests are limited (Druart et al., 2010). A few studies were performed using the terrestrial slugs *Deroceras reticulatum* (Iglesias et al., 2002; Iglesias et al., 2000) and snails *Monacha obstructa* (Shoaib et al., 2010) to assess embryotoxic effects of metals and pesticides and for *Helix aspersa* (syn. *Cantareus aspersus)* an embryotoxicity test has been proposed (Baurand et al., 2014; Druart et al., 2010). In comparison, for the aquatic environment there are several embryotoxicity studies available for invertebrates (Druart et al., 2012) and for vertebrates the test with *Danio rerio* (zebrafish) is standardized (OECD, 2013).

Furthermore, a very important aspect refers to the added value of mechanistic data to unravel adverse outcome pathways (Kramer et al., 2011). By integrating mechanistic data with data from existing testing programs one can produce more cost-effective, timely and more comprehensive evaluations (Villeneuve and Garcia-Reyero, 2011) and improve the identification of risk mitigation measures.

Aiming for an embryotoxicity test with soil species we have used *Enchytraeus crypticus* (Oligochaete), which represents a group of organisms with important ecological functions, e.g. organic matter decomposition and soil bioturbation (Didden, 1993). This species has already standardized ecotoxicity tests to evaluate survival, reproduction and bioaccumulation (ISO, 2004; OECD, 2004a, 2010) and is a good model due to its short life cycle (46 days), and embryonic development (9-11 days) (Bicho et al., 2015; Westheide and Müller, 1996). Further, the cocoons of this species are transparent which facilitates the visualization and study of the various stages of development. In the present study we addressed the following aspects for *E. crypticus*: 1) to study the embryonic development in detail, 2) to propose an embryotoxicity test, 3) to validate results with Cadmium (Cd) as a test substance (given its known embryotoxicity) (Brasfield et al., 2004; Coeurdassier et al., 2003; Druart et al., 2010; Gomot, 1998; Hwang et al., 1995; Itow et al., 1998; Middaugh and Dean, 1977) and 4) to analyse the known mechanism of competition between Cd and Calcium (Ca) for Ca channels via immunohistochemistry and Ca staining (Blazka and Shaikh, 1991; Braeckman et al., 1999; Craig et al., 1999; Li et al., 2010). The endpoints

assessed were embryonic development (via macroscopic monitoring), number of embryonic structures (via microscopic monitoring) and hatching success.

2. Materials and Methods

2.1. Test organisms

The species *Enchytraeus crypticus* (Oligochaete: Enchytraeidae) was used. Cultures are kept in agar plates prepared with a salt solution of $CaCl_2$, $MgSO_4$, KCl and $NaHCO_3$, fed *ad libitum* with oatmeal and maintained in laboratory under controlled conditions at 20ºC and a photoperiod of 16:8 (light: dark). Synchronized age cocoons were obtained following the procedures described by Bicho et al. (2015). In short, adults with well-developed clitellum are transferred to new plates, allowing cocoon deposition which occurs during a period of 2 days. Cocoons with 1-2 days old were selected from synchronized cultures.

2.2. Test soil

All tests were performed using the standard natural soil LUFA 2.2 (Speyer, Germany). The properties of this soil can be summarized as follows: pH ($CaCl_2$) of 5.5, 46% of maximum water-holding capacity (WHC_{max}), 4% organic matter content and a particle size distribution of 6% clay, 14% silt and 80% sand.

All soils were moistened to 50% of WHC with deionized water (or spiked with aqueous solution) and used immediately after preparation. In all experiments pH and moisture content was measured at the beginning and at the end of the experiment.

2.3. Test chemical and spiking

Cadmium ($CdCl_2 \cdot 2^{1/2}H_2O$, Fluka, Sigma-Aldrich, 98% purity) was tested in five concentrations: 0 – 1.6 – 5 – 16 - 50 mg Cd/kg soil dry weight (mg/kg soil DW). Solutions were prepared with deionized water, serially diluted and added onto the pre-moistened soil and homogeneously mixed. Test soil was allowed to equilibrate for 3 days previous test start. Test vessels consisted of 6-well plates (35 mm ø), containing 5 g of the test soil each, where treatments and replicates were distributed randomly. Three replicates were used per treatment per sampling day.

2.4. Test procedures

2.4.1. Exposure

Cocoons were randomly selected and introduced, as pools of 10, into each well containing the test soil. During the exposure procedure of adding the cocoons to the test soil it is important to distribute these evenly and to cover them with soil to avoid dehydration and ensure exposure.

The test was maintained at a photoperiod of 16:8 h light:dark and at 20ºC. The soil moisture content was checked by weighing and replenished every two days with deionized water. The exposure time was 9 days (to allow hatching) and samples were collected at days 0, 1, 2, 3, 6 and 9, corresponding to 1-2 days, 3, 4, 5, 7 and 11 days after cocoon laying.

2.4.2. Hatching test (Ht)

At the end of the exposure (9 days) the number of hatched juveniles was counted. To extract organisms from soil, cocoons were fixated with 96% ethanol and Bengal rose (1% solution in ethanol). The following day, soil samples were sieved through a 500 µm mesh to separate individuals from most of the soil and facilitate counting using stereo microscope (Zeiss Stemi 2000-C).

2.4.3. Embryo development

2.4.3.1. Macroscopic

Sampling procedure consisted in transferring the soil with cocoons from each well to a Petri dish with ISO water (OECD, 2004b), to remove the soil particles. With the help of a binocular (50X) and Dino-Eye camera attached and Dino-Lite software (Corp, 2010) photographs were taken for embryo macroscopic analysis. After this, two pools of five cocoons per replicate were stored in Eppendorf's (1.5 mL) with 10% formaldehyde until further analysis (histology).

2.4.3.2. Histology

Sampled cocoons (n=5) were placed on a square of filter paper and covered with another square, as an envelope. This was left to dry for 2 min and was transferred into histology

cassettes with sponge inside. Histology cassettes were introduced in an automated tissue processor (Microm STP 120) for paraffin embedding. The cocoons were transferred to embedding workstation to obtain paraffin blocks. Sections (2 μm) were cut on a rotary microtome (Leica RM 2035). Sections of cocoons were stained with Hematoxylin and Eosin (H&E) and Von Kossa (calcium staining) for light microscopic examination. Observations and registrations of the number of embryonic structures and photographs were made using an Olympus BX51 microscope with an Olympus camera attached.

In each concentration in each sampling day, the average number of embryonic structures that occurred in the embryos was presented (list of structures is shown in results). The membrane thickness of cocoons was assessed in Von Kossa staining (because membranes are more evident with this staining) by measuring height in four opposite parts of cocoon membrane (Fig. S1A, supplementary data). Measurements were carried out using ImageJ software (Rasband, 1997-2008). The average number for the four parts was used to compare differences in membrane thickness.

2.5 Data analysis

Normality and homogeneity of variances were checked. To assess significant differences between control and Cd treatments one-way analysis of variances (ANOVA) was used or alternatively the Mann-Whithey Rank Sum Test was used (SigmaPlot, 1997). Effect Concentrations (ECx) calculations were performed for the hatching success using the logistic 2 parameters regression model (TRAP software).

3. Results

3.1. Hatching success

Results can be observed in Fig. 1A. The number of hatched juveniles was significantly reduced (for all concentrations; ANOVA; $p<0.05$) and in a dose-related manner. Effect concentrations are summarized in Table 1.

Table 1: Summary of the effect concentrations (ECx) for *Enchytraeus crypticus* when exposed to $CdCl_2$ (mg Cd/kg DW soil) in LUFA 2.2 standard natural soil. Information includes the 95% confidence intervals (CI) shown in brackets and the model used to fit data and parameters: slope (S) and intercept (Y0). n.d. = not determined.

Test	endpoint	EC_{10} (mg/kg)	EC_{20} (mg/kg)	EC_{50} (mg/kg)	EC_{80} (mg/kg)	Model (parameters)
Ht	hatching	(n.d.)	1.0 (0.2<CI<1.8)	3.1 (2.5<CI<3.7)	5.2 (4.2<CI<6.2)	Logistic 2 param (S:0.16; Y0:22)

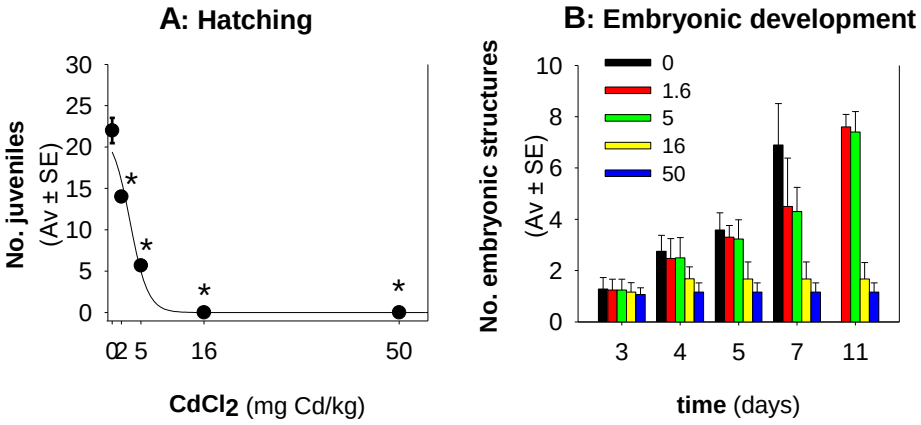

Figure 1: Results from hatching success, histological and immunohistochemistry analysis for *Enchytraeus crypticus* when exposed to $CdCl_2$ (mg Cd/kg DW soil) in LUFA 2.2 standard natural soil. All values are expressed in average ± standard error (Av ± SE). **A:** hatching success. **B:** embryonic development. The solid line represents the model fit to data. (*Dunnets': p<0.05).

3.2. Embryo development

3.2.1. Macroscopic

From macroscopic analysis of cocoons in control conditions (Fig. 2), it is possible to observe that cocoons are lemon shaped, transparent, the wall is membranous and the operculum symmetrically positioned on each side is also visible. Given the transparency of cocoons it is possible to visualize the eggs/embryos (2 to 5) within the cocoon fluid media.

Embryo development is similar between days 3 and 4 with embryo in morula stage (round shape), confirming the beginning of embryonic development and by day 5 progress can be seen by the invaginations in embryos, which will differentiate into anterior and posterior parts. On the 7th day, there is further differentiation, being already possible to observe the fusiform shape and movement of the juveniles (Fig. 2).

Macroscopic analysis of cocoons exposed to the Cd concentration range (Fig. S4, supplementary data) shows the development along days 3-11. At the 11th day, the juveniles had hatched.

Hatching was increasingly reduced with Cd concentration increase and not occurring by day 11. For 50 mg Cd/kg, embryos did not differentiate in juveniles by the 11th day and the presence of blastocoel (double black arrows) is observed indicating that embryos remain at blastula stage (Fig. S4, supplementary data).

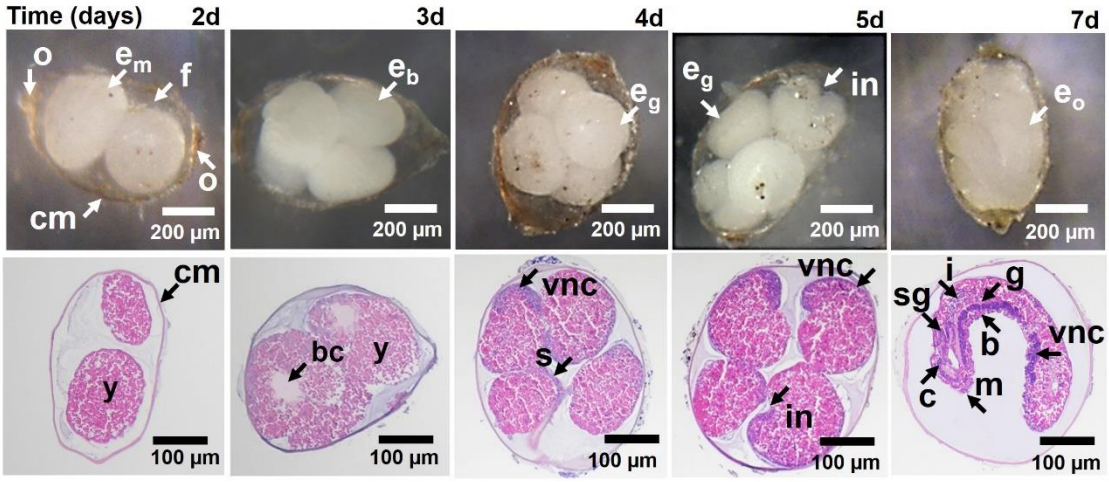

Figure 2: Macroscopic visualization (top row) and histological analysis with Hematoxylin and Eosin staining (bottom row) of embryo development of *Enchytraeus crypticus* in control conditions in LUFA 2.2 standard natural soil along time (2-7 days). b: body muscles; bc: blastocoel; c: cerebral ganglion; cm: cocoon membrane; e_b: embryo in blastula stage (round shape); e_g: embryo in gastrula stage; e_m: embryo in morula stage (round shape); e_o: embryo in organogenesis (fusiform shape); f: cocoon fluid; g: gut muscles; i: intestine; in: invagination; m: mouth; o: operculum; s: somatopleure; sg: septal glands; vnc: ventral nerve cord; y: yolk proteins.

3.2.2. Histology

From observations of Hematoxylin and Eosin stained slides from cocoons of control group (Fig. 2) the following structures were observed: yolk proteins, ventral nerve cord, somatopleure, mouth, cerebral ganglion, intestine, and body. Embryogenesis of *E. crypticus* was as follows: 2 days after cocoon deposition yolk proteins were uniformly distributed and embryos present the first cell divisions – morula stage. At 3 days large gaps were observed revealing that a blastocoel has been formed and ectoderm covers the embryo completely and begins the development and migration of cells that will originate the ventral nerve cord; the morphological changes are based on the elongation of the germ-band, and the formation of the anterior part begins – blastula stage. On the 4th day, differentiation of cells continues and somatopleure is now visible; anterior part becomes more developed than the posterior part, showing an increase in the number of cells forming the head structures – gastrula stage. On 5th day, the ventral nerve cord is differentiated into two different parts: the ventral part of the nerve cord and the dorsal compartment of the cerebral ganglion; the progression of tissue and organ development differs significantly between anterior and posterior parts; the ventral nerve cord begins to extend for about half of the body length while the anterior part of the embryo shows the first signs of organ differentiation, the posterior part is still less differentiated – morphogenesis. At the 7th day is the end of gastrulation, and organogenesis and segmentation begins; the mouth opening and septal glands in the anterior part is already visible and differentiation of internal structures in the posterior part starts (e.g. intestine and muscle tissue). During the next days of embryonic development until hatching, additional structures differentiate and embryos grow.

The presence of the various embryonic structures were recorded and counted during embryonic development for all treatments. Structures include 1: yolk proteins, 2: ventral nerve cord, 3: somatopleure, 4: mouth, 5: cerebral ganglion, 6: septal glands, 7: intestine, 8: body muscles, 9: gut muscles. In the control the number of structures (Fig. 1B) increased along time; for concentrations ≤5 mg Cd/kg this is similar to the control until day 5 and at day 7 there is a decreasing trend; for concentrations ≥ 16 mg Cd/kg there is no progress after day 3.

Representative pictures of the histological analysis are shown in Fig. S5 (supplementary data). For 1.6 and 5 mg Cd/kg only at day 11 it was possible to observe the structures

present at day 7 in control organisms: intestine, body and gut muscles, organization of all structures and a reduction of yolk proteins. For 16 and 50 mg Cd/kg, cell divisions are observed at the 3rd day, and from days 4 to 7 although the ventral nerve cord cells begin to develop is still possible to observe the blastocoel so cell divisions are ongoing; from 7th day onwards cells degenerate and disorganized tissue is observed by day 11. In addition, the presence of fungi is observed in more than half of the cocoons (62%) exposed to 16 mg Cd/kg and 100% when exposed to 50 mg Cd/kg, likely due to cell death.

Results of Von Kossa staining (Fig. 3) showed an increase of Ca staining with increasing Cd concentrations. Embryos treated with 0, 5 and 50 mg Cd/kg are shown to illustrate the differences. For controls and 5 mg Cd/kg Ca staining was observed from the 4th and 5th day as a brown Ca staining. At a further stage of development (7th day) most of the cocoons did not show Ca staining. For 50 mg Cd/kg, results are similar for days 3 and 4. At the 5th day, staining is more intense and from day 7-11 the staining is clearly more intense.

In addition, measurements of cocoon membrane thickness at the 7th day showed that Cd exposure significantly increased membrane thickness, i.e. values were for control organisms 0.038 ± 0.002 nm and for organisms exposed to 50 mg Cd/kg 0.064 ± 0.004 nm (Av ± SE).

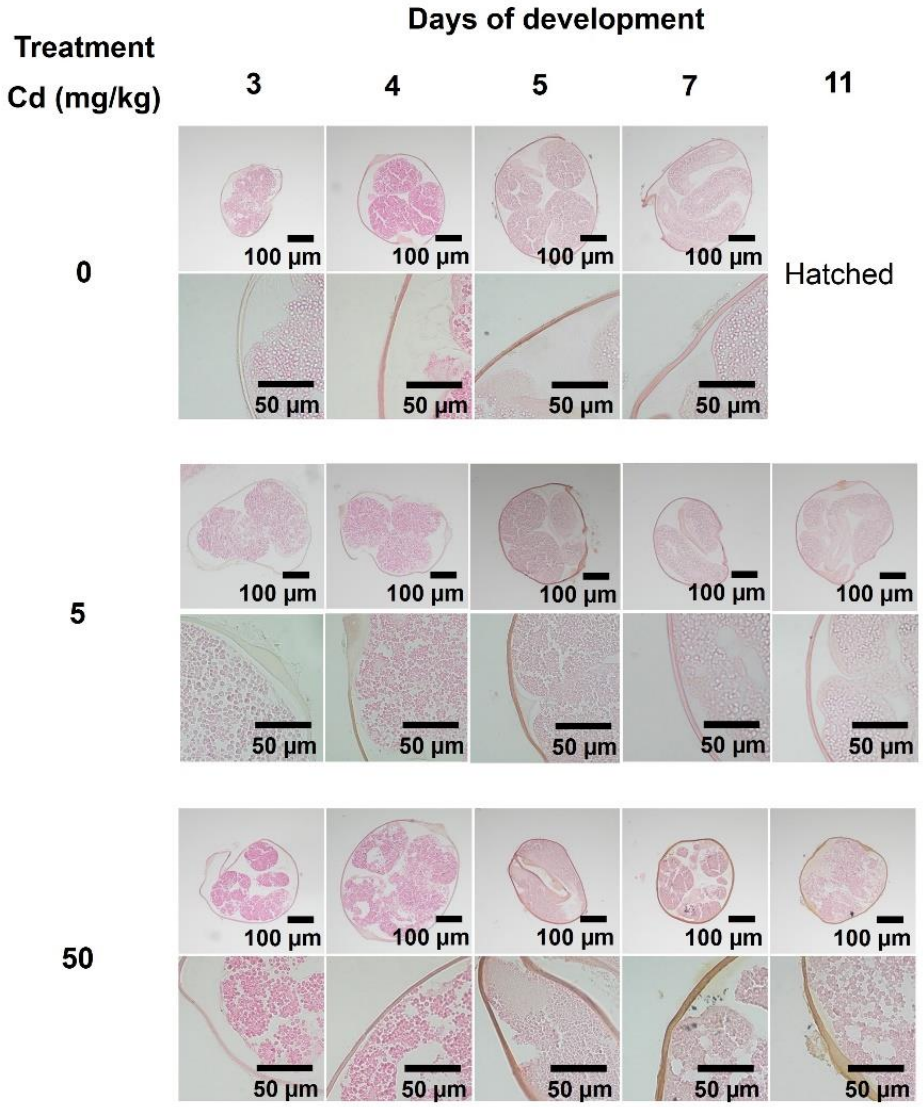

Figure 3: Results from the histological analysis with Von Kossa staining of embryo development of *Enchytraeus crypticus* exposed to $CdCl_2$ (mg Cd/kg DW soil) in LUFA 2.2 standard natural soil for 3, 4, 5, 7 and 11 days.

4. Discussion

Results allowed a comprehensive knowledge of *E. crypticus* embryogenesis. Previous information was provided by Westheide and Müller (1996) although less detailed. Information on *Enchytraeus coronatus* as given by Bergter et al. (2004) shows the similarity between these species.

The experimental test design as proposed here can be used as draft for an embryotoxicity test for *E. crypticus*. Such an embryonic development test brings much added value to the standard test (ISO, 2004; OECD, 2004a, 2010). This test starts with cocoons with synchronized age, which reduces the variability between organisms compared with the standard test (where adults with developed clitellum, including different ages are used) and it is shorter (11 days) [e.g. *Lymnaea stagnalis* is 15 days and *Helix aspersa* is 12 days (Druart et al., 2010; Gomot, 1998)]. Further, the test was shown to be reproducible (confirmed consistency among various tests, e.g. similar hatching EC_{50} in Bicho et al., (2015)). Not surprisingly, for Cd, the embryo development was a more sensitive endpoint compared to reproduction from a standard test (Bicho et al., 2015), and as confirmed here: $EC_{50\_hatching}$=3.1 mg Cd/kg) (hatching test), $EC_{50\_reproduction}$=35 mg Cd/kg (standard test) (Castro-Ferreira et al., 2012).

Results from the present study allowed to discriminate the specific day and/or affected stage of development to Cd toxicity. The reduced hatching with increasing Cd concentrations reflects the delay in embryogenesis at ≤5 mg Cd/kg and the disruption at ≥16 mg Cd/kg. For 16 mg Cd/kg in most (62%) of the cocoons, cells were degenerated and tissue disorganized, being consequently more prone to contamination by fungi. The effect of Cd on embryonic development and hatching was concentration dependent and for concentrations higher than 16 mg Cd/kg the effects seem to irreversibly stop embryogenesis, as confirmed in full life cycle test (Bicho et al., 2015).

The embryotoxicity of Cd is known to other invertebrate species, like aquatic molluscs (Gomot, 1998; Pietrock et al., 2008). The particular stage of embryonic development that is delayed or disrupted depends on the Cd concentration, as also observed for *Helix aspersa* by Druart et al. (2010). On the other hand, several authors claim that the effect of Cd is dependent on the stage of exposure, i.e., the earlier the exposure, the larger the effect (Marc et al., 2005; Pennati et al., 2006). In our study, the worst case scenario was studied since cocoons were exposed during first cell divisions (morula stage).

Results from Von Kossa staining in non-exposed cocoons, showed Ca deposition on the membrane during the first stages of embryonic development (morula and gastrula stages), but at organogenesis stage this Ca deposition decreased. To our knowledge this is the first study that indicates Ca homeostasis/dynamics during embryogenesis of an oligochaete (and possibly of invertebrates). Similarly, studies with vertebrate species, e.g. *Lepidochelys*

olivacea, *Coluber constrictor* and *Zootoca vivipara* have shown that Ca available in the eggshell decreases during embryogenesis (Packard et al., 1984; Sahoo et al., 1998; Stewart et al., 2011). The role of Ca in early embryogenesis is also well described in *Xenopus laevis* and *Danio rerio* (Drean et al., 1995; Whitaker, 2012). However in our study, for Cd exposed cocoons, this Ca deposition was not reduced. Additionally, an increase in the thickness of membranes at 50 mg Cd/kg for cocoons at organogenesis stage was observed (7^{th} day), which confirms the persistence of Ca deposition or accumulation.

Results from immunohistochemistry in controls support our observations of Von Kossa staining for the same conditions (see supplementary data for details). The presence of L-type Ca channels during early development is confirmed and a decrease of expression of these Ca channels was observed at later stages (7^{th} day) of embryonic development. Again studies with vertebrates are in agreement with these observations, where the expression of L-type Ca channel was showed during early development in *Xenopus laevis* (Drean et al., 1995). In our study, we observed the presence of Ca and L-type Ca channels proteins in the cocoon membrane at early stage of embryogenesis, followed by its reduction at a later stage. This indicates that Ca transport occurs through the extraembryonic membrane during the embryonic development. Regarding this mechanism, to the best of our knowledge, this has not been described for annelids before. For vertebrates (reptiles and birds), studies have shown that the mechanism of calcium transport by both oviparous and viviparous embryos does not differ between modes of parity, the calcium used in embryo development is either from eggshell or the placenta (Gabrielli and Accili, 2010; Stewart et al., 2011). The described mechanisms of embryonic uptake of Ca from extraembryonic membranes involve e.g. Ca channels, Ca binding proteins and Ca ATPases (Fregoso et al., 2010, 2012; Gabrielli and Accili, 2010; Stewart et al., 2011). It is shown that the uptake of Ca is low in early development, reaching the maximum absorption point during mid-development followed by a decrease at the end of development, for which the pattern of expression of these proteins follows the Ca uptake.

It is known that Cd and Ca compete for Ca channels in different species, e.g. zebrafish *Danio rerio* (Hen Chow and Cheng, 2003), aquatic insect (Braeckman et al., 1999; Craig et al., 1999), and soil invertebrates species like *Helix aspersa* (Druart et al., 2010) and *Eisenia andrei* (Li et al., 2010) or in mammalian cells (Blazka and Shaikh, 1991; Hinkle et al., 1987), and that Cd has a high affinity binding site in the L-type Ca channels (Marchetti,

2013; Misra et al., 2002). Additionally, in mammalian cells, it was shown that Cd induces gene transcription with consequent protein translation (Misra et al., 2002).

Based on the known mechanisms and the observation in our study of increased Ca staining with higher Cd concentration, we formulate the following mechanism hypothesis: Cd competes with Ca and enters embryos via the L-type Ca channels in the cocoon membrane; gene regulation mechanisms are activated to synthesize more Ca channel proteins; for higher Cd concentrations the compensatory mechanism is probably not enough, hence the disruption in the Ca homeostasis and embryo development.

To integrate data we illustrate results in a draft AOP (Fig. 4).

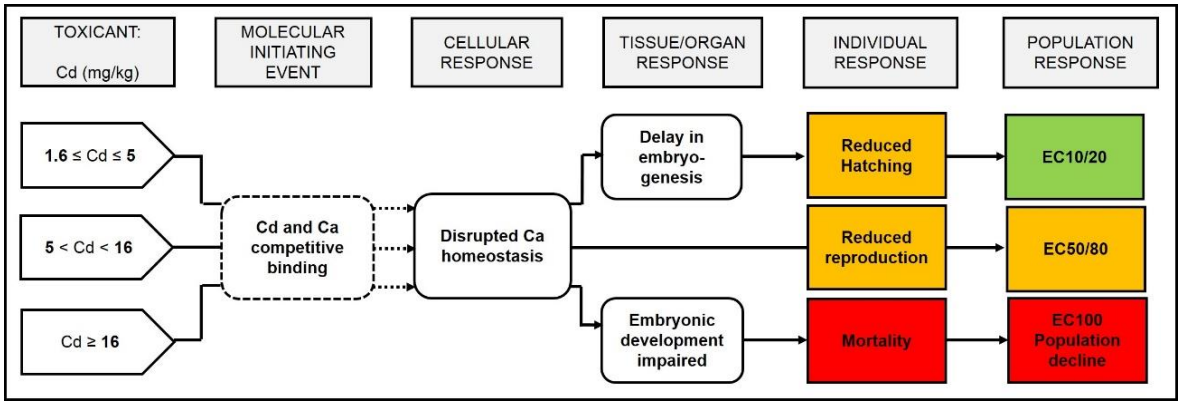

Figure 4: Adverse Outcome Pathway (AOP) for *Enchytraeus crypticus* when exposed to Cd in LUFA 2.2 soil. Red: adverse effect; Orange: semi-adverse effect; Green: no adverse effect; Square boxes represent final states for the organism and rounded boxes represent intermediate states. Dashed line represents relationships hypothesized.

This AOP combines results from the present study with the results observed in Bicho et al. (2015). As can be depicted, the initial reduced hatching (11 days) at <5mg/kg Cd exposure is partly *compensated* with time (hatching occurred later in time between 11 and 25 days, as measured in the study of Bicho et al. (2015), whereas for the highest concentration this was a true reduction, and concentrations between 5 and 16 mg Cd/kg caused intermediate effects (with higher uncertainty since values were interpolated within tested concentrations).

5. Conclusions

The present study provided the development of an embryotoxicity test for the soil species *Enchytraeus crypticus* (oligochaete). This means that new and relevant endpoints were added. We propose an embryo test where the macroscopic monitoring is performed. Additionally but not mandatory, histology can be included (as described). In particular we recommend further studies at the immunohistochemical level are done to confirm the mechanisms involving Ca transport. Further, this filled an existent gap in soil ecotoxicology regarding embryo development. The use of this test in future studies can provide helpful information for risk assessment and regulatory purposes.

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The authors declare that they have no conflict of interests.

7. Supplementary data

Materials and Methods

Immunohistochemistry

Immunostaining of calcium channels in cocoons was detected by immunohistochemistry using the primary antibody – Anti-Calcium channel L type DHPR alpha 2 subunit [20A] (Abcam. Cambridge, England). This is a mouse monoclonal antibody designed to detect 1.4-dihydropyridine (DHP) receptor alpha-2 subunit. This antibody detects a 220 kDa protein under non-reducing and a 143 kDa protein under reducing conditions representing DHP. The DHP receptor is part of the L-type Ca channel complex. Sections of cocoons of age 4, 5 and 7 days of all treatments were used (sampling times selected based on effects observed with macroscopy and histology). These were immersed in 10 mM sodium citrate (pH 6.0) buffer, and microwaved for 30 min at 700 watts for antigen retrieval. Antigen visualization was done with the Novocastra Novolink Polymer Detection System (Leica

Microsystems GmbH, Wetzlar, Germany) and involved the following steps: Sections were incubated with H_2O_2 (3%) for 10 min to eliminate endogenous peroxidase activity followed by a 5 min incubation with a protein blocking agent. Sections were subsequently incubated overnight at 4 ºC with the primary antibody diluted at 1:300 with BSA (5%), and on the following day, washed in TBS-buffered saline solution before incubation for 30 min with the secondary antibody system using diaminobenzidine (DAB) as a chromogen. Negative controls were obtained by omitting primary antibody to check antibody specificity. In the results one image representing the negative control is shown. Observations and photographs were made using an Olympus BX51 microscope with an Olympus camera attached. For the quantification process of the expression of Ca channels, the cocoon membrane was divided in eight equal parts (Fig. S1B). For each part a semi quantitative scoring system was used: 0 = no positive cells, 1 = positive cells in ≤ 50% of the section, and 2 = positive cells in > 50% of the section, adapted from Amaral et al (2012).

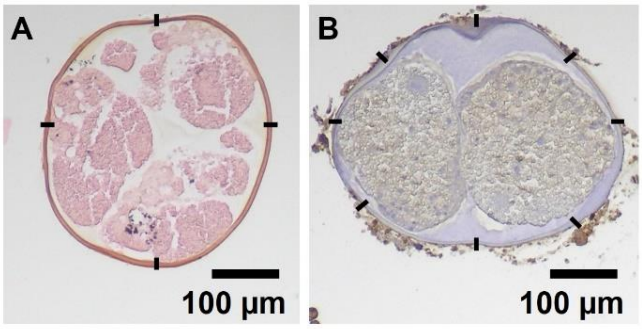

Figure S1: Schematic representation of the divisions of the cocoon membrane of *Enchytraeus crypticus*. **A:** measurement of membrane thickness **B:** visualization and semi-scoring of Ca channels.

Results

Immunohistochemistry

The expression of Ca channels was classified using an index (described above). Results regarding the index of expression of Ca channels are shown in Fig. S2. In controls the index of positive immunostaining tend to decrease during embryogenesis, whereas in the Cd treated cocoons for concentrations ≤ 16 mg Cd/kg, it was observed a slight increasing trend. Cocoons exposed to the highest concentration (50 mg Cd/kg) showed the highest

index of membrane with positive expression at the 7th day of development (Fig. S2) and can be observed in Fig. S3. It is important to note that further studies are needed to confirm the species specificity of the antibody.

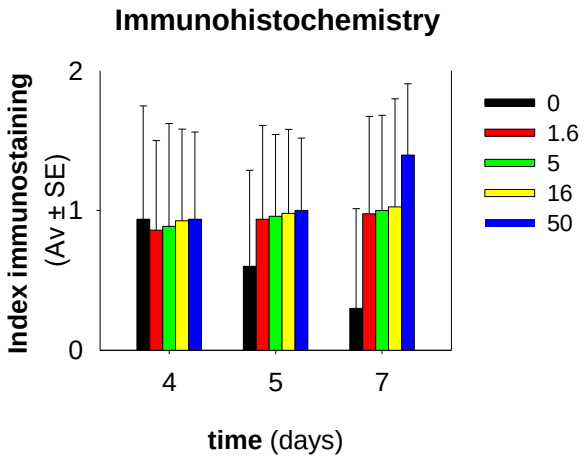

Figure S2: Results from immunohistochemistry analysis for *Enchytraeus crypticus* when exposed to CdCl2 (mg Cd/kg DW soil) in LUFA 2.2 standard natural soil. All values are expressed in average ± standard error (Av ± SE).

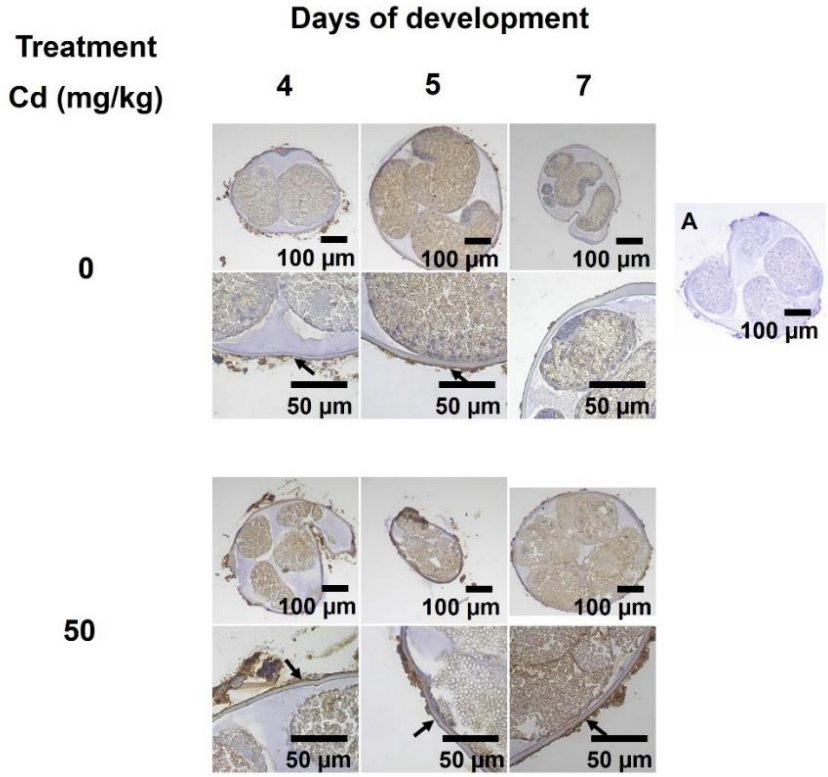

Figure S3: Results from the immunohistochemistry of embryo development of *Enchytraeus crypticus* exposed to $CdCl_2$ (mg Cd/kg DW soil) in LUFA 2.2 standard natural soil for 4, 5 and 7 days. Black arrows: positive immunostaining. A: negative control without positive staining. This section represents a control at 4 days.

Macroscopic and histological

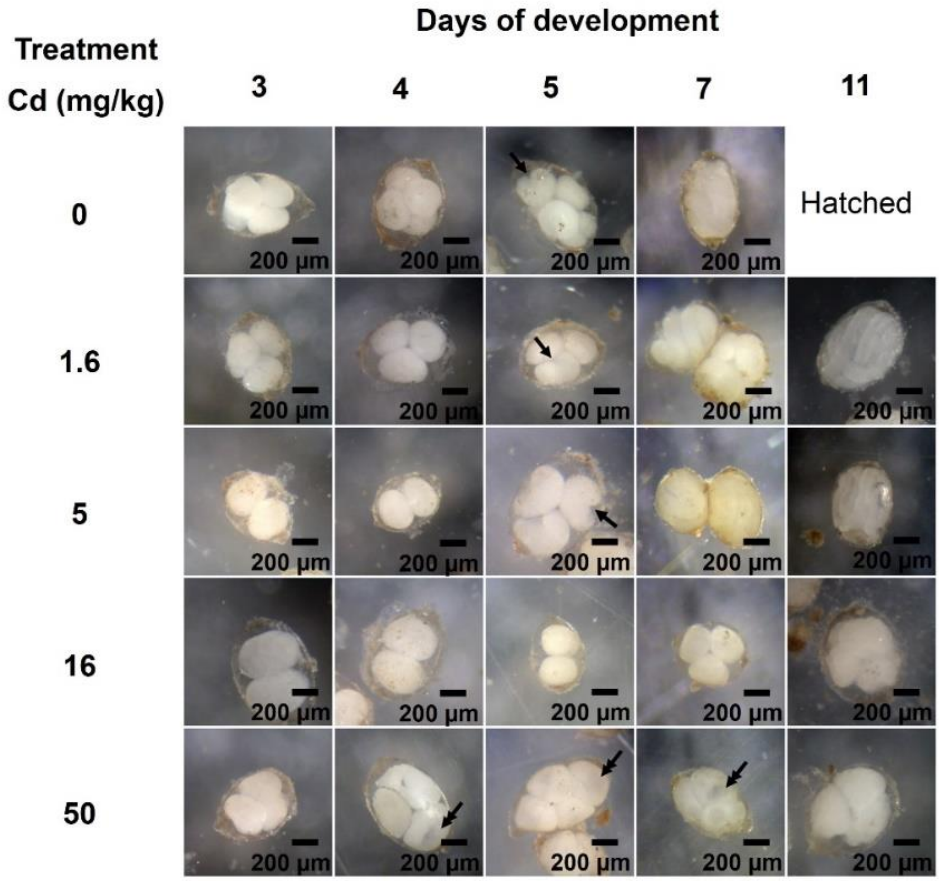

Figure S4: Results from the macroscopic visualization of embryo development of *Enchytraeus crypticus* exposed to $CdCl_2$ (mg Cd/kg DW soil) in LUFA 2.2 standard natural soil for 3, 4, 5, 7 and 11 days. Black arrows: invaginations; double black arrows: blastocoel.

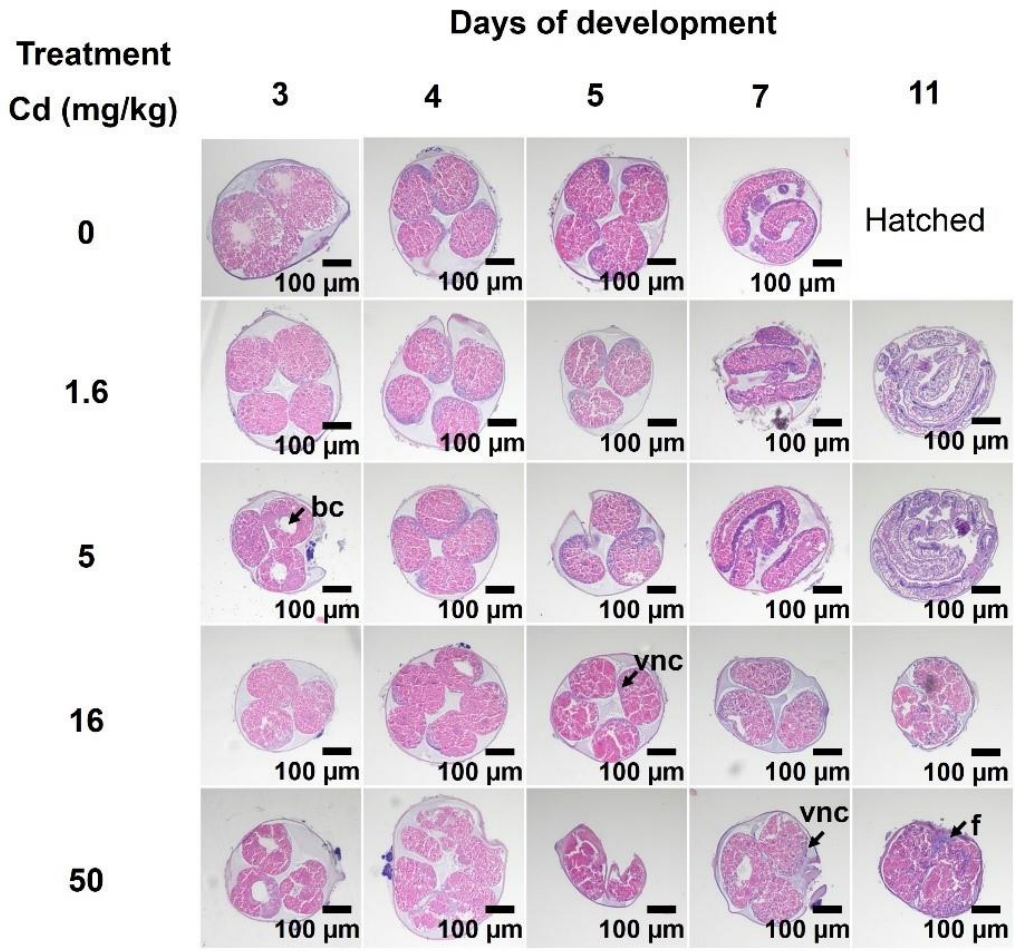

Figure S5: Results from the histological analysis with Hematoxylin and Eosin staining of embryo development of *Enchytraeus crypticus* exposed to $CdCl_2$ (mg Cd/kg DW soil) in LUFA 2.2 standard natural soil for 3, 4, 5, 7 and 11 days. bc: blastocoel; f: fungi; vnc: ventral nerve cord.

Reference

Amaral, M.J., Sanchez-Hernandez, J.C., Bicho, R.C., Carretero, M.A., Valente, R., Faustino, A.M.R., Soares, A.M.V.M., Mann, R.M., 2012. Biomarkers of exposure and effect in a lacertid lizard (*Podarcis bocagei Seoane*) exposed to chlorpyrifos. Environ. Toxicol. Chem. 31, 2345–2353.

8. References

Amaral, M.J., Sanchez-Hernandez, J.C., Bicho, R.C., Carretero, M.A., Valente, R., Faustino, A.M.R., Soares, A.M.V.M., Mann, R.M., 2012. Biomarkers of exposure and effect in a lacertid lizard (*Podarcis bocagei Seoane*) exposed to chlorpyrifos. Environ. Toxicol. Chem. 31, 2345–2353.

Baurand, P.-E., Capelli, N., Scheifler, R., de Vaufleury, A., 2014. An assessment of the embryotoxicity of cadmium in the terrestrial mollusk *Cantareus aspersus*: From bioaccumulation to impacts at different levels of biological organization. Ecotoxicol. Environ. Saf. 110, 89-94.

Bergter, A., Beck, L.A., Paululat, A., 2004. Embryonic development of the oligochaete *Enchytraeus coronatus*: an SEM and histological study of embryogenesis from one-cell stage to hatching. J. Morphol. 261, 26–42.

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

Blazka, M.E., Shaikh, Z.A., 1991. Differences in cadmium and mercury uptakes by hepatocytes role of calcium channels. Toxicol. Appl. Pharm. 110, 355–362.

Braeckman, B., Smagghe, G., Brutsaert, N., Cornelis, R., Raes, H., 1999. Cadmium Uptake and Defense Mechanism in Insect Cells. Environ. Res. 80, 231–243.

Brasfield, S.M., Bradham, K., Wells, J.B., Talent, L.G., Lanno, R.P., Janz, D.M., 2004. Development of a terrestrial vertebrate model for assessing bioavailability of cadmium in the fence lizard (*Sceloporus undulatus*) and in ovo effects on hatchling size and thyroid function. Chemosphere 54, 1643–1651.

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

Coeurdassier, M., De Vaufleury, A., Badot, P.M., 2003. Bioconcentration of cadmium and toxic effects on life-history traits of pond snails (*Lymnaea palustris* and *Lymnaea stagnalis*) in laboratory bioassays. Arch. Environ. Contam. Toxicol. 45, 102–109.

Corp, A.E., 2010. Dino-Lite Digital Microscope.

Craig, A., Hare, L., Tessier, A., 1999. Experimental evidence for cadmium uptake via calcium channels in the aquatic insect *Chironomus staegeri*. Aquat. Toxicol. 44, 255–262.

Didden, W. A. M., 1993. Ecology of the terrestrial Enchytraeidae. Pedobiologia 37, 2-29.

Drean, G., Leclerc, C., Duprat, A.M., Moreau, M., 1995. Expression of L-type Ca2+ channel during early embryogenesis in *Xenopus laevis*. Int. J. Dev. Biol. 39, 1027–1032.

Druart, C., Scheifler, R., Millet, M., Vaufleury, A., 2012. Landsnail eggs bioassays: A new tool to assess embryotoxicity of contaminants in the solid, liquid or gaseous phase of soil. Appl. Soil Ecol. 53, 56–64.

Druart, C., Scheifler, R., Vaufleury, A., 2010. Towards the development of an embryotoxicity bioassay with terrestrial snails: screening approach for cadmium and pesticides. J. Hazard. Mater. 184, 26–33.

Fregoso, S.P., Stewart, J.R., Ecay, T.W., 2010. Embryonic mobilization of calcium in a viviparous reptile: evidence for a novel pattern of placental calcium secretion. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 156, 147-150.

Fregoso, S.P., Stewart, J.R., Ecay, T.W., 2012. Expression of calcium transport proteins in the extraembryonic membranes of a viviparous snake, *Virginia striatula*. J. Exp. Zool. B Mol. Dev. Evol. 318, 250-256.

Gabrielli, M.G., Accili, D., 2010. The chick chorioallantoic membrane: a model of molecular, structural, and functional adaptation to transepithelial ion transport and barrier function during embryonic development. J. Biomed. Biotechnol. 2010, 940741.

Gomot, A., 1998. Toxic effects of cadmium on reproduction, development, and hatching in the freshwater snail *Lymnaea stagnalis* for water quality monitoring. Ecotoxicol. Environ. Saf. 41, 288–297.

Hen Chow, E.S., Cheng, S.H., 2003. Cadmium affects muscle type development and axon growth in zebrafish embryonic somitogenesis. J. Toxicol. Sci. 73, 149–159.

Hinkle, P.M., Kinsella, P.A., Osterhoudt, K.C., 1987. Cadmium uptake and toxicity via voltage-sensitive calcium channels. J. Biol. Chem. 262, 16333–16337.

Hwang, P.P., Lin, S.W., Lin, H.C., 1995. Different sensitivities to cadmium in tilapia larvae (*Oreochromis mossambicus*; teleostei). Arch. Environ. Contam. Toxicol. 29, 1-7.

Iglesias, J., Castillejo, J., Ester, A., Castro, R., Lombardia, M.J., 2002. Susceptibility of the eggs of the field slug *Deroceras reticulatum* to contact with pesticides and substances of biological origin on artificial soil. Ann. Appl. Biol. 140, 53–59.

Iglesias, J., Castillejo, J., Parama, R., Mascato, R., Lombardia, M.J., 2000. Susceptibility of the eggs of the pest slug *Deroceras reticulatum* to contact with metal salts. J. Mollus. Stud. 66, 171–176.

ISO, 2004. ISO: 16387 Soil quality - Effects of pollutants on Enchytraeidae (Enchytraeus sp.) - Determination of effects on reproduction and survival. ISO (International Organization for Standardization). Geneva, Switzerland.

Itow, T., Loveland, R.E., Botton, M.L., 1998. Developmental Abnormalities in Horseshoe Crab Embryos Caused by Exposure to Heavy Metals. Arch. Environ. Contam. Toxicol. 40, 33–40.

Kramer, V.J., Etterson, M.A., Hecker, M., Murphy, C.A., Roesijadi, G., Spade, D.J., Spromberg, J.A., Wang, M., Ankley, G.T., 2011. Adverse outcome pathways and ecological risk assessment: bridging to population-level effects. Environ. Toxicol. Chem. 30, 64–76.

Li, L.-Z., Zhou, D.-M., Peijnenburg, W.J.G.M., Wang, P., Van Gestel, C.A.M., Jin, S.-Y., Wang, Q.-Y., 2010. Uptake pathways and toxicity of Cd and Zn in the earthworm *Eisenia fetida*. Soil Biol. Biochem. 42, 1045–1050.

Marc, J., Le Breton, M., Cormier, P., Morales, J., Bellé, R., Mulner-Lorillon, O., 2005. A glyphosate-based pesticide impinges on transcription. Toxicol. Appl. Pharm. 203, 1-8.

Marchetti, C., 2013. Role of calcium channels in heavy metal toxicity. ISRN Toxicol. 2013, 184360.

Middaugh, D.P., Dean, J.M., 1977. Comparative sensitivity of eggs, larvae and adults of the estuarine teleosts, *Fundulus heteroclitus* and *Menidia menidia* to cadmium. Bull. Environ. Contam. Toxicol. 17, 645–652.

Misra, U.K., Gawdi, G., Akabani, G., Pizzo, S.V., 2002. Cadmium-induced DNA synthesis and cell proliferation in macrophages: The role of intracellular calcium and signal transduction mechanisms. Cell. Signal. 14, 327-340.

OECD, 2004a. Test No. 220: Guidelines for testing of chemicals - Enchytraeid Reproduction Test. OECD (Organization for Economic Cooperation and Development). OECD Publishing, Paris. France.

OECD, 2004b. Test No. 202: Daphnia sp Acute Immobilisation Test. OECD Guideline Test Chemical Section 2. OECD Publisher, Paris, France.

OECD, 2010. Test No. 317: Guidelines for testing of chemicals - Bioaccumulation in Terrestrial Oligochaetes. OECD (Organization for Economic Cooperation and Development). OECD Publishing, Paris, France.

OECD, 2013. Test No. 236: Fish Embryo Acute Toxicity (FET) Test. OECD Guideline Test Chemical Section 2. OECD Publisher, Paris, France.

Packard, M.J., Packard, G.C., Gutzke, W.H., 1984. Calcium metabolism in embryos of the oviparous snake *Coluber constrictor*. J. Exp. Biol. 110, 99–112.

Pennati, R., Groppelli, S., Zega, G., Biggiogero, M., De Bernardi, F., Sotgia, C., 2006. Toxic effects of two pesticides, Imazalil and Triadimefon, on the early development of the ascidian *Phallusia mammillata* (Chordata, Ascidiacea). Aquat. Toxicol. 79, 205–212.

Pietrock, M., Meinelt, T., Marcogliese, D.J., 2008. Effects of cadmium exposure on embryogenesis of *Stagnicola elodes* (Mollusca, Gastropoda): potential consequences for parasite transmission. . Arch. Environ. Contam. Toxicol. 55, 43–48.

Rasband, W.S., 1997-2008. ImageJ. National Institutes of Health, Bethesda.

Sahoo, G., Sahoo, R.K., Mohanty-Hejmadi, P., 1998. Calcium metabolism in olive ridley turtle eggs during embryonic development. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 121, 91–97.

Schirling, M., Bohlen, A., Triebskorn, R., Köhler, H.R., 2006. An invertebrate embryo test with the apple snail *Marisa cornuarietis* to assess effects of potential developmental and endocrine disruptors Chemosphere 64, 1730–1738.

Shoaib, M.A., Mahmoud, M.F., Loutfy, N., Tawfic, M.A., Barta, M., 2010. Effect of botanical insecticide Nimbecidine® on food consumption and egg hatchability of the terrestrial snail Monacha obstructa. J. Pest Sci. 83, 27–32.

SigmaPlot, 1997. Statistical Package for the Social Sciences e SigmaPlot for Windows 11.0 ed. . Chicago, IL, USA.

Stewart, J.R., Ecay, T.W., Heulin, B., Fregoso, S.P., Linville, B.J., 2011. Developmental expression of calcium transport proteins in extraembryonic membranes of oviparous and viviparous *Zootoca vivipara* (Lacertilia, Lacertidae). J. Exp. Biol. 214, 2999-3004.

Villeneuve, D.L., Garcia-Reyero, N., 2011. Vision & strategy: Predictive ecotoxicology in the 21st century. Environ. Toxicol. Chem. 30, 1-8.

Westheide, W., Müller, M.C., 1996. Cinematographic documentation of enchytraeid morphology and reproductive biology. Hydrobiologia 334, 263-267.

Whitaker, M., 2012. Calcium at fertilization and in early development. Physiol. Rev. 86, 25–88.

Chapter 4

Lifespan (all life) term test exposure using *Enchytraeus crypticus* – the effect of CuO NMs – longevity as novel endpoint

Lifespan (all life) term test exposure using *Enchytraeus crypticus* – the effect of CuO NMs – longevity as novel endpoint

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Abstract

Chemicals' toxicity is assessed during a certain exposure period of organisms' life. It is not feasible to conduct tests that last the all life duration and hence long term tests usually include one reproductive cycle. In the present study we optimized and propose a lifespan (all life) term test using *Enchytraeus crypticus* (Oligochaeta). The effect of copper oxide nanoparticles (CuO-NPs) was assessed in this lifespan test and compared to copper salt ($CuCl_2$), using the same effect concentrations on reproduction (EC_{50}). Monitored endpoints included survival and reproduction over-time (202 days). Results from survival showed that CuO-NMs caused shorter life of the adults compared to $CuCl_2$ (control LT_{80}: 260 days > $CuCl_2$ LT_{80}: 237 days > CuO-NPs LT_{80}: 228 days). The effect was even more amplified in terms of reproduction (control ET_{80}: 188 days > $CuCl_2$ ET_{80}: 155 days > CuO-NPs ET_{80}: 135 days). Results suggest that CuO-NPs may cause a higher Cu effect via a *trojan horse* mechanism. The use of lifespan tests brings a novel concept in ecotoxicity, the longevity. This is a particularly important aspect when the subject is NMs toxicity, where longer term exposure time is expected to reveal unpredicted effects via the current short/long-term tests.

Keywords: Longevity; All life exposure, Lifespan; Reproductive output

1. Introduction

Organisms' longevity is a complex process that can be influenced by various environmental events (Vanhooren and e Libert, 2012). Long term studies like lifespan tests are very important because such effects cannot be predicted based on short term tests, at least not yet given the shortage level of information. Chemicals' toxicity is commonly assessed during a certain exposure period of organisms' life; it is not feasible to conduct tests that last the lifespan duration and hence long term tests usually include one reproductive cycle. In terms of risk assessment, lifespan tests represent a continuous exposure to toxicants during the whole life, similar to what can occur in the natural environment, thus recreating a highly relevant scenario of exposure and unique consequent adverse effects (Coutellec and Barata, 2013; Van Gestel, 2012). There are very few studies within lifespan range and the species used include *Mus musculus*, *Drosophila melanogaster*, *Saccharomyces cerevisiae* and *Caenorhabditis elegans* (Buffenstein et al., 2008), not including any soil dwelling invertebrate. Most of these studies (using *M. musculus*, *D. melanogaster*, S. cerevisiae and *C. elegans*) were performed to discover genetic, environmental and pharmacologic modulators of aging for the lifespan extension purpose, providing new insights for human therapy (Hamilton and Miller, 2016; Lucanic et al., 2013). Studies that assess the effects of contaminants in lifespan are still limited, the few examples use *C. elegans* to investigate lifespan effects of metals and detergents (Harada et al., 2007; Wang et al, 2010).

Effects of NMs have been investigated for ca. 2 decades, and there has been increasing alert regarding the need for longer term exposure tests due to the potential long term effects of NMs. So far, results on acute toxicity on e.g. aquatic organisms produced the classification, of Ag-NPs as 'extremely toxic' and CuO-NPs as 'very toxic' (Kahru and Dubourguier, 2010). However, most of the data so far generated is on short-term/acute effects which is not advised. For instance Diez-Ortiz et al. (2015) found that 52 weeks aged Ag-NPs in LUFA 2.2 soil were more toxic to *Eisenia fetida* than Ag-NPs freshly spiked soil (1 week aged) (reproduction EC_{50} of 34 and 1420 mg Ag/kg, respectively); and Waalewijn-Kool et al. (2013) report that a release of Zn ions to soil, from ZnO-NPs, continued over one year. In fact, the need of more long-term toxicity studies to obtain a better understanding of NMs effects is fully recognized and pointed out as a current gap

and future priority in the knowledge on nanotoxicology (Baun et al., 2008; Kumar et al., 2014).

Copper oxide nanoparticles (CuO-NPs) are used in a wide range of industrial and commercial applications, such as, gas sensor (Chowdhuri et al., 2004), metallic and plastic coatings (Hernández Battez et al., 2010), circuits, batteries and semiconductor devices (Zhang et al., 2005) and solar energy conversion (Yin et al., 2005). Due to their antimicrobial and antifungal properties these NPs are also used in textiles industries, paints, plastics, food preservation, additives in lubricants, skin products and medical science (Dastjerdi and Montazer, 2010; Delgado et al., 2001; Gabbay et al., 2006; Jin and Ye, 2007). The increased production of CuO-NPs increased the risk of their introduction into the environment and human health (Buffet et al., 2013; Siddiqui et al., 2013). Therefore, its potential toxicity combined with its relatively low dissolution rate should not be ignored and it is important to investigate the toxicity of these materials (Blinova et al., 2010; Buffet et al., 2011; Saison et al., 2010; Stone et al., 2010).

Most of the information regarding the ecotoxicity of CuO-NPs is in the aquatic compartment (Chang et al., 2012), and is mostly based on "short-term"/acute effects (Mortimer et al., 2010; Nations et al., 2011; Pradhan et al., 2012; Zhao et al., 2011). Studies on chronic effects of CuO-NPs showed *Daphnia magna* growth and reproduction (21 days test) inhibition (Adam et al., 2015; Rossetto et al., 2014); and induced mortality and decreased growth in *Xenopus laevis* (Nations et al., 2015). Regarding soil compartment, most of the literature available is relative to plants (Da Costa and Sharma, 2015; Peng et al., 2015; Shi et al., 2014) with less information on soil dwelling invertebrates (Gomes et al., 2015a; 2015b; Gomes et al., 2012a; Amorim et al., 2012; Amorim et al., 2011; Heckmann et al., 2011; Unrine et al., 2010).

In the present study we propose a lifespan test i.e., all life term for the soil living oligochaeta *Enchytraeus crypticus*. *E. crypticus* is a model standard species where many endpoints are assessed: survival (ISO, 2005), reproduction (OECD, 2004), bioaccumulation (OECD, 2010a), embryo development (Gonçalves et al., 2015), or via a full life cycle with hatching, growth, maturity (Bicho et al., 2015a).

The procedures for a lifespan test using *E. crypticus* were here optimized using control conditions (un-spiked soil) by monitoring the survival and reproduction of the organism

over the entire time of its lifespan. Further, the developed assay was used to study the longevity effects of CuO-NPs in comparison to $CuCl_2$.

2. Materials and Methods

2.1. Test organisms

The test organism used belongs to the species *Enchytraeus crypticus*, Westheide and Graefe, 1992. Cultures were kept in agar plates fed *ad libitum* with grinded and autoclaved oats and maintained in laboratory under controlled conditions, e.g. photoperiod of 16:8 hours (light: dark) and temperature of 20 ± 1ºC. Juveniles of synchronized age (11 days) were used. For details on culture synchronization see Bicho et al. (2015a).

2.2. Test soil

The standard natural soil LUFA 2.2 (Speyer, Germany) was used. Main properties of the soil can be summarised as follows: pH (0.1 M $CaCl_2$) of 5.5, 43.3% of maximum water-holding capacity (WHCmax), 1.61% organic carbon and a particle size distribution of 7.9% clay, 16.3% silt and 75.8% sand.

2.3. Test procedures

2.3.1. Optimization of the lifespan assay: control conditions

The optimization of the lifespan assay was done in un-spiked soil, moistened to 50% of the WHC_{max}. Juveniles of synchronized age (11 days) were randomly selected and placed in each well (of the 6-well plates) at two densities: 1 (D1) and 20 (D20) organisms per replicate, ten replicates were used. After 25 days (11 plus 14 days to allow growth and reaching maturity) adults' survival was recorded and the surviving adults (25 days old) were transferred to new test plates, in the same conditions, i.e. D1 or D20 respectively. To ensure that no juveniles were transferred together with the adults, prior the transference to the new test plates, the organisms were cleaned in a petri-dish with distilled water and checked under a stereo microscope (Zeiss Stemi 2000-C). Every 15 days, the survival of the adults was recorded and the surviving adults were transferred to new test plates as described above. After each transfer, the previous test plates were left during 11 more days to ensure that the cocoons laid have time to hatch; after that, the soil in the well plates was

transferred to glass vials and fixated with 96% ethanol and Bengal rose (1% in ethanol) and the juveniles were counted using a stereo microscope (Zeiss Stemi 2000-C).

Food (grinded and autoclaved oats) was added weekly (2 and 10 mg for D1 and D20 exposed organisms, respectively). Water was added every 3 days. The test was maintained at a photoperiod of 16:8 hours light:dark and at 20 ± 1 ºC. The test ran until all the adults were dead (370 days).

2.3.2. Lifespan assay: exposure to CuO-NPs and $CuCl_2$

For the test with CuO-NPs and $CuCl_2$, organisms (juveniles of synchronized age) were exposed at density D1 following the procedures described above. 20 replicates per test condition were used, 2 mg of food was added weekly and water adjusted every 3 days. The test was maintained at a photoperiod of 16:8 hours light:dark and at 20 ± 1 ºC. The test ran for 202 days (plus 11 more days to allow the cocoons to hatch); the test duration was selected based on the results from the optimization of the lifespan assay in control conditions (≈ LT_{80}).

2.4. Test chemicals and spiking

Copper-salt ($CuCl_2 \cdot 2H_2O$) and Copper Oxide Nanoparticles (FP7 SUN pristine materials) were used (Table 1). The tested concentrations were selected based on the EC_{50} for reproduction effect ($CuCl_2$ = 180 mg Cu/kg and CuO-NPs = 1400 mg Cu/kg soil dry weight) as known from previous Enchytraeid Reproduction Test (ERT) results (Bicho et al., 2015b). $CuCl_2$ was added to pre moistened soil (20% w/w) as serially diluted aqueous solutions. For CuO-NPs, the NPs were added as dry powder to the soil as recommend by OECD for the testing of insoluble substances (OECD, 2010b). In short, CuO-NPs were thoroughly mixed manually with the dry soil to obtain the corresponding concentration range. After that, deionized water was added to reach 50% of the soil WHC. All soils were homogeneously mixed and allowed to equilibrate for 1 day before test start. Soil was spiked and renewed every 15 days during sampling.

Controls correspond to un-spiked LUFA 2.2 soil moistened until 50% of WHC. Test vessels consisted of 6-well plates (35 mm ø), each well containing 5 g of moistened soil. Treatments and replicates were distributed randomly in the test plates.

Table 1: Characteristics of the tested CuO-NPs and $CuCl_2$ including manufacturer, CAS, size, nominal surface area, density, purity and solubility/dispersability.

	CuO-NPs	**$CuCl_2$**
Manufacturer	Plasma Chem	Sigma–Aldrich
CAS Number		10125-13-0
Size (nm)	15-20	-
Nominal Surface Area (m^2/g)	47	-
Density (g/cm^3)	6.3	-
Purity (%)		99
Solubility/Dispersability	Not dispersible in water	Water soluble

2.5. Data analysis

To assess significant differences between treatments at each sampling day One-Way ANOVA (using Tukey Test or Dunn's method for multiple comparisons) was used (SigmaPlot 11.0).

Lethal Time (LTx) as time to reduce survival in x% and Effect Time (ETx) as time to reduce reproduction in x% calculations were performed for survival and reproduction, respectively, using the logistic equation or threshold sigmoid 2 or 3 parameters regression models (TRAP software).

3. Results

3.1. Optimization of the lifespan assay: control conditions

Results on survival at D1 and D20 are shown on Figure 1A and the ETx values are summarized in Table 2.

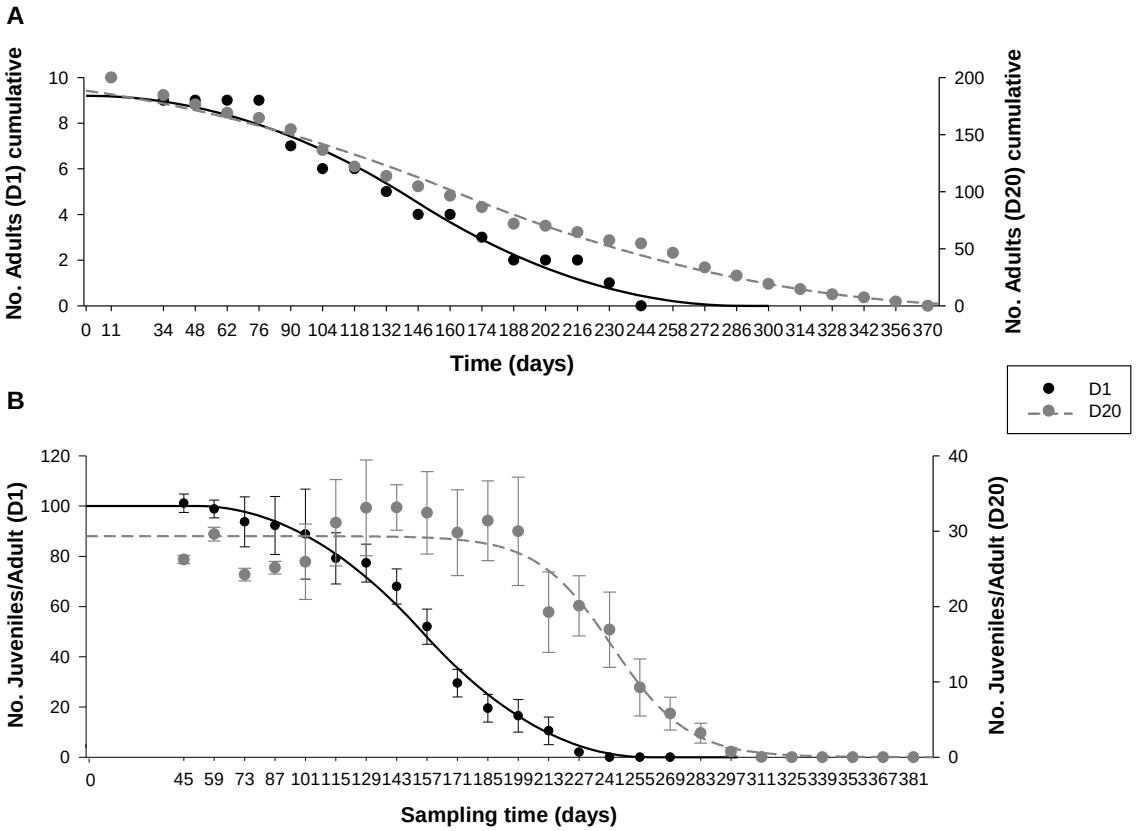

Figure 1: Lifespan test of *Enchytraeus crypticus* at two different organisms' densities (1 organism (D1) and 20 organisms (D20)) in LUFA 2.2 soil, over-time. A) Adults survival; all values are expressed as cumulative number (N = 10), B) Reproductive output; all values are expressed as average ± standard error (N = 10). The lines represent the model fit to data.

The lifespan at D1 is lower than at D20 (D1 LT_{50}: 145 days, D20 LT_{50}: 162 days). Results in terms of reproduction can be observed in Figure 1B: the number of juveniles produced per adult at D1 is higher than at D20, but D1 has a reproduction EC_{50} earlier than D20 (e.g. D1 ET_{50}: 154 days, D20 ET_{50}: 242 days, Table 2).

Table 2: Summary of the Effect Time (ETx) for survival (LTx) and reproduction for *Enchytraeus crypticus* in control conditions in LUFA 2.2 soil at two different organisms' densities (1 organism (D1) and 20 organisms (D20)).

	Survival					Reproduction				
	LT_{10} (95%-CI)	LT_{20} (95%-CI)	LT_{50} (95%-CI)	LT_{80} (95%-CI)	Model	ET_{10} (95%-CI)	ET_{20} (95%-CI)	ET_{50} (95%-CI)	ET_{80} (95%-CI)	Model
D1	62 (49-81)	92 (80-103)	145 (137-152)	183 (17-194)	Threshold 2 param (S:0.007; Y0:9.2)	97 (78-117)	117 (102-131)	155 (142-168)	182 (160-204)	Threshold 2 param (S:0.010; Y0:100)
D20	26 (14-39)	72 (63-80)	162 (157-167)	227 (219-235)	Threshold 2 param (S:0.004; Y0:200)	204 (180-229)	218 (201-236)	242.4 (23-254)	267 (249-285)	Logistic 2 param (S:0.014; Y0: 29.3)

3.1. Lifespan assay: exposure to CuO-NPs and $CuCl_2$

The effects of CuO-NPs and $CuCl_2$ on *E. crypticus* lifespan (survival) can be depicted in Figure 2A and the ETx calculated are summarized in Table 3.

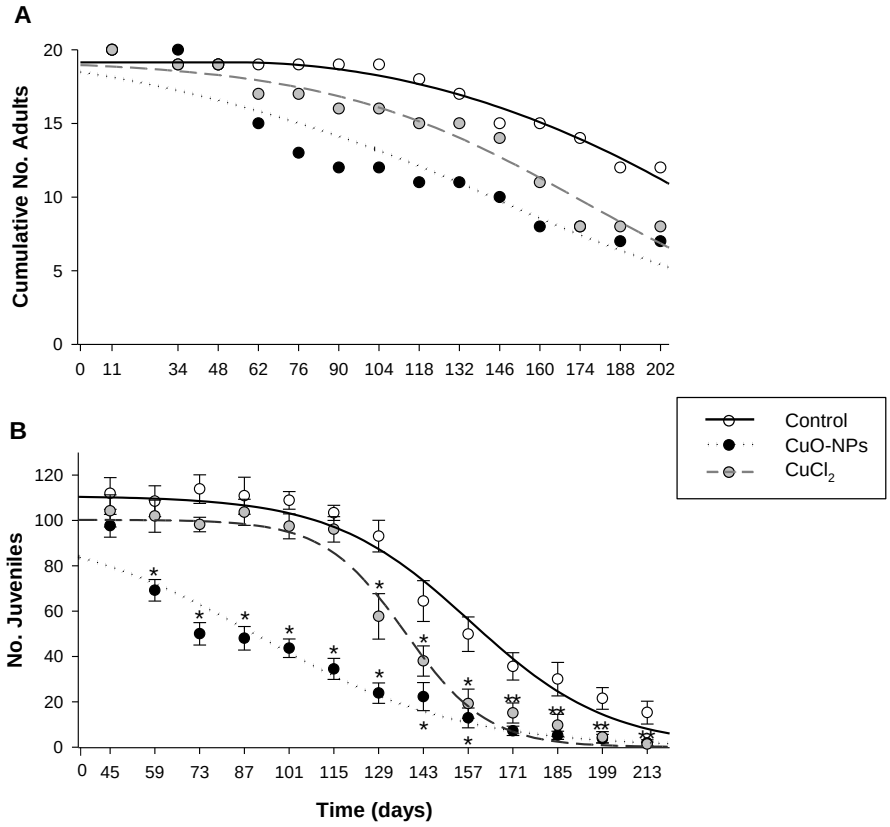

Figure 2: Lifespan test of *Enchytraeus crypticus* when exposed to CuO-NPs and $CuCl_2$ (mg Cu/kg DW soil) in LUFA 2.2 soil, over-time. A) Adults survival; all values are expressed as cumulative number (N = 20), B) Reproductive output; all values are expressed as average ± standard error (N = 20). Asterisks indicate significant differences between control and treatments at each sampling day ($p<0.05$ Tukey Test or Dunn's method). The lines represent the model fit to data.

CuO-NPs exposure caused a more severe lifespan decrease than $CuCl_2$: control LT_{50}: 218 days > $CuCl_2$ LT50: 175 days > CuO-NPs LT_{50}: 145 days. Results in terms of reproduction (Fig. 2B) show that CuO-NPs exposure caused higher effects on reproduction in *E. crypticus*, with a 50% reduction in reproduction occurring earlier than $CuCl_2$ (e.g. control ET_{50}: 158 days > $CuCl_2$ ET_{50}: 138 days > CuO-NPs ET_{50}: 92 days, Table 3).

Table 3: Summary of the Effect Time (ETx) for survival (LTx) and reproduction for *Enchytraeus crypticus* when exposed to CuO-NPs and $CuCl_2$ (mg Cu/kg DW soil) in LUFA 2.2 soil. n.d. = not determined.

	Survival					Reproduction				
	LT_{10} (95%-CI)	LT_{20} (95%-CI)	LT_{50} (95%-CI)	LT_{80} (95%-CI)	Model	ET_{10} (95%-CI)	ET_{20} (95%-CI)	ET_{50} (95%-CI)	ET_{80} (95%-CI)	Model
Control	127 (117-137)	157 (151-164)	218 (207-227)	260 (242-278)	Threshold 2 param (S:0.006; Y0: 19.1)	110 (99-121)	128 (120-135)	158 (153-163)	188 (179-197)	Logistic 2 param (S:0.012; Y0:110.9)
$CuCl_2$	77 (59-95)	113 (102-125)	175.3 (167-184)	237 (218-256)	Logistic 2 param (S:0.006; Y0:19.3)	110 (101-118)	120 (114-126)	138 (134-142)	155 (149-162)	Logistic 2 param (S:0.020; Y0:100.23)
CuO-NPs	23 (n.d.)	64 (40-88)	145 (130-160)	204 (175-233)	Threshold 2 param (S:0.005; Y0:19.7)	23 (10-36)	48 (39-57)	92 (86-97)	135 (125-145)	Logistic 2 param (S:0.008; Y0:97.7)

4. Discussion

4.1. Optimization of the lifespan assay: control conditions

This is the first study where the entire lifespan of an enchytraeid was monitored in soil. Previous knowledge on enchytraeids' lifespan (in agar media) showed: 120 days for *Enchytraeus albidus* (Ivleva, 1953), 127 days for *Enchytraeus doerjesi* (Westheide and Graefe, 1992) and 224 days for *Enchytraeus coronatus* (Rodriguez et al., 2002). Westheide and Graefe (1992) also reported an 85 days lifespan for *E. crypticus* which is considerably less than the 244 and 370 days we observed for D1 and D20, respectively. Possibly the differences in terms of test media, soil (in our study) and agar media influence the longevity. Hence, this indicates that *E. crypticus* can live longer in soil compared to agar which is not surprising given that agar is an artificial substrate.

The experimental test design as proposed here can be used as draft for a lifespan test in soil for *E. crypticus*. Results showed that the selected sampling points to assess the survival and reproductive output over-time were adequate. Although it is a very long test, the associated cost are relatively low, except time consumption, and the level of information is of very high level. The majority of the studies that assess endpoints like survival, reproduction, bioaccumulation or growth are based on much shorter exposure periods, covering up to 4 weeks of duration (about 28-30 days) (Diez-Ortiz et al., 2015; Peters and Granek, 2016) and cannot predict the effects of longevity.

Analysis of organism survival over time showed that at D1 enchytraeids died earlier compared to D20. The results on reproduction (number of juveniles per adult) at D1 and D20 showed a higher reproductive output at D1 than at D20 and are in agreement with results from a detailed study using the same species (Gonçalves et al., submitted) where the authors observed smaller cocoons and less embryos at D20 compared to D1. This has been observed in other studies: lower reproductive output at higher densities compared to lower densities, for instance in *Lumbricus terrestris* (Butt et al., 1994) the cocoon production was 1.5, 0.6, 0.1, 0.06, 0.04 and 0.0 at D1, D2, D3, D4, D6 and D8 respectively and *Biomphalaria alexandrina* showed four times as many eggs per week in lower than in high densities (Mangal et al. 2010).

Regarding reproduction, we observed a decrease in reproductive output over time, possibly age related. Changes in fertility in relation to age (reproductive senescence) have been reported in other organisms and can vary among these (Jones et al., 2014). For example, in

Caenorhabditis elegans the fast decline in the reproduction begins at young to middle age due to sperm depletion (Hughes et al., 2007) whereas in *Drosophila melanogaster* is due to apoptosis of ageing egg chambers (Zhao et al., 2008). In *E. crypticus* the reproductive output showed a variation along the lifespan of the organisms and decreased with aging. This further reiterates the importance of using organisms with synchronized age in ecotoxicological testing as recommended for this species and implemented in the full life cycle test (Bicho et al., 2015a).

4.2. Lifespan assay: exposure to CuO-NPs and $CuCl_2$

Results in controls were similar to those obtained in the optimization test, although, the endpoint reproduction ("optimization test" D1 ET_{50}= 155 days and "controls of Cu exposure tests" ET_{50}= 158 days) was more stable than survival ("optimization test" D1 LT_{50}= 145 days and "controls of Cu exposure tests" LT_{50}= 217 days), which confirms the increased relevancy of chronic effects compared to acute.

Results showed that CuO-NPs were more toxic than $CuCl_2$, i.e. exposure to CuO-NPs caused shorter longevity and reproduction. Gomes et al. (2012b, 2011) reported, in *Mytilus galloprovincialis*, that $CuCl_2$ was easily eliminated, whereas CuO-NPs had slower elimination rate resulting in an increased accumulation with time of exposure. In short, even though Cu concentrations in the digestive gland of mussels were higher for $CuCl_2$ than for CuO-NPs in the first week of exposure, Cu accumulation decreased for $CuCl_2$ at the end of experiment (15 days) whereas it increased for CuO-NPs exposure. The observed differences in terms of longevity (59-143 days period) could be related with different accumulation/elimination rates between NPs and salt.

From day 157 onwards effects became more similar between CuO-NPs and $CuCl_2$. This could mean that, after prolonged exposure, Cu elimination (from $CuCl_2$) was less efficient (also linked to the age of the organisms, note that from day 143 there is a reduction in reproduction, also in control) and the effects caused by $CuCl_2$ meet those caused by CuO-NPs.

Another study with *Daphnia magna* also showed higher internal concentrations of Cu from exposured to CuO-NPs compared to $CuCl_2$, but in this case, $CuCl_2$ caused higher toxicity in terms of reproduction (Adam et al., 2015).

The mechanism of Cu uptake from CuO-NPs is not fully understood. Some authors (Studer et al., 2010 and Karlsson et al., 2008) explain the higher citoxicity of CuO-NPs (in comparison to $CuCl_2$) via a *trojan horse* mechanism, i.e. NPs can release a boom of metal ions inside the cells, possibly due to lower pH which causes a higher dissolution. Shi et al. (2011) report higher toxicity of CuO-NPs (in comparison to $CuCl_2$) to *Landoltia punctate* due to the high uptake of ions released from the NPs, but question the intra-cellular form of Cu and if the CuO-NPs themselves are taken up into the cells. Pradhan et al. (2012) suggest the intake of CuO-NPs in *Allogamus ligonifer*, and also state that the Cu ions released from the CuO-NPs may contribute to the toxicity of CuO-NPs. A study by Navratilova et al. (2015) showed that it was possible to detect CuO-NPs by Single Particle ICP-MS, in natural soil extracts mixed with a suspension of CuO-NPs, indicating that CuO-NPs persist in the nano form (even though in the form of agglomerates) and do not completely solubilize in the presence of soil components, i.e. organic matter.

In our case, enchytraeids ingest soil containing CuO-NPs, and once the NPs reach the organisms' digestive track the low pH would increase the dissolution of NPs and release of Cu ions. Additionally the direct uptake of CuO-NPs into the cells with increased Cu bioaccumulation can play a role and produce effects via a *trojan horse* mechanism.

4. Conclusions

A lifespan test was developed for the first time in soil for an enchytraeid and includes longevity as an additional endpoint. The proposed lifespan term test will be extremely useful to assess the prolonged effects of toxicants, e.g. very important for nanomaterials. Results showed precisely that longevity was more affected for CuO-NPs compared to $CuCl_2$, which would not be predictable based on the current standard long term testing. We understand that the test length may be an issue but highly recommend the performance of longevity test for selected cases and design, in particular for the testing of nanomaterials.

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References

Adam, N., Vakurov, A., Knapen, D., Blust, R., 2015. The chronic toxicity of CuO nanoparticles and copper salt to *Daphnia magna*. J. Hazard. Mater. 283, 416–422.

Amorim, M.J.B., Scott-Fordsmand, J.J., 2012. Toxicity of copper nanoparticles and $CuCl_2$ salt to *Enchytraeus albidus* worms: survival, reproduction and avoidance responses. Environ. Pollut. 164, 164–168.

Amorim, M.J.B., Gomes, S.I.L., Soares, A.M.V.M., Scott-Fordsmand, J.J., 2011. Energy Basal Levels and Allocation among Lipids, Proteins, and Carbohydrates in *Enchytraeus albidus*: Changes Related to Exposure to Cu Salt and Cu Nanoparticles. Water, Air, Soil Pollut. 223, 477–482.

Baun, A., Hartmann, N.B., Grieger, K., Kusk, K.O., 2008. Ecotoxicity of engineered nanoparticles to aquatic invertebrates: a brief review and recommendations for future toxicity testing. Ecotoxicology 17, 387–395.

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

Bicho, R.C., Ribeiro, M.J., Fordsmand, J.J, Amorim, M.J.B. 2015b Tracing effects of NMs along their life cycle – toxicity in soil *(Enchytraeus crypticus*) (Poster Presentation) 25th Annual Meeting of the Society of Environmental Toxicology and Chemistry – SETAC Europe 2015. 3-7 May, Barcelona, Spain.

Blinova, I., Ivask, A., Heinlaan, M., Mortimer, M., Kahru, A., 2010. Ecotoxicity of nanoparticles of CuO and ZnO in natural water. Environ. Pollut. 158, 41–47.

Buffenstein, R., Edrey, Y.H., Larsen, P.L., 2008. Animal Models in Aging Research - A Critical Examination, in: Sourcebook of Models for Biomedical Research. pp. 499–506.

Buffet, P.-E., Richard, M., Caupos, F., Vergnoux, A., Perrein-Ettajani, H., Luna-Acosta, A., Akcha, F., Amiard, J.-C., Amiard-Triquet, C., Guibbolini, M., Risso-De Faverney, C., Thomas-Guyon, H., Reip, P., Dybowska, A., Berhanu, D., Valsami-Jones, E., Mouneyrac, C., 2013. A mesocosm study of fate and effects of CuO nanoparticles on endobenthic species (*Scrobicularia plana*, *Hediste diversicolor*). Environ. Sci. Technol. 47, 1620–1228.

Buffet, P.-E., Tankoua, O.F., Pan, J.-F., Berhanu, D., Herrenknecht, C., Poirier, L., Amiard-Triquet, C., Amiard, J.-C., Bérard, J.-B., Risso, C., Guibbolini, M., Roméo, M., Reip, P., Valsami-Jones, E., Mouneyr, C., 2011. Behavioural and biochemical

responses of two marine invertebrates *Scrobicularia plana* and *Hediste diversicolor* to copper oxide nanoparticles. Chemosphere 84, 166–174.

Chang, Y.-N., Zhang, M., Xia, L., Zhang, J., Xing, G., 2012. The Toxic Effects and Mechanisms of CuO and ZnO Nanoparticles. Materials (Basel). 5, 2850–2871.

Chowdhuri, A., Gupta, V., Sreenivas, K., Kumar, R., Mozumdar, S., Patanjali, P.., 2004. Response speed of SnO2-based H_2S gas sensors with CuO nanoparticles. Appl. Phys. Lett. 84, 1180–1182.

Coutellec, M.A., Barata, C., 2013. Special issue on long-term ecotoxicological effects: an introduction. Ecotoxicology 22, 763–766.

Da Costa, M.V.J., Sharma, P.K., 2015. Effect of copper oxide nanoparticles on growth, morphology, photosynthesis, and antioxidant response in *Oryza sativa*. Photosynthetica 54, 110–119.

Dastjerdi, R., Montazer, M., 2010. A review on the application of inorganic nanostructured materials in the modification of textiles: focus on antimicrobial properties. Colloids Surf B Biointerfaces 79, 5–18.

Delgado, K., Quijada, R., Palma, R., Palza, H., 2001. Polypropylene with embedded copper metal or copper oxide nanoparticles as a novel plastic antimicrobial agent. Lett Appl Microbiol 53, 50–54.

Diez-Ortiz, M., Lahive, E., George, S., Ter Schure, A., Van Gestel, C.A.M., Jurkschat, K., Svendsen, C., Spurgeon, D.J., 2015. Short-term soil bioassays may not reveal the full toxicity potential for nanomaterials; bioavailability and toxicity of silver ions ($AgNO_3$) and silver nanoparticles to earthworm *Eisenia fetida* in long-term aged soils. Environ. Pollut. 203, 191–198.

Gabbay, J., Borkow, G., Mishal, J., Magen, E., Zatcoff, R., Shemer-Avni, Y., 2006. Copper oxide impregnated textiles with potent biocidal activities. J Ind Text 35, 323–335.

Gomes, S.I.L., Murphy, M., Nielsen, M.T., Kristiansen, S.M., Amorim, M.J.B., Scott-Fordsmand, J.J., 2015a. Cu-nanoparticles ecotoxicity – Explored and explained? Chemosphere 139, 240–245.

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

Gomes, S.I.L., Novais, S.C., Gravato, C., Guilhermino, L., Scott-Fordsmand, J.J., Soares, A.M.V.M., Amorim, M.J.B., 2012a. Effect of Cu-nanoparticles versus one Cu-salt: analysis of stress biomarkers response in *Enchytraeus albidus* (Oligochaeta). Nanotoxicology 6, 134–143.

Gomes, T., Chora, S., Pereira, C.G., Cardoso, C., Bebianno, M.J., 2014. Proteomic response of mussels *Mytilus galloprovincialis* exposed to CuO NPs and Cu^{2+}: an exploratory biomarker discovery. Aquat. Toxicol. 155, 327–36.

Gomes, T., Pereira, C.G., Cardoso, C., Pinheiro, J.P., Cancio, I., Bebianno, M.J., 2012b. Accumulation and toxicity of copper oxide nanoparticles in the digestive gland of *Mytilus galloprovincialis*. Aquat. Toxicol. 118-119, 72–79.

Gomes, T., Pinheiro, J., Cancio, I., Pereira, C.G., Cardoso, C., Bebianno, M., 2011. Effects of Copper Nanoparticles Exposure in the Mussel *Mytilus galloprovincialis*. Environ. Sci. Technol. 45, 9356–9362.

Gonçalves, M.F.M., Bicho, R.C., Rêma, A., Soares, A.M.V.M., Faustino, A.M.R., Amorim, M.J.B., 2015. Development of an embryotoxicity test for *Enchytraeus crypticus* – The effect of Cd. Chemosphere 139, 386–392.

Hamilton, K.L., Miller, B.F., 2016. What is the evidence for stress resistance and slowed aging? Exp. Gerontol. 82, 67–72.

Harada, H., Kurauchi, M., Hayashi, R., Eki, T., 2007. Shortened lifespan of nematode *Caenorhabditis elegans* after prolonged exposure to heavy metals and detergents. Ecotoxicol. Environ. Saf. 66, 378–383.

Heckmann, L.-H., Hovgaard, M.B., Sutherland, D.S., Autrup, H., Besenbacher, F., Scott-Fordsmand, J.J., 2011. Limit-test toxicity screening of selected inorganic nanoparticles to the earthworm *Eisenia fetida*. Ecotoxicology 20(1), 226–233.

Hernández Battez, A., Viesca, J.., González, R., Blanco, D., Asedegbega, E., Osorio, A., 2010. Friction reduction properties of a CuO nanolubricant used as lubricant for a NiCrBSi coating. Wear 268, 325–328.

Hughes, S.E., Evason, K., Xiong, C., Kornfeld, K., 2007. Genetic and Pharmacological Factors That Influence Reproductive Aging in Nematodes. PLoS Genet. 3, e25.

ISO, 2005. Soil Quality - Effects of pollutants on Enchytraeidae (*Enchytraeus* sp.). Determination of effects on reproduction and survival. Guideline No 16387. International Organization for Standardization. Geneva, Switzerland.

Ivleva, I. V., 1953. Growth and reproduction of the potworm (*Enchytraeus albidus*). Zool. Zh. 32, 394–404.

Jin, S., Ye, K., 2007. Nanoparticle-mediated drug delivery and gene therapy. Biotechnol. Prog. 23, 32–41.

Jones, O.R., Scheuerlein, A., Salguero-Gómez, R., Camarda, C.G., Schaible, R., Casper, B.B., Dahlgren, J.P., Ehrlén, J., García, M.B., Menges, E.S., Quintana-Ascencio, P.F., Caswell, H., Baudisch, A., Vaupel, J.W., 2014. Diversity of ageing across the tree of life. Nature 505, 169–173.

Kahru, A., Dubourguier, H.C., 2010. From ecotoxicology to nanoecotoxicology. Toxicology 269, 105–119.

Karlsson, H.L., Cronholm, P., Gustafsson, J., Moeller, L., 2008. Copper Oxide Nanoparticles Are Highly Toxic: A Comparison between Metal Oxide Nanoparticles and Carbon Nanotubes. Chem. Res. Toxicol. 21, 1726–1732.

Kumar, A., Kumar, P., Anandan, A., Fernandes, T.F., Ayoko, G.A., Biskos, G., 2014. Engineered Nanomaterials: Knowledge Gaps in Fate, Exposure, Toxicity, and Future Directions. J. Nanomater. 2014, 16p.

Lucanic, M., Lithgow, G.J., Alavez, S., 2013. Pharmacological lifespan extension of invertebrates. Ageing Res. Rev. 12, 445–58.

Manusadžianas, L., Caillet, C., Fachetti, L., Gylytė, B., Grigutytė, R., Jurkonienė, S., Karitonas, R., Sadauskas, K., Thomas, F., Vitkus, R., 2012. Toxicity of copper oxide nanoparticle suspensions to aquatic biota. Env. Toxicol Chem 31, 108–114.

Mortimer, M., Kasemets, K., Kahru, A., 2010. Toxicity of ZnO and CuO nanoparticles to ciliated protozoa *Tetrahymena thermophila*. Toxicology 269, 182–189.

Nations, S., Long, M., Wages, M., Maul, J.D., Theodorakis, C.W., Cobb, G.P., 2015. Subchronic and chronic developmental effects of copper oxide (CuO) nanoparticles on *Xenopus laevis*. Chemosphere 135, 166–174.

Nations, S., Wages, M., Cañas, J.E., Maul, J., Theodorakis, C., Cobb, G.P., 2011. Acute effects of Fe2O2, TiO2, ZnO and CuO nanomaterials on *Xenopus laevis*. Chemosphere 83, 1053–1061.

Navratilova, J., Praetorius, A., Gondikas, A., Fabienke, W., von der Kammer, F., Hofmann, T., 2015. Detection of Engineered Copper Nanoparticles in Soil Using Single Particle ICP-MS. Int. J. Environ. Res. Public Health 12, 15756–15768.

OECD, 2010a. Test No. 317: Guidelines for the Testing of Chemicals - Bioaccumulation in Terrestrial Oligochaetes. OECD (Organization Econ. Coop. Dev. Publ. Paris, Fr.)

OECD, 2010b. Test No. 25: Guidance for the Testing of Manufactured Nanomaterials: OECD Sponsorship Programme: First Revision. OECD (Organization Econ. Coop. Dev. Publ. Paris, Fr.)

OECD, 2004. Test No. 220: Guidelines for the Testing of Chemicals - Enchytraeid Reproduction Test. OECD (Organization Econ. Coop. Dev. Publ. Paris, Fr.)

Peng, C., Duan, D., Xu, C., Chen, Yongsheng, Sun, L., Zhang, H., Yuan, X., Zheng, L., Yang, Y., Yang, J., Zhen, X., Chen, Yingxu, Shi, J., 2015. Translocation and biotransformation of CuO nanoparticles in rice (*Oryza sativa* L.) plants. Environ. Pollut. 197, 99–107.

Peters, J.R., Granek, E.F., 2016. Long-term exposure to fluoxetine reduces growth and reproductive potential in the dominant rocky intertidal mussel, *Mytilus californianus*. Sci. Total Environ. 545-546, 621–628.

Pradhan, A., Seena, S., Pascoal, C., Cássio, F., 2012. Copper oxide nanoparticles can induce toxicity to the freshwater shredder *Allogamus ligonifer*. Chemosphere 89, 1142–1150.

Ramskov, T., Selck, H., Banta, G., Misra, S.K., Berhanu, D., Valsami-Jones, E., Forbes, V.E., 2014. Bioaccumulation and effects of different-shaped copper oxide

nanoparticles in the deposit-feeding snail *Potamopyrgus antipodarum*. Environ. Toxicol. Chem. 33, 1976–1987.

Rodriguez, P., Arrate, J.A., Martinez-Madrid, M., 2002. Life history of the oligochaete *Enchytraeus coronatus* (Annelida, Enchytraeidae) in agar culture. Invertebr. Biol. 121, 350–356.

Rossetto, A.L. de O.F., Melegari, S.P., Ouriques, L.C., Matias, W.G., 2014. Comparative evaluation of acute and chronic toxicities of CuO nanoparticles and bulk using *Daphnia magna* and *Vibrio fischeri*. Sci. Total Environ. 490, 807–814.

Ruiz, P., Katsumiti, A., Nieto, J. a, Bori, J., Jimeno-Romero, A., Reip, P., Arostegui, I., Orbea, A., Cajaraville, M.P., 2015. Short-term effects on antioxidant enzymes and long-term genotoxic and carcinogenic potential of CuO nanoparticles compared to bulk CuO and ionic copper in mussels *Mytilus galloprovincialis*. Mar. Environ. Res. 111, 107–120.

Saison, C., Perreault, F., Daigle, J.C., Fortin, C., Claverie, J., Morin, M., Popovic, R., 2010. Effect of core-shell copper oxide nanoparticles on cell culture morphology and photosynthesis (photosystem II energy distribution) in the green alga, *Chlamydomonas reinhardtii*. Aquat. Toxicol 96, 109–114.

Shi, J., Abid, A.D., Kennedy, I.M., Hristova, K.R., Silk, W.K., 2011. To duckweeds (*Landoltia punctata*), nanoparticulate copper oxide is more inhibitory than the soluble copper in the bulk solution. Environ. Pollut. 159, 1277–1282.

Shi, J., Peng, C., Yang, Y., Yang, J., Zhang, H., Yuan, X., Chen, Y., Hu, T., 2014. Phytotoxicity and accumulation of copper oxide nanoparticles to the Cu-tolerant plant *Elsholtzia splendens*. Nanotoxicology 8, 179–188.

Siddiqui, M.A., Alhadlaq, H.A., Ahmad, J., Al-Khedhairy, A.A., Musarrat, J., Ahamed, M., 2013. Copper oxide nanoparticles induced mitochondria mediated apoptosis in human hepatocarcinoma cells. PLoS One 8, e69534.

Stone, V., Nowack, B., Baun, A., van den Brink, N., Kammer, F., Dusinska, M., Handy, R., Hankin, S., Hassellöv, M., Joner, E., Fernandes, T.., 2010. Nanomaterials for environmental studies: classification, reference material issues, and strategies for physico-chemical characterisation. Sci Total Env. 408, 1745–1754.

Studer, A.M., Limbach, L.K., Van Duc, L., Krumeich, F., Athanassiou, E.K., Gerber, L.C., Moch, H., Stark, W.J., 2010. Nanoparticle cytotoxicity depends on intracellular solubility: comparison of stabilized copper metal and degradable copper oxide nanoparticles. Toxicol. Lett. 197, 169–174.

Unrine, J.M., Tsyusko, O. V, Hunyadi, S.E., Judy, J.D., Bertsch, P.M., 2010. Effects of Particle Size on Chemical Speciation and Bioavailability of Copper to Earthworms (*Eisenia fetida*) Exposed to Copper Nanoparticles. J. Environ. Qual. 39, 1942–1953.

Van Gestel, C.A.M., 2012. Soil ecotoxicology: State of the art and future directions. Zookeys 176, 275–296.

Vanhooren, V., Libert, C., 2013. The mouse as a model organism in aging research: usefulness, pitfalls and possibilities. Ageing Res. Rev. 12, 8–21.

Waalewijn-Kool, P.L., Diez Ortiz, M., van Straalen, N.M., van Gestel, C.A., 2013. Sorption, dissolution and pH determine the long-term equilibration and toxicity of coated and uncoated ZnO nanoparticles in soil. Environ. Pollut. 178, 59–64.

Wang, D., Liu, P., Yang, Y., Shen, L., 2010. Formation of a combined Ca/Cd toxicity on lifespan of nematode *Caenorhabditis elegans*. Ecotoxicol. Environ. Saf. 73, 1221–1230.

Westheide, W., Graefe, U., 1992. Two new terrestrial *Enchytraeus species* (Oligochaeta, Annelida). J. Nat. Hist. 26, 479–488.

Yin, M.., Wu, C.-K.., Lou, Y.., Burda, C.., Koberstein, J.T.., Zhu, Y.., O'Brien, S., 2005. Copper oxide nanocrystals. J. Am. Chem. Soc. 127, 9506–9511.

Zhang, D.-W., Yi, T.-H., Chen, C.-H., 2005. Cu nanoparticles derived from CuO electrodes in lithium cells. Nanotechnology 16, 2338–2341.

Zhao, J., Wang, Z., Liu, X., Xie, X., Zhang, K., Xing, B., 2011. Distribution of CuO nanoparticles in juvenile carp (*Cyprinus carpio*) and their potential toxicity. J. Hazard. Mater. 197, 304–310.

Zhao, R., Xuan, Y., Li, X., Xi, R., 2008. Age-related changes of germline stem cell activity, niche signaling activity and egg production in *Drosophila*. Aging Cell 7, 344–354.

Chapter 5

General discussion and final considerations

General discussion and final considerations

Throughout this thesis some aspects of *Enchytraeus crypticus* biology were deeply investigated towards the development of new assays to assess chemicals toxicity.

E. crypticus can regenerate, but only the anterior fragment, i.e. formation of the tail (Chapter 2). The posterior regeneration took 6 days after which the organisms grew (for about 16 days) until they reach sexual maturity and were able to release cocoons (sexual reproduction). Regeneration is probably an additional survival strategy to deal, for instance with mechanical stress/injuries that can have implications for the interpretation of ecotoxicity results. Despite not pursued in the present thesis, regeneration ability could be further implemented as endpoint to assess effects of toxicants.

The embryonic development of *E. crypticus* was described and an embryotoxicity test was developed and validated (using cadmium as test substance) (Chapter 3). The development of the embryotoxicity test filled an existing gap in soil ecotoxicology, where the study of effects in the embryonary life-stage was not possible. Thus, endpoints such as embryo development and hatching success were added to field of soil ecotoxicology.

By monitoring survival and reproductive output of *E. crypticus* over its lifespan the understanding of the relationship of longevity and reproductive biology of these organisms was improved; and allowed the design of a long-term (life-span) assay (Chapter 4). The investigation of effects of contaminants on long-term exposure regimes has been long recommended for hazard assessment. This gained even more importance in the case of nanomaterials for which the effects might not be detected under the standard toxicity test time frame.

To understand the relationships between contaminants and species, more studies using chemical, biochemical, and molecular tools (ecotoxicogenomics) are needed, like epigenetic studies, as well as proteomic and metabolomic analyses. The combination of this tools and endpoints covering effects is the key for an effective ecotoxicology approach.

Finally, it is necessary add new tests or adjust the standard assays available in order to enable them to address the new endpoints with several contaminants (e.g. nanomaterials).